PEPTIDES

Structure and Biological Function

Proceedings of the Sixth
American Peptide Symposium

Erhard Gross
Johannes Meienhofer

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PREFACE

The Sixth American Peptide Symposium, held June 17-22, 1979, at Georgetown University in Washington, D. C., was attended by six hundred scientists from twenty four countries. Topics discussed ranged from peptide mediated ion transport to the challenging question of peptide receptor isolation and characterization.

Discussion of peptide chemical and physical analysis was followed by broad coverage of the assembly of biologically active peptides in solution and on solid supports or via semisynthesis. The making of peptides in 1979 has advanced to techniques now feasible with the developments in the field of Recombinant DNA.

Roderich Walter, whose untimely death ended a most successful career in peptide chemistry and biology, was among those to recognize early the potential of Recombinant DNA techniques for the man-made peptide. In one of his contributions to these Proceedings he addressed himself to this question and compared conventional peptide synthesis with the DNA-based approach.

John Baxter chaired a special session on applications of Recombinant DNA techniques to the synthesis of peptides of physiological significance. Annie Chang reported on the structure of the cloned cDNA that encodes for the bovine corticotropin-β-lipotropin precursor protein.

Josef Martial and Arthur Riggs demonstrated to us how close we are to the realization of access via Recombinant DNA techniques to peptides of therapeutic importance. The messages in cloned DNA for human growth hormone and insulin have been expressed in *Escherichia coli* and much effort is underway to advance these syntheses to larger scale technology and apply the products to be derived to the good of necessary medical treatment.

Vincent du Vigneaud — eminent scholar in the field of neurohypophyseal hormones and Nobel Prize Winner in 1955 — died in 1978. The Symposium paid tribute to the man and his scientific accomplishment in a Memorial Lecture presented by Klaus Hofmann.

The Second Alan E. Pierce Award was given to R. Bruce Merrifield for his concept of assembling the peptide chain on insoluble supports. In the Award Lecture Dr. Merrifield discussed new developments in Solid Phase Peptide Synthesis.

George W. Anderson retired in 1978 from a most productive and rewarding scientific career which among many other accomplishments saw his contributions to carboxyl group activation and the clarification

of questions related to racemization. The Symposium awarded Dr. Anderson the Honorary Chairmanship.

Organizing this Symposium was an immensely enjoyable experience. The cooperation of so many participants from all over the world was the finest there is. Associates, colleagues, and friends were always ready to help when called upon. It is with pleasure that I recognize their fine cooperation, assistance, and support. Dean Rose and Dean Beaudreau of the Medical and Dental Schools of Georgetown University responded most favorably to my request for lecture room and laboratory space. Dr. Nemes and his associates endeavored constantly to bring about the best of possible logistics for the Symposium. Dean Sullivan of the School of Continuing Education at Georgetown and Susan Goldsmith assisted the Symposium in numerous ways and made available Dormitory Space on Georgetown's Lower Campus. Driscoll and Associates were Conference Coordinators and saw to it that day to day operations advanced smoothly. Kay Bolls, my secretary, Karin Rose, and Dorian Lozell directed every effort at the efficient operation of the Organizing Office.

The Publisher of the Proceedings of this Symposium is Pierce Chemical Company of Rockford, Illinois. It is a pleasure to acknowledge the unfailing enthusiasm of Roy Oliver in this venture and the enduring efforts in the Editorial Office of Melba Rinaldo, Cheryl Roettger, and last, but not least, Robert (Bob) A. Vigna.

To Johannes Meienhofer, I should like to express my gratitude for his invaluable help throughout the editorial process. With this Proceedings Issue Hans will retire from the Chairmanship of the Publication Committee of the American Peptide Symposium. Dr. Meienhofer's many years of dedication and service to this publication are deeply appreciated.

Contributions from Industry and Grants from Government Agencies have done much to make possible the considerable student and international participation in this Symposium. This support is gratefully acknowledged and identified elsewhere in the Proceedings.

By mid-1979, so it appears, the Analysis, Synthesis, and Biology of Peptides were advancing as strongly as ever before. With just measure of confidence one may look forward to years ahead of challenging endeavors with novel and biologically ever more intriguing assemblies of amino acids.

Bethesda, December 1979 Erhard Gross

SIXTH AMERICAN PEPTIDE SYMPOSIUM

Georgetown University
Washington, D.C.
June 17-22, 1979

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PEPTIDE RECEPTORS

ABBREVIATIONS

A

Aad	aminoadipic acid
ACE	angiotensin-converting enzyme
AChR	postsynaptic acetylcholine receptors
Acm	acetamidomethyl
ACT	actinomine
ACTH	adrenocorticotropic hormone (adrenocorticotropin)
Acy	α-aminooctanoic acid
Ada	adamantylalanine
ADH	antidiuretic hormone
Adoc	adamantyloxycarbonyl
ADPOC	1,(1-adamantyl)-1-methylethoxycarbonyl
Aeh	2-amino-5,6-epoxyhexenoic acid
Aha	ω-aminoheptanoic acid
Ahp	2-amino-5-*p*-hydroxyphenylpentanoic acid
Aib	α-aminoisobutyric acid
AMD	actinomycin D
Amp	2-amino-5-*p*-methoxyphenylpentanoic acid
ANG'	angiotensin II amide
Aoe	2-amino-8-oxo-9,10-epoxydecanoic acid
apo A-I	apolipoprotein A-I
aPP	avian pancreatic polypeptide
App	2-amino-5-phenylpentanoic acid
Asu	aminosuccinyl
AT	angiotensin
ATx	adult thymectomyzed
AVP	8-arginine vasopressin
AVT	arginine vasotocin
Azoc	α,α-dimethyl-4-phenylazobenzyloxycarbonyl
Azo-Tac	*p*-phenylazophenylsulfonylaminocarbonyl

B

BHA	benzhydrylamine
BK	bradykinin
β-LPH	β-lipotropin
BN	bombesin
Boc	*tert*-butyloxycarbonyl
Boctf	2,2,2-trifluoro-*tert*-butyloxycarbonylaminoethyl
Bpoc	2-(4-biphenylyl)propyl(2)oxycarbonyl
bPP	bovine pancreatic polypeptide
BPTI	bovine pancreatic trypsin inhibitor
BTI	bovine trypsin-kallikrein inhibitor
*t*Bu	*tert*-butyl
Bug	*t*-butyl-glycine (β-methylvaline)

Bz	benzoyl
Bzl	benzyl

C

Car	carboranylalanine
4CC	four component condensation
CCD	countercurrent distribution
CCK	cholecystokinin
CD	circular dichroism
CDI	*N,N'*-carbonyldiimidazole
cDNA	complementary DNA
Cha	cyclohexylalanine
CI	chemical ionization
CLIP	corticotropin-like intermediate lobe peptide; ACTH (18-39)
CMC	critical micelle concentration
CNS	central nervous system
ConA	concanavalin A
CP	cholecystokinin-pancreozymin
CS	chorionic somatomammotropin
CRF	corticotropin releasing factor
CSF	cerebrospinal fluid

D

DABA	diaminobutyric acid
DCC	dicyclohexylcarbodiimide
DCC-HOBt	dicyclohexylcarbodiimide — 1-hydroxybenzotriazole
DCHA	dicyclohexylamine
Ddz	2-(3,5-dimethyloxyphenyl)propyl(2)oxycarbonyl
Dha	dehydroalanine
DIC	diisopropyl carbodiimide
DIEA	diisopropylethylamine
DMAE	dimethylaminoethanol
DMF	dimethylformamide
DMPC	dimyristoylphosphatidylcholine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dncp	dinitrocarboxyphenyl
Dnp	2,4-dinitrophenyl
Dns	dansyl, 1-dimethylaminonaphthalene-5-sulfonyl
DPPA	diphenylphosphoryl azide
DSIP	delta-sleep inducing peptide
Dts	dithiasuccinoyl

E

ECF-A	eosinophil chemotactic factor of anaphylaxis
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EI	electron impact
EK	enkephalin
EP	endorphin

F

FA	formamide
FDMS	field desorption mass spectrometry
FD	field desorption
FDNB	fluorodinitrobenzene
FITC	fluorescein isothiocyanate
Fmoc	fluorenylmethoxycarbonyl
Form	formyl
FSH	follicle stimulating hormone
FT	fourier transform
FTS	facteur thymique serique (thymic serum factor)

G

GABA	γ-aminobutyric acid
GALV	gibbon ape leukemia virus
GC/HREIMS	gas chromatography/high resolution electron impact mass spectrometry
GCMS	gas chromatography mass spectrometry
GDMP	guanyldimethylpyrazole
GH	gastrin, human
GH	growth hormone (somatotropin)
Gla	γ-carboxyglutamic acid
GLC	gas liquid chromatography
Glc	galactose
GLC-MS	gas liquid chromatography-mass spectrometry
Gn-RH	gonadotropin-releasing hormone (gonadoliberin)
GPC	gel permeation chromatography
GPI	guinea pig ileum

H

hCG	human chorionic gonadotropin
hCS	human chorionic somatomammotropin
HDL	high density lipoprotein
HDVal	hexadecavalinomycin
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
hGH	human growth hormone
HMPA	hexamethylphosphoramide

HOBT	*N*-hydroxybenzotriazole
HONBI	*N*-hydroxy-5-norbornene-2,3-dicarboximide
HOSu	*N*-hydroxysuccinimide
HPCD	high performance countercurrent distribution
HPLC	high pressure liquid chromatography
hPP	human pancreatic polypeptide
hPTH	human parathyroid hormone
HPTLC	high performance thin layer chromatography
HRFDMS	high resolution field desorption mass spectrometry

I

I-CPY	immobilized carboxypeptidase Y
IEF	isoelectric focusing
Ioc	isobutyloxycarbonyl
Iph	β-phenyl-β-alanine
IRA	internal reference amino acids
Ise	isoserine
Iva	isovaline

J

J	spin-spin coupling constant

L

Lac	lactic acid
Lal	lysinoalanine
LAP	leucine aminopeptidase
LβNA	leucyl-β-naphthylamide
LCAT	lecithin-cholesterol acyltransferase
LH	luteinizing hormone
LRH	luteinizing hormone releasing hormone (luliberin)
β-LPH	β-lipotropin
LSC	liquid scintillation counting
LVP	8-lysine vasopressin
LVT	lysine vasotocin

M

MA	mixed anhydride
Mbh	4,4'-dimethyloxybenzhydryl
MCD	mast cell degranulating
MDP	*N*-acetylmuramyl-L-alanyl-D-isoglutamine
med	minimum effective dose
MES	*N*-2-hydroxyethylpiperazine
Mlal	N^{ϵ}-monomethyllysinoalanine
MPO	myeloperoxidase

MS	mass spectrometry
Ms	methanesulfonyl
Msc	2-(methylsulfonyl)ethyloxycarbonyl
MSH	melanophore stimulating hormone (melanotropin)

N

β-NA	β-naphthylamide
NBS	*N*-bromosuccimide
NCA	*N*-carboxyanhydride
NDH	neurodepressing hormone
NMM	*N*-methylmorpholine
NMP	*N*-methylpyrrolidone
NMR	nuclear magnetic resonance
NOE	nuclear Overhouser enhancement
Np	nitrophenyl
Npa	*p*-nitrophenyl acetate
NPD	*p*-nitrophenyldiazomethane
Nps	2-nitrophenylsulfenyl
Npys	3-nitro-2-pyridinesulfenyl
NT	neurotensin

O

OBzl	benzyl ester
O*t*Bu	*tert*-butyl ester
ODS	octadecasilyl
OMI	oocyte maturation inhibitor
OMPA	oxymethylphenylacetic
ONp	4-nitrophenyl ester
OP	oxypressin
OPcp	pentachlorophenyl ester
OPfp	pentafluorophenyl ester
OPht	*N*-hydroxyphthalimide ester
oPP	ovine pancreatic polypeptide
ORD	optical rotatory dispersion
OT	oxytocin
OTcp	2,4,5-trichlorophenyl ester

P

P	polystyrene (resin)
PAGE	polyacrylamide gel electrophoresis
Pam	phenylacetamidomethyl
PAP	*N*-acrylylpyrrolidine (resin)
PCA	passive cutaneous anaphylaxis
PCP	pentachlorophenyl
PEG	polyethyleneglycol
Pen	penicillamine

PFC	plaque forming cells
Pfp	pentafluorophenyl
PHA	phytohaemagglutinin
ΔPhe	dehydrophenylalanine
PLL	poly-L-lysine
PMSF	phenylmethylsulfonyl fluoride
PMSG	pregnant mares'serum gonadotropin
pMZ	*p*-methyloxybenzyloxycarbonyl
pNA	*p*-nitroanilide
Pon	phenylacetoxymethyl-3-nitrobenzamidomethyl
Pop	phenylacetoxypropionyl
PP	pancreatic polypeptide
pPP	porcine pancreatic polypeptide
PPRS	pineal prolactin regulating substance
pr	prolactin
PRP	proline rich phosphoprotein
PTH	parathyrine (parathyroid hormone)
PTH	phenylthiohydantoin

Q

QNB	quinuclidinyl benzilate
Qxc	quinoxalinecarbonyl

R

REMA	repetitive excess mixed anhydride
R_f	front ratio
rGH	rat growth hormone
RIA	radioimmunoassay
RIEF	recycling isoelectric focusing
RP-HPLC	reversed phase-high pressure liquid chromatography
RPLC	reversed phase liquid chromatography

S

SBTI	soybean trypsin inhibitor
Sbz	sulfobenzyl
SP	substance P
Spe	spermidine
SS	somatostatin
SSAV	simian sarcoma associated virus
Suc	succinyl
sulfmoc	9-(2-sulfo)fluorenylmethyloxycarbonyl

T

Tac	*p*-toluylaminocarbonyl
Tcp	2,4,5-trichlorophenyl
TDO	thiophene dioxide
TFA	trifluoroacetic acid
Tfa	trifluoroacetyl
TFE	2,2,2-trifluoroethanol
Thi	β-(2-thienyl)alanine
TLC	thin layer chromatography
TLCK	*N*-α-*p*-tosyl-L-lysylchloromethyl ketone
Tml	N^{ϵ}-trimethyllysine
TMS	trimethylsilyl
TMV	tobacco mosaic virus
TMV-SH	mercaptosuccinyl tobacco mosaic virus
TN-I	cardiac troponin
TNP	trinitrophenyl
Tos	4-toluenesulfonyl
TP	2-thiopyridone
TPCK	L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone
2-TPTFA	2-thiopyridone trifluoroacetate
TRH	thyroliberin (thyrotropin releasing hormone)
TRIS	tris(hydroxymethyl)aminomethane
Trt	trityl, triphenylmethyl
Tyr(I)	3-(iodo)tyrosine
Tzl	thiazolidine-4-carboxylic acid

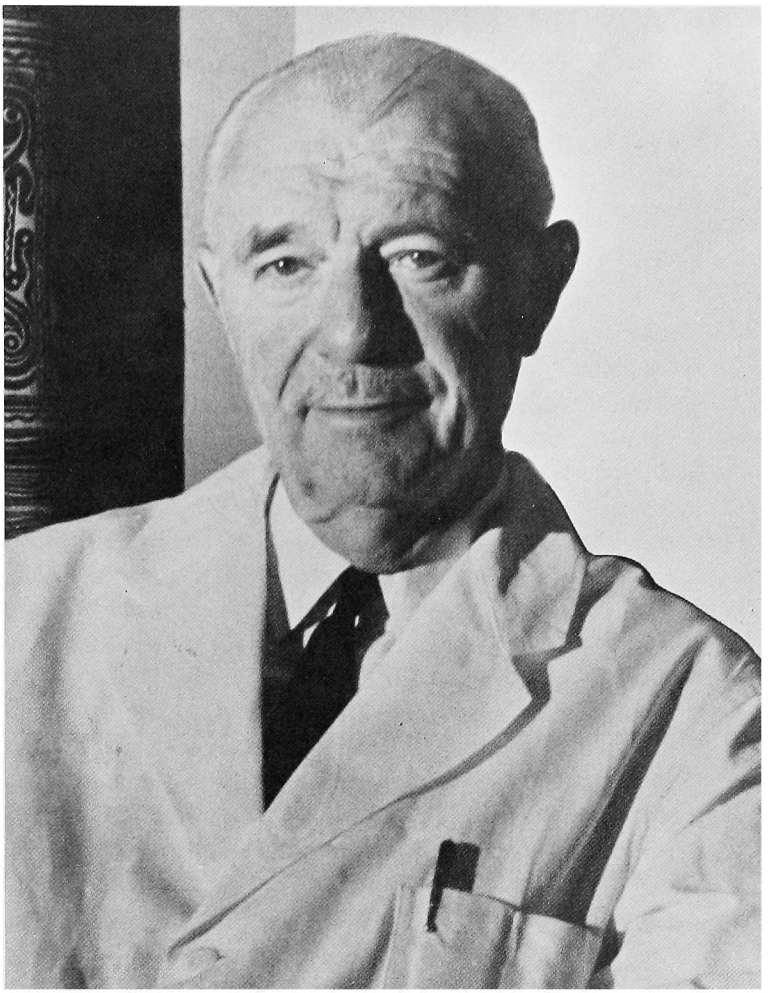
V

VIP	vasoactive intestinal polypeptide
V_l	light chain variable region
Val	valinomycin

Z

Z	benzyloxycarbonyl
Z-8-OQ	benzyl-8-hydroxyquinolylcarbonate
Ztf	2,2,2-trifluoro-N-benzyloxycarbonylaminoethyl

IN MEMORIAM
VINCENT DU VIGNEAUD
(1901-1978)



VINCENT DU VIGNEAUD
(1901-1978)

A TRIBUTE TO VINCENT DU VIGNEAUD
(1901-1978)

K. HOFMANN, *Protein Research Laboratory*
University of Pittsburgh School of Medicine
Pittsburgh, PA 15261

Vincent du Vigneaud passed away on December 11, 1978 after a long illness but his scientific heritage is preserved. It is a fitting tribute to him to set aside time at this meeting in his honor. However, as we shall note, his scientific contributions go beyond the field of peptide chemistry; he addressed many pertinent biological problems and found solutions to them. If one views the totality of his scientific contributions one recognizes a thread of continuity connecting sulfur containing biologically important compounds. This thread extends from insulin to cysteine to homocysteine to methionine to cystathionine to biotin to oxytocin and vasopressin and their analogs. In the Messenger lectures delivered at Cornell University in 1950[1] he likens his scientific work to a trail in research. I quote: "An attempt was made to retrace the research trails originating from a study of insulin that I have had the pleasure of working out in association with various collaborators over a period of twenty-five years. I attempted to present not only the findings encountered, but also in many instances the stepwise evolution of these findings, including the accidents of fate that played part".

It is indeed an honor and a genuine pleasure for me to pay tribute to a former mentor and friend by revisiting certain landmarks along his "trail" with you today.

Some of du Vigneaud's earliest researches dealt with the chemical nature of insulin. Abel[2] crystallized insulin in 1926 and Jensen, Wintersteiner and du Vigneaud[3] investigated the composition of acid hydrolysates of the crystalline hormone. With the rather primitive analytical methods available at the time, the presence in such hydrolysates of cystine and various amino acids such as tyrosine, histidine, arginine, lysine and leucine was established. Based on this evidence it was concluded that insulin was a protein. Much later du Vigneaud commented as follows regarding this conclusion: "It may seem strange to speak of work establishing insulin as a protein because it is now a generally accepted fact that a hormone can be a protein or that a

protein can be a hormone, yet at that time (in 1928) there was great reluctance in accepting this viewpoint." The thinking at that time was strongly influenced by Willstatter's concepts regarding the chemical nature of enzymes. These were assumed to consist of a small functional coenzyme and a protein carrier called an apoenzyme. Insulin was believed to be a small molecule which was attached or adsorbed to a high molecular carrier.

In 1930 du Vigneaud became acquainted with L. F. Audrieth, a faculty/colleague, at the University of Illinois who was an expert in the liquid ammonia field. He became impressed with liquid ammonia as a remarkable solvent for insulin and the sparingly soluble cystine. Audrieth's use of metallic sodium as a reducing agent in liquid ammonia prompted du Vigneaud to apply this method to the reduction of cystine to cysteine[4]. He devised the technique of *S*-benzylation of cysteine by adding benzyl chloride to sodium in liquid ammonia reduced cystine and along similar lines developed procedures for the *S*-methylation[5] and ethylation of thiols[6]. The observation that the *S*-benzyl group was removed from *S*-benzylcysteine by reduction with sodium in liquid ammonia[7] represents a significant contribution to peptide chemistry

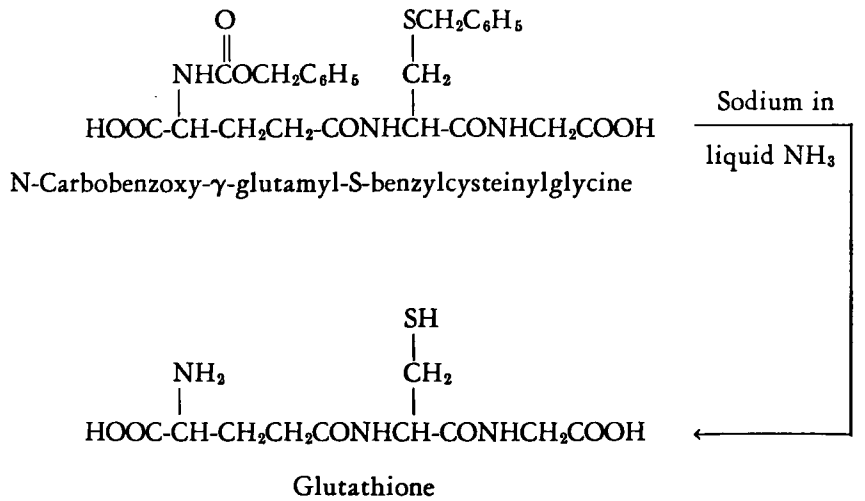


Fig. 1. A synthesis of glutathione

since it made possible the transient protection of the thiol group of cysteine during peptide syntheses. In 1932 Bergmann and Zervas[8] introduced the benzyloxycarbonyl group (carbobenzoxy group) into amino acids and with the discovery that this group could be cleaved by

catalytic hydrogenolysis laid the groundwork for the development of modern peptide chemistry. The discovery with Sifferd[7] that benzyloxycarbonyl groups can be removed from cysteine and cysteine containing peptides by sodium in liquid ammonia broadened the scope of the carbobenzoxy method and opened its application to sulfur containing peptides. The utility of these various techniques was amply demonstrated by a synthesis of glutathione (Figure 1)[9]. This involved the assembly of *N*-benzyloxycarbonyl-γ-glutamyl-*S*-benzylcysteinylglycine followed by simultaneous sodium in liquid ammonia cleavage of the *N*-benzyloxycarbonyl and *S*-benzyl protecting groups.

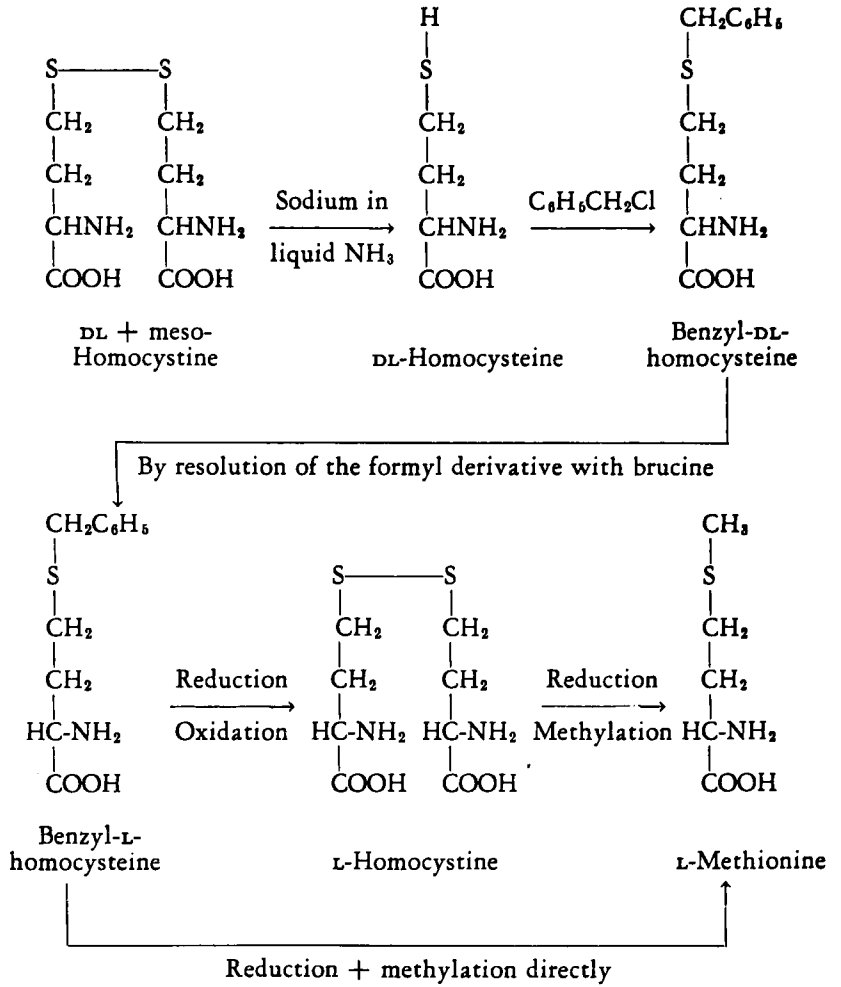


Fig. 2. Preparation of the L isomer of homocystine and methionine

Another series of investigations at this time dealt with the preparation of homocystine and homocysteine. In collaboration with Butz[10] he prepared racemic homocystine by heating methionine with strong sulfuric acid. This amino acid had not been encountered in nature. The chemistry and sterochemistry of both homocystine and homocysteine were extensively investigated and a practical synthetic route (Figure 2) to these compounds was perfected which relied on sodium in liquid ammonia reactions[11-13]. DL-*S*-benzylhomocysteine was resolved and the L-form was identified by conversion to L-methionine, again applying the sodium in liquid ammonia procedure.

As we proceed along the "trail" we will come to realize that the techniques which du Vigneaud developed early in his career provided solutions in his later investigations on the chemical nature of the neurohypophysial principles oxytocin and vasopressin.

I now wish to turn to a section of the "trail" that is concerned with the formation of cysteine in the animal body and to the metabolic relations between methionine, cysteine, homocysteine, cystathionine and choline. Du Vigneaud called the basic principles underlying these relationships transulfuration and transmethylation. Much of the early information in this field was derived from nutrition experiments in which the weight increase of rats on a variety of dietary regimes served as the endpoint. Jackson and Block[14] showed that methionine could support growth of rats on a cysteine-free diet and Womack, Kemmerer and Rose[15] established methionine as an essential amino acid. In other words the rat has the ability to synthesize cysteine but not methionine. With Dyer and Harmon, du Vigneaud observed that synthetic homocystine like methionine was able to support the growth of rats on diets deficient in cystine[16]. These observations pointed to a metabolic relation between methionine and homocysteine and suggested demethylation of methionine as the initial step in cysteine biosynthesis.

Brand, Block, Kassell and Cahill[17] proposed a scheme (Figure 3) for the conversion of methionine to cysteine which involved demethylation with formation of homocysteine followed by a condensation of the homocysteine with α-aminoacrylic acid to form a mixed thioether. Cleavage of this thioether on the homocysteine side they postulated would result in the formation of cysteine. This hypothesis attracted du Vigneaud's attention and in collaboration with Brown and Chandler[18] he set out to synthesize the L-isomer of this thioether (Figure 4) which he called L-cystathionine. The synthetic compound was fed to rats in a diet

in which methionine was present at a level such that no growth occurred unless cystine, or some compound capable of forming cystine was present. The experiment depicted on Figure 5 shows that under these dietary conditions L-cystathionine as well as cystine were capable of supporting growth[18].

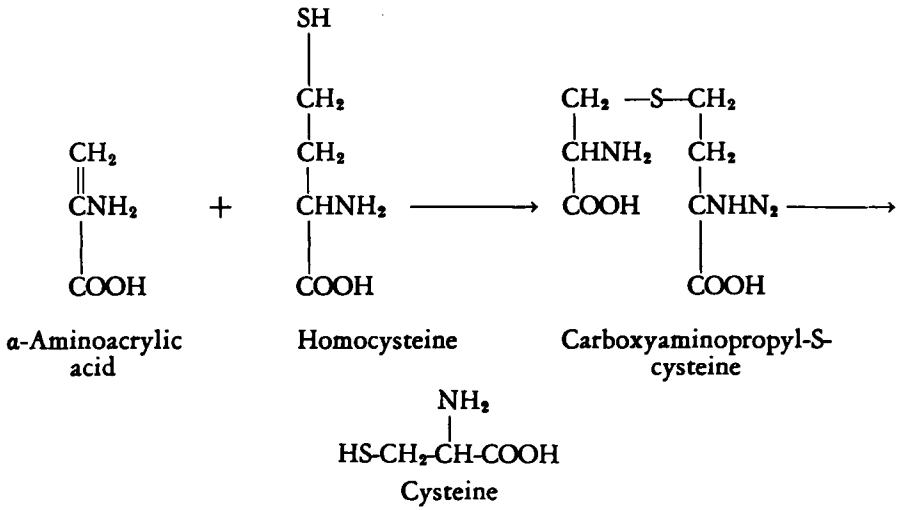


Fig. 3. Hypothesis suggested by Brand, Block, Kassell, and Cahill for the conversion of homocysteine to cysteine

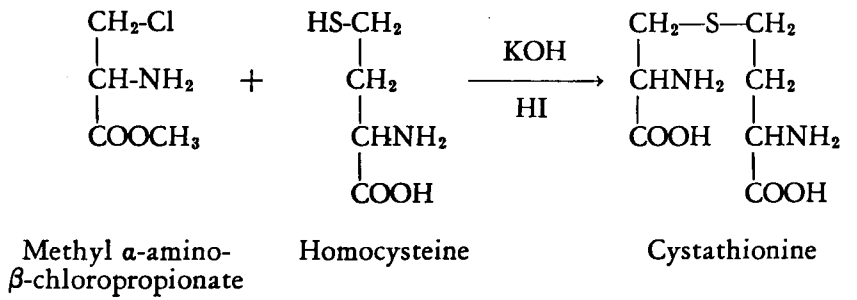


Fig. 4. Synthesis of cystathionine

The observation that L-cystathionine supported growth of rats on a cystine deficient diet indicated that the thioether was cleaved to form L-cysteine. Additional feeding studies had demonstrated that cystathionine did not give rise to homocysteine and this finding was supported by *in vitro* studies with liver slices. It was observed also that the addition to liver slices of a mixture of homocysteine and serine resulted in a 60% conversion of the homocysteine sulfur to cysteine[19]. This result provided

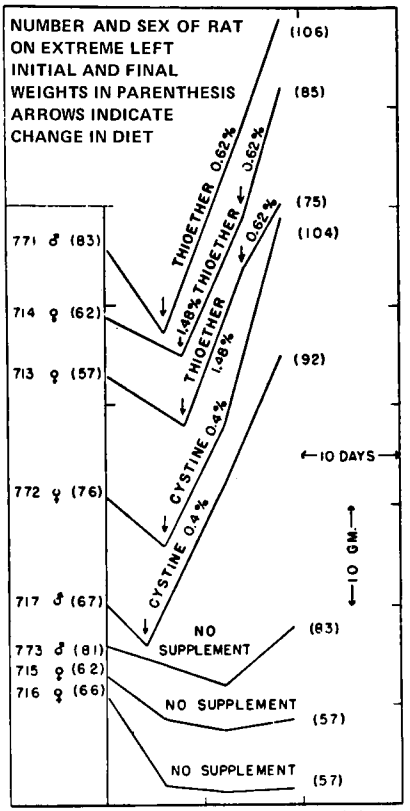


Fig. 5. Growth curves of rats receiving a basal diet containing 0.2% DL-methionine, with additions of L-cystine or the thioether (L-cystathionine) indicated on the curves.

strong evidence in support of the hypothesis that homocysteine was indeed an intermediate in the formation of cystathionine. The importance of serine as a precursor of cysteine derives from the work of Stetten[20] who fed ^{15}N labeled serine to rats and found label in the cystine isolated from the animals.

Before continuing the discussion of du Vigneaud's work on intermediary metabolism of sulfur compounds it seems fitting to have a brief look at the status of biochemistry at the time. In the 1930's the biochemistry department at the College of Physicians and Surgeons in New York City was one of the outstanding departments in the country and one that made scientific history. It was in that department where Rudolf Schonheimer and his colleagues performed the by now classical experiments pointing to "The dynamic state of the body constituents"[21]. The application of isotopes to the solution of biochemical problems provided the key to these developments which revolutionized biochemical thinking. Harold C. Urey had developed methodology for the preparation of deuterium oxide and other compounds enriched with respect to stable isotopes and the availability of such materials for biochemical studies opened far reaching biochemical frontiers.

Since the growth experiments had severe limitations du Vigneaud began to apply tracer techniques to gain more detailed information regarding the conversion of methionine to cysteine.

With Kilmer[22] he synthesized DL-methionine labeled in the β and γ positions with ^{13}C and containing ^{34}S. A closer look at this synthesis (Figure 6) provides some insight into the difficulties that were encountered during these pioneering days of tracer methodology.

Processor Harold C. Urey made available the isotopes in the form of sodium cyanide enriched with respect to ^{13}C and sodium sulfite containing ^{34}S. Through a complex multistep synthesis the doubly labeled methionine (VI) was prepared from these starting materials. Not only was the introduction of label into a desired compound beset with difficulties, but the determination of the isotope content of isolated metabolic products was not an easy task. The isotopically labeled

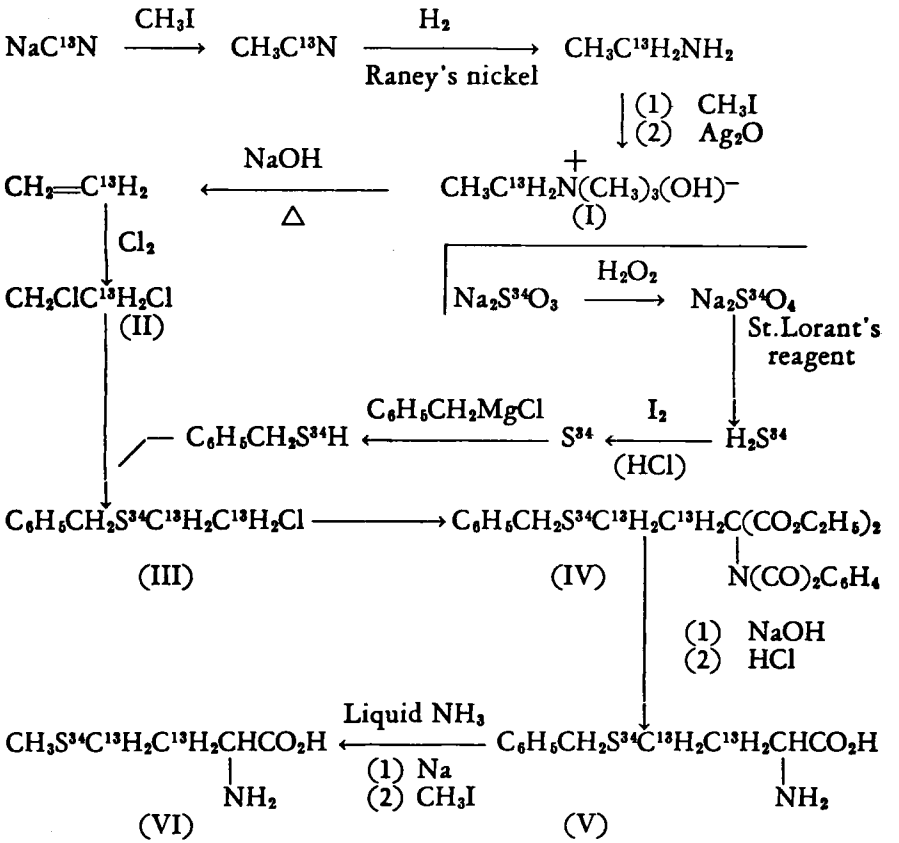


Fig. 6. Compounds and reactions involved in the synthesis of labeled methionine.

elements had to be converted into a volatile form for mass spectrometric analysis; carbon to CO_2, nitrogen to elemental N_2 or ammonia.

The doubly labeled methionine was fed to rats on a cysteine-free diet. The rats were shaved at the beginning of the experiment and after 38 days on the experimental ration received another haircut. The cystine isolated from the hair contained ^{34}S but no isotopically labeled carbon[23]. From this result it was concluded that only the sulfur of methionine was

utilized for cysteine synthesis. The results of this tracer experiment supported the hypothesis (Figure 7) regarding the synthesis of cysteine from methionine. Methionine is demethylated, the resulting homocysteine condenses with serine to form cystathionine which undergoes cleavage with formation of cysteine. As was found later, the carbon chain of homocysteine is converted to α-ketobutyric acid in this process[24]. In view of the fact that the biosynthesis of cysteine from methionine involves the transfer of the sulfur atom from homocysteine to serine du Vigneaud referred to this process as transulfuration.

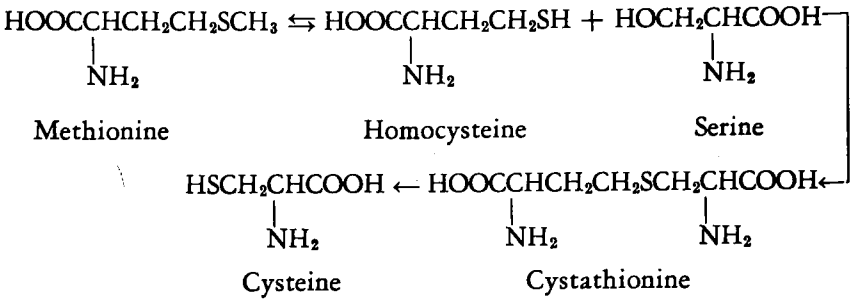


Fig. 7. Scheme for the conversion of methionine to cysteine in the animal body.

An interesting coincidence led to the concept of transmethylation. Here again the crucial observations were based on rat feeding experiments. Rose at the University of Illinois obtained good growth of rats fed a methionine-cysteine free diet that was supplemented with homocysteine. Similar experiments were carried out by du Vigneaud with Dyer and Kies[25] with negative results in that the animals failed to grow. The animals in both laboratories grew well when methionine in lieu of homocysteine was added to the diet. A comparison of the diets used in the two laboratories revealed differences in the vitamin supplements employed. Rose used rice bran extract (tikitiki) and milk concentrates; du Vigneaud employed crystalline B complex vitamins that were available at the time. In order to resolve this discrepancy Rose and Rice[26] repeated du Vigneaud's experiment and *vice versa*[27] and each laboratory confirmed the other's results. It was concluded that the supplements employed by Rose must have contained a factor or factors that were missing in du Vigneaud's diet. Du Vigneaud noted that rats on his diet had developed fatty livers a characteristic symptom of choline deficiency. This led him to the conclusion that choline was the missing factor in his diet and this reasoning turned out to be correct. Accordingly, diets containing both choline and homocysteine were fed to rats and this regimen supported growth equally as well as did methionine[27]. Du

Vigneaud speculated on the basis of these findings that choline, a compound rich in methyl groups, could act as a methyl donor for the conversion of homocysteine to methionine. These early findings led to the concept of transmethylation and that of labile methyl groups. The observation that rats grew well and failed to develop fatty livers on a choline-free diet supplemented with methionine suggested that the methionine methyl-group may serve as a methyl donor in the biosynthesis of choline. These concepts were confirmed by the use of tracer experiments. Du Vigneaud synthesized methionine in which the methyl group was enriched with respect to deuterium and fed this compound to rats. The choline isolated from the tissues of these animals contained deuterium in its methyl groups thus demonstrating transmethylation from methionine to choline. Conversely deuteriomethyl labeled choline was prepared and fed to rats with the result that the methyl group of methionine became enriched with deuterium[28]. Thus the hypothesis that methionine was biosynthesized from homocysteine *via* a methyl transfer from choline was substantiated. It was also found that the transfer of methyl groups was a continual process. With Cohn, Chandler, Schenk and Simmonds, du Vigneaud could demonstrate by tracer methodology that the methyl group of creatine also was derived from the methyl group of methionine[28]. The transfer of methyl groups from methionine to choline and from choline to methionine was a reversible process but the methyl group of creatine could not serve as a methyl donor for the methylation of homocysteine. These various reactions are illustrated on Figure 8. In 1941 du Vigneaud concluded "From our study, we know only that the methyl group of methionine and choline can be transferred, but we do not know whether methionine or choline react directly or whether they are precursors of derivatives from which the methyl groups are released. Although methionine can be demethylated *in vitro,* the conditions required are drastic. Attention must therefore be directed to any possibility whereby the bond between the CH_3 group and the S atom may be weakened. The formation of a sulfonium ion would be expected to effect such a labilization". It remained for Cantoni[29] to identify the methyl donor in biological systems as the sulfonium ion *S*-adenosylmethionine (Figure 9).

Another vantage point along the "trail" was the work carried out between 1940 and 1942 on the isolation and structure proof of the yeast growth factor biotin. This had an interesting history. Biotin in the form of its methyl ester was isolated from egg yolks in 1936 by Kögl and

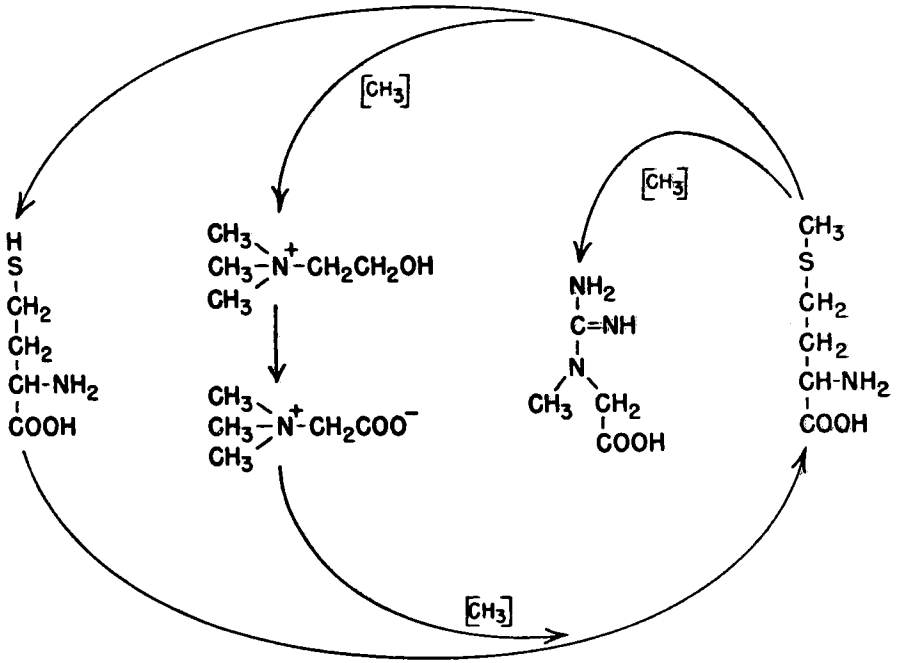


Fig. 8. Hypothetical scheme showing metabolic interrelationships between homocysteine, choline, betaine, creatine and methionine.

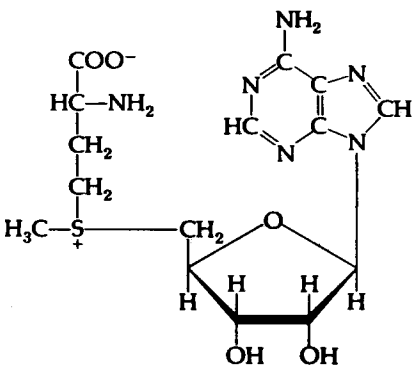


Fig. 9. The structure of S-adenosylmethionine.

Tönnis[30]. In 1940 du Vigneaud, Melville, György and Rose[31] identified biotin as the curative factor for egg white injury (vitamin H) thus adding biotin to the list of the B-complex vitamins. A short resume of the background which stimulated du Vigneaud to enter this field seems of interest. It was known from the work of Boas[32] and of Lease and Par-

sons[33] that rats receiving diets rich in raw egg white as a source of protein develop a severe dermatitis and nervous disorders. In the later stages of this disease (Figure 10) the rats assume a kangaroo-like posture due to spasticity of the hind legs and eventually die if the condition is not relieved. Certain foodstuffs such as liver and yeast contain a substance which is able of preventing or curing this egg white injury. This substance was named vitamin H (H standing for the German word Haut meaning skin). György, working on the isolation of vitamin H from liver concentrates, turned to du Vigneaud for help with this problem and a collaboration ensued. The similarity in certain properties of biotin and vitamin H suggested to György, Melville, Burk and du Vigneaud[34] that vitamin H and biotin could be one and the same compound. When a vitamin H concentrate from liver was subjected to electrodialysis, it was found that the vitamin H activity (assayed on rats) and the biotin activity (assayed with yeast) distributed identically in the cells of the dialysis apparatus. A sample of biotin methyl ester, obtained from Kögl, was then assayed with vitamin H deficient rats and proved to be highly potent in preventing egg white injury[31]. These results demonstrated conclusively the identity of the two substances. It is amusing to note that in the laboratories of a German pharmaceutical house one chemist prepared liver concentrates for György while another, just across the hall, prepared egg yolk concentrates for Kögl. These men did not realize that they were working on the same problem.

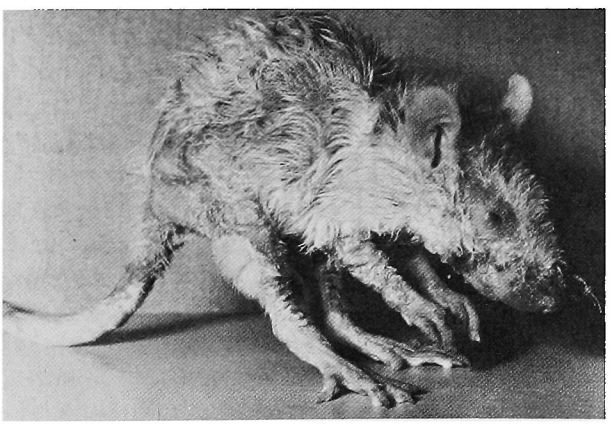


Fig. 10. Biotin deficient rat.

Crystalline biotin methyl ester was isolated from liver concentrates by du Vigneaud, Hofmann, Melville and György in 1941[35] and the structure of the vitamin was established soon thereafter in the Cornell laboratory. (Figure 11) The first synthesis of biotin by Harris and Collaborators at Merck[36] corroborated the postulated structure.

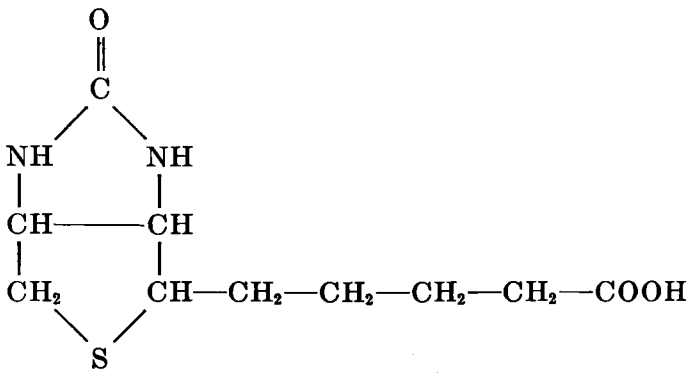


Fig. 11. The structure of biotin.

We have noted that the feeding of raw egg-white to rats results in biotin deficiency. The material in raw egg-white that is responsible for its toxicity has been isolated and characterized. It is a protein called avidin (because of its great avidity for biotin) which binds biotin very firmly[37]. The ensuing highly stable avidin-biotin complex passes through the alimentary canal of rats thus depriving the animals of this vitamin.

Du Vigneaud's work on the posterior pituitary principles oxytocin and vasopressin was started in 1932 and continued until 1940 when it was interrupted by the second World War. Some progress had been made in purification of these principles mainly by the application of precipitation and electrophoretic techniques. Of prime importance were some preliminary results indicating that these hormones were cystine derivatives. During the second World War new techniques were developed which influenced critically progress in posterior pituitary hormone research. The first of these was the Craig countercurrent procedure published in 1944[38], the second was the development by Moore and Stein of the starch column chromatographic technique for the quantitative separation and determination of amino acids in very small samples of protein hydrolysates[39].

In 1947 du Vigneaud returned to work on the isolation of the active principles in posterior pituitary extracts. A concentrate he had received

from Parke-Davis and Company in 1940 was stored during the war years and when reassayed in 1947 had lost 50% of its oxytocic potency. Using the Craig countercurrent technique he was able with Livermore to isolate from this old sample homogeneous oxytocin of high biological activity[40]. The amino acid composition, determined by the Stein-Moore method, of acid hydrolysates of this material showed the presence of aspartic acid, cystine, glutamic acid, glycine, isoleucine, leucine, proline and tyrosine in the molar ratios of 1:1[41]. The hydrolysate also contained ammonia in a ratio of 3:1 with respect to individual amino acids. The amino acids and ammonia accounted for 97% of the hydrolyzed material. Molecular weight determinations of oxytocin indicated the presence of a monomer[42]. In order to determine if the molecule was a cyclic or a straight chain peptide oxytocin was oxidized with performic acid[43]. A single compound was obtained having the same amino acid composition as the starting material except that in lieu of cystine two molecules of cysteic acid were present. Thus oxytocin was a cyclic peptide.

Earlier in my presentation I alluded to the fact that du Vigneaud's work followed a "trail" and the desulfurization of oxytocin to be discussed next provides an example.

During the elucidation of the structure of biotin the establishment of the size of the sulfur ring became a difficult problem. The supply of the vitamin prepared at Cornell was very limited thus hampering the degradation work. Merck and Company at Rahway was interested in the isolation of biotin and they had available to them a large quantity of biotin concentrates; however, they had not succeeded in obtaining the pure compound. When they became aware of the method used at Cornell to secure the crystalline compound they applied this same procedure to their concentrates and produced sizable amounts of the vitamin. This material was offered to du Vigneaud under the condition that further work on biotin structure would be carried out as a cooperative venture. This was agreed upon. About the same time a young investigator at Merck by the name of Mozingo had developed a novel method for removing sulfur from organic molecules by the use of Raney nickel. This procedure was applied to biotin in collaboration with the Merck Group and led to the formation of the compound desthiobiotin[44]. Turner, Pierce and du Vigneaud[45] exposed oxotocin to Raney nickel and obtained a compound in which the cystine sulfur was replaced by two hydrogen atoms, i.e. the two cysteines forming the disulfide bridge were converted to alanines. Hydrolysis of the desthiooxytocin followed by amino acid

analysis of the hydrolysate showed this to be true. The results of performic acid oxidation and desulfurization supported the cyclic peptide structure of oxytocin. Using the dinitrofluorobenzene technique of Sanger, du Vigneaud and his colleagues[46] were able to show that oxytocin contained a free amino group which was derived from one of the cysteine residues. The hormone did not contain a free carboxyl group. By a combination of the Edman technique and analysis of peptides obtained by partial hydrolysis of the hormone the amino acid sequence was arrived at (Figure 12). The final question which had to be solved was the source of the three ammonia molecules. It seemed logical to assume that two of the ammonias were derived from the sidechains of aspartic and glutamic acid but the source of the third remained to be established. The finding that the C-terminus of oxytocin was not cleaved by carboxypeptidase located the third amide group at the C-terminal glycine. The correct structure (Figure 13) of oxytocin was proposed in 1953[47]. In his characteristically critical approach to laboratory results du Vigneaud commented as follows: "It is obvious that, in spite the fact that this was the only structure we could arrive at through the realization of the results from our degradative work, synthetic proof of structure was mandatory."

H–Cys–Tyr–Ile–Glu–Asp–Cys–Pro–Leu–Gly–OH

Fig. 12. The amino acid sequence of oxytocin.

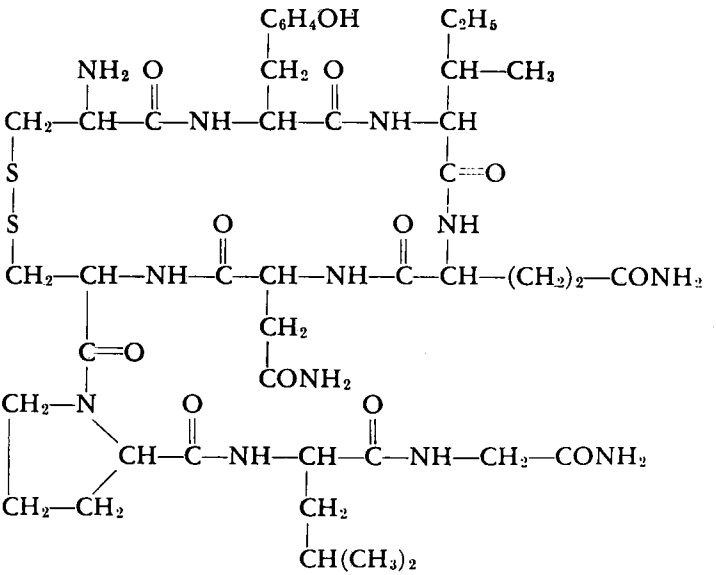


Fig. 13. The structure of oxytocin.

The synthetic approach to oxytocin was based on reactions in liquid ammonia discussed previously. Early observations with Sealock[48] showed that oxytocin was a disulfide that could be reduced with cysteine to the sulfhydryl form and reoxidized by aeration without appreciable loss in biological activity. Through knowledge of the oxytocin structure it became clear that the benzylated derivative of reduced oxytocin (oxytoceine) has the structure shown on Figure 14. Du Vigneaud reasoned that reduction of this dibenzyl derivative with sodium in liquid ammonia should afford oxytoceine which on aeration was expected to form the 20-membered ring-compound oxytocin. In collaboration with Gordon, he subjected high potency oxytocin to reduction with sodium in liquid ammonia and added benzyl chloride to the reaction product[49]. Analysis of the resulting material showed it to be the desired S,S-dibenzyloxytoceine. Debenzylation with sodium in liquid ammonia followed by air oxidation regenerated biologically active oxytocin. These experiments pointed to a conformation of the open chain peptide in which the two sulfhydryl groups are in close proximity otherwise polymerization rather than ringclosure would have taken place. The facile ringclosure to the 20-membered ring, so elegantly demonstrated by du Vigneaud, is the first example of the concept that the amino acid sequence endows proteins with the thermodynamic information necessary for folding into a specific conformation.

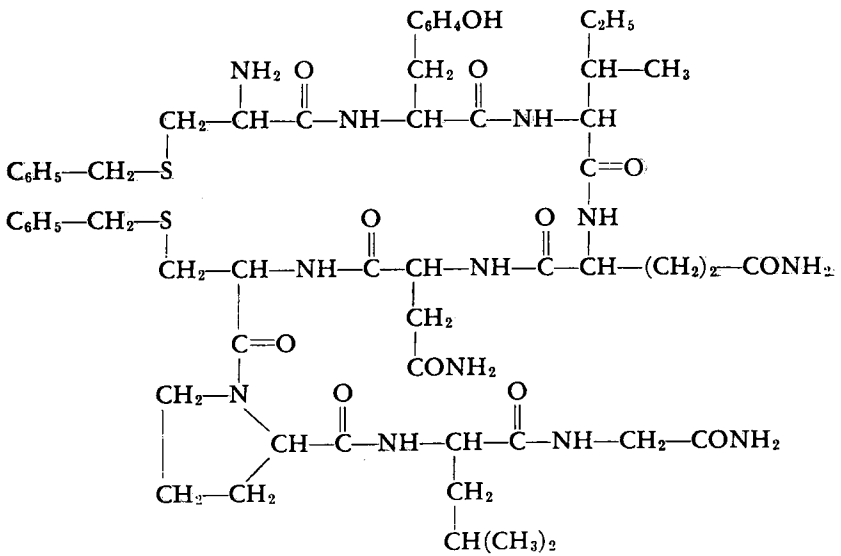


Fig. 14. Benzylated derivative of reduced oxytocin.

Since it was known from the work of Sifferd and du Vigneaud[7] that the benzyloxycarbonyl group as well as the *S*-benzyl group are cleaved by sodium in liquid ammonia the strategy for a synthesis of oxytocin was directed toward construction of the protected peptide *N*-benzyloxycarboyl- *S* -benzylcysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl- *S*-benzylcysteinyl-prolyl-leucyl-glycine amide (Figure 15). This peptide was assembled, deprotected with sodium in liquid ammonia and the reaction product on aeration afforded oxytocin. This first oxytocin synthesis was communicated to the Journal of the American Chemical Society on July 13, 1953 by du Vigneaud, Ressler, Swan, Roberts, Katsoyannis and Gordon[50]. The paper concluded with the statement "If the synthetic product truly represents oxytocin, which it does so far as we are concerned, this would constitute the first synthesis of a polypeptide hormone. What effect slight changes in the structure of a compound of such complexity might have on chemical, physical and biological properties must be investigated".

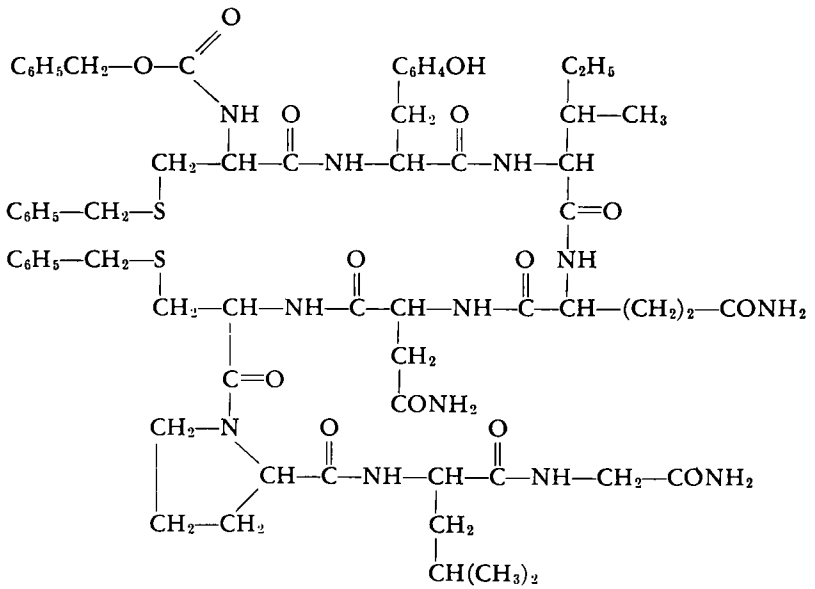


Fig. 15. Proposed intermediate for the synthesis of oxytocin.

While the work on oxytocin was under way the structure of the vasopressins was also determined. By means of a wealth of data derived from degradation studies, paralleling those outlined for oxytocin, a structure for arginine-vasopressin was arrived at with Popenoe and Lawler[51] which is very similar to that of oxytocin (Figure 16). The

synthesis of a material possessing lysine-vasopressin activity was published in 1956[52], homogeneous lysine-vasopressin was synthesized by Meienhofer and du Vigneaud in 1960[53].

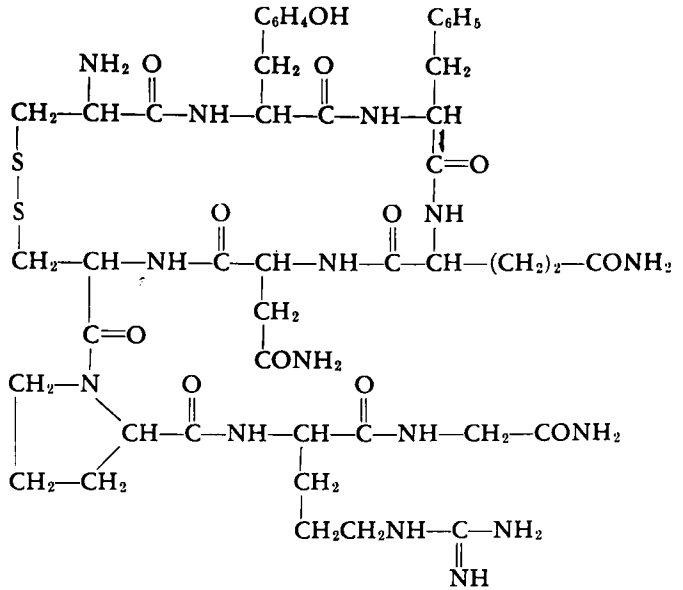


Fig. 16. The structure of arginine vasopressin.

The observation by du Vigneaud and his colleagues that a certain combination of amino acids into peptides can result in compounds exhibiting potent physiological activities opened a vast field of biological and chemical research. He stated, "It is a little startling to think that the amino acids when put together in a certain way, in a particular architecture, can lead to such an array of compounds exhibiting such a variety of physiological and pharmacological properties".

His findings with oxytocin and vasopressin were of great fundamental importance since they demonstrated for the first time that the substitution of certain amino acid residues in the sequence of a physiologically active peptide can bring about significant changes in biological specificity. The exchange in oxytocin of isoleucine for phenylalanine and of leucine for arginine (or lysine) alters the physiological activity of the molecule from one of mainly oxytocic activity to one with mainly vasopressor potency. These findings have stimulated much research into the relations between peptide structure and physiological function.

Thus far we have been concerned with the scientific legacy of Vincent du Vigneaud. We may now ask the question who was this man and what was the atmosphere in his laboratory that spawned so much fundamental work? I was privileged to spend two years at the Biochemistry Department at Cornell Medical College as a postdoctoral fellow and thus was able to catch some of the spirit of his organization. It was a very busy place indeed and hard laboratory work was mandatory. Graduate students were expected to spend several evenings a week in the laboratory and papers were frequently written until late at night. Professor du Vigneaud lived in the suburbs of New York but he maintained a room in the department where he spent many a night during the week. These were the evenings when he came to visit with his collaborators smoking a White Owl cigar and sharing a cold drink with us during discussions of the latest research developments. He was always highly interested in the day's results and was truly devoted to his scientific work. He had a highly critical attitude toward laboratory results and this permeates his writings. Every possible angle of a certain project was carefully discussed and new approaches and ideas, which could clarify a certain issue were sought. Papers were usually written in collaboration with those who actually did the work. A secretary was present and while discussions went on she was typing the latest version of the manuscript. A great many versions were hammered out before the Boss was satisfied. Unquestionably du Vigneaud was in command and he was highly respected by his collaborators. "Dee" as he was known by his colleagues was associated with a great number of graduate students, postdoctoral fellows and visiting professors during his scientific career. For example, Fritz Lipmann worked in the department for several years during which he wrote his famous paper entitled, "Metabolic Generation and Utilization of Phosphate Bond Energy".

All the people who ever worked in Dee's laboratory belonged to the V du V club. He kept in constant touch with us and each year during the Federation meeting we all got together for beer and pretzels to share time with former colleagues and the boss and his charming wife, Zella. Du Vigneaud received many honors during his scientific career but the triumph came with the 1955 Nobel prize in chemistry. In answer to a congratulatory note I sent him on this occasion; he answered, "The real thrill of such an award is sharing the pleasure with one's friends, and particularly with those who have been associated with me on the trail".

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SECOND ALAN E. PIERCE AWARD LECTURE

R. B. MERRIFIELD

SOLID PHASE PEPTIDE SYNTHESIS

SOLID PHASE PEPTIDE SYNTHESIS

R.B. MERRIFIELD, S.B.H. KENT, J.P. TAM, F.S. TJOENG, V. SARIN, S. MOJSOV, M.W. RIEMEN, T.W. WONG and C. VOSS, *Rockefeller University, New York, New York 10021*

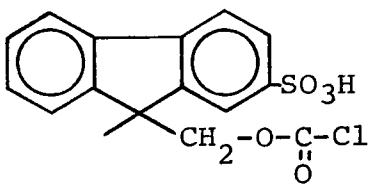
Our aim has been to modify and refine solid phase peptide synthesis so that we can more nearly achieve our goals of a general synthetic method for peptides and small proteins.[1] This has required the development of some new methods for the attachment of the peptide to the resin support and of improved ways to remove the free or protected peptide at the end of the synthesis. It has also involved studies on ways to obtain more homogeneous products. That can be done either by eliminating side reactions and avoiding the formation of by-products or by improving purification methods and removing the by-products after they have formed. I would like to discuss some recent work along these various lines that is now being carried out by members of our laboratory.

Affinity Purification

One of my own interests has been the development of an affinity chromatography method to select out the target molecule that is still growing at the end of a stepwise synthesis and separate it from all of the non-growing, terminated chains.[2] This goal has also been achieved by others in a variety of ways. We have devised a selective purification method that depends on the addition of a reversible protecting group, bearing a strongly acidic function, to the amino group of the growing peptide chains. After cleavage from the support, the two classes of peptide chains are separated chromatographically and the derivatized non-terminated peptides are then regenerated to the free peptides. The 9-(2-sulfo)fluorenylmethyloxycarbonyl (Sulfmoc) group fulfills the requirements for a reagent compatible with such a scheme.

The Sulfmoc group is a modification of the 9-fluorenylmethyloxycarbonyl (Fmoc) group developed by Carpino and Han,[3] and is based on their finding that this group is stable toward acid but is readily removed by base. For the present purpose it was necessary to introduce a strongly

acidic function into the molecule to facilitate the ion-exchange separation of the derivatized product from the underivatized impurities. We have found that ring sulfonation of Fmoc-Cl readily gives the required reagent, Sulfmoc-Cl *1*.



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The general scheme for the application of this technique is shown in Figure 1. The Sulfmoc group has the advantage that it will provide the only anion in the peptide at a pH below 2. Therefore, the Sulfmoc-peptide can be bound to a weakly basic (DEAE) ion-exchange column under acidic conditions in which the underivatized peptides, even those with more than one carboxyl group will not be retained. The Sulfmoc-peptide can then be eluted at higher ionic strength. Finally, the Sulfmoc group can be removed by treatment with a mild base such as morpholine or aqueous triethylamine to give the purified peptide. Alternatively, the Sulfmoc-peptide can be bound to a quaternary amine ion-exchanger and deprotected directly on the column by aqueous base. The free peptide can then be eluted by acidification. This technique has been applied to several small neutral, acidic and basic peptides and to synthetic des-acetyl-thymosin α_1, a 28-residue peptide containing several acidic and basic residues.[4] In the latter case, the homogeneity of the peptide was dramatically improved by this simple one-step procedure (Figure 2). A second synthesis of thymosin α_1 by Tai Wong, using our new Pam-resin support has given a crude product of greatly improved homogeneity, which was further purified by the Sulfmoc procedure. It should be pointed out that long Sulfmoc-peptides or those containing free arginine are sometimes quite insoluble and require special procedures.

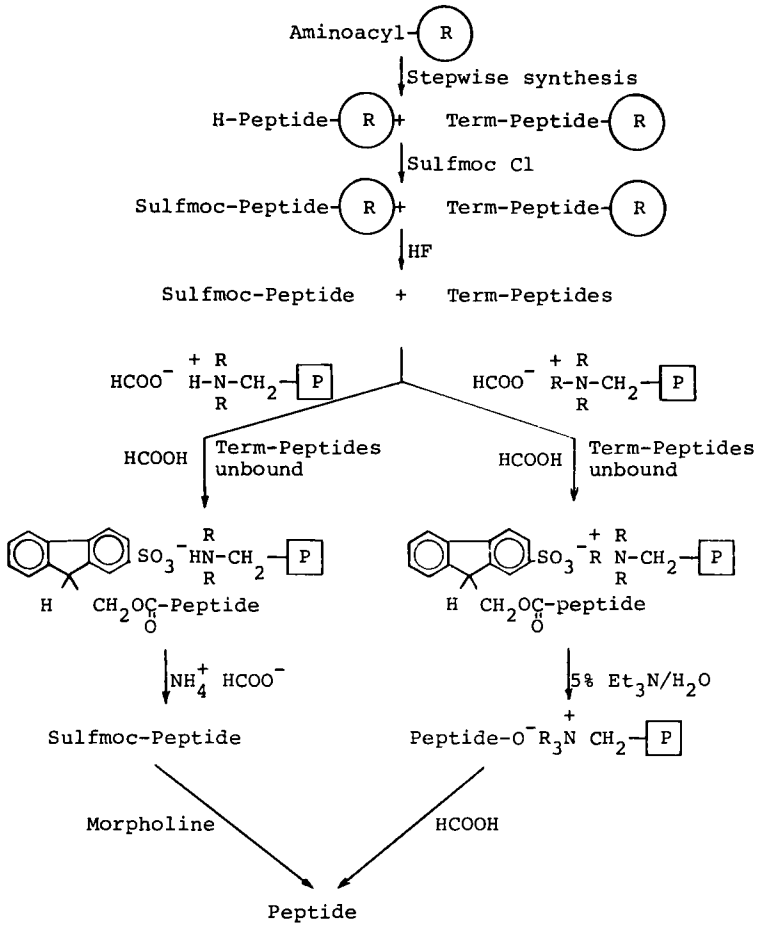


Fig. 1. A general scheme for the application of the 9-(2-sulfo)fluorenylmethyloxycarbonyl group to the purification of synthetic peptides. R is the resin support for solid phase synthesis; P is the ion-exchange polymer for chromatographic purification; Term-peptide represents the peptide chains that have been terminated at the α-amino group and are no longer growing.

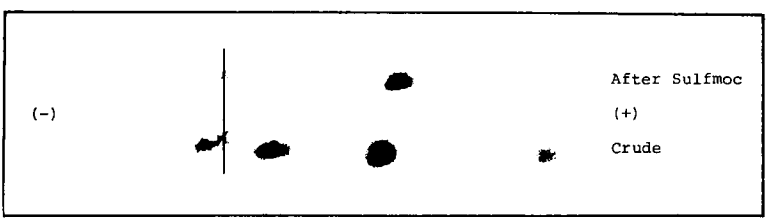


Fig. 2. Purification of des-acetyl-thymosin α_1 by the Sulfmoc method.

We have recently found that under special conditions the imidazole group of a histidine residue can be used as an anchor for the Sulfmoc group. Figure 3 shows that the group is readily removed by HF, but is sufficiently stable in TFA to be used in conjunction with the alkoxybenzyl alcohol-resin.

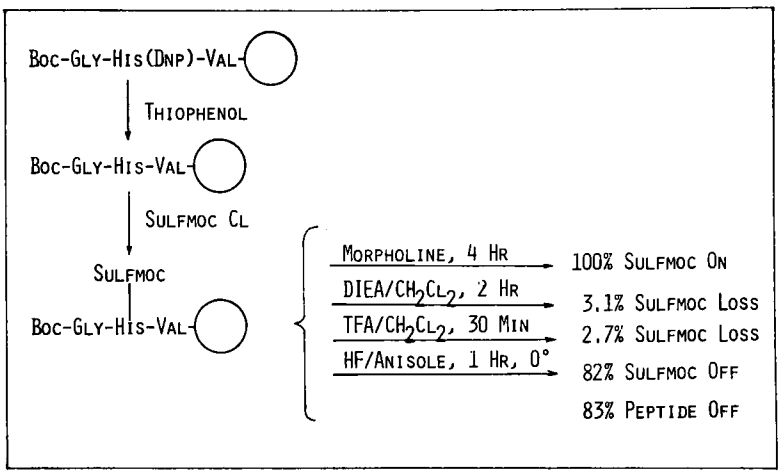
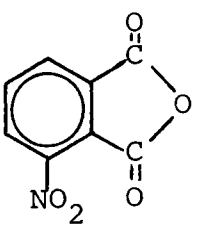


Fig. 3. Attachment of the Sulfmoc group through the side chain of histidine.

The technique described to here does not remove deletion peptides that are still growing at the end of the synthesis. We, therefore, have purposely terminated peptide chains that are still unreacted at the end of each coupling reaction to eliminate this potential source of deletion peptides.[5] The reagents 2-nitrophthalic anhydride *2* and sulfobenzoic anhydride *3* recommended by Wieland and Birr[6] were examined because they introduce strongly acidic groups into the terminated chains, which aid in the removal of these unwanted components.



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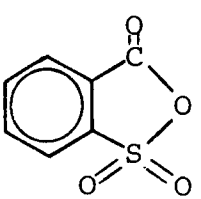

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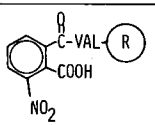
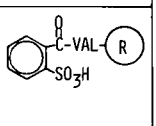
Table I shows that 2-carboxy-3-nitro-benzoyl-valine-resin was cleaved approximately 5 to 8% per hour in 5% diisopropylethylamine, 50% trifluoroacetic acid or HF. This may be acceptable for many

Table I. The Stability of 2-Carboxy-3-nitro-benzoylvaline-resin and 2-Sulfo-benzoylvaline-resin to Acids and Bases.

TREATMENT	LOSS (%/HR)	
		
5% DIEA/CH_2Cl_2	5	0.14
5% Et_3N/H_2O		0.20
10% MORPHOLINE/CH_2Cl_2		0.05
50% TFA/CH_2Cl_2	8	0.11
HF/ANISOLE	8	0.15

applications, but 2-sulfo-benzoyl-valine-resin was much more stable and is the reagent of choice. It was deprotected by these reagents at rates of only 0.1 to 0.2%/hour. We also confirm Birr's belief that the carboxyl and sulfonic acid groups are not activated by dicyclohexylcarbodiimide and do not couple with neighboring free amino groups. Thus, Val-Resin was 1/2 substituted by reagents *2* and *3* and then treated with DCC. There was no decrease in the picrate titration after such treatment. The combined Sulfmoc-Sulfobenzoyl method was applied to a radiolabeled tetrapeptide as outlined in Figure 4.

The peptide was first synthesized on the regular $ClCH_2$-resin. Figure 5, upper, shows the chromatographic separation of *free* peptides (derived from steric inaccessibility or temporary termination), *Tfa-peptides* (permanent termination), *Sbz-peptides* (representing potential deletions) and *Sulfmoc-peptides* (desired product). Figure 5, lower, shows the corresponding results for a similar synthesis, but using the Pam-resin support!

On this improved support all three classes of by-products were reduced by an order of magnitude, and in our experience on this and a number of larger peptides in the Pam-resin represents an important practical improvement solid phase synthesis.[7] Even with this im-

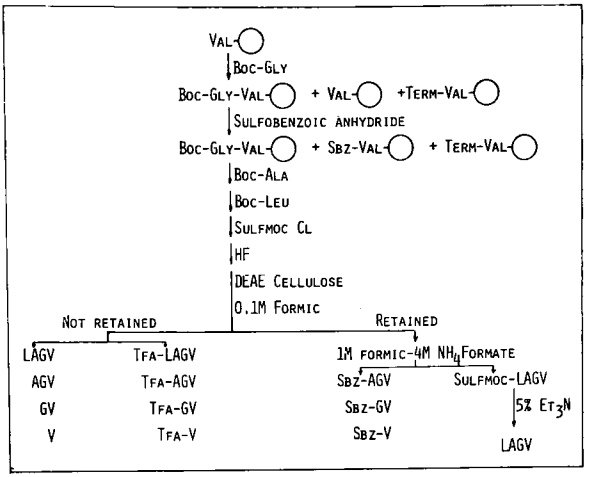


Fig. 4. The Sulfmoc-Sulfobenzoyl method for purification of synthetic peptides. LAGV; Leu-Ala-Gly-Val, Sbz; Sulfobenzoyl.

provement, however, the Sulfmoc-Sulfobenzoyl-procedure made a measurable further improvement. When the Sulfmoc peptide peak was collected, deprotected, and run on an amino acid analyzer column in a system designed to separate all of the potential termination and deletion peptides the product was extremely clean. The data before and after Sulfmoc are given in Table II. The alternate work-up procedure is shown in Figure 6. Here, the cleaved peptide mixture was washed onto an AG1x2 quaternary column in 0.1*N* formic acid. The free- and Tfa-peptides passed through, while the Sbz- and Sulfmoc-peptides were retained. The latter was deprotected with 5% aqueous Et_3N and upon acidification the purified LAGV was eluted. The base-stable Sbz-peptides were only eluted at much higher ionic strength.

Table II. Chromatographic Analysis of the Homogeneity of Synthetic Leu-Ala-Gly-Val Before and After Purification by the Sulfmoc Method.

Peptide	% of Total			
	$-OCH_2$-Resin		$-OCH_2$-Pam-Resin	
	No Sulfmoc	After Sulfmoc	No Sulfmoc	After Sulfmoc
V	0.10	0.05	0.10	0.00
AGV	0.51	0.01	0.03	0.01
LAGV	97.48	99.90	99.59	99.98
GV	0.71	0.01	0.18	0.00
LAV	0.79	0.04	0.00	0.00
LGV	1.41	0.00	0.10	0.01
LV	0.00	0.00	0.00	0.00

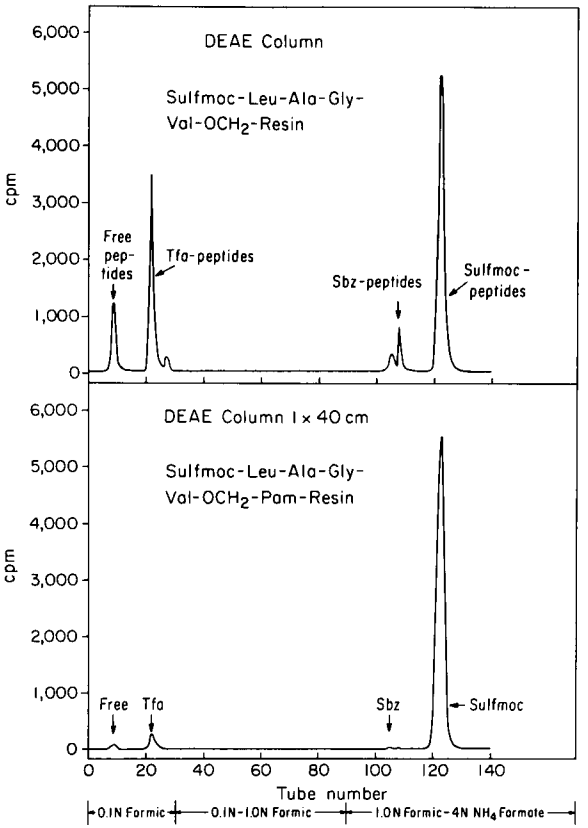


Fig. 5. Chromatographic purification on a tertiary amine resin of synthetic peptides by the Sulfmoc-Sulfobenzoyl method.

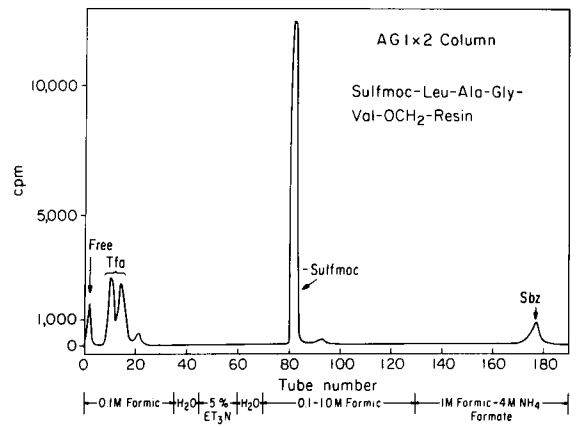


Fig. 6. Chromatographic purification on a quaternary amine resin of synthetic peptides by the Sulfmoc-Sulfobenzoyl method.

Reduction of Side Reactions

Instead of attempting to obtain pure products by the separation of impurities, the ideal approach would be to avoid the formation of all by-products. We obviously cannot do that, but along with many other people we are attempting to reduce the level of their formation. I think the work of Stephen Kent and Alexander Mitchell on the mechanism of trifluoroacetylation is an important step in that direction.[8] It has generally been assumed that trifluoroacetylation is due to carry over of the acid into the coupling step. However, we have shown that the major cause of this side reaction does not involve the coupling reaction at all, but is due to an intrabead nucleophilic transfer of Tfa groups from oxygen to nitrogen (Figure 7). When hydroxymethyl groups are present on the resin, they are readily esterified by trifluoroacetic acid. These Tfa-esters are then slowly attacked by the amino groups of neighboring peptides chains on the same resin bead to give the Tfa-amides and, hence, terminated chains. Hydroxymethyl sites are already present on some commercial $ClCH_2$-resins and additional sites can be generated by gradual acidolytic cleavage of the benzyl ester bonds anchoring peptide chains to resins. Pam-resins are made from aminomethyl-resins by a

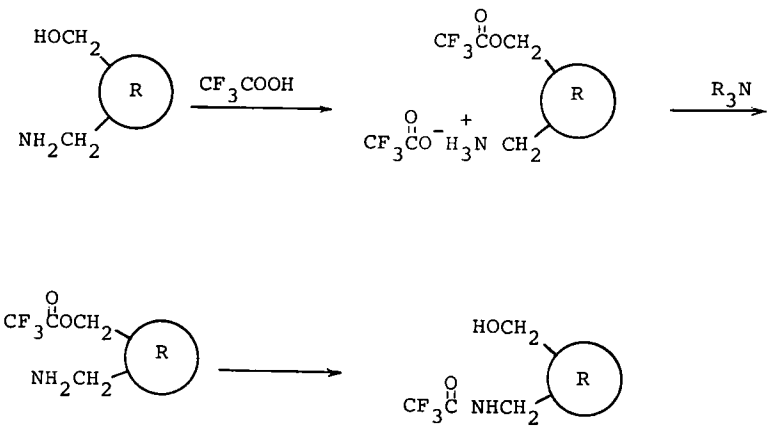


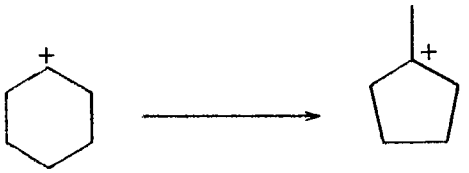
Fig. 7. The nucleophilic transfer mechanism of trifluoroacetylation.

process that does not generate such groups and the ester bond is so much more stable in acid than the usual benzyl ester there is virtually no generation of $HOCH_2$-groups by acidolysis. Levels of Tfa-terminated chains produced by this mechanism can, therefore, be reduced from about 2%/cycle to 0.02%/cycle. This means that a total of only 2% of terminated peptides would be generated during the synthesis of a 100-residue protein. Trifluoroacetylation due to DCC activation of TFA that is mechanically carried over into the reaction vessel can still occur and must be carefully guarded against.

In addition to the by-products that are formed at the repetitive steps of the synthesis, there are a host of side reactions associated with the side chains of individual amino acids. I would like to report on three such problems. It was recently shown in our laboratory[9] that protection of tyrosine with a cyclohexyl ether significantly reduced the extent of the HF-catalyzed rearrangement to the *3*-alkyltyrosine. The secondary ether had adequate stability toward TFA for use in stepwise synthesis but was readily cleaved by HF with formation of a cyclohexyl cation, which could be intercepted by scavengers before significant ring alkylation occurred. The value of the cyclohexyl group was demonstrated in a synthesis of angiotensin II.

It has been recognized for a long time that β-benzyl aspartyl residues can readily cyclize to the imide upon treatment with acid or base and then re-open to a mixture of α- and β-aspartyl-peptides. This is especially true if the next residue is Gly, Ser, or His. Dr. Tam reasoned that a β-cyclohexyl ester would greatly reduce this reaction and, together with Mark Riemen, Tai Wong and Siong Tjoeng, was able to show experimentally that the idea was correct.[10] In strong acid such as HF, the protonated ester will slowly close to the imide with loss of ROH by an $A_{AC}2$ mechanism. In a competing reaction the alkyl carbonium ion is formed by an $A_{AL}1$ mechanism. Normally a rapid return will keep the concentration of ester high and favor imide formation. In the case of cyclohexyl, however, the secondary carbonium ion *4* may rapidly rearrange[11] to the much more stable tertiary methylcyclopentyl carbonium ion *5* and effectively reduce imide formation.

The secondary ester is also much more resistant to base catalyzed cyclization. Thus, the tetrapeptide Boc-Glu(OBzl)-Asp(OR)-Gly-Thr(Bzl)-resin was synthesized with R = benzyl or cyclohexyl. The peptide-resin was cleaved with HF, -20°, 30 minutes, before and after treatment with tertiary amine, and the extent of imide formation was



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determined chromatographically, Table III. The cyclohexyl group gave some reduction in HF-catalyzed imide formation (2.8% to 0.8%) and a large reduction in the amount catalyzed by DIEA (51% to 0.3% in 24 hours). Blake has just reported very similar results with the cyclopentyl ester of aspartyl peptides.[12]

Table III. Acid and Base-Catalyzed Imide Formation from Benzyl and Cyclohexyl Esters of Aspartyl Peptides. a. Corrected for loss in HF.

Condition			Product (%)					
			R = Bzl			R = cHex		
Reagent	Temp °C	Time Hr	α-Asp	β-Asp	imide	α-Asp	β-Asp	imide
HF	0	0.5	87.5	0	12.5	95.6	0	4.4
HF	-20	0.5	97.2	0	2.8	99.2	0	0.8
TEA[a]	20	24	0.0	0	100.0	86.0	0	14.0
DIEA[a]	20	24	49.0	0	51.0	99.7	0	0.3

The third side chain problem we have been examining is the rearrangement of asparaginyl peptides during solid phase synthesis. The general mechanism of nitrile formation during the coupling of asparagine has been studied in detail.[13-17] The main reactions for the coupling of Boc-Asn with Gly-Resin are illustrated in Figure 8. The activated Boc-Asn-X can react with Gly-resin to give the Asn-Gly product, but in a competing reaction the isoimide ring can close and then reopen in base to give β-cyanoalanine. This by-product can then be reactivated by DCC (or anhydride) and couple with the amino component to form the nitrile-containing peptide. The isoimide can react to give Asn-Gly or, if it opens in the other direction, an amidine would be formed. The reports that in solid phase synthesis the dehydration did not occur when activation was with DCC-HOBt[18] or when the amide was protected with the dimethoxybenzhydryl (Mbh) group[19] seemed convincing, but reports that it did not occur with short DCC coupling time or if symmetrical anhydrides were used for activation seemed unreasonable. In any case, no

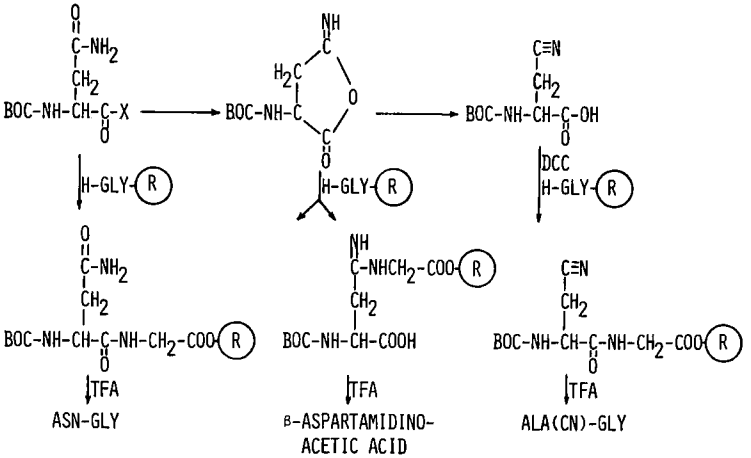

Fig. 8. The mechanism of nitrile formation during coupling of Boc-asparagine.

highly sensitive and quantitative data were available on the product distribution following any coupling method. Therefore, Dr. Mojsov synthesized Boc-Asn-Gly-Resin by various coupling methods and, after cleavage from the resin support, fractionated chromatographically and quantitated the spectrum of products. Since we suspected that cleavage with HF had complicated previous interpretations, the amino acid was anchored by a *p*-alkoxybenzyl ester bond which could be cleaved in TFA. The results are tabulated in Table IV. Both DCC and preformed

Table IV. Product Distribution Following Coupling of Boc-Asn and Gly-*p*-alkoxybenzyl-resin by Various Methods.

COMPOUND	ELUTION TIME (MIN)	MOL % OF TOTAL PRODUCT			
		DCC	ANH	DCC/HOBT	ASN(MBH) + DCC
β-ASP-GLY	70	0.2	1.2	0	0
α-ASP-GLY	185	0.3	1.1	0.1	0.2
ASPARTOYL-GLY	200	0	0	0	0
ASN-GLY	270	27.0	50.1	98.9	99.8
ISO-ASN-GLY	283	0	0	0	0
ALA(CN)-GLY	295	60.4	40.1	0.7	0
β-ASPARTAMIDINO ACETIC	360	12.1	7.5	0.2	0

symmetrical anhydride coupling caused large amounts of nitrile formation and significant amounts of a second by-product that is tentatively identified as β-aspartamidino acetic acid. Reduction of reaction time or alteration of proportions of reactants did not change the pattern appreciably. Addition of HOBt to the DCC reaction nearly eliminated the by-products and a DCC coupling with Boc-Asn(Mbh) gave essentially pure Asn-Gly. When Ala(CN)-Gly was treated with HF (30 minutes, 0°) and worked up in H_2O it was quantitatively rehydrated to Asn-Gly. Presumably nitrile formation had actually occurred in the experiments reported earlier, but was obscured by this rehydration process.

The Development of New Resin Attachments

One of the more active lines of development of solid phase synthesis has been the investigation of new materials for the supports and of new ways to attach the peptide chains. Among the new materials, the polyamides of the Sheppard[20] and Walter[21] laboratories appear to be especially useful. Among the new attachments, one can include nucleophile-labile phenyl-[22] and sulfonylethyl esters,[23] photolabile phenacyl[24] and o-nitrobenzyl[25] esters and several substituted benzyl esters with increased acid stability. In our laboratory the oxymethyl-phenylacetamidomethyl-resin (Pam-resin) was developed as a more acid-resistant support,[7] and Sparrow devised a chemically similar linkage for the purpose of introducing a spacer or handle between the peptide chain and the polystyrene resin.[26] Dr. Tam is currently developing some new supports, which he has termed multipurpose, multidetachable resins.[27] Their structures and relations to the standard resin and Pam-resin are shown in Figure 9. They are all peptide benzyl esters. The Pam-resin has a

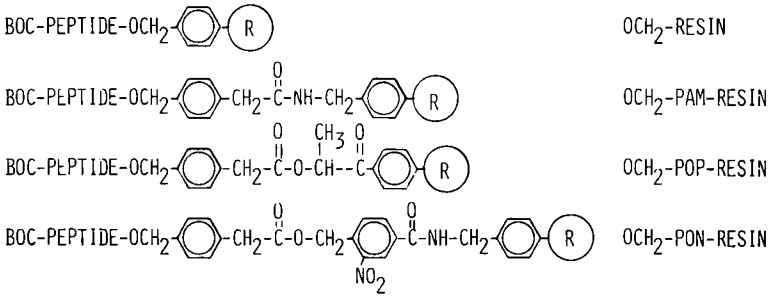

Fig. 9. Some new resin supports for solid phase synthesis.

phenyl-acetamidomethyl group inserted between the resin and the ester bond and this electron withdrawing group makes the ester 100 times more acid stable than the simple polystyrene-benzyl ester; although still readily cleavable by HF. The new linkages, called Pop- and Pon-resins, introduce two new features. They also contain an electron withdrawing group para to the benzyl ester, but this time it is an ester, rather than an amide. They both make the peptide-benzyl ester bond about 100 times more acid stable, but still cleavable by HF. In addition, they provide a second cleavable bond. In both resins this second bond is photolabile and can also be cleaved by various nucleophiles. Thus, cleavage at the second ester gives the fully protected peptide-oxymethylphenylacetic acid, which is useful for fragment synthesis. It can also be purified and then converted to the protected peptide acid or to the free peptide. It can be reattached to a photolabile resin such as 2-bromopropionyl-resin or to an aminomethyl-resin to give an acid resistant peptidyl-oxymethyl-Pam-resin. Direct acidolysis at the benzyl ester gives the free peptide in one step.

I would like now to describe some recent examples of the application of these various new supports to a project in which we are attempting to prepare the V_H domain of M603 mouse myeloma immunoglobulin.[28] The groups of Givol and Wilchek[29] and Haber, Burton and Matsueda[30] have been working on the total stepwise synthesis of similar V_L domains. We decided to examine a different approach as illustrated in Figure 10. Our plan is to combine stepwise and fragment methods, and in the process to get some experience with the new resin supports. Six amino-terminal protected fragments have been prepared on photolabile resins and most have been purified satisfactorily. Notice the convenient location of Gly residues. These are being assembled by stepwise fragment coupling of the first five units onto the sixth one which has been reattached to another support. The resulting 1-68 peptide will then be combined with 69-120 which has been synthesized stepwise on a Pam-resin. Let us now look specifically at three of these peptides.

The protected fragment 27-42 was initially synthesized stepwise after attachment of Boc-Gly-O^-Cs^+ to 2-bromopropionyl-resin. However, sizeable chain losses or terminations occurred at the mono, di, tri and tetrapeptide stages, resulting in incorrect amino acid ratios. The side reactions included cyclization of Gly to the dihydro-oxazinone, diketopiperazine formation at Pro-Gly and at Pro-Pro, and some pyroglutamic termination. Dr. Tjoeng then devised and carried out the

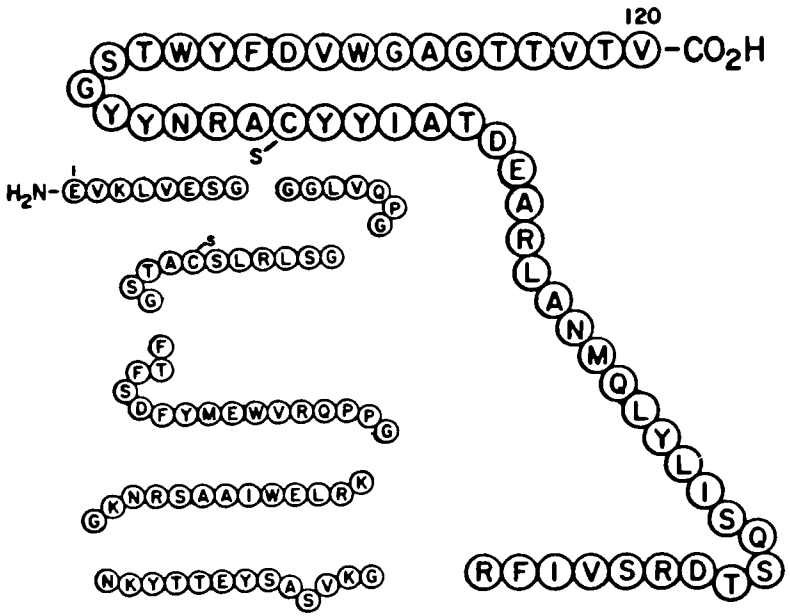

Fig. 10. A solid phase — fragment strategy for the synthesis of the V_H domain of mouse myeloma M603.

scheme shown in Figure 11, in which the first two additions were with dipeptides and the remainder of the couplings were with single amino acids.[31] The amino acid analyses were in excellent agreement with theory in the latter synthesis.

The protected fragment 56-68 was synthesized in two ways by Christoph Voss. He first assembled it stepwise on the 4-bromomethyl-(3-nitro)-benzamidomethyl-resin of Rich.[25] It could be removed in 84% yield by photolysis at >350 nm in DMF, and was purified by repeated precipitation from N-methylpyrrolidone by addition of water. It was then reattached to the same resin support in 24% yield. This preparation is being used to assemble the 1-68 peptide by fragment coupling. The 56-68 fragment was also prepared by the modified route shown in Figure 12. Boc-Gly-oxymethylphenylacetic acid was prepared and coupled with 2-bromopropionyl-resin to give Boc-Gly-OCH_2-Pop-resin. Because there is a spacer between the peptide and the α-methyl-phenacyl ester bond the cleavage will be independent of the nature of the C-terminal residue. A 52% yield of the Boc-peptide-oxymethylphenylacetic acid was obtained.

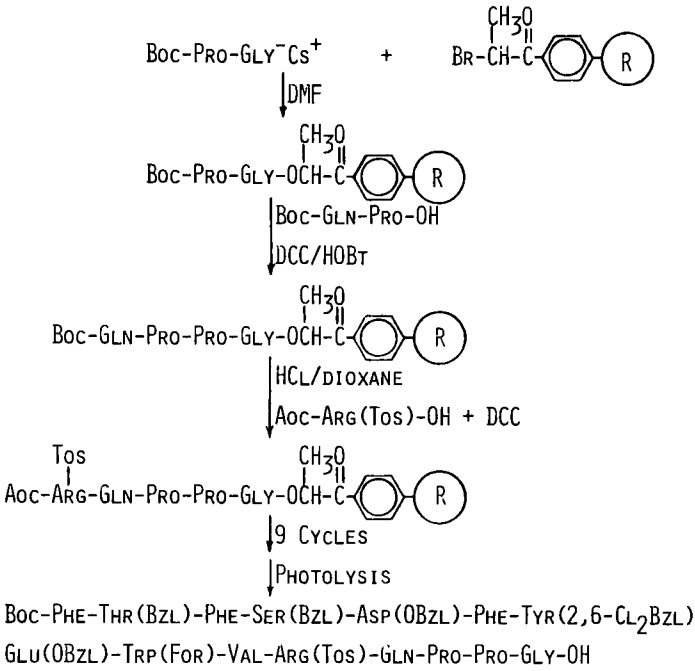

Fig. 11. Synthesis of protected M603 fragment 27-42 on 2-bromopropionyl-resin.

This product was purified by repeated precipitation with water from N-methylpyrrolidone. The purity of the fully protected peptide was assessed indirectly by cleaving and deprotecting in HF and then examining the free peptide on the amino acid analyzer column. A single major peak at 69 min was found plus one small impurity at 52 min. For the proposed fragment synthesis of the 1-68 peptide, the purified protected peptide was reattached in 43% yield to an aminomethyl resin, giving Boc-Peptide (56-68)-OCH_2-Pam-resin.

The final example is the use of the Pam-resin by Dr. Tjoeng for the stepwise synthesis of the 52-residue peptide V_H-(69-120) half domain and the 120-residue M603 V_H-(1-120) whole domain. Figure 13 shows the synthetic scheme and some of the results. The plan was to carry out a stepwise synthesis on the acid stable Pam-resin, under conditions where side reactions due to termination by trifluoroacetylation or to premature loss of chains would be minimal. [^{14}C]Gly was introduced at step 6 and [^{3}H]Val was coupled at the 49th step in order to be able to follow the course of the synthesis of the half domain and to assess the homogeneity

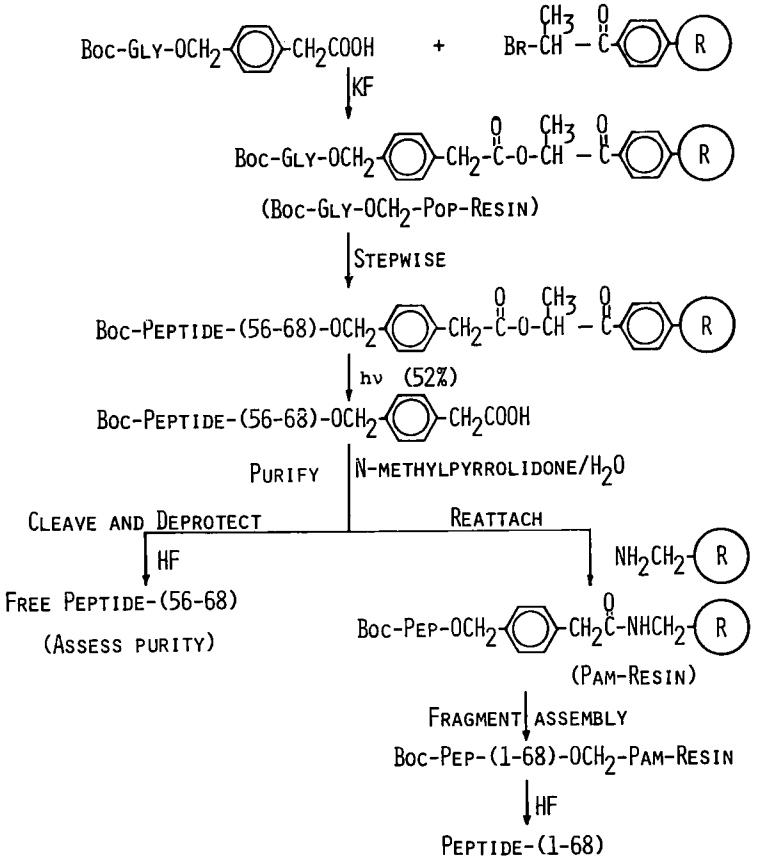

Fig. 12. Synthesis of protected M603 fragment 56-68 on Pop-resin and reattachment to Pam-resin.

of the product. By counting the filtrates after TFA deprotection it was found that an average of 0.013% of the chains were lost per step. This is about 100 times less than usually found on regular benzyl ester resins and agrees well with the previously determined acid stabilities. At the DIEA neutralization step the losses were only 0.0026% per step. Based on the specific activity of [^{3}H]Val, it could be estimated that only 80% of the original chains were still growing at step 49. This gives an average of 0.5% termination per step, which is much higher than expected if it were all due to trifluoroacetylation. It may, of course, be due to a few large losses at residues such as glutamine, but that has not been established. After the 52-residue peptide was assembled, half of it was cleaved in HF/10%

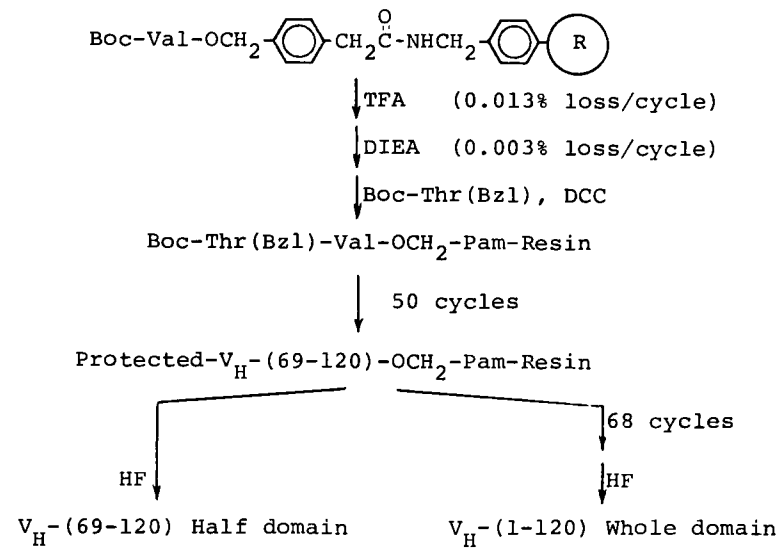

Fig. 13. Synthesis of M603 fragment 69-120 on Pam-resin.

anisole for 1 hour at 0°. Based on recovery of ^{14}C, the overall yield from [^{14}C]Gly^{6} was 83%. The peptide was passed through a Sephadex G-75 column in 50% acetic acid and a main fraction was obtained in which the $^{14}C/^{3}H$ mole ratio was 1.02. Because this peptide was rather insoluble in solvents other than 50% acetic acid it was somewhat difficult to purify. The Sulfmoc-peptide was also quite insoluble in all but high concentrations of acetic acid or trifluoroacetic acid and this technique could not be applied in the usual way to its purification. However, Dr. Tjoeng discovered that the HF-cleaved Sulfmoc-peptide was soluble in 70% phenol, and in that solvent about 75% of the crude mixture was bound to an AG 1 x 2 column. After washing sequentially with ethanol and H_2O, the Sulfmoc was removed by washing the column with 5% Et_3N and the purified free peptide was then eluted with 50% HOAc. This is the largest peptide that has been purified by the Sulfmoc method. Stepwise extension of the chain to the full 120 residues of the V_H domain is now under way.

It is our hope that the various fragments described here can be assembled into a complete V_H domain which can be compared in detail with the product of stepwise synthesis. Each can then be combined with natural light chain components and examined for its hapten binding properties.

Finally, I would like to describe some recent experiments of Dr. V. Sarin on the swelling of polystyrene resin beads before and after the synthesis of a long peptide chain. It has generally been assumed that the observed swelling of an unsubstituted bead in a particular solvent would represent a maximum volume increase of that bead and that when this space was filled by growing peptide chains there would be no more room for further chain growth. Since a 1% crosslinked bead swells in methylene chloride to about 5 times its dry volume, there would be enough room to accommodate a 4-fold increase in mass. This level is not normally reached even in a long synthesis and the maximum capacity of such beads has not been fully tested. For example, the weight of the protected-peptide-resin at the end of our ribonuclease synthesis (MW 19,791) was only about 1.7 times that of the starting bead.[32] Dr. Sarin assembled a repeating Leu-Ala-Gly-Val sequence by stepwise coupling onto a 1% crosslinked styrene-divinylbenzene support at a substitution of 0.9 mmol/gm, so that the final product was 80% peptide. Swelling measurements in CH_2Cl_2 and DMF showed that the starting dry resin increased in volume by factors of 6.2 and 3.3 respectively. The interesting numbers, which have not been reported before, are the following: at the end of the synthesis the dry volume had increased to 4.5 times the original volume of the unsubstituted beads; in CH_2Cl_2 the swollen volume reached 10.5 units and in DMF it was 24! Thus, the maximum expansion of the polystyrene network had by no means been reached when the unsubstituted bead was fully swollen. Depending on the model and the assumptions that are made, it can be estimated that the fully extended 1% crosslinked polystyrene network can increase in volume by more than 100 times. If that is true, then we are far from having reached the ultimate capacity of the resins as supports for solid phase peptide synthesis. It is clear that both the polystyrene and the peptide become solvated and contribute to the swelling of the bead. The relative contributions will depend on the hydrophobicity of the peptide. It should be pointed out, however, that we are not talking about highly polar free peptides, but about relatively nonpolar, fully protected peptides which are not grossly different from polystyrene itself. That, of course, is why this support has worked so well in the first place.

I think some of the data presented here represent real improvements in solid phase synthesis, and together with the results presented by others at this meeting they encourage me to believe that the effective general methods we are all seeking will eventually be found.

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AFFINITY AND CARRIER-MEDIATED PEPTIDE PURIFICATION

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Introduction

Many reviews have been published on protein sequence analysis[1,2]. In these reviews methods are given for the cleavage of a protein into overlapping peptides and for the partial sequencing of the amino acids in each peptide using amino acid analyses[3] and a degradation procedure developed by Edman[4]. Until recently hardly any attention was paid to the problem of isolation of the cleaved peptides. Most of the authors state that the principles and practice of peptide purification are similar to those of protein purification and suggest the use of known methods of protein isolation based on size, charge, etc. But this is not so; in enzyme purification the proteins can be followed by their biological activity, which can also be used for purification of the proteins by affinity chromatography, while isolated peptides from a protein digest are usually monitored by ultraviolet absorption. Therefore it seems that the peptide purification step, which today is completely empirical, is the rate-limiting step in the whole process of protein sequencing.

A powerful method of peptide isolation involves the labeling of the protein at specific residues with a suitable group, before or after degradation. The group can be colored or radioactive. Antibodies to the introduced group, bound to a carrier, will enable the isolation of the labeled peptides in one step using biological recognition affinity chromatography[5]. The color or radioactivity is required mainly as a double check or when a very small amount of peptide is being isolated.

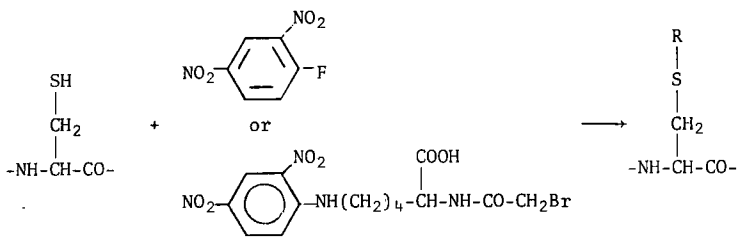
Reversible polymeric reagents which label specific residues can also be used to facilitate peptide isolation[6]. The quantity of protein required for such an operation is much higher. Avidin columns can be used for the same purpose, provided that the biotin is attached to specific residues on the protein[7]. In contrast to the classical methods of peptide isolation, which are based on size and charge discrimination, the methods des-

cribed here are based on the amino acid constitution of the peptides. Their purification is achieved by biological recognition and specific chemical reactions[5].

Isolation of Modified Peptides by Affinity Chromatography on Antibody Columns

In this section we shall describe the use of immobilized antibodies to dinitrophenyl (DNP) groups, or myeloma protein MOPC 315 which binds DNP[5], for the isolation of peptides to which a DNP group had been covalently bound to different residues by various means. We use the DNP group only as a model, since it has a high molar extinction coefficient and can be easily monitored, but the method is not limited to DNP. Any other group, including fluorescence or radioactivity can be introduced to obtain higher sensitivity.

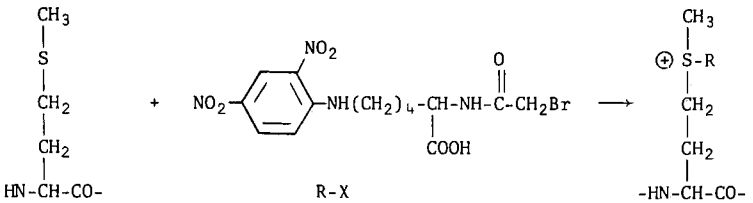
Cysteine Peptides — Several DNP derivatives were checked for their ability to modify cysteinyl residues selectively. The best reagents were *N*-bromoacetyl-N$^{\epsilon}$-DNP-lysine (BADL), fluorodinitrobenzene (FDNB), and dinitrobenzylchloride. The reaction with BADL was performed at pH 8.5, while with the other two reagents the pH was 6-6.5.



Glutathione, papain, and tobacco mosaic virus protein were reacted with either BADL or FDNB. The modified compounds were applied to the anti-DNP column to remove unreacted protein. The DNP-modified proteins retained on the column were eluted with acetic acid or guanidine from antibody columns or with 0.1 M NH_4OH from the MOPC column. The eluted proteins were digested with trypsin and chymotrypsin and applied again to the columns. A peptide containing the cysteine residue from papain and one from tobacco mosaic virus were isolated in pure form and in high yields. When this method was applied to proteins which have more than one SH group, before or after reduction, further steps of chromatography were required to separate them according to their molecular weight or charge.

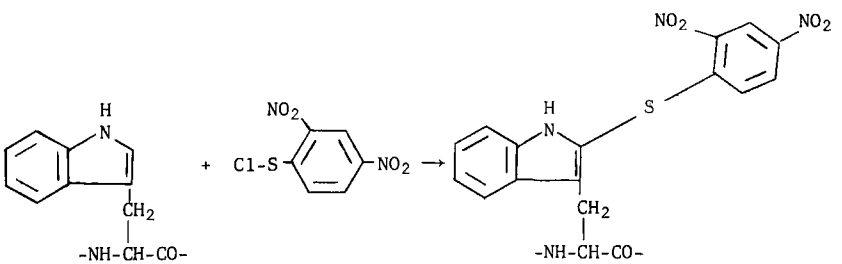
Both reagents were found satisfactory. The BADL has the advantage that it gives stable products and the cysteine can be identified as carboxymethyl cysteine while the FDNB has the advantage that the reaction is reversible with mercaptoethanol and the peptide can be eluted from the column with this reagent. This advantage lost its importance, however, when we worked with MOPC columns, since all peptides were eluted with dilute ammonia.

Methionine — Methionine-containing peptides and proteins were reacted with BADL in 30% acetic at 55°C for 4 hours or at room temperature for 24 hours. The reacted peptides and proteins were applied



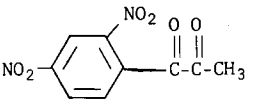
to the antibody columns to separate the modified peptides and proteins from the unmodified. The modified proteins were digested with proteolytic enzymes and the methionine-containing peptides were adsorbed to the column and eluted with ammonia, formic acid or acetic acid, depending on which column was used. The peptides could also be eluted with mercaptoethanol, and in this case the peptides were isolated without the DNP group which remained on the column. Methionine-containing peptides from ribonuclease and lysozyme were isolated by this proce--dure. In combination with CNBr, overlapping peptides were isolated.

Tryptophan — Tryptophan-containing peptides and proteins were reacted with dinitrophenylsulfenylchloride in 30% acetic acid for 1 hour at room temperature[8]. The DNP-modified tryptophan peptides from



human serum albumin and cytochrome C were isolated on the antiDNP-antibody column. This procedure was also used to isolate overlapping tryptophan peptides by modification of the tryptophan with dinitrophenylsulfenylchloride before and after chymotryptic cleavage of the proteins.

Arginine — The work with arginine is still in progress. The guanidine group of arginine-containing peptides is modified with nitromalondialdehyde or dinitrophenylglyoxal. The modified peptides are adsorbed on the antibody columns.



Aspartic and Glutamic Acid — Two methods are being used for the modification of carboxyl groups. One involves the reaction of the carboxyl groups with dinitrophenylethylamine and water-soluble carbodiimide; the other method is the conversion of the acids to hydroxamates followed by their reaction with FDNB.

Other Amino Acids — The isolation of tyrosine[9], lysine[10] and histidine-containing peptides using this approach have been reported. Many advantages are evident in applying affinity chromatography to peptide isolation. The chromatographic pattern of each peptide type would be much less complicated than that of the entire mixture, and isolation of each peptide would be facilitated. Categorization of peptides according to their amino acid content implies that high molecular weight proteins would be more conducive to study.

Purification of Synthetic Peptides

Purification of large peptides prepared by the stepwise solid-phase method[11] is very difficult to achieve, due to the small differences in the physical and chemical properties of peptides with the wrong sequences. In recent years several methods for the purification of synthetic peptides have been developed. These methods are based on the introduction of charge groups[12] or on specific chemical reactions with polymeric reagents[13] at the end of the synthesis. Another approach is to block free peptide chains, remaining after each coupling step, with anhydrides containing strongly acidic groups[14]. The acidic properties of the resulting peptides make possible their removal at the end of the synthesis.

In our studies we have used both these approaches with antibodies. We block each step during the synthesis with one reagent, and at the end of the reaction we reversibly block the N-terminal group with another reagent. Antibodies to the groups introduced during the synthesis bind and remove the shorter peptides, while antibodies to the reversible groups enable us to catch the required peptides. After each coupling step of the peptide synthesis the product was reacted with trinitrobenzenesulphonic acid or FDNB. At the end of the reaction we introduced biotinylmethionine, as suggested by Krieger *et al*[13]. After deprotection and removal from the support, the peptides were applied to an antiDNP-antibody column and all the peptides containing DNP or trinitrobenzenesulphonic acid were adsorbed. The unadsorbed peptides were collected and passed through an avidin column. The required peptide was adsorbed to the avidin column and could be eluted with guanidine. The final step is the CNBr cleavage[13]. This approach was checked with several model peptides with encouraging results. One of the problems we encountered was the conditions required to remove the biotinyl peptides from the avidin column and, of course, the methionines in the peptide chain. We are therefore adopting the 9(2-sulfo)-fluorenylmethyloxycarbonyl group (SUFMOC)[15] for this purpose and are preparing antibodies against this group. Using this approach we hope to progress in our continuous efforts to chemically synthesize an antibody[16].

The Avidin-Biotin Complex

The high affinity constant between the glycoprotein avidin and the vitamin biotin renders this system an extremely effective tool in protein chemistry. This system represents a complementary approach for antibodies and lectins in biological interactions which exploits the specific binding between a protein and a ligand. It has been shown[17] that biotinyl peptides from transcarboxylases are adsorbed to an avidin-Sepharose column and can be eluted only with 6M guanidine hydrochloride at pH 1.5 and in low yields[18]. This study shows that, in principle, the avidin-biotin complex can even be used as a tool for purification of systems in which the biotin is not a native component. We have prepared a selection of biotinyl derivatives which can be covalently bound to a variety of functional groups in proteins (Figure 1), similarly to the DNP-containing derivatives. Peptides containing the biotinyl group were adsorbed to the avidin column and were eluted with 6M guanidine hydrochloride. Even though this system is useful in some cases, it is not recommended due to

the drastic conditions required for elution and the extra step of removal of guanidine from the isolated peptides.

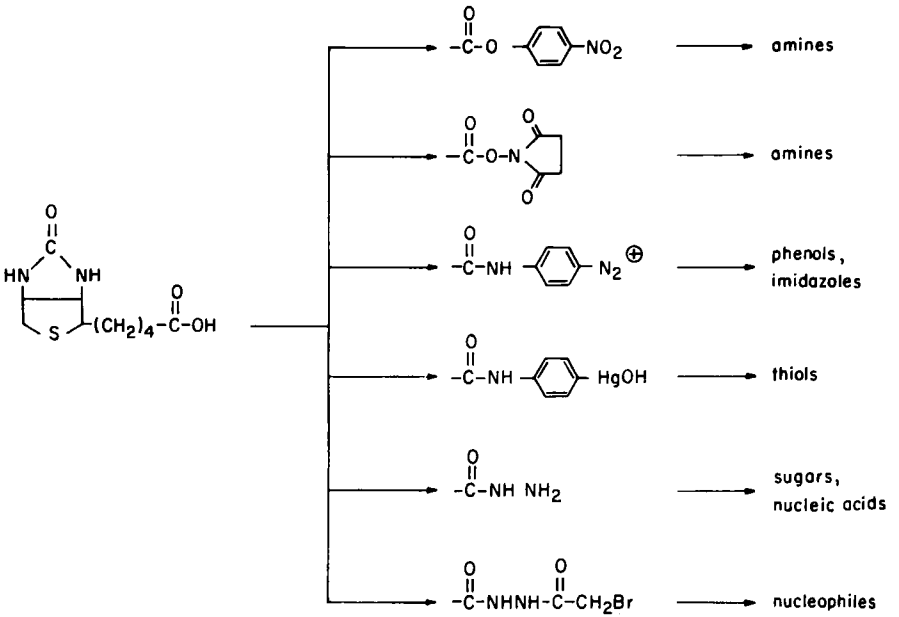


Fig. 1. Biotinyl derivatives for coupling to proteins.

Carrier-Mediated Isolation of Peptides

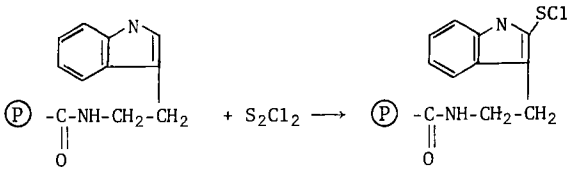
Polymeric compounds containing functional groups similar to the DNP reagents described above, and binding reversibly to proteins, were used to selectively fish out peptides from the mixture of peptides in a protein digest. This kind of reaction is termed "covalent affinity chromatography". The isolation of peptides with reactive polymers has an advantage whenever unlimited amounts of peptides are available, since they have high capacities of binding. On the other hand, it is not useful whenever a small amount of peptide has to be purified since there is no label through which the peptide can be identified and followed. So far, the carrier-mediated peptide isolation has been used for the purification of peptides containing cysteine[6], methionine[19], tryptophan[20] and tyrosine[21]. The purification of synthetic peptides using the cysteine-specific column has also been described[13]. It was also used to discriminate between exposed and buried tyrosine residues in proteins[21].

The following is a brief description of the different columns that were used:

i) Cysteine-containing peptides were isolated on columns containing mercury[13] or S-S-bridges[6]. The peptides were removed from the column with a reducing agent such as mercaptoethanol. By this method different cysteine peptides from parvalbumin and mercaptalbumin were isolated. The method is fast and very specific.

ii) Methionine-containing peptides were isolated on polymers containing chloroacetyl groups, P -$NHCOCH_2Cl$ in the presence of sodium iodide and under acidic conditions for 12 days at 37° C. The peptides were liberated from the column with mercaptoethanol[19]. This method was applied to model compounds as well as to lysozyme. The sodium iodide was used to convert the chloroacetyl to iodoacetyl groups. We have worked for many years with bromoacetyl and iodoacetyl polymers for the same purpose. These include iodoacetyl-polylysyl-Sepharose and iodoacetamidoethyl-polyacrylamide. The best conditions for reaction were 30% acetic acid for 24 hours at 37° or for 70 hours at 24° C. Small peptides such as Gly-Met-Gly were quantitatively bound but high molecular weight peptides were not always adsorbed. We could not bind glucagon and only one methionine peptide containing residues 6-13 from carboxymethyl-lysozyme was bound. We encountered many problems during the removal of the peptides from the column whether with mercaptoethanol or by boiling in water for 2 hours. We attribute our difficulties to the excess of iodide present.

Tryptophan-containing peptides were isolated on polymers with the following structure P -Aryl-S_xCl[20]. The peptides were removed from the column with mercaptoethanol resulting in thiotryptophan peptides[8]. The tryptophan peptide from human serum albumin and from glucagon were isolated. Due to the hydrophobic nature of these polymers, nonspecific adsorption of peptides was observed; but these peptides could be removed by different washings since they were not covalently bound. We have worked with polymers based on dinitrophenylsulfenylchloride and tryptamine coupled to agarose. These polymers were prepared by coupling tryptamine to aragose or to polyacrylamide. The indole ring was reacted with either dinitrophenylsulfenylchloride followed by mercaptoethanol and sulfur monochloride (S_2Cl_2), or directly with S_2Cl_2. The structure of the polymers is as follows:



We used these polymers for the isolation of model tryptophan peptides.

Tyrosine-containing peptides were isolated on a column containing diazonium salts P -Aryl-N_2^{+}[21]. Agarose columns containing diazonium salts were able to quantitatively catch model tyrosine peptides, as well as enkephalin and all the tyrosine peptides obtained from ribonuclease after tryptic digestion. The same polymer was also used to identify exposed tyrosines in RNAse and chymotrypsin. We have shown that RNAse binds to these columns via Tyr 73, 76 and 115, while chymotrypsin is bound through Tyr 146 and 228. The peptides were removed from the column with $Na_2S_2O_4$. It is interesting to note that the bound proteins on the column could be cleaved by enzymes in order to get the desired peptides.

Conclusions

We have discussed here three different methods for the selective isolation of peptides. Each method has its advantages and pitfalls. We anticipate increased applications of affinity chromatography to peptide purification using antibodies, since specific antibodies can be raised to any hapten introduced into specific sites on the protein.

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MASS SPECTROMETRIC STUDIES OF PEPTIDE STRUCTURES

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In the past five years our laboratory has developed a generalized procedure for the study of the structures of peptides employing high resolution mass spectrometric techniques, especially field desorption mass spectrometry (HRFDMS) and gas chromatography/electron impact mass spectrometry (GC/HREIMS). The elements of this generalized procedure can be summarized as follows:

General Procedure

1. Total hydrolysis of the antibiotic to amino acids, identified by

   a. HRFDMS,
   b. derivatization and HREIMS, and
   c. derivatization and GC retention times on a chiral column

2. Quantitation of the amino acids by GC and amino acid analyzer

3. Assignment of a molecular formula to the antibiotic by molar ratios of amino acids and by HRFDMS on the molecular ion

4. Assignment of a partial sequence to the antibiotic from fragment peaks in its HREI mass spectrum

5. Partial hydrolysis of the antibiotic to oligopeptides, identified by

   a. derivatization and GC/HREIMS and GC/FIMS, and
   b. HRFDMS

6. Overlapping of the oligopeptides, combined with HREIMS on the intact antibiotic, to assign the structure.

Peptaibophol Antibiotics

This procedure was developed in the course of our study of the structure of antiamoebin I[1], one of the peptaibophol antibiotics, defined as antibiotics containing several α-aminoisobutyric acid (Aib) residues and a C-terminal phenylalaninol unit (Phol). Once developed, the procedure was then applied to other members of the peptaibophols—antiamoebin II[2], emerimicins III and IV[3], and alamethicins I and II[4]. Structures of these members of the peptaibophol class of antibiotics are shown in Figure 1.

```
          1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16  17  18  19
1:  Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-α-Gln-Phol

2:  Ac-Aib-Pro-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-α-Gln-Phol

          1   2   3   4   5   6   7   8   9  10  11  12  13  14  15
3:  Ac-Phe-Aib-Aib-Aib-Iva-Gly-Leu-Aib-Aib-Hyp-Gln-Iva-Hyp-Aib-Pro-Phol

4:  Ac-Phe-Aib-Aib-Aib-Iva-Gly-Leu-Aib-Aib-Hyp-Gln-Iva-Pro-Aib-Pro-Phol

          1   2   3   4   5   6   7   8   9  10  11  12  13  14
5:  Ac-Phe-Aib-Aib-Aib-Val-Gly-Leu-Aib-Aib-Hyp-Gln-Iva-Hyp-Ala-Phol

6:  Ac-Phe-Aib-Aib-Aib-Val-Gly-Leu-Aib-Aib-Hyp-Gln-Iva-Hyp-Aib-Phol
```

Fig. 1. Structures of peptaibophol antibiotics. 1, alamethicin I; 2, alamethicin II; 3, antiamoebin I; 4, antiamoebin II; 5, emerimicin III; 6, emerimicin IV. All optically active amino acids and phenylalaninol units have the *L* configuration. Aib, α-aminoisobutyric acid; Iva, isovaline (α-ethylalanine); Phol, phenylalaninol; Ac, acetate.

Antiamoebin and the other members of the class were chosen initially for study since it would be difficult to assign their structures employing the classical techniques for peptide structure elucidation due to the N-terminal acetyl group, the C-terminal phenylalaninol group, and the large number of α-alkyl amino acids present in the peptide chain. Thus, we regard the mass spectrometric procedure as a complementary technique, particularly useful for derivatized peptides, peptides containing unusual amino acids, and other specialized cases.

The general procedure requires modification or augmentation in individual studies and the specialized techniques employed or developed during the course of our studies of peptaibophol antibiotics include the dehydration of a peptide-bound glutamine and reduction of the resulting nitrile to a peptide-bound ornithine, and the related conversion of a peptide-bound glutamic acid to its methyl ester and the latter's reduction to a peptide-bound 2-amino-4-hydroxypentanoic acid, also identified by GC/MS[4].

Another ancillary technique developed for the peptaibophols and other antibiotics containing Aib (or Iva)-Pro (or Hyp) units is the selective cleavage of such links in trifluoroacetic acid[1], which with antiamoebin I gives the four peptides Ac-Phe-(Aib)$_3$-Iva-Gly-Leu-Aib, Hyp-Gln-Iva, Hyp-Aib and Pro-Phol.

Following our initial reports on the mass spectrometric assignment of structures to peptaibophol antibiotics, we have directed our efforts both toward improvements in the mass spectrometric techniques and toward applications of the techniques to different classes of peptides. We shall deal first with the improvements in the mass spectrometric procedures.

Improvements in Field Desorption Mass Spectrometry

It is obvious from a cursory examination of the general procedure described above that FDMS has played a central role in our studies of peptides by mass spectrometry. Thus, it seems appropriate to review briefly here the general characteristics of the field desorption technique[5]. In the FD technique a sample is deposited in solution on a specially prepared emitter wire, either by dipping the emitter wire in the solution or by adding one or more drops of the solution to the emitter wire *via* a microsyringe (freezeloading)[6]. Solvent is then evaporated and the emitter is inserted into the ion source of the mass spectrometer, where a high field (*ca.* 12,000 volts) is applied to the wire. Ions are desorbed from the emitter and focused as usual in the mass spectrometer. The emitter wire may be heated as necessary by passing an electric current through it.

Field desorption ionization is a gentle technique, giving mainly molecular ions, although fragment ions can be observed when the emitters are heated. It is an excellent technique for qualitative detection of relatively non-volatile or unstable compounds and has been used for quantitation of isotopically labeled materials and rough quantitation of mixtures of related compounds, including antibiotics, though it does not successfully quantitate mixtures of compounds of differing polarity. It is often a sensitive technique also, giving repetitive spectra with as little as 40 nanograms of neomycin, for example[6]. The measurement of compounds at high masses has proven successful both at low resolution and high resolution following development of suitable mass marking substances, the hexakis (polyfluoroalkoxy)phosphazenes, which give good molecular ions by field ionization and do not desensitize the ion source[7]. Similarly, the technique of cation replacement was developed in order to

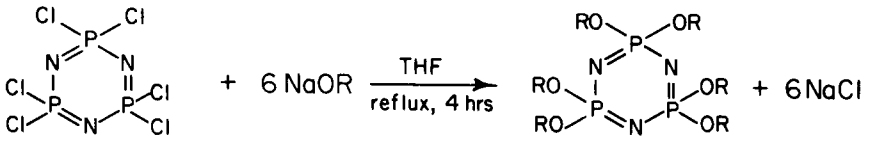
distinguish molecular ions from M + Na ions in spectra of high molecular weight compounds[8]. The upper mass limit of field desorption appears to be, as with other mass spectrometric techniques, a function of the volatility (and polarity) of a compound and of the upper mass range of the particular mass spectrometer employed (which for newer Varian MAT 731 spectrometers is approximately 1800 amu) at maximum accelerating potential (and greatest sensitivity), and for the Kratos MS 50 with an extra large magnet is approximately 2800 amu at maximum accelerating potential.

A recent modification of FDMS developed in an attempt to enhance the polarity range of the compounds amenable to study is laser-assisted field desorption mass spectrometry, first reported by Schulten, *et al.*,[9] and subsequently investigated in our own laboratory. The German workers reported that they were able to obtain by laser-assisted FDMS a spectrum of vitamin B_{12}, a compound whose spectrum they were unable to obtain with FDMS alone. Employing the same type of laser as that used by Schulten, we find that laser-assisted field desorption does indeed give somewhat better spectra for compounds at the borderline of detection (for example, saxitoxin and streptomycin), but in the limited experiments thus far we have not observed dramatic improvement in the range of applicability of FDMS through laser assistance. Nevertheless, the technique is still exceedingly promising, since numerous parameters can be varied. We are, for example, studying a variety of laser sources and feel that a pulsed laser should have greater utility than the previously employed continuous lasers. We also are optimistic that a laser with absorption in the infrared should be helpful. Since many of the compounds studied thus far lack absorption at the ultraviolet wavelength of the argon laser, it is difficult to see how an argon laser (previously employed by our group and that of Schulten) can be effective other than by black body absorption by the emitter with consequent indirect heating of the adsorbed material.

A second improvement in FDMS that we are currently investigating involves the phosphazenes developed for mass markers as described above[7]. These have been used in the past for peak matching individual ions in the mass spectrometer, but for complete high resolution and low resolution spectra this is a laborious procedure. Computerized techniques would be far superior, but to be successful the necessary programs must be written employing phosphazenes instead of perfluoroalkanes, and requisite mixtures of phosphazenes must be synthesized with adequate ion intensities over a broad range. The latter goal has been

achieved, as seen in the accompanying spectrum (Figure 2) of a blend of four mixtures of phosphazenes prepared as shown in the equation below from mixtures of alkoxides. Adequate ion intensity is observed over the mass range 700 to 2000 and this blend of phosphazenes would be suitable in that range for measuring either the complete spectrum of one compound or molecular ions of the components of a mixture.

SYNTHESIS OF MULTIFLUOROALKOXYCYCLOTRIPHOSPHAZENES



$$R = -CH_2(CF_2)_nF$$

or

$$-CH_2(CF_2)_nH$$

An outgrowth of this investigation is the recent observation that the same phosphazenes are useful as mass markers in the chemical ionization (CI) mode, and we anticipate using them, either by peak matching or with computer techniques, for high resolution CIMS.

Applications to Peptide Antibiotics

Peptaibophols — Structure assignments of the peptaibophol antibiotics, in particular of alamethicin, have been accompanied by synthesis of the assigned structure of alamethicin[10,11]. However, the synthesized material has had neither the complete pore-forming activity nor the complete antibacterial activity of native alamethicin[10,11]. In a study of the cause of this discrepancy, both Dr. Marshall's group and our group have reinvestigated the components of alamethicin by high pressure liquid chromatography (HPLC). An HPLC curve for alamethicin is shown in Figure 3, and HPLC traces for antiamoebins I[1] and II[2], and emerimicins III and IV[3] and the previously unstudied emerimicin II[12] in Figure 4. Similar curves were obtained for zervamicins I and II[13]. The emerimicins

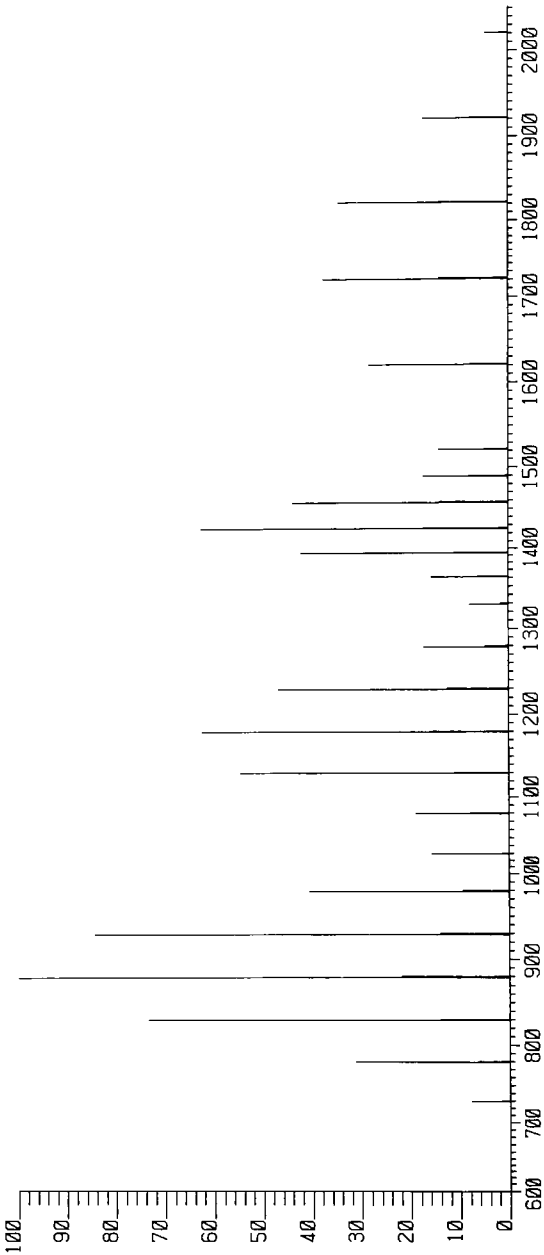


Fig. 2. Field desorption mass spectrum of a blend of 4 mixtures of hexakis(polyfluoroalkoxy)cyclotriphosphazenes prepared as mass markers.

and zervamicins are especially interesting because of the enhanced antibacterial activity of some of the components, coupled with their variable pore-forming activity.

The HPLC traces show that all of the materials previously investigated are, in fact, mixtures, as had been indicated earlier in their field desorption mass spectra. The solvent system, which does not employ a buffer, provides samples which are amenable to field desorption mass

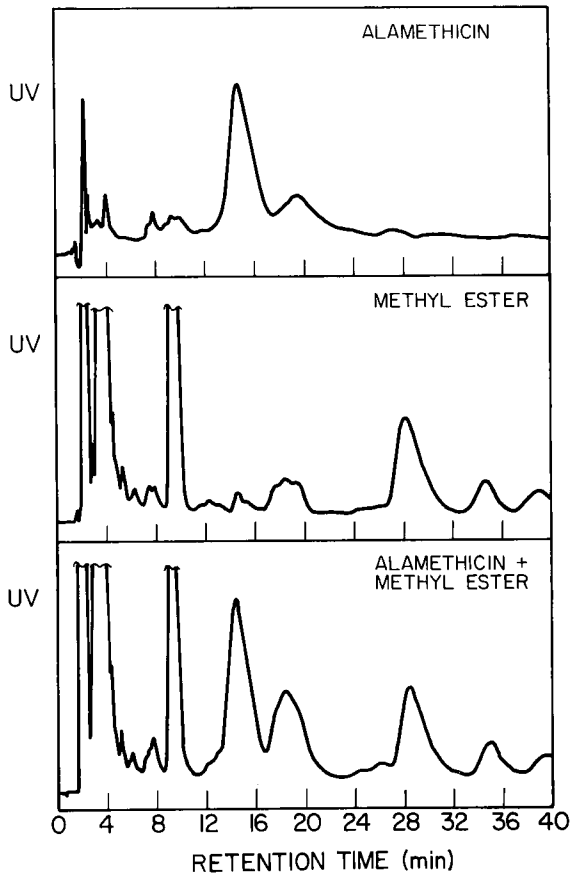


Fig. 3. HPLC curve for alamethicin and its methyl ester. Conditions: Waters HPLC, 254 nm detector, μ-Bondapak C_{18} column, MeOH-H_2O-i-PrOH (50:30:20) solvent, 1.0 ml/min flow rate, 250 μg sample in 25 μl.

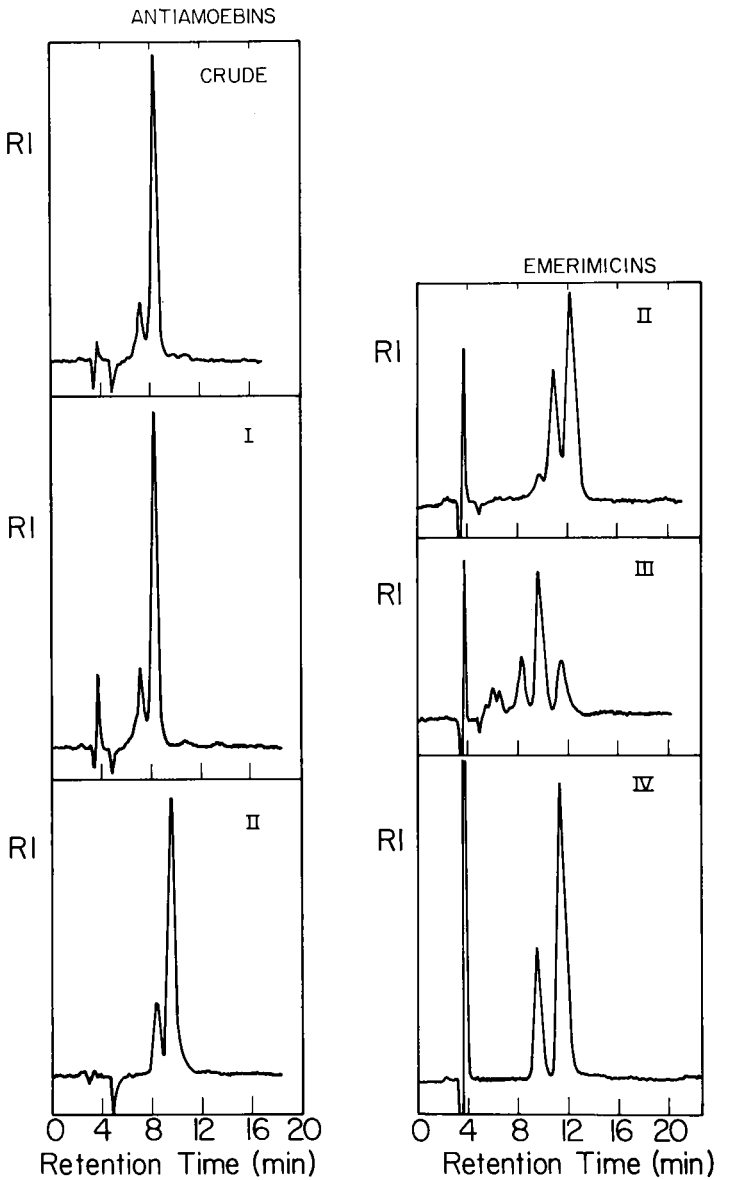


Fig. 4. HPLC curves for antiamoebins I and II, and for emerimicins II, III and IV. Conditions: same as Figure 3, except refractive index detector, 0.8 ml/min flow rate, 140-250 μg sample in 14-25 μl.

spectrometric investigation. The major component of antiamoebin I has the molecular formula previously assigned (M = 1669) and the minor component (antiamoebin III) is a homolog (M = 1655), which recently has been shown to have Aib instead of Iva at position 5 (Figure 1) by interpretation of its EI mass spectrum We are presently reinvestigating all of the other separated components by FDMS and the general procedure with an eye toward studying their relative bioactivities.

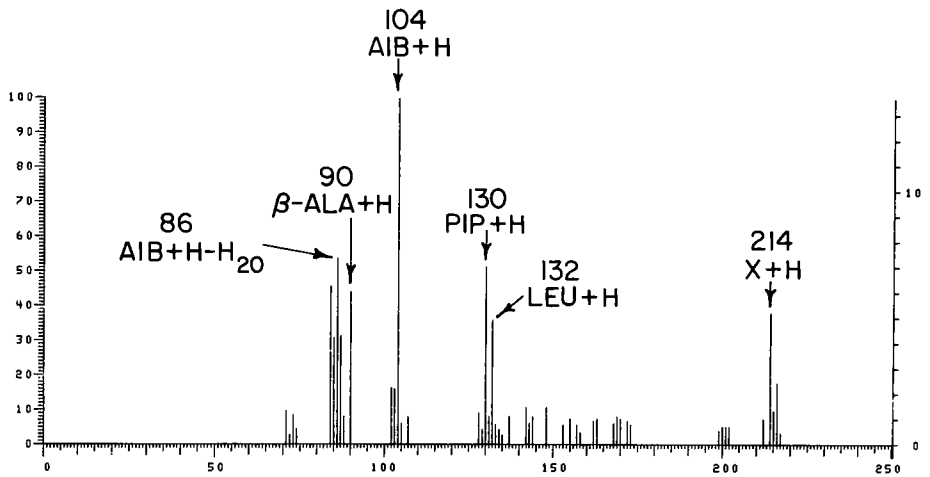


Fig. 5. Laser-assisted field desorption mass spectrum of the mixture of amino acids resulting from hydrolysis (6 *N* HCl, 110°, 18 hr) of antibiotic CC 1014.

CC 1014 — In addition to reinvestigating the peptaibophol antibiotics we are also studying the applicability of the general procedure to a number of other peptides, including other antibiotics. Among these compounds is the antibiotic CC 1014[14], which was originally isolated by Dr. P. F. Wiley in the antitumor screening program at The Upjohn Company from *Paecilomyces abruptus* nov. sp. NRRL 11110 and which inhibits L1210 cells *in vitro* (ID_{90} 0.08 μg/ml) as well as P388 tumors *in vivo*. It seemed of interest to see how far the general procedure could go in assigning the structure of CC 1014 by mass spectrometry alone.

Hydrolysis of CC 1014 gives a mixture of amino acids with the laser-assisted FD mass spectrum shown in Figure 5. The M + H ions at *m/e* 90, 104, 130, 132, and 214 have been identified by GC/EIMS (Figure 6) of the derivatized mixture as those of β-alanine, α-aminoisobutyric acid, pipecolic acid, leucine and a novel C_{11} amino acid.

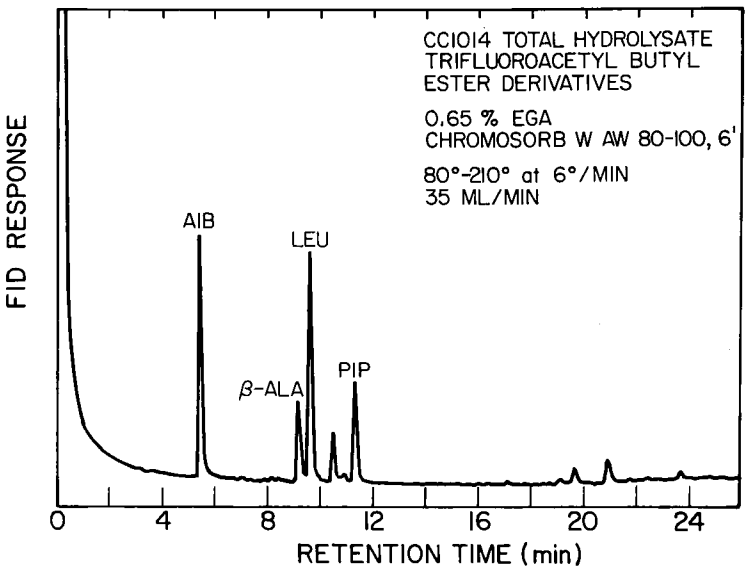
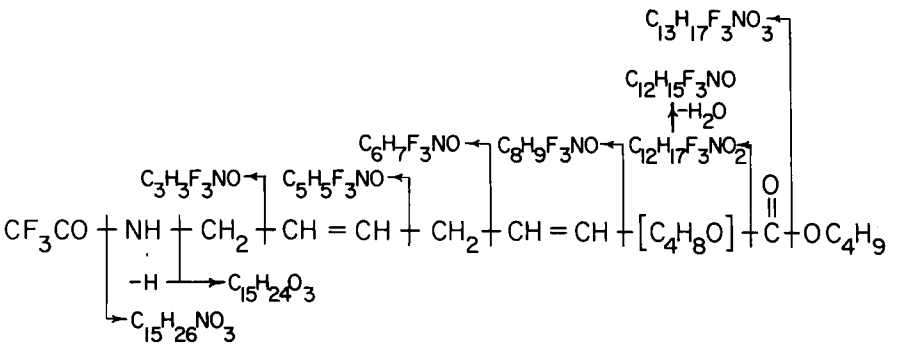


Fig. 6. Gas chromatogram of a derivatized mixture of the amino acids whose FD mass spectrum is found in Figure 5. The GC peaks with retention times 19.6, 20.9 and 23.7 min all give the same mass spectrum for the C_{11} amino acid.

The molecular formula of the C_{11} amino acid was established as $C_{11}H_{19}NO_3$ by HRFDMS (214.1429, $C_{11}H_{20}NO_3$, M + H), which agrees with high resolution data obtained on the N-trifluoroacetyl butyl ester by GC/HREIMS and with the molecular ion (*m/e* 366, M + H) in the GC/CI mass spectrum (isobutane reagent gas). The partial structure shown below, with fragmentation, was also assigned on the basis of GC/HREIMS data. The C_4H_8O unit could be located at any of several positions in the chain.



Quantitation of the GC trace and conventional amino acid analysis gave conflicting molar ratios of the amino acids (Aib:Leu:β-Ala:Pip : 4:3:1:1 *vs.* 6:2:1:4, respectively), making a molecular formula based on these analyses meaningless. However, the molecular weight established by the FD mass spectrum of CC 1014 (Figure 7), its methyl ester and acetate was 1217 and the molecular formula suggested by high resolution FD data for intact CC 1014 was $C_{62}H_{112}N_{11}O_{13}$ (1218.8497, M + H).

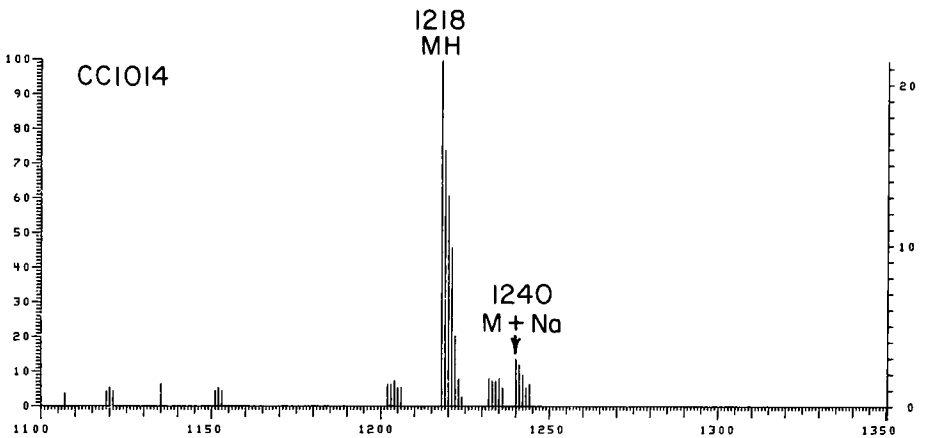


Fig. 7. Field desorption mass spectrum of antibiotic CC 1014.

Sequencing of CC 1014 was also carried out according to the general procedure. The high resolution electron impact mass spectrum of intact CC 1014 gave measurable ions up to *m/e* 1199 (M - H_2O), an ion whose composition (1199.8297, $C_{62}H_{109}N_{11}O_{12}$) agreed with that of CC 1014 assigned by HRFDMS. The partial sequence assigned from the high resolution electron impact spectrum is Aib-Leu-Leu-Aib-Aib, shown above the low resolution spectrum (Figure 8).

In addition, CC 1014 was partially hydrolyzed to a mixture of oligopeptides. Derivatization of the mixture and GC/MS study of the derivatives identified the peptides Aib-Leu, Leu-Aib, Leu-Leu-Aib-Aib, and Aib-Aib, which confirm the partial structure assigned from the high resolution electron impact spectrum on the intact antibiotic.

While a complete structure of antibiotic CC 1014 cannot be assigned by mass spectrometry alone, the general procedure developed is clearly of value in study of this antibiotic and compounds like it.

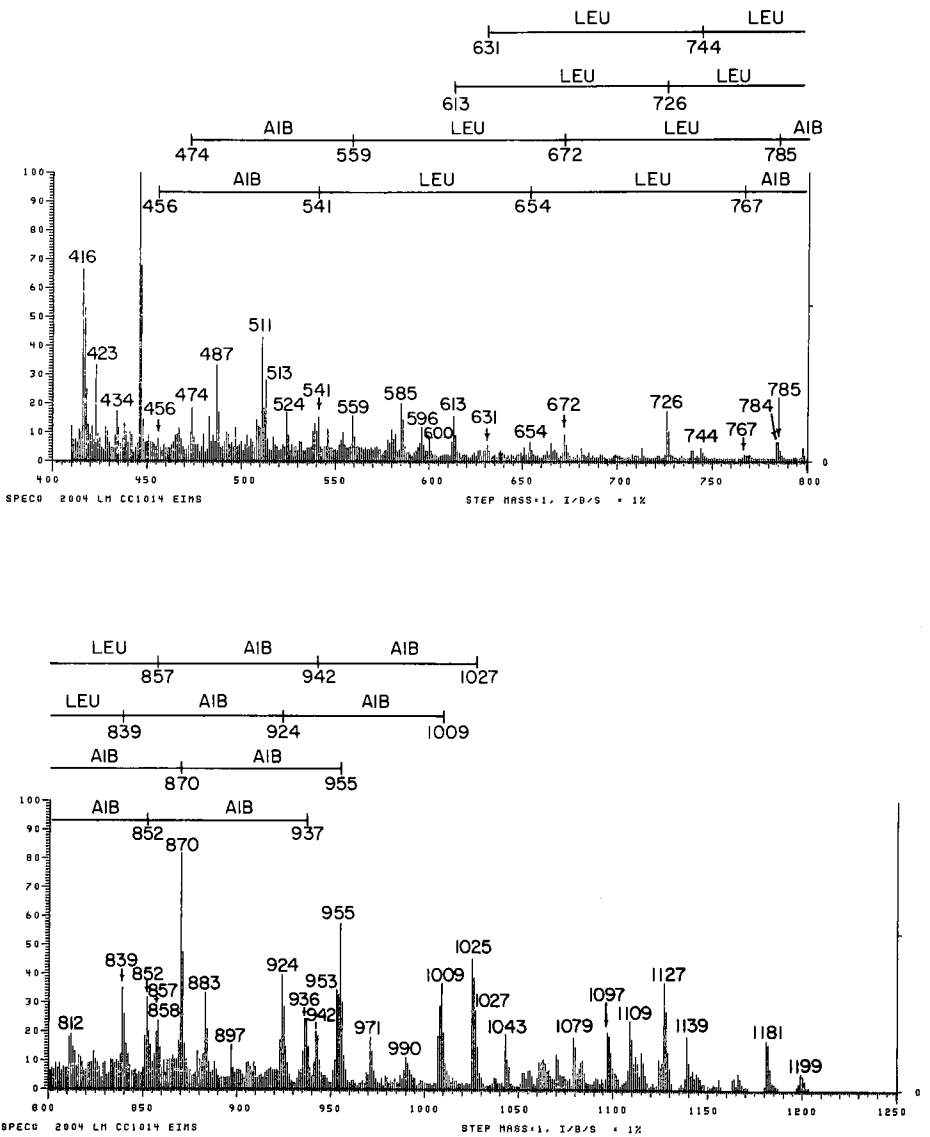


Fig. 8. Electron impact mass spectrum of antibiotic CC 1014. The partial sequence shown above the spectrum is based on high resolution measurement of the ions indicated.

Acknowledgments

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ULTRAMICRO TECHNIQUES FOR THE ISOLATION AND ANALYSIS OF PEPTIDES AND PROTEINS

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High performance liquid chromatography (HPLC) has been used in combination with a bioassay, radioimmunoassay or radioreceptor assay for the identification and quantitation of specific peptides, as well as for their isolation in pure form. A sensitive fluorometric detection system for amino acids, peptides and proteins has enabled the chemical analysis of small quantities of these purified peptides. The advantage of HPLC over conventional chromatography is that the individual components remain in narrow bands while eluting from the column. Therefore, components with minor structural differences may be completely separated in one or two chromatographic runs. Column effluents are monitored with the reagent fluorescamine, which reacts with primary amines to form highly fluorescent adducts[1]. Fluorescamine is ideally suited for analysis of the effluent of HPLC columns, since the reaction is completed within seconds, thereby minimizing post-column band spreading. Since fluorescamine and its hydrolysis by-products are nonfluorescent, the reagent may be added in a continuous fashion to the column effluent. However, in order to further utilize the peptide it is necessary to stream split[2] so that one portion of the column effluent is reacted with fluorescamine, while the remainder goes to a fraction collector. For purely analytical applications, such as amino acid analysis, all of the column effluent is reacted with fluorescamine[3].

The limit of detection with the current instrumentation is 10-100 pmol. It is, therefore, possible to carry out the isolation of a peptide or protein on the nanomole scale and use only a small percentage of the peptide for column monitoring. Detection of peptide fragments, generated as a first step in sequence analysis, and amino acid analysis are both possible at the picomole level. However, the main limiting factors so far have been amine contamination in the buffers and solvents, as well as the spurious loss of peptide encountered at these low levels. The steps taken to deal with these problems will be discussed later. HPLC has also been

used with sensitive ultraviolet absorption at short wave lengths (200-215 nm)[4,5], but this technique is inherently non-specific. The highly specific fluorometric method is more versatile and practical.

Isolation and Analysis of Opioid Peptides

Biologically active peptides and proteins are usually present at low concentrations and investigators have required tissues from a great many large animals for their isolations. The sensitivity of the present methodology allows the isolation of active peptides from laboratory animals, which are the species used for physiological and pharmacological studies. This is important, since slight differences in the primary structure of the same active peptide may occur among species and these same differences can have profound effects on radioimmunoassays or bioassays. For this reason, we have isolated and characterized the opioid peptides from the rat.

The first two opioid peptides were isolated by Hughes and coworkers[6] from pig brain. These peptides, which mimic morphine in two different bioassays, have the structures Tyr-Gly-Gly-Phe-Met (Met-enkephalin) and Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin). Larger peptides containing the Met-enkephalin sequence within their structures were subsequently isolated from pituitary tissues[7,8]. With the aid of a radioreceptor assay for opiates, we isolated 2 nmol of rat β-lipotropin (10,000 daltons) from 40 anterior pituitaries[9]. We determined that the amino acid composition of rat β-lipotropin differs appreciably from those of human and ovine β-lipotropins. Rat β-endorphin (3,500 daltons) as isolated from 200 rat pituitaries with a yield of 6 nmol[10]. Its amino acid composition was found to be the same as ovine and camel β-endorphin. Comparative tryptic peptide mapping using HPLC with fluorescent detection demonstrated that the sequences of rat and camel (and ovine) β-endorphin are identical[10].

During these studies on the rat pituitary, we detected a protein (ca. 30,000 daltons) that appeared to be the precursor of β-lipotropin[9,11]. Chemical analysis was used to establish this point in the following manner. When β-lipotropin (or β-, γ-, or α-endorphin) is digested with trypsin, only one of the fragments, β-lipotropin 61-69, contains the enkephalin sequence and is active in the radioreceptor assay. Trypsin digestion of the partially purified protein from the rat pituitary yielded only one active peptide fragment which eluted at the same position from an HPLC column as the nonapeptide β-lipotropin 61-69. We have succeeded in purifying this protein, which we named pro-opiocortin, to

homogeneity from a single camel pituitary[12] and partial chemical characterization was conducted on the pure protein.

Based on immunocytochemical observations[13], we used our methodology to investigate the opioid peptides in the adrenal medulla. Granules were isolated from bovine adrenal medulla and lysed with a dilute acid solution. The released peptides and proteins were separated by gel filtration and by HPLC[14]. Both Met-enkephalin and Leu-enkephalin were found to be present in approximately the same concentration and relative proportions as in brain striatal tissue. However, a previously unknown series of opioid peptides and putative enkephalin precursors were discovered[14]. One peptide, approximately the size of β-endorphin, was shown not to be β-endorphin as it eluted from the HPLC column at a position differing from any of the known opioid peptides. Another large peptide, approximately the size of β-lipotropin, was digested with trypsin. The fragment possessing activity did not elute at the same position on HPLC as the nonapeptide β-lipotropin 61-69, demonstrating that the peptide was not β-lipotropin.

Since slaughterhouse or autopsy tissue is usually not processed until hours or days after death, the possibility of nonspecific proteolysis must be considered. This can lead to degradative loss of the physiologically active components and to artifactual generation of components with *in vitro* biological activity. When working with laboratory animals it is possible to remove the tissues of interest within minutes after sacrifice. Tissues are immediately frozen or homogenized in cold, dilute acid (pH 2) in the presence of protease inhibitors. During the work on the low molecular weight opiod peptides in rat pituitary[9], we were able to identify Met-enkephalin, β-endorphin and α-endorphin, but there was no opioid activity detectable at the elution positions of Leu-enkephalin or γ-endorphin.

In an analytical application, Met-enkephalin and Leu-enkephalin were measured in different brain regions of mice[15]. The tritiated forms of both enkephalins were obtained from commercial sources and purified by HPLC. An aliquot of each, equivalent to a few thousand counts per minute and representing a fraction of a picomole was added as an internal standard to each homogenate. The deproteinized extracts were chromatographed on the HPLC column. Elution conditions were optimized for the separation of the enkephalins[16] and samples were processed at the rate of one per hour. An aliquot of the fraction from every run corresponding to each enkephalin was taken for quantitation by the radioreceptor assay and another aliquot was taken for counting radioac-

tivity in order to determine recovery through the procedure. The HPLC provided the specificity and the radioreceptor assay provided the quantitation.

Guidelines for HPLC

Volatile solvents and buffers are used as eluants whenever possible to facilitate the recovery of sample after column chromatography. The fluorescent detection system does not allow the use of amine containing buffers. Furthermore, commercially available chemicals typically contain amine contaminants, which must be removed by distillation over ninhydrin. In order to avoid peptide loss during transfer or storage, due to adsorption to glass, it is advisable to allow solutions of the peptide to come in contact only with polypropylene tubes or pipet tips. Lyophilization of proteins is risky and near the end of an isolation samples are only concentrated *in vacuo* rather than taken to dryness. Likewise, ultrafiltration or dialysis may have to be avoided.

Tissues are homogenized and extracted under conditions inhibitory to proteolytic activity. Initial fractionation is often by size on Sephadex columns. However, for the isolation or analysis of low molecular weight peptides, removal of proteins by trichloroacetic acid precipitation or boiling is often easier. Large volumes of the sample solution can be applied to the column (provided the component of interest is not eluted by the sample diluent) without loss of resolution. Milligram amounts of peptides may be applied to a standard (25 x 0.46 cm) HPLC column without a significant loss of resolution. The effect of concentration of the sample at the top of the resin bed can be deleterious with the few peptides that tend to aggregate or precipitate. One result of this problem is a memory effect, in which only a portion of the material injected into the column elutes during the initial run and the remainder elutes at the same position in later runs. Another result is a loss of resolution.

The peptides and proteins studied in this laboratory so far have all been non-enzymatic in nature and are stable with respect to their biological activity. In fact, pro-opiocortin is digested with trypsin in order to release the peptide that is active in the radioreceptor binding assay. The HPLC of enzymes has been performed using gel permeation and ion-exchange[17]. The problems of enzyme denaturation on the reverse-phase column are now being addressed in this laboratory.

Selection of Chromatography Conditions

Reverse phase chromatography has proven to be most useful for the separation of peptides. In this technique the solute partitions between the mobile phase and a hydrophobic stationary phase bonded to a silica matrix. A gradient of increasing 1-propanol in buffer has been used for both small and large peptides. For the best resolution the column is typically run at an acidic pH by incorporating formic acid and pyridine into the eluant. By varying the pH values between 3 and 5.5 it is possible to obtain an entirely different separation pattern. The selectivity is such that a change of only 2% in the propanol content of the eluant can mean the difference between a component being strongly retarded or rapidly eluted from the column. Several resins, including the functional groups C_{18}, C_8, CN and phenyl can be used in the reverse phase mode. The selectivity of each resin is slightly different and the sequential use of two resin types with the same gradient can achieve great purification.

HPLC of proteins requires special considerations. Proteins above 40,000 daltons may be excluded from the 6 to 10 nm pores of the standard commercial resins. 50 nm pore C_8 and CN resins have been prepared in this laboratory (R. Lewis, to be published) and used for the chromatography of proteins as large as collagen chains (95,000 daltons) and tyrosinase (128,000 daltons). The use of a low eluant flow rate is also important in order to obtain the best resolution from an HPLC column. A flow rate of 15 ml/hr with a 25 x 0.46 cm column approaches the optimal efficiency of several hundred theoretical plates for the chromatography of a protein (as opposed to up to several thousand for a small peptide). High recoveries (70-90%) are typically obtained from the reverse-phase column even for the chromatography of a few micrograms of protein.

It is apparent that HPLC is becoming a standard research technique. The procedures described in this article, and given in more detail in the references from this laboratory, have been developed in accordance with the design of the fluorescent detection system. This combination allows for the isolation and characterization of peptides and proteins in a rapid fashion and on a scale requiring far less starting material than by conventional chromatographic procedures. HPLC is also a valuable technique when used in conjunction with a radioimmunoassay or bioassay for quantitative analysis, since it provides an important criterion of specificity.

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NEW DEVELOPMENTS IN ISOELECTRIC FOCUSING

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Introduction

Isoelectric focusing (IEF) is generally recognized as a powerful method for the analysis and micropreparative separation and purification of proteins and larger peptides. In this paper we wish to report on our multidisciplinary effort to develop a new system for preparative IEF potentially applicable to large scale purification of peptides. The core of the system is an apparatus based on a new recycling principle[1]. The solution to be fractionated is continuously recycled between a multichannel heat-exchange reservoir and a multichannel focusing apparatus. Fluid flow through the focusing apparatus is stabilized by a parallel array of filter elements which streamline the flow of fluid and eliminate the problems of boundary distortions commonly observed in other types of continuous flow electrophoresis instruments. Joule heat is dissipated in the heat-exchange reservoir, rather than the focusing apparatus itself. This eliminates the throughput constraints inherent in other types of electrophoresis instruments and virtually unlimited quantities of sample can be processed.

The apparatus has been complemented by automated data collection sensors which periodically monitor the pH and ultraviolet (UV) absorption in all recirculating channels. These sensors are interfaced with a Hewlett-Packard desk top computer and plotter which provide real-time data outputs.

The usefulness of preparative IEF is at present limited by the need to employ Ampholine for the establishment of stable pH gradients. This results in product contamination with this ill-defined buffer. Several attempts have been made to develop stable pH gradients using well defined buffer systems[2-4], but none has yet been quite successful. Our approach to this problem is guided by computer simulation of the isoelectric process using an explicit theory of IEF developed in our

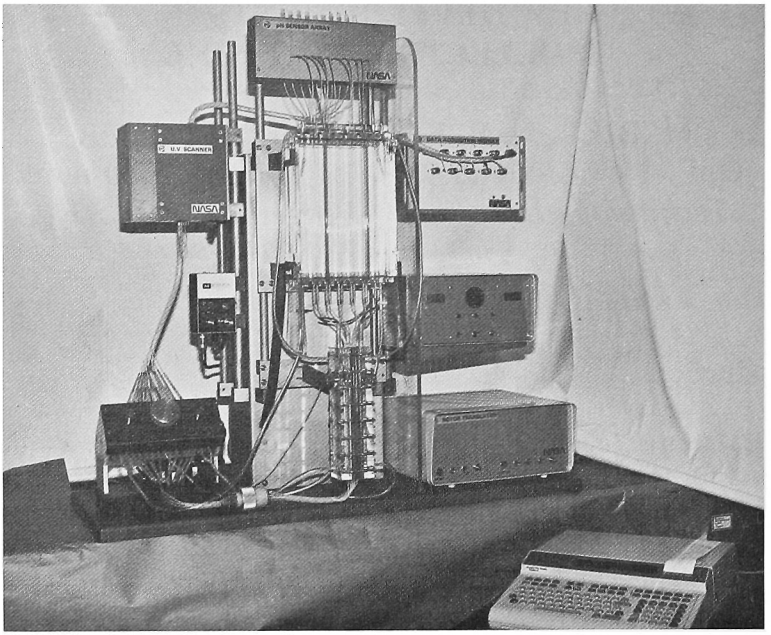

Fig. 1. Recycling isoelectric focusing apparatus (RIEF).

laboratory[5]. Careful choice of two or three ampholytes, typically amino acids or dipeptides, will generate stable gradients over a narrow pH range overlapping their isoelectric points. Several such buffer systems capable of giving useful separations are in current use.

Apparatus

A modular approach was taken in the design of the IEF system, as shown in Figure 1. The photograph illustrates a separation of two colored proteins, albumin blue stained and red hemoglobin, narrowly confined by the focusing process to two channels of the heat-exchange reservoirs. The components of the system are represented schematically in Figure 2. The system can be envisaged as being composed of two interlinked parts.

The Recycling Isoelectric Focusing Apparatus (RIEF) — comprises the essential components for the focusing process:

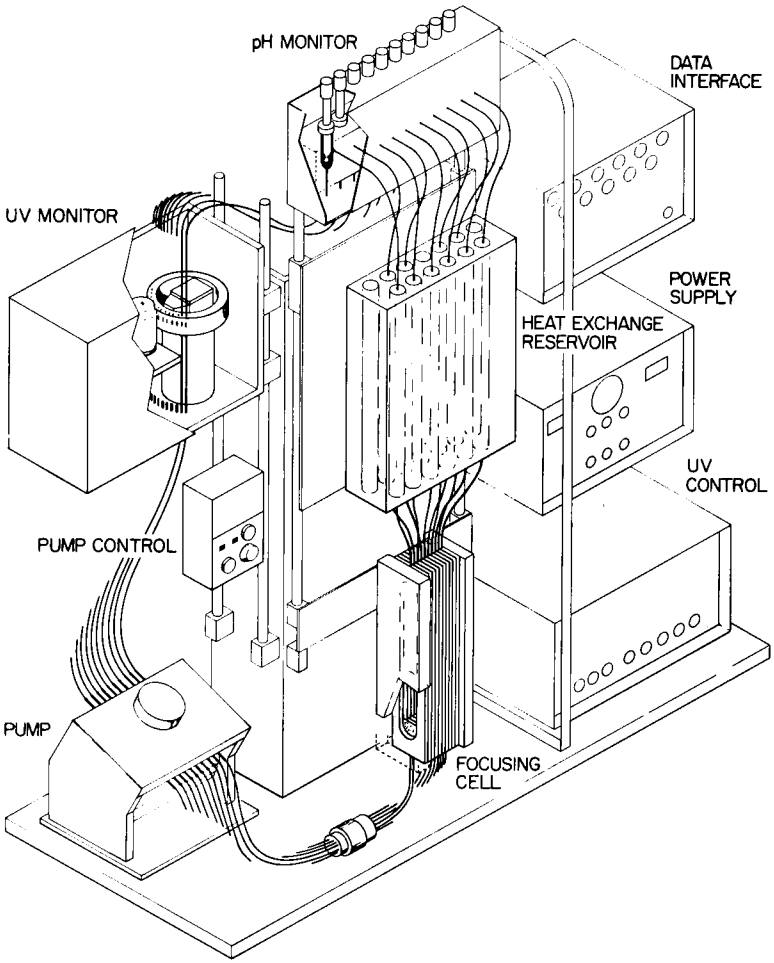

Fig. 2. Schematic presentation of the RIEF apparatus.

1. The multichannel heat-exchange reservoir is the holding container for the solution to be fractionated. It comprises 10 individual glass tubes for the sample solution, two tubes for the electrode electrolytes, and a tube for the reference solution used in the calibration of the UV monitor. Each sample tube feeds directly into a corresponding channel of the focusing cell, from where it is pumped through the UV and pH monitors and back into the same tube.

2. The focusing cell consists of a number of narrow channels separated from each other by filter elements, the purpose of which is to streamline the flow of liquid through the cell. The cell comprises two plastic end plates with recessed platinum electrodes between which any number of spacers can be assembled. These spacers have an internal cavity of 20 x 2 x 0.2 cm, coextensive with the electrodes and have means for attachment of input and output tubing. Neither the chemical nature of the filter elements nor their porosity is critical and a variety of filters are being used. In the present apparatus, we use arbitrarily an assembly of ten spacers, corresponding to the ten sample tubes in the reservoir. The spacer assembly is separated from the electrode compartments by ion-exchange membranes to avoid mixing of the sample with the anode and cathode electrolytes. The electrical transport is transversal to the downward flow of the fluid and in every pass through the cell only a small drift of solutes across the filters will suffice to gradually establish final equilibrium distribution of all components.

3. A multichannel peristaltic pump with a planetary gear drive is used for the recirculation of all fluids. The pump controller permits the regulation of the flow to about 10 ml/channel/min and the direction of flow is reversible.

The Multiplexed Electronic Data Acquisition System (MEDAS) is not an essential part of the focusing system, but was designed to facilitate data acquisition with the RIEF apparatus. It is not within the scope of this article to describe the design and construction of this part of the apparatus[1], but it will suffice to mention that it is capable of measuring periodically the UV absorption and pH in all flowing channels of the RIEF apparatus. MEDAS is under the control of a Hewlett-Packard Model 9825A desk top computer, which receives raw data from the MEDAS interface at preset time intervals, converts these raw data into optical density and pH units, performs a variety of statistical analyses, provides printouts of data in real time, and stores them on a magnetic tape for future manipulations. In addition, the computer is interfaced with a Hewlett-Packard Model 9872A x-y plotter, which is programmed to graphically display the pH, UV, current and statistical evaluation of pH and UV data in three separate plots, as is shown in Figure 3.

MEDAS can also be used for feed-back control of the separation process, as the pH gradient in the system can be manipulated by the addition or withdrawal of buffering components in the recycling appar-

atus. Withdrawal of Ampholine from either or both of the two extreme subcompartments of the RIEF apparatus will result in a progressive flattening of the pH gradient. The computer can be programmed to establish or maintain a desired pH gradient, by relay mediated control of infusion pumps. Alternatively, if the mixture contains a major component sensed by the UV monitor, it can be made to focus into a desired channel, by similar buffer manipulations.

Operation

The computer is programmed to instruct the operator in the proper sequence of operations and requests pertinent data for entry into its memory. The monitors are calibrated first with a buffer of pH 7 with no UV adsorption, followed by a buffer of pH 4 with an OD of approximately 1. The scaling factors are automatically computed and stored in the computer memory for conversion of raw monitor data into OD and pH values. Each recorded measurement is actually the average of 25 measurements taken at close time intervals (2 msec) and the complete cycle of 10 pH, 10 UV, and 1 current measurement (actually 525 measurements) requires less than 2 sec.

Following calibration, the Ampholine containing solution (or mixture of amino acids as discussed later on) is introduced into the heat-exchange reservoir and circulation through the cell established. Air purging is accomplished by brief reversals of flow direction at rapid flow rates. It is important to emphasize that all electrophoretic transport occurs only within the focusing cell itself, across its filter elements. The pH gradient is also established within the focusing cell, by focusing of the buffer components. As the final equilibrium distribution of all components is independent of the starting distribution, the sample can be added to any or all of the channels in the heat-exchange reservoir. Nevertheless, it is advantageous to first prefocus the buffer alone, and then add a concentrated sample solution to one of the center channels. This avoids the exposure of the sample to extreme pH values, as the pH gradient is formed faster than the sample equilibrates, and minimizes the exposure of the sample.

The progress of focusing can be followed by the computer printouts and graphic displays. Usually, a set of 24 measurements at preset time intervals is taken during any focusing experiment. Mostly, 2 h total time are sufficient, and the computer will take the measurements at 5 min intervals. At the end of the experiment, all channels are collected simul-

taneously by rechanneling of all flows into a test tube array held in an appropriate device.

Because of the modular nature of the design of the apparatus, there is great flexibility in the number of fractions collected or volume capacity. The apparatus shown has only 10 channels, but this number can be increased or decreased at will. The volume capacity of the instrument depends mainly on the volume of the heat-exchange reservoir. The reservoir shown in Figure 1 has a total capacity of 400 ml and another reservoir of 1,000 ml is also frequently used. The focusing process has been carried out also at the 10 liter level, using larger spacers with an effective area of 100 cm^2.

Implicit in the focusing process is the desalting of the sample, all salts ending up in the two electrode electrolytes. Precipitation of some proteins insoluble at their isoelectric point may be avoided by the addition of 8 M urea or various non-ionic detergents.

Results

The RIEF system is presently being studied for the separation of a variety of proteins and peptides, including glucagon and various synthetic derivatives, myosin peptides, synthetic ACTH, thymosin, cobra venom, hementerin, β-MSH, acid phosphatase, and others. Time will permit the description of only a few results.

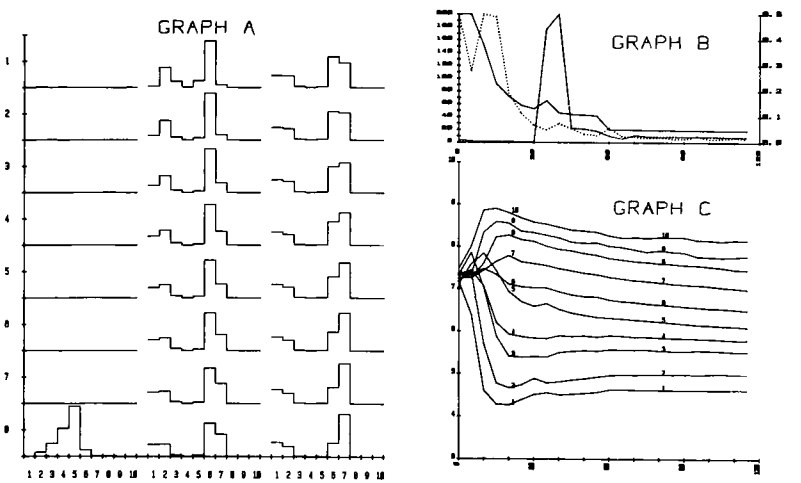

Fig. 3. MEDAS plots documenting the separation of hemoglobin and albumin (see text).

Figure 3 exemplifies a real-time MEDAS plot obtained with two colored proteins, hemoglobin and albumin stained with bromphenol blue. Fractionation of 1 g total protein was carried out at 200 volts in 1% Ampholine, pH 3.5-10. Graph A shows 24 sequential profiles of the UV absorption across the 10 channels, taken at 5 min intervals. The first 7 profiles were flat, this marking the prefocusing of Ampholine. The protein mixture was added in channel 5, as seen from plot 8, column I. The progress of focusing can be followed by scanning column II & III, albumin finally concentrating in channels 1 & 2, hemoglobin in channels 6 & 7.

The solid line in graph B records the decrease of the focusing current with time to a final value of 18 mamp. The dotted line illustrates the variance between subsequent sets of pH measurements, and the dashed line represents the same data for UV measurements. All three lines settle down to their minimum at about 70 min focusing, inclusive of prefocusing, which indicates the equilibration of the system. Thereafter, there is only a small cathodic drift, found in all isoelectric focusing.

Graph C plots the pH values as a function of time and shows that the pH gradient is developed within the 35 min of prefocusing, followed by a slow drift towards lower pH values.

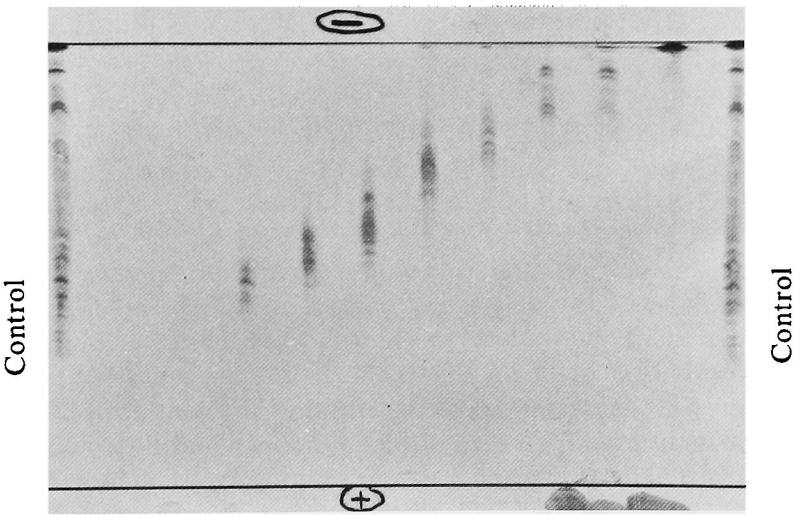

Fig. 4. RIEF fractionation of myosin peptides. Myosin CNBr peptides, RIEF 1% Ampholine, pH 3.5-7, PAG 2% Ampholine pH 3.5-7, 2/23/79

To assess the resolution achievable, more complex systems are necessary. Peptides obtained by cyanogen bromide cleavage of myosin (250 mg) were fractionated in 400 ml of 8M urea, with 2 ml each of Ampholine, pH 3.5-5 and pH 5-7. Figure 4 shows the analytical gel patterns of the 10 RIEF fractions and of the original mixture. Excellent resolution has been obtained with only minor overlap of components between subsequent fractions.

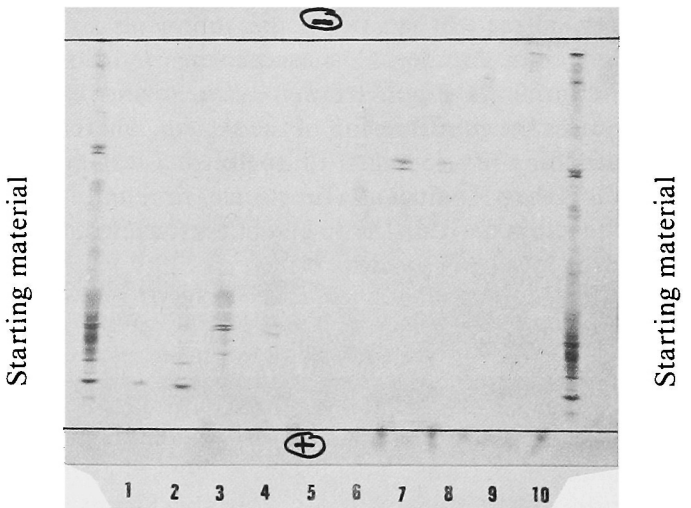

Fig. 5. RIEF fractionation of cobra venom. Cobra venom, Agkistradon Halys Blomhofii pH 4-6.5, PAG IEF pH 4-6.5, 4/15/79

Figure 5 presents comparable data for the fractionation of 250 mg of cobra venom in 250 ml distilled water containing 4 ml Pharmalyte, pH 4-6.5. The pH values of the ten fractions were 4.09, 4.31, 4.54, 4.78, 5.04, 5.37, 5.69, 6.01, 6.56, and 7.42. Phospholipase activity was associated with the first two fractions, and the specific activity increased by a factor of 6.2.

Development of New Buffer Systems

IEF requires the generation of stable pH gradients, usually achieved by means of synthetic mixtures of polyaminopolycarboxylic acids, commercially available under a variety of tradenames, such as Ampholine, Pharmalyte, etc. While these are well suited for analytical purposes, they cause contamination of purified products in preparative applications. Several authors[2-4] have attempted to develop stable pH gradients using simpler buffer systems of clearly defined composition.

In trying to achieve the same objective, we thought it necessary to first develop an explicit theory of isoelectric focusing of simple ampholytes describing their steady-state distribution in an electric field[5]. This mathematical model of IEF rests on the following basic concepts: (A) the concentration of component subspecies is defined by equations of chemical equilibria; (B) in the steady state, a balance exists between the mass transports resulting from electromigration and from diffusion; and (C) the condition of electroneutrality prevails in the physical scale of the system under consideration. From the classic relationship describing these concepts and the values entered or dissociation constants of the individual components, their electrophoretic mobility, initial concentration, and current density, the model computes the pH, conductance, and concentration of each component along the axis of an assumed IEF column.

The model is too complex to be described in the present paper, but it has permitted the compilation of a library of simulated IEF runs with a variety of ampholyte mixtures. This is presently used as the basis for a rational selection of components for the establishment of stable pH gradients. These are being evaluated in three experimental systems: polyacrylamide gels, density stabilized columns and the automated RIEF apparatus. Separation of hemoglobin and albumin in the RIEF apparatus, comparable to that shown in Figure 3, is obtainable using 3 component systems with glutamine and arginine or lysine as acidic and basic ampholytes and glycyl-glycine or glycyl-glycyl-glycine as the intermediate buffering ampholyte, all in the 2.5 to 15 mM concentration range. The pH gradient is not linear, but is adequate for this separation. The proteins have to be added at the beginning of the fractionation and not after prefocusing, as otherwise the so-called conductivity gap prevents equilibration.

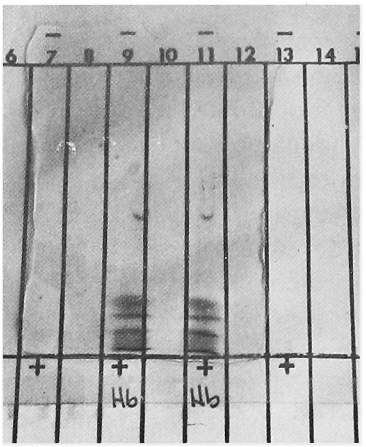

Fig. 6. Polyacrylamide gel focusing of hemoglobin in Ampholine-free buffer (see text).

Narrow pH gradients are also obtainable, and in Figure 6 is shown the focusing of hemoglobin in a polyacrylamide gel containing only 16 mM histidine (pI 7.54) and 16 mM β-alanyl-histidine (pI 8.17). The resolution of the hemoglobin bands is comparable to that obtainable with narrow pH range Ampholine. Other comparable ampholyte systems are presently under investigation.

Discussion

The RIEF system described in this paper represents a significant innovation in preparative IEF. While multicompartmented cells are among the oldest electrophoretic devices[6], this is the first IEF apparatus in which recycling is employed. In this respect, it bears closest analogy to forced-flow electrophoresis[7]. Due to the modular nature of the apparatus, virtually unlimited quantities of proteins or peptides can be processed. Much remains to be learned about optimization of the separations, expansion of the pH scale by reprocessing of individual or pooled fractions in a second run across all ten channels, feedback control, etc. These studies are currently under way.

Acknowledgments

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HIGH PERFORMANCE COUNTERCURRENT DISTRIBUTION (HPCD)

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People ask: Why Countercurrent Distribution (CCD)? Why not Chromatography (C)? The answer is: CCD and especially High Performance CCD (HPCD) is more powerful than people realize and less complicated than people think. There is, however, a condition: We must not use CCD in the way we use C.

Let us look in that context at a C-column separating a mixture, e.g. A & B (Figure 1). The process of chromatographic separation is clearly limited to a narrow moving band. It is the working zone of the column. Its width passes through a maximum which represents a small fraction of the total column length. This means that most of the column's separation potential is wasted, corresponding plates being used only for storage and transport. C can afford that waste. CCD cannot! It can, however, avoid the waste. The respective principle is simple and not even new[1]. It says: change the stationary phase and the moving working zone into a moving phase and a stationary working zone. We then have two moving phases, two sites of elution, and need not store material on either side beyond the working zone.

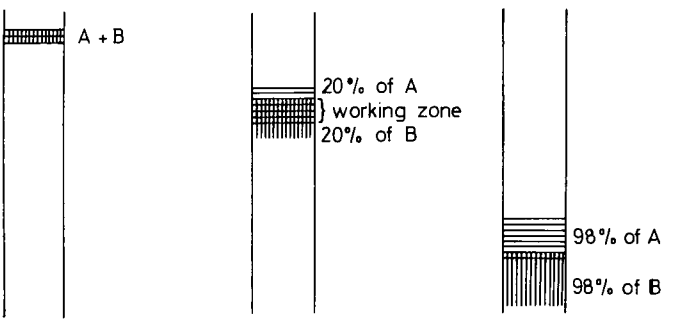

Fig. 1. The "working zone" in a chromatographic column.

Application of this principle brings us half way to HPCD. The other half must be based on simplified design and mechanics, as compared to existing CCD-machines.

A Machine for HPCD

In this paper we briefly present the prototype of a machine conceived to bring CCD back into the arsenal of preparative chemistry, for example as a means of dealing with the purification problems in batchwise or continuous manufacture of peptides. Figure 2 represents a photograph of the heart of the machine. Construction and function are, however, better explained by the schematic drawings in Figure 3-5. Note the recurring motive which is *simple design* and *simple mechanics*!

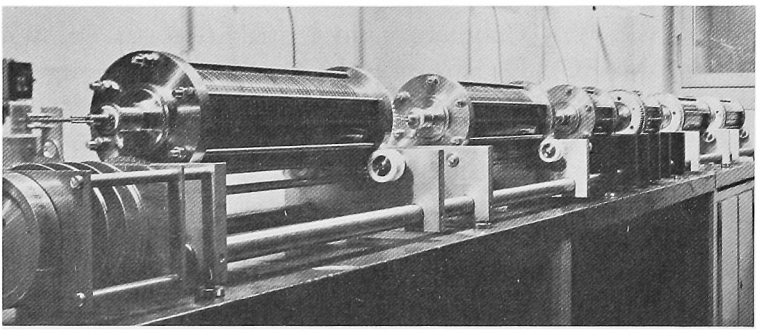

Fig. 2. Separation train on roller bearings (240 chambers).

The separation chamber (Figure 3) is a flat cylinder (axis horizontal, radius 5 cm, length 1 cm); 20 or 50 adjacent chambers make a separation cylinder (Figure 4); communication between neighboring chambers is provided for by a small hole drilled in each dividing wall (Figure 3, 4, and 5); two or more separation cylinders make a separation train (Figure 5).

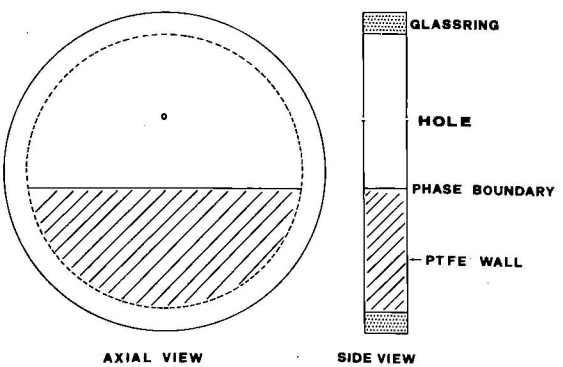

Fig. 3. Separation chamber.

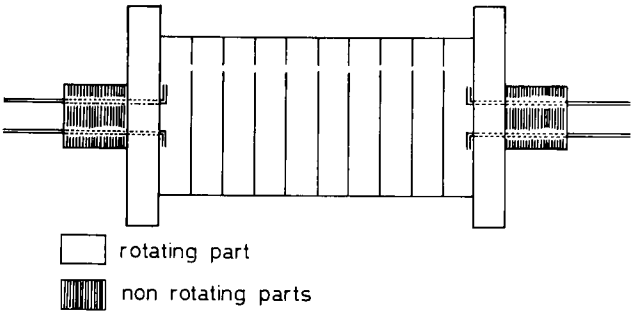

Fig. 4. Separation cylinder with inlets and outlets for the phases and the holes between the individual chambers.

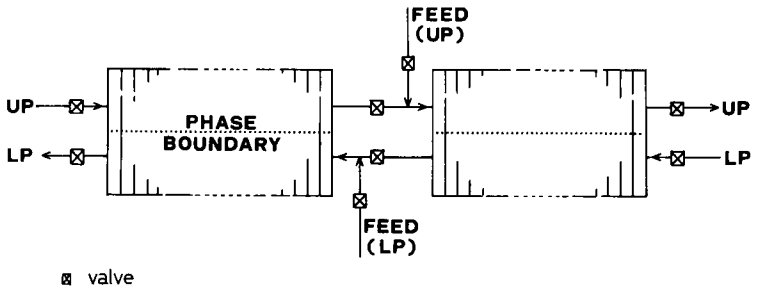

Fig. 5. Separation train with phase and feed transport lines. UP upper, LP lower phase.

Along the train the holes are located on a single straight line, running parallel to the axes of the cylinders. A pair of axially mounted tubings at each end of a cylinder connects the first and the last chamber to the outside. Their fitting is such that they remain at rest while the cylinder is being rotated. These tubings serve as inlets and outlets while the flow of phases is controlled by valves. The same inlets controlled by additional valves allow for feed inflow in place of upper or lower phase flow, the feed point (or area) being placed between any two cylinders or even at one end of the train. For batchwise operation, feed solutions may be introduced directly into one or more separation chambers. Up to 20% of the chambers may be loaded with saturated solutions of feed in either or both phases. Openings in the cylinder wall provide access to every separation chamber; these openings are shut when the chambers are at work. The separation train rests on roller bearings (Figure 2) and is driven continuously by a motor.

Rotation causes one phase to form a film which "wets" the chamber walls to be drawn through the bulk of the other phase thus distributing the solute between the upper and lower phases. This type of phase contact is not only simple, but also minimizes phase emulsification. Solute distribution throughout the train is effected by injection of upper phase at the left and lower phase at the right end of the train. These injections take place when the holes between the chambers are moving through the upper or the lower phase, respectively (Figure 5). They cause alternate countercurrent phase movement through every chamber, and corresponding phase ejections (eluates) from each end of the train.

Operation

There are numerous possible choices of operational parameters and ways in which they affect solute behavior; however, these effects are well understood. Suffice it to say that the direction of migration of solutes in a given phase pair is uniquely governed by the respective partition coefficients and the ratio R of upper phase to lower phase flow, and that R is readily readjustable at any moment during a run, for example on the basis of analytical controls of solutes in the train. Such controls and subsequent adjustments are of course amenable to automation. Automated performance becomes insensitive to drifting partition coefficients and fluctuations of temperature.

Separation by Elution

There are important practical consequences for both batch and continuous operation: any solute S fed near the center of the train can by suitable choice of R′ be made to reappear quantitatively as the slowest moving species in the outflowing upper or lower phase (elution). S will of course be accompanied by all additional solutes that move in the same direction and at a faster rate. However, solutes moving in the opposite direction will be wholly or partly removed from S, depending on separation factors and on the plate number of the train. Repetition of the same operation is approximately equivalent to doubling the plate number. Further repetition of the operation with a suitably altered flow ratio R″ will reverse only the direction of S so that it now is separated from solutes which still move in the original direction. As a corollary, a machine pair operated simultaneously with flow ratios R′ and R″, respectively, will continuously produce purified solute S; such a combination of two

machines may necessitate some additional manipulation (e.g. concentration) of the S-carrying eluate to be fed into the second machine. Ratios R′ and R″ and required plate numbers for a given degree of purification may be calculated from simple formulae. However, they must not be calculated if the operator prefers to program his machine empirically on the basis of direct analytical controls. Neither resolution nor eluate concentrations are affected by the phase ratio within the train; equal volumes of each phase are most convenient.

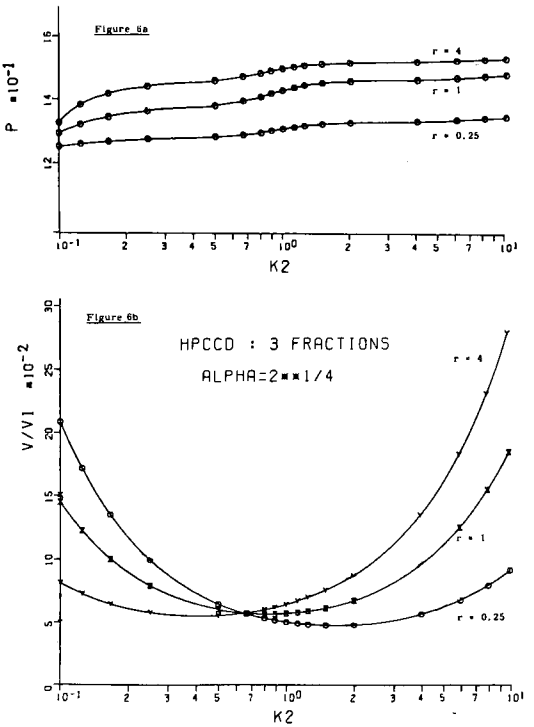

Fig. 6. HPCD separation of a mixture of S_1, S_2, S_3 with partition coefficients K_1, K_2, K_3, separation factors $\alpha = K_2/K_1 = 4\sqrt{2}$, $\gamma = K_3/K_2 = \sqrt{2}$, using phase ratios r = 1/4, 1, 4 and flow ratios $1/K_1$: Total elution of S_3 with upper phase, partial elution of S_1 with lower phase while S_2 remains in the train. a: Required plate number P in the train; feed point at P/3 from UP inflow. b: Ratio V/V_1 of required total elution volume and total chamber volume. Separation is then completed by changing the flow ratio to $R = (K_1K_2)^{-0.5}$ which will cause fast elution of residual S_1 with the lower phase and slow elution of S_2 with the upper phase.

Separation by Retention

Work-up of S from a single batch may also be accomplished in a more direct manner, that is to say by keeping S nearly stationary in the machine (retention) while its nearest neighbors are eluted from each end of the train. The program for the most economical approach to this type of problem can be read from computerized graphs. Examples are given in Figure 6. Note the dependence of performance on the phase ratio, typical for batch operation.

Enrichment

Continuous central feed of a dilute solution of contaminated S into the machine operated with a flow ratio $R = 1/K_S$ will cause S to remain in the train as stationary peak.

The Quantitative Meaning of HPCD

The degree of plate saving must be considered in the context of yield and/or resolution, and of plate efficiency. For example, for a 1:1 binary mixture plate saving is greatest with batchwise central feed and symmetrical elution. The respective condition is that $R = (K_A K_B)^{-0.5}$. The separation is practically complete (purities 99%) with the plate number at least equal to $P = 1 + 4.5(\sqrt{\beta} + 1)/(\sqrt{\beta} - 1)$, where $\beta = K_B/K_A$ (plate efficiency 100%). The minimum plate number N required for corresponding chromatographic separation depends on the capacity factor k′: within the limits k′ = 10 and $k' = 1/\sqrt{\beta} \approx 1$ the value of N goes from N_{10} to N_1, cf. Table I. Compare with the value of P.

β	P	N_{10}	N_1
2.0	28	63	184
1.41	53	229	734
1.19	105	925	2800
1.09	209	3560	11200
1.044	417	13700	46500

(Purity of recovered A and B is 99%)

With an average plate efficiency of 50% (BuOH/H_2O, 5-10 rpm) we must in practice double the estimated value of P. This is acceptable in view of the simple and space saving construction, and of the volume (100 ml) per chamber, the machine being conceived for preparative use.

Applications

Machine performance (200 chambers) was as expected in isolation of peptides (batch), alkaloids (continuous, 100 hours) and glycosides (continuous, 1000 hours).

Acknowledgments

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HUMAN LEUKOCYTE INTERFERON PRODUCTION AND PURIFICATION TO HOMOGENEITY BY HPLC

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Introduction

Production and purification of human interferon has attracted much attention since its discovery by Isaacs and Lindenmann in 1957[1]. Since that time, a vast literature on interferon has accumulated[2]. Until recently, no clear and reliable data on its chemical structure has been available. Despite many programs directed toward its production and purification, interferon is available in limited quantities only.

In the past few years, analysis and detection of amino acids, peptides, and proteins at the picomole level by fluorescence has been developed by Udenfriend and coworkers.[3-5] These techniques were later combined with high performance liquid chromatography (HPLC) for the rapid fractionation of large peptides[6].

Recently, we have used HPLC to obtain for the first time human leukocyte interferon in a pure form and have determined its amino acid composition.[7,8] The purification procedure included selective precipitations, gel filtration and four HPLC steps. The HPLC steps were reverse-phase chromatography at pH 7.5 on Lichrosorb RP-8 (octyl groups chemically bound to silica microparticles), normal-partition chromatography on Lichrosorb Diol (glycol groups on silica) and reverse-phase chromatography at pH 4.0 on Lichrosorb RP-8. Gradients of n-propanol were used for elution of interferon from these columns. The overall purification was 80,000-fold and the specific activity of pure interferon was 2-4 x 10^8 units/mg. Interferon prepared by the above procedure

yielded a single band of molecular weight 17,500 ± 1,000 on polyacrylamide gel electrophoresis. The antiviral activity was shown to be associated with this protein band. The amino acid composition of interferon was obtained by analysis of 50 to 100 pmoles with a fluorescamine analyzer.

So far, most of the interferon used for clinical studies has been produced by induction of leukocytes obtained from blood donors. Other sources of interferon include lymphoblastoid cell cultures and fibroblast cells. Leukemic patients with high leukocyte counts are a rich source of white blood cells. Leukocytes can be selectively removed from such patients by leukapheresis[9]. By this method, an amount of leukocytes equivalent to that obtained from 100 to 300 units of blood can be obtained from a single patient with a count of 10^5 leukocytes/μl.

Results

Leukocytes from various sources were tested for their ability to produce interferon on induction with virus. We found that leukocytes obtained from patients with chronic myelogenous leukemia (CML) produced higher levels of interferon than leukocytes from patients with chronic lymphocytic leukemia or the acute leukemias as previously reported by Lee *et al.*,[10] and Hadhazy *et al.*[11] Leukocytes from patients with CML yielded 5-80 x 10^3 units of interferon/10^7 cells/ml. Leukocytes from patients with acute or chronic lymphocytic leukemia yielded interferon titers from 0 to 1,000 units/10^7 cells/ml. Leukocytes from normal blood donors produced 5,000 to 10,000 interferon units/10^7 cells/ml.

Interferon produced by leukocytes from CML patients was compared to that produced by leukocytes from normal donors. The elution profiles from the various HPLC columns were highly reproducible and therefore could serve as an analytical tool for comparing interferon preparations. The first HPLC step, performed on Lichrosorb RP-8 (Figure 1), produced almost identical profiles of proteins and activity. Pooled fractions 11-15 from the Lichrosorb RP-8 column were applied to and fractionated on Lichrosorb Diol (Figure 2). Three major peaks of activity, labelled α, β, and γ, were observed. As can be seen, the protein profiles exhibited by interferon preparations from both sources were almost identical. The activity profiles were very similar except that the amount of activity under peak α is lower in preparations from leukemic cells compared to those from normal leukocytes. The ratio of peaks α, β, and γ from normal leukocytes varies from one preparation to another. Peak γ of the Diol columns was then further chromatographed as shown

in Figure 3. Several peaks of antiviral activity were detected. Fraction 44 from each chromatography (Figure 3) contained homogeneous interferon and was used for amino acid analysis.

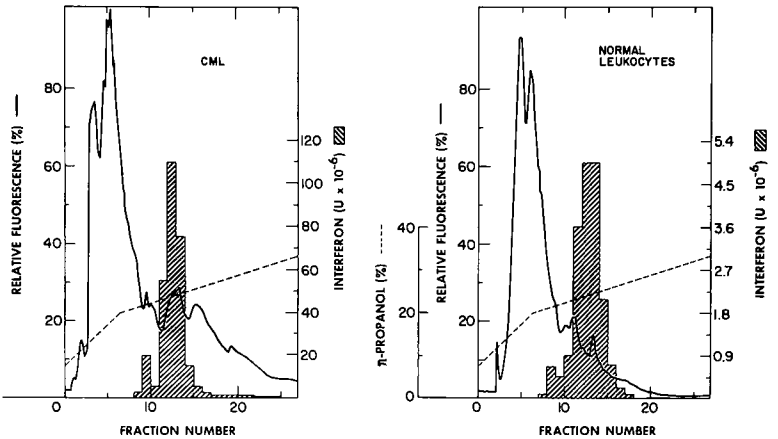

Fig. 1. Chromatography of human leukocyte interferon from normal and leukemic leukocytes on Lichrosorb RP-8 (4.6 x 250 mm) at pH 7.5. CML refers to chronic myelogenous leukemia. A gradient of n-propanol in 1 M sodium acetate was used. The flow rate was 0.25 ml/min and 1.5 ml fractions were collected.

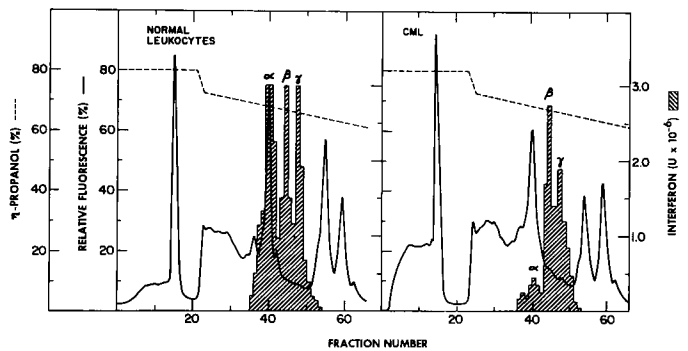

Fig. 2. Chromatography of human leukocyte interferon on Lichrosorb Diol (4.6 x 250 mm). A gradient of decreasing n-propanol concentration in 0.1 M sodium acetate was used. The flow rate was 0.25 ml/min and 0.75 ml fractions were collected.

Amino acid analyses were performed with a fluorescamine analyzer on 2 μg of pure interferon. Under these conditions, all amino acids except for tryptophan were measured at 300 pmoles or above. This is 12 times higher than the practical low limit of the fluorescamine analyzer. Interferons from normal and leukemic cells did not show any significant difference in amino acid composition (Table I).

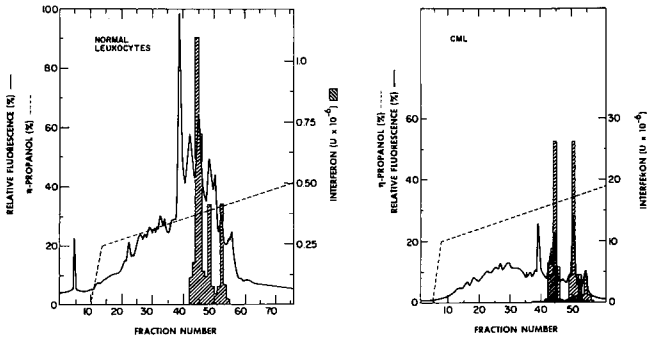

Fig. 3. Chromatography of human leukocyte interferon (peak γ) on Lichrosorb RP-8 (4.6 x 250 mm). A gradient of n-propanol in pyridine (1M)-formic acid (2M) was used. The flow rate was 0.25 ml/min and 0.75 ml fractions were collected.

Table 1. Amino Acid Composition of Leukocyte Interferons

Amino Acid	Interferon Source	
	Normal Leukocytes	CML Leukocytes
ASX	15	13
SER	8	9
THR	8	8
GLX	24	24
PRO	6	5
GLY	5	5
ALA	8	8
VAL	8	8
MET	4	4
ILE	9	8
LEU	22	21
TYR	5	5
PHE	9	9
HIS	3	3
LYS	12	11
ARG	7	8
CYS	3	3

Conclusion

High resolution chromatography of proteins has been developed and coupled with a high sensitivity monitoring system based on fluorescamine. This combination of high resolution and sensitivity enabled the isolation and chemical characterization of pure human leukocyte interferon for the first time. With this methodology, both for analytical and preparative applications, a new source of human leukocyte interferon was established. It was found that interferon obtained from leukemic leukocytes exhibits the same elution characteristics on various HPLC columns and the same amino acid composition as that from normal leukocytes. We, therefore, conclude that these two interferons are identical.

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PREPARATIVE PURIFICATION OF PEPTIDES BY REVERSED PHASE LIQUID CHROMATOGRAPHY ON INEXPENSIVE COLUMNS

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The purification of peptides by high performance liquid chromatography (HPLC) has been well documented in recent years (see Reference 1 for a review). We reported the purification of human β-endorphin (β_h-EP) by reversed phase HPLC on a 100 mg scale.[2] Most of the published work, however, requires typical high performance hardware; i.e. high pressure pumps, loop injectors, etc. We have developed a low pressure (< 150 psi) system employing lyophilizable mobile phases, transparent at 206 nm, for purifying peptides on inexpensive prepacked columns containing silica alkylated with C-8 groups.

The liquid chromatographic equipment used is basically that reported by us for the purification of protected peptides.[3] A variable wavelength monitor, a Laboratory Data Control SpectroMonitor II, was used to monitor the effluent from the column. In one example, a 50 mg sample of crude Tyr-D-Thr-Gly-Phe-Thz-NH_2[4] (Thz = thiazolidine-4-carboxylic acid) in 1 ml of 0.01 *M* triethyl ammonium acetate, was loaded onto a 2.5 x 31 cm (Size B) RP-8 LOBAR column (E.M. Laboratories) which had been equilibrated with 0.01 *M* acetic acid adjusted to pH 4.5 with triethylamine. Elution was carried out at 2 ml/minute with a linear gradient to 4% acetonitrile over 30 minutes, held at 4% for 20 minutes, then increased to 20% over 3 hours. Fractions were collected every 3 minutes. Figure 1 shows the 215 nanometer tracing of the column effluent. Homogeneous product (tlc, HPLC) was found in the fractions indicated. Lyophilization, followed by gel filtration on Sephadex G-10 in 0.01 *M* HCl yielded 13 mg peptide as the hydrochloride.

Amino acid analysis (6 *M* HCl, 32 hours, 110°C) showed: Thr 1.01, Thz 0.90, Gly 1.00, Tyr 0.97, Phe 0.98, NH_3 0.93. Elemental analysis indicated 2 moles of water: $C_{28}H_{37}N_6O_7S\,Cl{\cdot}2\,H_2O$: Theory C 49.96, H 6.14, N 12.48, S 4.76, Cl 5.23. Found C 49.55, H 6.27, N 11.90, S 4.89, Cl

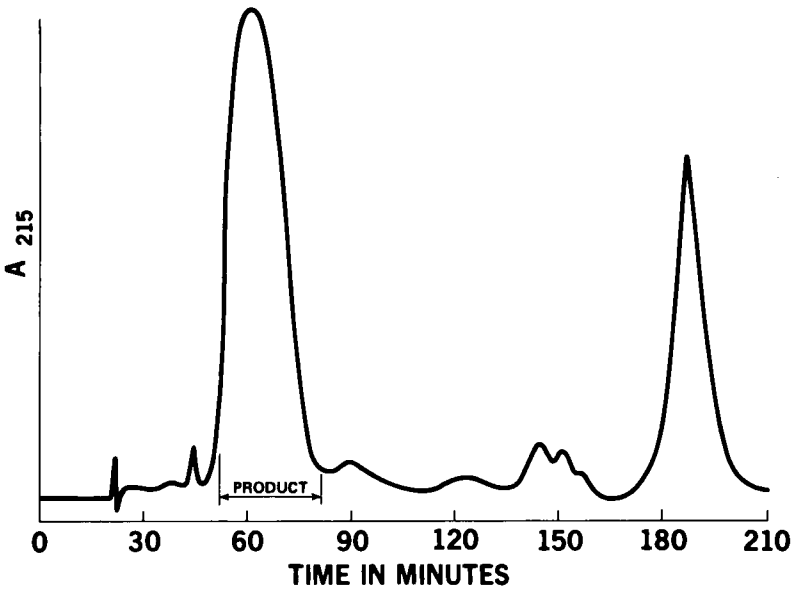

Fig. 1. Purification of Tyr-D-Thr-Gly-Phe-Thz-NH_2 on LOBAR RP-8, 2.5 x 31 cm. System: 0.01 *M* AcOH pH 4.5-acetonitrile, 0%-20% over 230 minutes at 2 ml/minute.

5.60. Total recovery of the load, based on the 275 nanometer absorption of the load and pooled fractions, was 96.7%.

Human β-endorphin, synthesized by solution phase methods[2] is routinely purified on a 3.7 x 44 cm LiChroprep RP-8 LOBAR column (Size C). In a typical run, crude β_h-EP (150 mg, obtained after Na—Liquid NH_3 reduction of protected 31-peptide, Sephadex G-10 desalting [0.01 N HCl] and lyophilization) was dissolved in 0.01 *N* HCl (5 ml) and injected or pumped on a 3.7 x 44 cm LiChroprep RP-8 LOBAR column. The mobile phase program consisted of successive 500 ml portions of 0.01 *N* HCl, 10%, 12%, and 15% acetonitrile in 0.01 *M* HCl, followed by a linear gradient from 15 to 25% acetonitrile in 0.01 *N* HCl during which the major β_h-EP-containing peak emerged from the column. The chromatogram was completed by a step gradient of 500 ml each of 25%, 40%, and 90% acetonitrile in 0.01 *N* HCl. A flow rate of 10 ml/minute was used. Figure 2 shows a typical chromatogram of a preparative run monitored at 254 nm. Each 20 ml fraction was analyzed by injecting 100 μl onto an analytical column. In this case, a 0.38 x 30 cm μ Bondapack C_{18} column (Waters Associates) with 29% acetonitrile in 0.01 *M* HCl, at a flow rate of 2 ml/minute was used. Figures 3a, b, and c show analytical HPLC of early, center, and late fractions from the human β-endorphin peak of Figure 2.

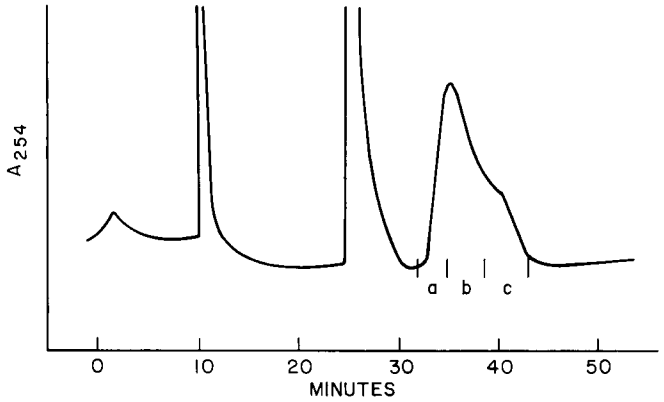

Fig. 2. Purification of 150 mg crude human β-endorphin on 3.7 x 44 cm LOBAR RP-8 with 0.01 *M* HCl-acetonitrile gradient at 10 ml/min. Fractions marked a, b, and c were analyzed by HPLC (Figure 3).

A typical run produced 27 mg of β_h-EP, 68 mg of β_h-EP contaminated with other species, and 50 mg of non-EP material. Rechromatography of the contaminated pool produced an additional 11 mg of β_h-EP, for a total of 38 mg. Amino acid analysis (6 *N* HCl—phenol, 24 hours, 100° C) showed Lys 5.00 (5), Asp 2.08 (2), Thr 2.97 (3), Ser 1.90 (2), Glu 3.13 (3), Pro 0.95 (1), Gly 3.20 (3), Ala 2.08 (2), Val 0.95 (1), Met 1.03 (1), Ile 1.70 (2, 72 hour hydrolysis), Leu 2.08 (2), Tyr 1.92 (2), Phe 1.93 (2). The figures in parentheses are the theoretical values. This material was identical to the natural material in all tests used; e.g. ORD, CD, chromatographic behavior and biological activity[5] (guinea pig ileum assay; carried out by C.H. Li, San Francisco, CA).

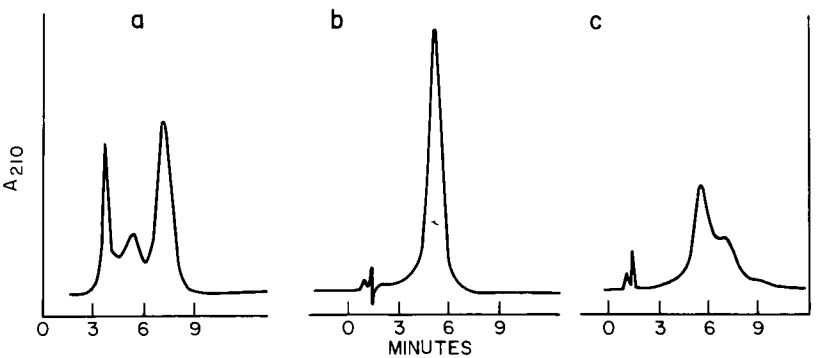

Fig. 3. Analytical chromatograms of fractions from preparative human β-endorphin chromatography. Column: 0.39 x 30 cm μ Bondapak C_{18}. Mobile phase: 29% acetonitrile in 0.01 *M* HCl at 2 ml/minute.

The combination of step and linear gradients employed was found empirically to give larger yields of clean β_h-EP than either a continuous or all-step gradient system. Column life-time has not been determined since after 12 runs no degeneration was seen.

These two examples were chosen from many runs to show the utility of this system. Other mobile phases, not necessarily UV transparent, have been used successfully. The low cost and wide applicability of the system extends the availability of preparative reversed phase HPLC to a larger number of laboratories engaged in synthesis and isolation.

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NEW MICROMETHODOLOGY FOR THE ISOLATION OF PEPTIDES

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Introduction

Biologically active peptides generally occur in animal tissues or fluids in small quantities. In the past their isolation and characterization have often required large amounts of starting material since the methods involved were relatively inefficient. Although powerful tools such as HPLC and fluorometric detection are now available for the final purification of small quantities of peptides, the preparation of crude extracts still relies on conventional techniques of often low efficiency. Based on previous observations by Bennett et al[1,2] we have developed a novel, highly effective micromethod for general purpose peptide extraction.

Experimental Procedures

Tissues were homogenized in 15% trichloroacetic acid or in a mixture of 50 ml formic acid, 150 ml trifluoroacetic acid (TFA), 10 g NaCl, and 90 ml HCl (37%) per liter.[2] Urine was deproteinized by addition of an equal volume of 30% TFA.[1] Precipitates were removed by centrifugation and the supernatant extracted with diethyl ether for lipid removal. For peptide extraction, the aqueous phase (carefully freed from residual ether) or a peptide standard solution (in 0.2M acetic acid) was passed through a column of octadecasilyl-silica (ODS) (Porasil C 18, 50-80μ, Waters Associates) using a simple chromatography system. The instrument consisted of a pump (Milton Roy), a medium pressure glass column ($\leq$500 psi), and a back pressure device (Nupro check valve, 25 psi) at the column exit to maintain a minimum pressure in the column. The pump inlet was protected with a bubble trap. Two column sizes were used for small and large samples, with dimensions of 4 x 0.9 and 15 x 2.5 cm and flow rates of 60 and 150 ml/h, respectively. The sample solution (up

to 2000 ml) was loaded by pumping it through the column. The column was then washed with 1% TFA in water until the effluent was free of salts and primary amines (fluorescamine test). Peptides (which are retained on the column under such conditions) were eluted with a solution of 80% methanol and 1% TFA in water and the eluate was dried. β-Endorphin, Leu^5-enkephalin, somatostatin, and ACTH were quantitated by specific radioimmunoassay (RIA).

Results and Discussion

The solvent-dependent interaction of peptides with ODS forms the basis of the proposed technique. Peptide molecules, though highly polar, usually contain at least some hydrophobic character and thus will interact with the hydrophobic ODS. This interaction is strongest in fully aqueous solution resulting in the binding of peptides to ODS. It can be overcome by less polar solutions such as water-alcohol mixtures which will elute peptidic material from the column. The efficiency of the method has been tested with a variety of synthetic peptides (Table I). All peptides except homocarnosine were retained by ODS, suggesting that even small peptides are sufficiently hydrophobic for extraction with this procedure. Bound peptides were recovered from the ODS column with virtually 100% yield. Even a protein such as serum albumin was recovered in good yield. Furthermore the capacity of the ODS material for peptides appears to be quite high. As much as 5 and 15 mg of Leu^5-enkephalin and γ-endorphin, respectively, were bound by a small ODS column. The capacity may be dependent on the degree of hydrophobicity of each peptide since it was substantially higher for the larger (and more hydrophobic) γ-endorphin than for Leu^5-enkephalin.

With this procedure peptide extracts can be completely separated from salts (Table I). It can therefore be applied to rapid desalting of peptide solutions. Amino acids are also completely and rapidly separated from peptides (Table I). Complete separation of amino acid is important for the isolation of peptides by means of fluorometric detection.[3] The present method compares favorably with another procedure utilizing Copper-Sephadex.[3,4]

We have used the method to demonstrate somatostatin- and β-endorphin-like immunoactivity in normal human urine. Peptide material from ½ liter of urine was extracted as indicated in the experimental section. Immunoreactive somatostatin and β-endorphin, corresponding to 30 and 31 pg/ml, respectively, of original urine were found in the

Table I. Performance of a Small Octadecasilyl-Silica (ODS) Column (4 x 0.9 cm).

Sample	Retention% (in 1% TFA)	Recovery% (With 80% MeOH) 1% TFA	Capacity (mg)
Amino acids	0	0	-*
Salts	0	0	-
GABA-His (Homocarnosine)	0	0	-
pGlu-His-Pro-NH_2 (TRH)	100	-	-
Val-Thr-Leu	100	-	-
Met-Enkephalin	100	94	-
Leu-Enkephalin	100	-	5
Arg-Vasopressin	100	107	-
Somatostatin	100	111	-
γ-Endorphin	100	99	15
β-Endorphin	100	111	-
Insulin	100	96	-
Bovine serum albumin	100	85	-

10 nmol of sample or more (in 0.5 ml acetic acid) was loaded. The column was washed with 20 ml 1% TFA followed by 20 ml 80% MeOH/1% TFA. Retention and recovery were determined by the amount of samples found in the aqueous and methanolic fractions, respectively. Peptides and amino acids were assayed with fluorescamine (TRH after acid hydrolysis).
*not determined.

peptide fraction. In a second experiment a single cow brain was extracted using the protein precipitation agent of Bennett.[2] The peptide extract was tested by RIA for Leu[5]-enkephalin, β-endorphin and ACTH which were found in amounts corresponding to 8, 9 and 1 nmol/brain respectively.

The preliminary results suggest highly efficient extraction of peptides present in small amounts of brain tissue and urine. In addition, the proposed procedure, consisting of a series of simple and rapid steps such as deproteinization, lipid removal, and passage through ODS (removing small polar molecules) provides a highly prepurified peptide extract which is suitable for final purification by HPLC techniques. The amounts of biologically active peptides extracted from relatively small quantities of tissues or fluids are sufficient for at least partial structural characterization by modern micromethodology. Finally the technique should be particularly well suited for the extraction of peptides from large volumes of solutions of low peptide concentration. By pumping large

volumes through the ODS column, peptides are concentrated on the column and can then be eluted in a small volume of acidic methanol. In this way sufficient quantities of peptides can be accumulated relatively easily.

Acknowledgements

We wish to thank Dr. R. Benoit, Salk Institute for the RIA of somatostatin and A. Mauss and G. Kleeman for technical assistance. This work was supported by NIH grants HD-09690-04 and AM-18811-04 and by the William Randolph Hearst Foundation.

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PURIFICATION OF SYNTHETIC PEPTIDES BY MEANS OF COUNTERCURRENT CHROMATOGRAPHY WITH THE HORIZONTAL FLOW-THROUGH COIL PLANET CENTRIFUGE

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The horizontal flow-through coil planet centrifuge uses two-phase solvent systems to produce partition chromatography in the absence of solid supports.[1,2] Studies with this instrument have included the separation of commercially available dipeptides[2] and dinitrophenyl amino acids.[1,2] Recently, this instrument was used to purify two synthetic peptides.

Materials and Methods

Peptides — Two peptides having the sequence Ser-Ser-Ile-Ile-Arg (I) and Tyr-Ala-Ala-Nle-Ala-Ala-Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys (II) were prepared by a modification of the Merrifield solid phase method[3] using a Vega Model 96 automated synthesizer. The Bzl group was used to protect Ser, Asp, and Glu; Arg, Tyr, and Lys were protected by Tos, o-Br-Bzl, and o-Cl-Z groups respectively. Carboxyl-terminal residues were coupled to the resin as their cesium salts.[4] The average load obtained was 0.4 mmoles amino acid/g resin. Peptides were custom-cleaved with HF by Peninsula Laboratories.

Horizontal Flow-Through Coil Planet Centrifuge — The principle, design and use of this apparatus have been previously described.[1,2] In our work, the n-butanol:acetic acid:water (4:1:5 by vol) system was employed, and the mobile phase was pumped through the instrument at 16 ml/hour while the apparatus was spun at 500 rpm.

Results and Discussion

Peptide I. — This peptide (100 mg crude) was purified on the planet centrifuge with the polar (lower) phase of the solvent system described above as the mobile phase. Aliquots from the centrifuge run were analyzed by TLC with cellulose plates and the same solvent system; detection was by a collidine-containing ninhydrin reagent.[5] The results (Figure 1) and amino acid analyses (not shown) indicate that the spot with the larger R_F is peptide I, and the spot with the smaller R_F is serine (from insufficient washing of the resin after the last coupling step). In four runs, pure peptide was found 24±2 ml after the solvent front. The yield was about 65% but should be improved by re-running tubes which contained both peptide and serine. Also, non-peptide contaminants which co-eluted with peptide I on gel filtration were separated by planet centrifugation. Results identical to the 16 ml/hour run were obtained with a 24 ml/hour flow-rate.

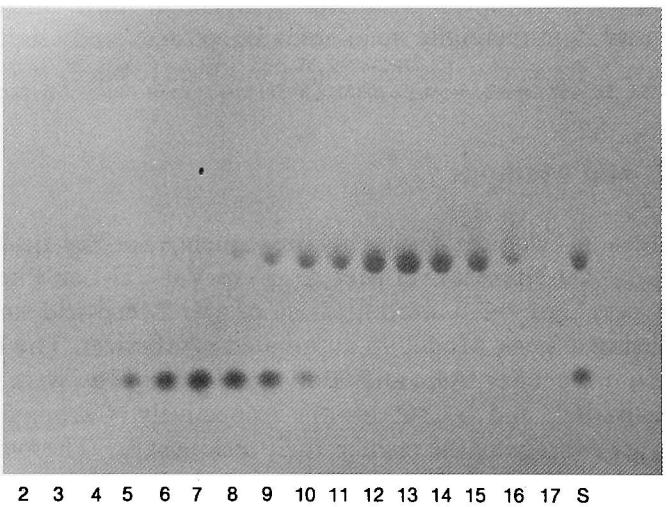

Fig. 1. TLC Monitoring of the Purification of Peptide I. Tube 1 is that which immediately follows the tube containing the solvent front; S refers to 16 μg of crude starting material.

Peptide II. — This peptide (100 mg crude) was purified on the planet centrifuge using the non-polar (upper) phase of the solvent system described above as the mobile phase. For appropriate tubes, levels of fluorescence (Figure 2A), yield of peptide (Figure 2B), amino acid composition (not shown) and behavior on TLC (Figure 3) were

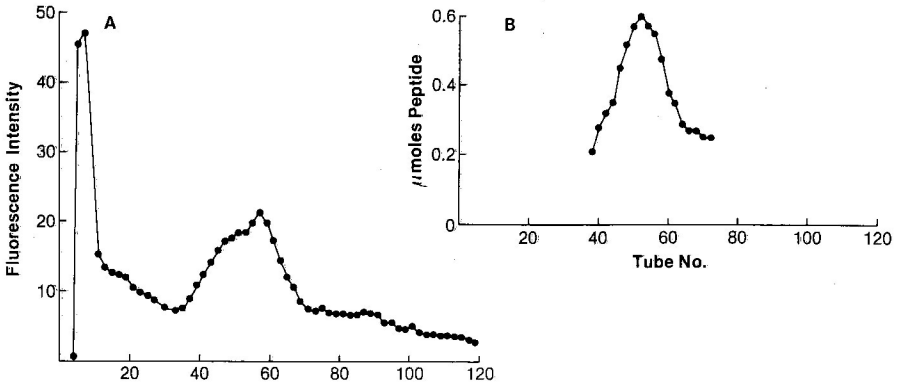

Fig. 2. Monitoring the Purification of Peptide II. Tubes are numbered as described for Figure 1. A. Fluorescence profile was obtained with an AMINCO SPF-500 spectrofluorometer in the single beam mode λ_{exc} = 280 nm; λ_{em} = 308 nm. B. Yields were determined by amino acid analysis with a Durrum D-500 analyzer.

determined. The results indicate that the run could be divided into three main areas of interest. The high levels of fluorescence for the contents of tubes 5-11 and their lack of reaction with ninhydrin when run on TLC suggest that these tubes contain blocked peptides and aromatic by-products of deblocking. (With the non-polar phase as the mobile phase, the least polar products appear in the early tubes.) The low molar ratios of Tyr, Nle, Ala and Arg to the rest of the residues suggest that this fraction also contains shortened sequences. Tubes 38-49 contain pure peptide II as

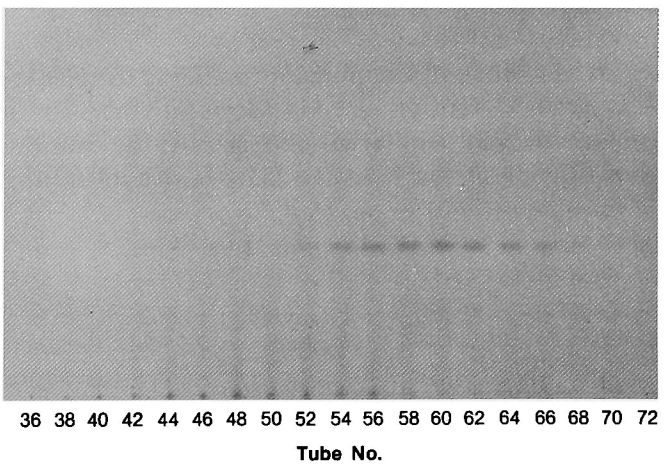

Fig. 3. TLC Monitoring of the Purification of Peptide II. TLC and spot detection were performed as described for Figure 1.

indicated by amino acid composition and their uniform behavior on TLC (Figure 3). Tubes 50-66 contain peptide contaminated with tyrosine (from insufficient washing of the resin following the last coupling). An increase in fluorescence for these tubes (Figure 2A) is accompanied by an increase in the number of moles of tyrosine/mole of peptide on the descending side of the peptide concentration curve (Figure 2B). With previous runs, the contents of tubes containing this spot were pooled and analyzed; tyrosine gave a molar ratio of 1.3. Finally, the distinct, fairly intense spot in tubes 50-66 migrates on TLC and thin-layer electrophoresis in 5% formic acid (not shown) identically to tyrosine. Pure peptide was obtained in only 12% yield. If the portion contaminated with tyrosine was included, the yield was raised to 33%. Solubility was a problem with peptide II and was probably responsible for both the low yield and the streaking on TLC (Figure 3). To begin the run, the peptide had to be dissolved in warmed mobile phase. Analysis of the column contents showed that the peptide precipitated when it came into contact with the stationary phase. An additional 17% of the pure peptide was found in the column contents. Also, a yield of 50% pure peptide was obtained with a 10 mg sample without measuring column contents.

General Conclusions — The planet centrifuge frees peptides of salts, resin-by-products, amino acids, and contaminating peptides in overnight runs with no attention needed. Separations can be performed on a preparative scale if solubility is maintained.

Acknowledgements

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A SIMPLE REVERSED-PHASE LOW-PRESSURE LIQUID CHROMATOGRAPHY SYSTEM FOR THE PURIFICATION OF PEPTIDES AND PREPARATIVE SEPARATION OF ENKEPHALIN DIASTEREOMERS

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Introduction

High pressure reversed-phase liquid chromatography (RPLC) is gaining widespread acceptance for chromatography of peptides;[1-5] however, the majority of applications report only small (<1 mg) sample loads. We were interested in the use of RPLC for the purification of gram quantities of synthetic peptides. A disadvantage of this technique is the cost of the high-pressure chromatography apparatus and the reversed-phase solid support. We now report our results with the RPLC purification of synthetic peptides utilizing a simple, inexpensive, low-pressure RPLC system.

Materials and Methods

The reversed-phase solid support utilized for this study was a C_{18}-silica gel prepared by reaction of 10-20 μ LP-1 silica gel (Whatman-Quantum Division) with octadecyltrichlorosilane (Aldrich Chem. Co.).[6] Excess free hydroxyl groups were capped with trimethylsilyl groups (final carbon content = 16-22%).

The main feature of this chromatography system is the refillable glass column (Figure 1) designed by Karl H. Michel and Robert F. Miller of Eli Lilly and Company.[7] The Michel-Miller columns, which are now available commercially (Ace Glass Co.), are sealed at each end with specially designed threaded teflon end fittings. The entire system can be used at pressures up to ca. 200 psi with organic as well as aqueous solvents. Aqueous acetonitrile with an ammonium acetate or formate buffer (pH 4-7) is typically used as elution solvent.

Fig. 1. Michel-Miller Refillable Columns.
A: ¼-28 standard teflon fitting, B: Michel-Miller teflon end fitting, C: aluminum safety shield cap, D: plastic safety shield, E: glass column. A typical complete system consists of teflon tubing connecting a glass solvent reservoir to an FMI explosion proof lab pump (Fluid Metering, Inc.), a stainless steel pressure gauge, and a slider injection valve (Altex Scientific, Inc.).

Results and Discussion

We have used the Michel-Miller RPLC system for over three years to purify gram quantities of synthetic peptides, e.g., enkephalin, somatostatin (SRIF), and substance P. Typically 1-5 mg of the crude peptide obtained after removal of all protecting groups is chromatographed on a column (1.0 x 50 cm) of C_{18}-LP-1 (30 g) to obtain the optimal solvent composition for purification. When the solvent composition has been established, the remaining crude peptide is chromatographed over a preparative size column (4.8 x 70 cm) of C_{18}-LP-1 (685 g) (Figure 2). To remove residual buffer salts, the purified peptide is chromatographed over Sephadex G-10 using 0.2*N* HOAc as elution solvent.

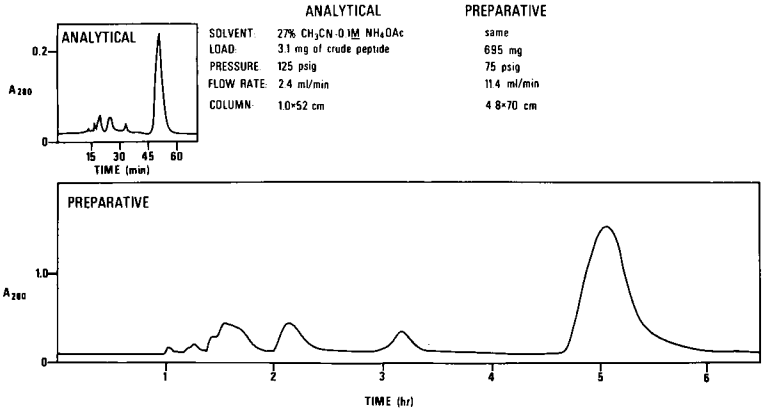

Fig. 2. RPLC of Tyr-D-Met-Gly-Phe-NMe-Met-NH_2

Similar to other reports[8,9] we have found that peptide diastereomers are separable using RPLC. Initial experiments with SRIF (10 mg) and D-Trp[8]-SRIF (10 mg) indicated that scale up from the analytical runs (Figure 3) was practical. Similar results were obtained with enkephalin analog diastereomers.

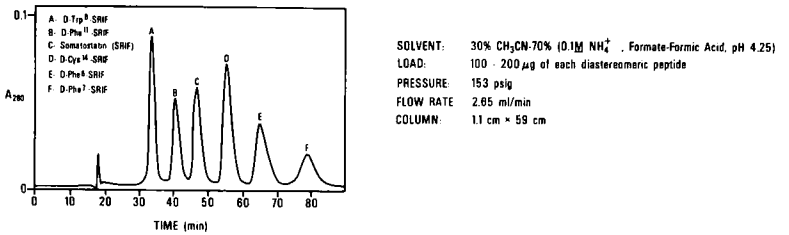

Fig. 3. Analytical RPLC of pure somatostatin diastereomers

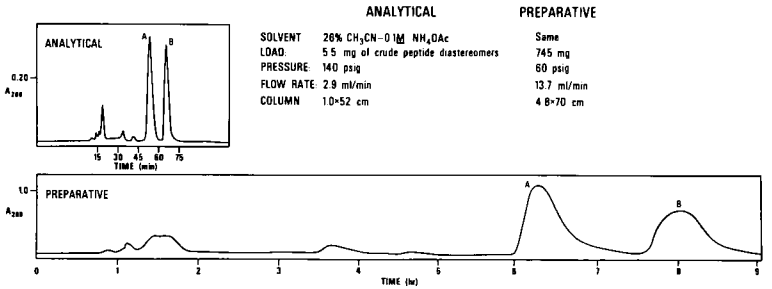

Fig. 4. RPLC of Tyr-D-Ala-Gly-L-*p*Cl-Phe-NMe-Met-NH_2 = A and Tyr-D-Ala-Gly-D-*p*Cl-Phe-NMe-Met-NH_2 = B

We now routinely synthesize enkephalin analogs using a racemic amino acid at either position 1, 2, 4, or 5 and separate the resultant pair of diastereomeric analogs utilizing RPLC (Figure 4). Examples of enkephalin diastereomer separations are presented in Table I. In all cases these separations were scaled up directly to give preparative separations. This process yields two enkephalin analogs in the time formerly required to synthesize one analog. More importantly, this procedure allows the use of a racemic amino acid without requiring enzymatic or chemical resolution.

Table I. Analytical[a] RPLC Separation of Enkephalin Diastereomers

Analog[b]	Elution Time (min) L	D	CH_3CN (%)[c]
D,L-3-OH-Phe[1]	77	64	23[d]
D,L-3-F-Tyr[1]	80	65	20
D,L-Allylgly[2]	70	83	20[e]
D,L-Ala[2]	182	206	18[d]
D,L-pCl-Phe[4][f]	54	66	26
D,L-pBr-Phe[4]	45	52	30
D,L-Phe[4]	42	55	25
Gly[2],D,L-Phe[4],L-Met-NH_2[5]	30	44	23
D,L-Phe[4],L-Met-NH_2[5]	34	46	23
D,L-Met-NH_2[5]	34	50	23
D,L-NMe-Met-NH_2[5]	42	59	25
D,L-Pro-NH_2[5]	44	69	20

[a]Peptide loads: 2.1-6.1 mg. [b]Based on H-Tyr-D-Ala-Gly-Phe-NMe-Met-NH_2. [c]Conditions: 1.0x50 cm column, 0.1*M* NH_4OAc, pH 7, 100-140 psig, 2.3-2.9 ml/min. [d]pH 4.5. [e]pH 4.1. [f]See Fig. 4.

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ISOLATION OF PEPTIDE HORMONES BY REVERSED-PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY

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A combination of the resolving power of reversed-phase high pressure liquid chromatography and the use of octadecylsilyl-silica (ODS-silica) in the batch extraction of tissue homogenates provides a means of rapidly purifying peptides from tissues and plasma.

Reversed-Phase High Pressure Liquid Chromatography (RP-HPLC) Using Trifluoroacetic Acid as a Hydrophobic Anion Pairing Reagent

RP-HPLC is proving to be the method of choice for the purification of both natural and synthetic peptides. The remarkable resolution obtainable with RP-HPLC columns can be enhanced by using acidic solvent systems.[1-4] At low pH the adsorption of peptides to underivatized silica (i.e. ion exchange effects) is reduced and column efficiency in terms of peak shape and theoretical plates is greatly improved. In addition a hydrophobic anion-pairing reagent such as trifluoroacetic acid (TFA)[1] can be used to associate with the free amino groups which become fully protonated at low pH. This causes increased affinity for the reversed-phase support and hence column efficiency can be improved still further. Using a C_{18} μ Bondapak column, 6000A pumps and a solvent programmer (Waters Associates) in combination with an aqueous acetonitrile solvent system containing 0.1% TFA throughout, it is possible to resolve many peptides and proteins. For instance, met-enkephalin, α-MSH, β-MSH, ACTH, calcitonin, cytochrome c, bovine serum albumin, bovine prolactin and bovine growth hormone are eluted in this order by a linear gradient of 20-60% acetonitrile containing 0.1% TFA over two hours. The solvent system will permit monitoring for UV absorbance at 210nm (Perkin Elmer LC55 spectrophotometer) and is volatile, facilitating sampling for biological and immunological assay.

Batch Extraction of Peptides from Tissue Homogenates Using ODS-Silica (Sep-Pak C_{18} Cartridges)

Peptides in aqueous solution have a very high affinity for ODS-silica. This property has been used to extract tritiated corticotropin, somatostatin and their metabolites from rat tissue homogenates and plasma in a batch procedure.[1,5] ODS-silica cartridges (Sep-Pak C_{18} cartridges, Water Associates) provide a convenient means of applying this methodology to the extraction of corticotropin-like peptides from rat pituitaries.

Rat pituitaries were removed, frozen in liquid nitrogen within 30 seconds of decapitation and stored at –40°C prior to extraction. Ten pituitaries (18 mg) were homogenized at 4° C in 20 ml aqueous formic acid (5% v/v), TFA (1% v/v), 1 *N* HCl and sodium chloride (1% w/v). The high acid content was present to ensure that all peptidases were inhibited, the formic acid and sodium chloride ensured a high yield by minimizing binding of peptide to structural protein and the TFA was added to increase the affinity of solubilized peptides for the ODS-silica. Following centrifugation of the homogenate the supernatant was passed four times through a C_{18} Sep-Pak cartridge. This was washed with 0.1% aqueous TFA and the peptide fraction was eluted with 3 ml of aqueous 80% acetonitrile containing 0.1% TFA.

The pituitary extract was immediately run on RP-HPLC by diluting the Sep-Pak eluate 4 fold with 0.1% TFA and pumping this directly onto the $C_{18}\mu$ Bondapak column. This was then eluted with a linear aqueous 20-60% acetonitrile gradient containing 0.1% TFA over 2 hours (Figure 1). Tritium labelled hACTH (100 ng; kindly provided by Dr. R. Wade, Ciba Labs, Horsham, U.K.[6]) was included in the extraction. It was observed that the structural integrity of the ^{3}H-ACTH was maintained and that the overall recovery was approximately 90%. Multiple peaks of ACTH immunoreactivity (midportion) including substantial amounts of an apparent high molecular weight form were observed. The main peak of immunoreactivity was contained in fractions 33-38. These fractions were combined, diluted 2 fold with 0.1% TFA and pumped back onto the same column. This was eluted with 25.6% acetonitrile containing 0.1% TFA (Figure 2). The main peak of ACTH immunoreactivity from Figure 1 was resolved into several components. Each peak coincides exactly with a uv absorbing material, indicating a high state of purity.

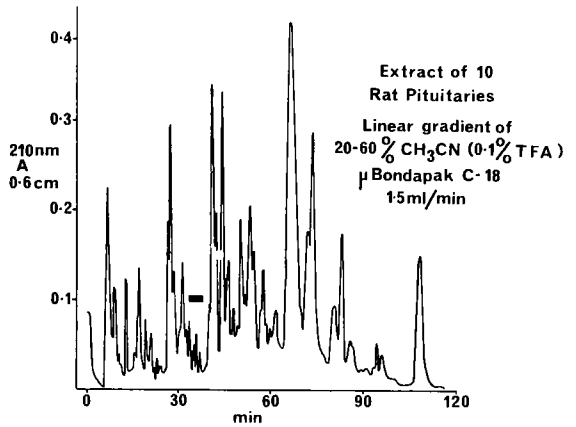

Fig. 1. Gradient elution RP-HPLC of an extract of 10 rat pituitaries; 1.5 ml fractions were collected. The solid bar represents fractions 33-38 which were rerun isocratically (see Figure 2).

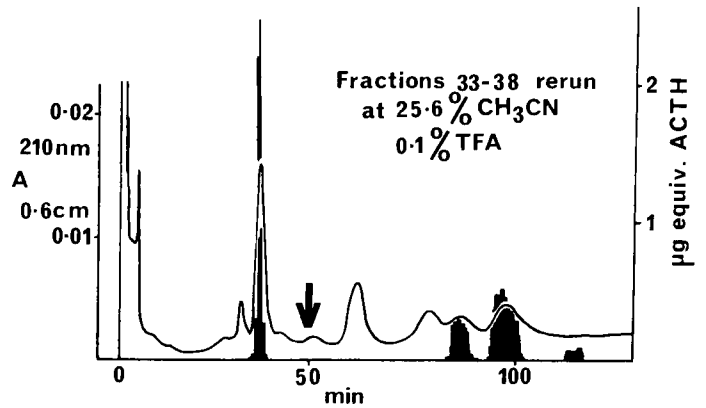

Fig. 2. Isocratic elution RP-HPLC of fractions 33-38 from Figure 1; 1.5 ml fractions were collected. Solid bars represent ACTH immunoreactivity. The arrow shows the elution position of ^{3}H-hACTH.

This preliminary study indicates that corticotropin-like peptides of the pituitary can be extracted and purified rapidly using only reversed-phase chromatography. The avoidance of lyophilization, gel-filtration, and ion-exchange chromatography ensures a high yield. The ODS-silica cartridge extraction method has been applied to the isolation of milligram quantities of high molecular weight forms of calcitonin from medullary thyroid carcinomas. Other applications include the extraction of immunoreactive ACTH from plasma and tissue culture media. These examples illustrate the potential of this methodology in both preparative and analytical studies.

Acknowledgements

We thank Ms. S. James for her skilled technical assistance. This work was supported by grants from the MRC of Canada (Grant No. 299-51) and the U.S. Public Health Service (Grant No. 293-83).

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PEPTIDE AND AMINO ACID ANALYSIS BY RP-HPLC

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Introduction

Reverse phase-high pressure liquid chromatography (RP-HPLC) has now become the tool of choice for the analysis of amino acid derivatives and synthetic peptides as well as for their isolation in pure form.[1-4] RP-HPLC has also been used for the isolation of naturally occurring peptides, such as the α-endorphin[1], β-endorphins, and related peptides[5] and pigeon pancreatic small somatostatin SS.[6]

While previous reports might not have addressed themselves to the recovery of peptides, it became obvious that certain families of peptides would elute from the commercially available columns almost quantitatively, whereas others would be unpredictably and partially displaced from the top of the column where they had been adsorbed. With the introduction of the TAAP buffers (Trialkyl ammonium phosphates) and more specifically the TEAP buffer (Triethylammonium phosphate[7]) these effects could be minimized. However, recoveries of larger proteins such as insulin or cytochrome C were not quantitative even when using TEAP with the more polar μBondapak CN columns. Independently, O-Hare and Nice,[8] studying hormonal polypeptides, have shown good resolution and recoveries while using an acidic phosphate buffer and a C_{18} derivatized Hypersyl column.

New techniques of derivatization of the silica (spherical 5 micron particles), high carbon loads, "capping" of the residual silanol groups and new packing technologies have led one of us (B.K., with support from Technicon Instrument Corporation) to develop state of the art columns, the performance of which, as applied to amino acids, peptides and proteins, is succinctly described here.

Materials and Methods

HPLC and solvent systems were those previously described.[7] TEAP, pH 2.25, was used for the separations described here even though dilute TEAP, (pH 6.50; dilution ½) was found to be significantly more appropriate for acidic peptides, such as human gastrin I (unpublished). HPLC columns (.46 x 15 or 25 cm) were packed with 5 micron spherical silica particles which had been derivatized (C_{18} coverage $> 3\ \mu M/m^2$: original surface area 200 m^2/g) and end capped with trimethyl chlorosilane. Original pore size *ca.* 100 A.

Results and Discussion

The main advantages of the columns which were used in the studies reported here are several:

1. Higher resolution, due to large plate counts/meter (60-80,000), was illustrated by three particularly difficult separations.

a) [D-Trp^8]-somatostatin and [D-Trp^8,D-Cys^{14}]-somatostatin which could not be separated under the conditions described in Reference 7, were resolved under the following conditions: one Supelcosil LC-18 (15 cm) column; 2 μg load of each tetradecapeptide; solvent system: A = TEAP, pH 2.25; B = 60% CH_3CN, 40% A; isocratic conditions 36% B; absorbance at 210 nm; flow rate 1.5 ml/min. Retention times were 18.4 and 19.8 min., respectively.

b) Phenylthiohydantoin (PTH) amino acids. Almost baseline separation of 24 PTH amino acids in one single run was achieved within 36 minutes. This separation is very sensitive to pH. At the optimum pH range of 3.10 to 3.20 PTH-Asp elutes just between PTH-Thr and PTH-Gly; it shifts toward the former at higher pH (3.27), toward the latter at lower pH (3.07). All other PTH amino acids are less sensitive to such small pH differences. Conditions were as follows: Column: Supelcosil LC-18 (0.46 x 15.0 cm); 50-800 pmoles load of each PTH amino acid in 10 μl MeOH/H_2O (90:10); solvent system: A = H_2O; B = TEAP pH 3.12, MeOH, CH_3CN (4:3:3); flow rate 1.5 ml/min.; Absorbance 268 nm; temp. 26 ± 1°C; gradient conditions: isocratic 31% B (6 min), 10 min linear gradient to 54% B, isocratic 54% B (6 min), 12 min linear gradient to 90% B, isocratic 90% B (5 min), 2 min linear gradient back to 31% B. The order of elution and retention times (sec) are as follows: PTH-Cys-SO_3H (274), PTH-His (350), PTH-Asn (372), PTH-Ser (432), PTH-Thr (468), PTH-Asp (518), PTH-Gly (548), PTH-Gln (576), PTH-Arg (618),

PTH-Glu (892), PTH-Ala (926), PTH-CMC (1006), PTH-HO-Pro (1102), PTH-Tyr (1222), PTH S-Me-Cys (1422), PTH-Pro (1522), PTH-Val (1632), PTH-Met (1660), PTH-Trp (1998), PTH-Ile (doublet: 2026), PTH-Phe (2068), PTH-Leu (2080), PTH-NLe (2128), PTH-Lys (2156). In developing a reliable system for the separation of PTH amino acids, we were conscious of the problems of repeatability without reproducibility common to most systems appearing in the literature. We addressed this problem by testing several Supelcosil-LC-18 columns and had the B buffer prepared by several investigators. Whereas the former endeavor has shown small variations from column to column, the second has shown that reproducibility in buffer composition could be achieved. A full report is in preparation.

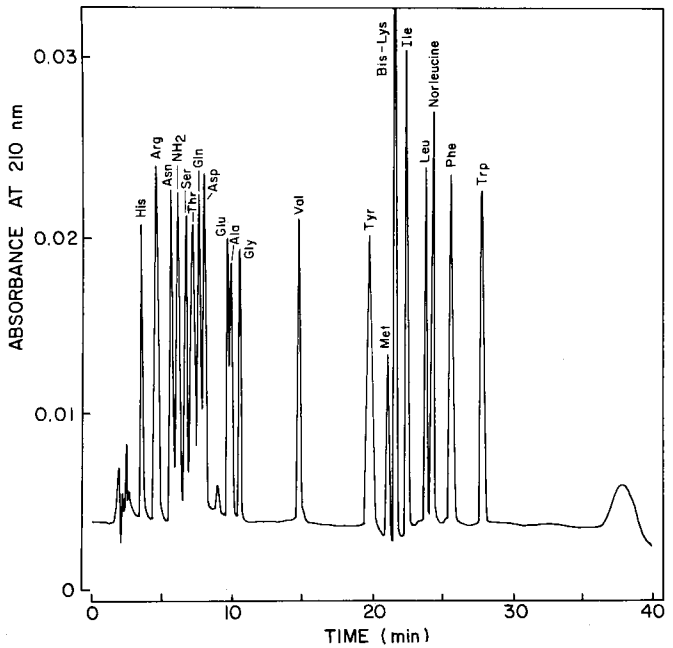

Fig. 1. RP-HPLC of L-Phe-L-X dipeptides. Column C_{18} — 2 #1 (0.46 x 25.0 cm); B.K.'s laboratory. Load *ca.* 1 nmole each L-Phe-L-X dipeptide; X = amino acid assigned to each peak. Solvent system: A = H_2O/TEAP pH 2.25 (1:1). B = H_2O/TEAP pH 2.25/CH_3CN (5:3:2). Gradient conditions: isocratic 10% B (40 sec) 6 min linear gradient to 45% B, isocratic 45% B (8 min) 2 min linear gradient to 100% B. Flow rate 1.5 ml/min. Sensitivity 0.04 aufs. Back pressure 3000 psi. Temperature 25 ± 1°C. Chart speed .5 cm/min.

c) Separation of L-Phe-L-X dipeptides. Two years ago[2] we showed that diastereoisomeric dipeptides of the type L-Phe-L-X and L-Phe-D-X, where X was one of the twenty common amino acids, could be separated thus allowing for a rapid quantitation of racemization. We also suggested

that such a system could be used for rapid amino acid analysis. Figure 1 demonstrates the feasibility of such analysis (see conditions in legend).

2. The high number of plates/meter which are available allow for shorter columns which in turn allow for shorter analysis time.

3. High recoveries have been observed for such oligopeptide classes as the hypothalamic releasing factors, glucagon, and insulin. The behavior of larger proteins on these columns is being investigated at the present time.

4. High column stability has been observed. It is probably due to the high C_{18} coverage and capping which protects the silica support against solvents which might otherwise slowly dissolve it. As a consequence, columns maintain high plate counts for long periods of time if proper care is taken.

Conclusion

Because of its inherent simplicity, versatility and resolutive power, RP-HPLC is one of the few analytical (preparative) techniques which is easily adaptable to many areas and whose potential is yet to be fully realized in the peptide/protein field.

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STRATEGIES FOR THE SEQUENCING OF PROTEINS BY GAS CHROMATOGRAPHIC MASS SPECTROMETRY

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Gas chromatographic mass spectrometry (GCMS) has been shown to be a powerful technique for the determination of the amino acid sequence of oligopeptides. The basic strategy is as follows:

Primary Degradation Fragment (2-50 amino acids)

↓ Partial hydrolysis

Complex mixture of di- to hexapeptides

1) 3N HCl/MeOH or CH_2N_2 3) B_2D_6/THF

2) CF_3-CO_2-CH_3 4) TMS-DEA

↓

Complex mixture of O-TMS polyamino alcohols

This mixture is injected into a gas chromatograph-mass spectrometer which generates the mass spectrum of each component. The sequence of each peptide component is deduced from the mass spectrum. As an example, the mass spectrum of the derivative of Ser-Met-Leu-Leu is shown in Figure 1.

The GCMS technique effectively complements the Edman degradation-based method. The GCMS data are equally reliable from all parts of the polypeptide, in contrast to Edman sequencing, which is very reliable at the N-terminus, but gets less so as one approaches the C-terminus.

A combination of GCMS sequencing and automated Edman degradation was used to sequence bacteriorhodopsin, the photosynthetic membrane protein from *Halobacterium halobium,*[2] in collaboration with H.G. Khorana, et al.[3,4] The hydrophobic membrane protein and its fragments were insoluble in aqueous buffers, restricting the use of

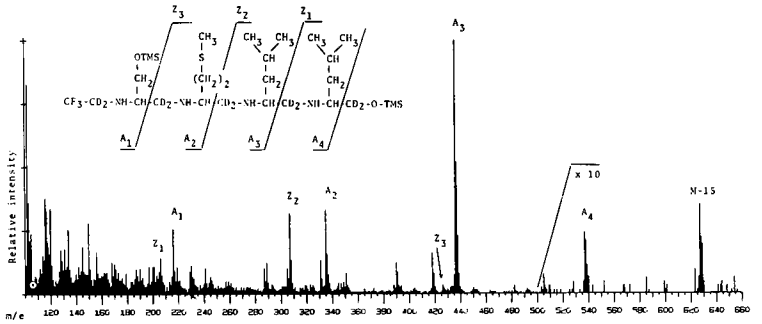

Fig. 1. Mass spectrum of Ser-Met-Leu-Leu derivative.

proteolytic enzymes. Therefore, the protein was cleaved chemically with CNBr. Each of the fragments was isolated and sequenced using a combination of GCMS and Edman techniques. Several of the CNBr fragments are shown in Figure 2.

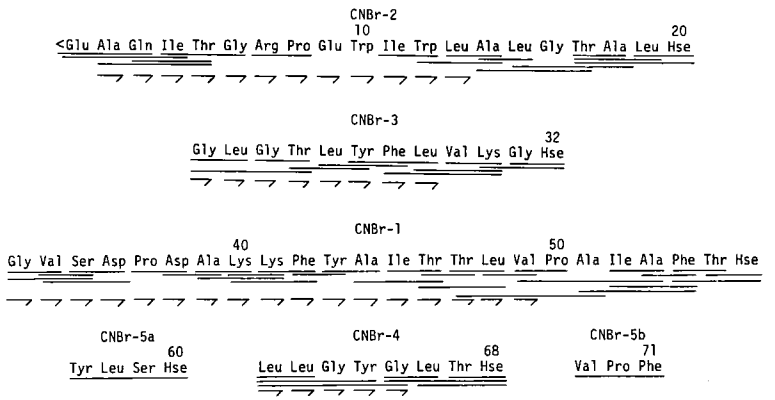

Fig. 2. CNBr fragments from the N-terminal region of bacteriorhodopsin. Peptides identified by GCMS are underlined; arrows indicate Edman cycles.

The N-terminal sequences were determined by Edman degradation; however, these sequences could not be extended further due to washout of the small hydrophobic peptides from the sequencer cup. The C-terminal sequences were determined from the GCMS data by searching for homoserine-containing peptides and constructing sequences toward the N-terminus using overlapping peptides. In addition, the GCMS data provided peptides from the N-terminal regions, which increased the reliability of these results.

New methodology, including prolonged enzymatic digestion of suspensions of fragments and multiple-time acid hydrolysis, had to be developed to generate fragments for GCMS.

Small CNBr fragments, e.g. CNBr-5a and -5b (Figure 2), were sequenced by GCMS as a mixture without purification or further fragmentation.

Having determined the sequences of the CNBr fragments, one must correctly order these peptides. This is usually done by isolating overlap peptides from a second digest. Because of the insolubility of bacteriorhodopsin, such an approach was difficult. Therefore, the CNBr fragments were ordered by specifically identifying Met-containing peptides by GCMS in a partial acid hydrolyzate of very large bacteriorhodopsin fragments. In this case, it is not necessary to identify every component in the resulting very complex mixture (Figure 3).

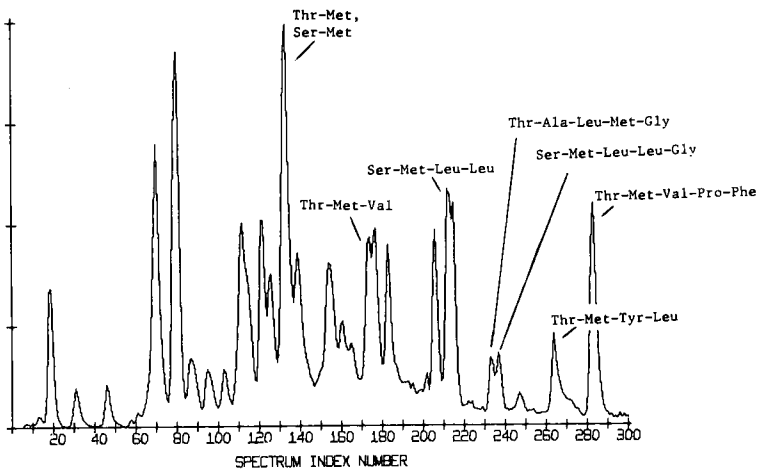

Fig. 3. Total ionization plot derived from res. 1-71 of bacteriorhodopsin. Only Met-containing peptides are labeled.

The complete sequence of 248 residues of bacteriorhodopsin has been determined in this way.[4] The use of these two independent sequencing methods increased the speed and reliability of the structure determination; difficulties due to the hydrophobic nature of the protein were overcome by use of primarily chemical cleavages.

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COMPLETE PRIMARY STRUCTURE OF A PROLINE-RICH PHOSPHOPROTEIN (PRP-4), A POTENT INHIBITOR OF CALCIUM PHOSPHATE PRECIPITATION IN HUMAN PAROTID SALIVA

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Human saliva is supersaturated with respect to apatitic calcium phosphate,[1] a property which provides important protection for the teeth. Of the approximately forty macromolecules present in human saliva,[2] two phosphorylated polypeptides have been found to inhibit precipitation of calcium phosphate salts.[3,4] This unusual property prevents the occurrence of certain undesirable consequences of supersaturation. The resulting stabilized, supersaturated saliva protects the teeth by providing driving forces which, firstly, suppress dissolution of enamel into its fluid environment, secondly, enhance mineral stability during attack of the teeth by the acidic end-products of microbial metabolism, and thirdly, are responsible for the frequently observed recalcification of early subsurface carious lesion.[5] The complete primary structure of one of these inhibitors, a tyrosine-rich phosphopeptide, named statherin,[6] has been reported and the structure-function relationships of both kinds of phosphoproteins have been discussed.[4,6,7] We now report the complete structure determination of the most potent of the second class of phosphoprotein inhibitors, a group of anionic proline-rich phosphoproteins (PRP), of which four major (PRP 1-4)[8,9] and a number of minor members exist in saliva.

Methods

1. **Detection and Purification of PRP-4:** PRP-4 was detected in human parotid saliva by an assay which depends on the ability of the PRP proteins to inhibit crystal growth of calcium phosphate salts and to inhibit hydrolysis of dicalcium phosphate dihydrate to more basic calcium phosphate salts.[10] Purified PRP-4 was judged homogeneous by

polyacrylamide gel electrophoresis[8] and automated NH_2-terminal sequence analysis.[7]

2. **Cleavage of PRP-4:** PRP-4 was digested with pyroglutamyl-amino peptidase for removal of the N-terminal residue prior to automated liquid phase sequence analysis. Tryptic and chymotryptic peptide fragments of PRP-4 were purified under conditions previously described for statherin[6] as were C-terminal analyses using carboxypeptidases A and B.[6] PRP-4 was chemically cleaved with 0.03N HCl for 2 hours at 105°C. Cleavage at both histidine residues was accomplished using N-bromosuccinimide.[11]

3. **Structural Analyses of PRP-4:** Amino acid analysis,[12] automated[13] and manual liquid phase sequence analysis,[14] with modification,[15] and determination of phosphate content[16] was performed under identical conditions as for statherin.[6] Automated solid phase sequence analyses were performed on a Sequemat model Mini 15 sequencer.[17] Peptides of PRP-4 produced by cleavage with 0.03N HCl were attached to diisothiocyanate glass (Pierce Chemical Company, Rockford, IL.) as described[18] without prior purification. Following Edman degradations, the PTH amino acids were identified by gas,[19] high pressure liquid,[20] and thin layer chromatography.[21]

Results and Discussion

The structure of PRP-4 was elucidated by a combination of sequencing techniques. Residues 2-54 were determined by automated liquid phase sequence analyses of PRP-4 (following enzymatic removal of pyroglutamate) and on peptides CT-1 and T-2. These analyses established the identity of the two phosphoserine residues at position 8 and 22. Automated solid phase sequencing techniques were employed to selectively sequence, without prior purification, the singular lysine containing peptide, L-1 (Figure 1), produced by dilute acid cleavage of PRP-4. After the first degradation cycle, only the lysine-containing peptide remained attached to DITC-glass in the solid phase sequencer. Sequence analysis of peptide L-1 established the PRP-4 sequence from residues 52-74. Peptides T-3 and T-4, composing the remaining C-terminal region of PRP-4, were sequenced manually and found to possess identical N-terminal 11 residue sequences (Figures 1 and 2).

PRP-4 was cleaved with N-bromosuccinimide at the two histidine residues to establish the order of peptides T-3 and T-4 and to complete the PRP-4 sequence. Peptides NBS-1 and NBS-2 (Figure 1) were purified

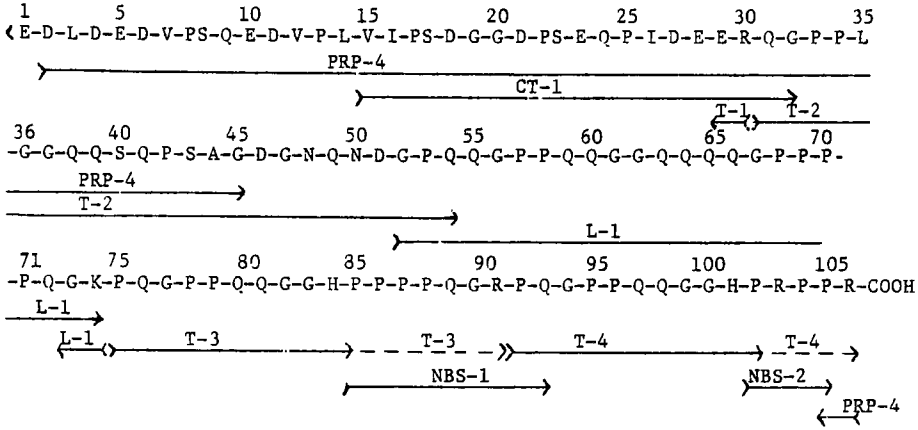
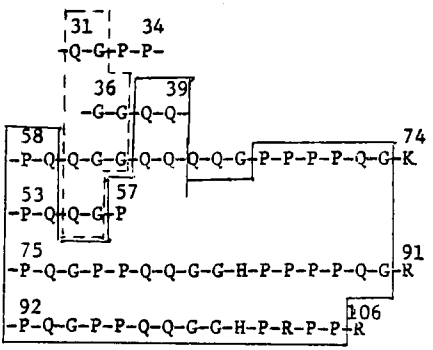


Fig. 1. The complete amino acid sequence of PRP-4. <E indicates pyroglutamate, PS, phosphoserine, CT, peptide fragment produced by digestion with chymotrypsin, T, peptides produced by tryptic digestion, L-1, peptide produced by cleavage of PRP-4 with 0.03N HCl, NBS — peptides produced by cleavage of PRP-4 with N-bromosuccinimide.

Fig. 2. Internal sequence homologies in PRP-4. Solid boxed area (□) indicates primary area of identity and broken area (⬚) indicates smaller secondary area of identity in PRP-4.



and sequenced for 9 and 5 residues, respectively. Manual sequence analysis of NBS-1 provided sequence overlaps for and established the order of peptides T-3 and T-4 since amino composition indicates that T-3 contains one additional Pro, Gly and Gln, but one less Arg. Sequence analysis of NBS-2 completed the PRP-4 sequence through residue 105. All residues found by sequence analysis of PRP-4 and its fragment plus the C-terminal Arg found by carboxypeptidase digestion of PRP-4 account for all the residues in PRP-4 found by amino acid analysis.

A number of structural features about the PRP-4 sequence are noteworthy. Firstly, PRP-4 possesses a highly charged amino terminal segment and inhibitory activity is associated with the two phosphoserine residues in positions 8 and 22. The negative charge of the amino terminal 29 residues contrasts strongly with the positive charge and hydrophobicity in the remainder of the molecule. Secondly, both the

Lys_{74}-Pro_{75} and the Arg_{91}-Pro_{92} bonds, surprisingly cleave readily with trypsin. This suggests some unusual conformational stress within the PRP-4 sequence. Thirdly, the C-terminal region and limited segments in the N-terminal half in PRP-4 show remarkable duplication of sequence (Figure 2) suggesting gene replication in the transcription of the PRP-4 protein; it is significant in this regard that Gln, Pro and Arg can be interchanged by a single point mutation.

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STRUCTURE AND BIOLOGICAL ACTIVITY OF VIROTOXINS

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The most common death cap in Europe, the green Amanita phalloides contains two groups of toxic peptides, the amatoxins and the phallotoxins. Similarily, the white species, Amanita virosa, contains α-amanitin and a few neutral phallotoxins, but lacks the variety of closely related, homologous toxins found in the green species. On the other hand, there are some toxins present in this white Amanita, which are unknown in the green species. Among them amaninamide,[1] a new amatoxin, and the virotoxins which differ from amatoxins and phallotoxins as well.[2] The main component, viroisin, has been elucidated in structure. It exhibits the same toxicity as phalloidin.

The virotoxins were detected by their unique UV absorption spectrum, indicating the presence of a 2′-alkylsulfonyltryptophan moiety (Figure 1). This spectrum was known from a synthetic oxidation product of phalloidin, the phalloidinsulfone.[3] It can be clearly distinguished from that of 2′-alkylthiotryptophan as in the phallotoxins; from that of 2′-alkylsulfoxytryptophan as present in amanin, amaninamide and in the synthetic phalloidinsulfoxides; as well as from that of the 2′-alkylsulfoxy-6′-hydroxy-tryptophan, which is found in the amatoxins. The chromophoric systems of all naturally occurring toxic peptides of the Amanita species are compiled in Figure 1, together with their UV absorption spectra. It must be noted that in the group of virotoxins some minor components show the spectrum of 2′-alkylsulfoxytryptophan, i.e. they must be sulfoxides.

The main component of the virotoxins was named viroisin. Its structure has been elucidated and is shown in Figure 2. Like the phallotoxins, the virotoxins possess a γ-hydroxylated leucine unit, which allows selective cleavage of the neighbouring peptide bond. By treatment of viroisin with trifluoroacetic acid, a secocompound was prepared which could be subjected to Edman degradation. For the first three positions, the PTH-derivatives of valine, threonine and serine, respectively, were

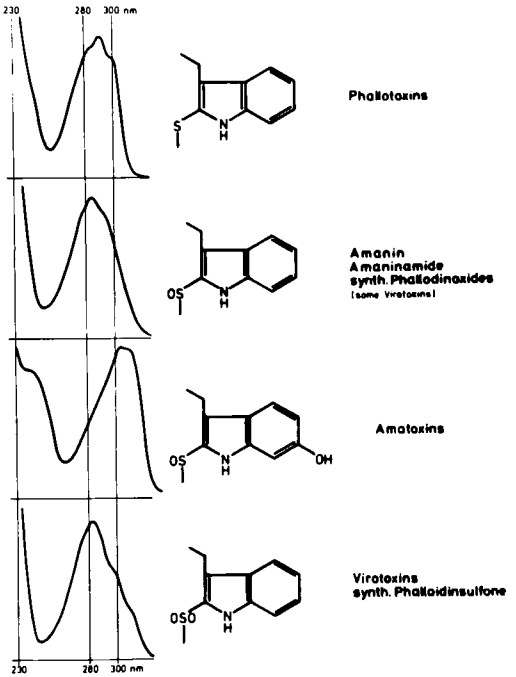

Fig. 1. Structure and UV absorption spectra in methanol of the chromophores of natural and synthetic Amanita toxins.

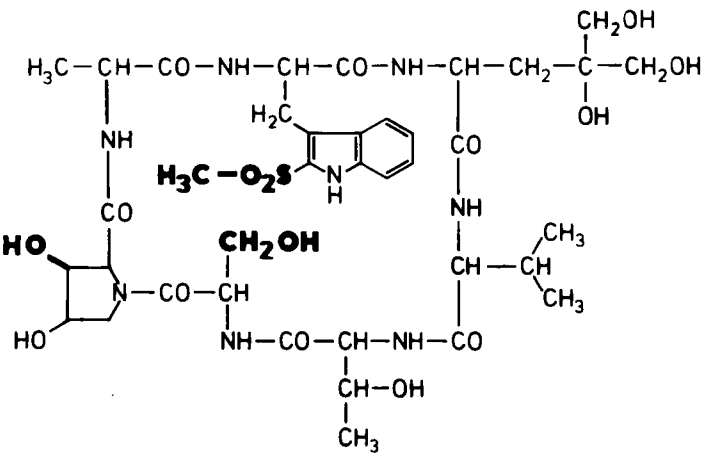

Fig. 2. Structure of viroisin. Those moieties differing from the structure of phallotoxins are in bold type.

identified by tlc as well as ms. The PTH-derivative of the amino acid in position 4 could not be identified by tlc. The unknown amino acid was separated from the hydrolysate and, after dansylation, was identified as dihydroxyproline. This result is in good agreement with the observation that the PTH-derivative of this amino acid as obtained by Edman degradation and analysed by mass spectrometry yielded the molecular ion $M^+ = 228$, corresponding to PTH-pyrrolcarbonic acid formed from PTH-dihydroxyproline by dehydration. In the following two degradation steps PTH-alanine and PTH-methylsulfonyltryptophan could be characterized by either tlc or ms. The last amino acid was identified as the same hydroxylated leucine present in phallisin, i.e. the γ, δ, δ'-trihydroxyleucine.

Further experiments became necessary to determine the stereochemistry of the dihydroxylated proline. The diol resisted IO_4^--oxidation and therefore could not be of a *cis* configuration (Figure 3, I and II). Among the two trans configurations, III and IV, compound III was excluded by direct comparison with an authentic sample isolated from diatom cell wall proteins and kindly provided by Dr. B. Volcani.[4] Proof that structure IV is present in viroisin was obtained by H^1nmr spectra at 360 MHz. The data for Hα, Hβ and Hγ in III indicate that the *cis* vicinal coupling constants are smaller than 1.5 Hz. Comparably, in the spectrum of IV there is only one large coupling of 3.8 Hz, all others being below 1.4 Hz. Since the configuration of one of the Hδ protons must be *cis* to Hγ, the larger coupling identifies the Hγ signal while the smaller couplings indicate *trans* relationships between Hγ and Hβ, and Hβ and Hα, respectively.

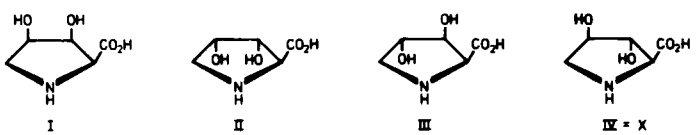

Fig. 3. The four possible isomers of 3,4-dihydroxy-L-proline

Like the phallotoxins, the virotoxins bind to rabbit muscle actin with high affinity. Using equilibrium dialysis, competitive binding experiments of a virotoxin mixture with an actin·[^{3}H]-demethylphalloin complex were performed. As shown in Figure 4, the concentration of the virotoxins necessary to displace 50 % the labelled toxin is about 1.5 times lower than that required of phalloidin. Since phalloidin is the toxin with highest affinity to actin, the virotoxins form even more stable complexes with actin than the phallotoxins do. This is remarkable, because the

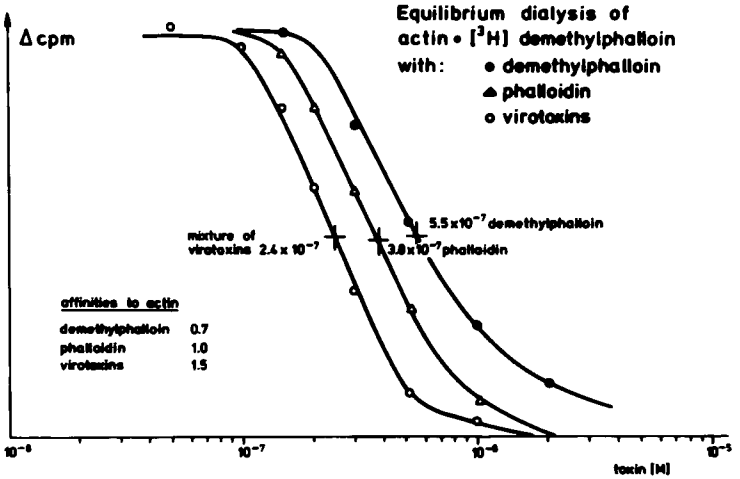

Fig. 4. Relative affinity to actin of two phallotoxins (•, Δ) and a mixture of virotoxins (o) as determined by equilibrium dialysis. High Δcpm values mean low extent of substitution of [^{3}H]-demethylphalloin bound to actin by the unlabelled toxin.

virotoxins lack the rigid bicyclic structure of the phallotoxins, which appears to be a prerequisite for their biological activity. Therefore, we assume that in the case of the virotoxins the "toxic" conformation is induced by the interaction with actin. The free energy for such conformational change is most probably provided by two additional hydrogen bonds involving the hydroxylic groups of serine and 3,4-dihydroxyproline, which are absent in phallotoxins. In fact, a monocyclic analogue of the virotoxins prepared from phalloidin, the 2′-methylsulfonyldethiophalloidin, which differs from the virotoxins by lacking the OH groups of serine and dihydroxyproline, proved to be devoid of any biological activity.

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CYCLIC PEPTIDE-METAL SALT COMPLEXES

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Interactions between cyclic peptides and metal ions can provide useful structural information on the stereochemical requirements for binding and determine factors responsible for coordination and the way in which it occurs. As a part of our research on peptide-type ion carriers, we report the crystal structure analyses of cyclodisarcosyl complexes with Cu(II) and Ag(I) and compare the results with those previously obtained for the lithium complex.[1]

Preparation of the Cu(II) and Ag(I) complexes from ethyl acetate solution[2] gave crystals with the following stoichiometries: $Cu(H_2O)_6 \cdot (ClO_4)_2 \cdot 2$(*cyclo*-$Sar_2$) and $AgNO_3 \cdot \frac{1}{2}$(*cyclo*-Sar_2). The Cu(II) complex crystallizes in the monoclinic $P2_1/c$ space group with cell dimensions a = 13.879A, b = 14.504A, c = 13.083A, β = 90.98° and Z = 4; the Ag(I) complex in the triclinic P^1 space group with c = 5.410A, b = 7.562A, c = 8.020A, α = 92.06°, β = 105.07°, γ = 104.60° and Z = 2. Data collection, structure determination and refinement were carried out for both structures on the CAD4 SDP11/34 diffractometric system of the Centro di Metodologie Chimico Fisiche of the University of Naples. Both structures have been solved with the aid of the Patterson map. The structures were refined by full-matrix least-squares, treating the thermal vibration of all non-hydrogen atoms anisotropically to a residual of 0.084 and 0.058 for 3802 and 939 observed data for the Cu(II) and Ag(I) complexes, respectively.

In Figures 1 and 2 the modes of packing for Cu(II) and Ag(I) complexes are reported.

The independent crystallographic unit in the Cu(II) complex is composed of six water molecules octahedrically coordinated to the Cu(II) ion, two tetrahedral perchlorate ions, and four independent halves of cyclodisarcosyl molecules lying on centers of symmetry. All available hydrogens of water molecules are involved in hydrogen bonding as donors and all carbonyl oxygens of the cyclic peptides functions as

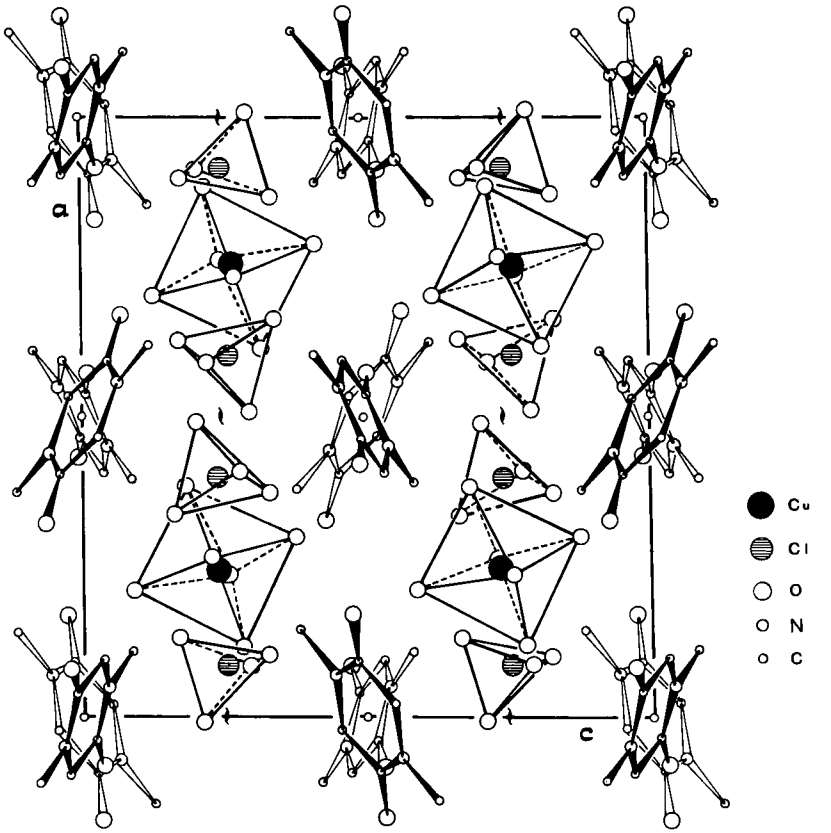

Fig. 1. Mode of packing as viewed along the *b* axis for the Cu(II)-complex.

acceptors. Four oxygen atoms, two for each perchlorate ion, participate also in the hydrogen bonding scheme. The O-H···O distances are in the range 2.66 - 3.01 A.

For the Ag(I) complex the independent unit consists of a metal ion surrounded by oxygen atoms either of the trigonal nitrate anions or the cyclodisarcosyl molecules. The cyclic peptide lies on a center of symmetry. Three oxygen atoms, one of the cyclosarcosyl and two of a nitrate ion, bridge two metal ions. In this rather complicated three-dimensional network, the metal ion experiences different types of contacts with the surrounding oxygen atoms: two short distances (2.35 and 2.41 A), four intermediate (between 2.57 and 2.68 A) and one rather long (2.90 A). The description of the Ag(I) coordination cannot be done simply in terms of regular geometries.

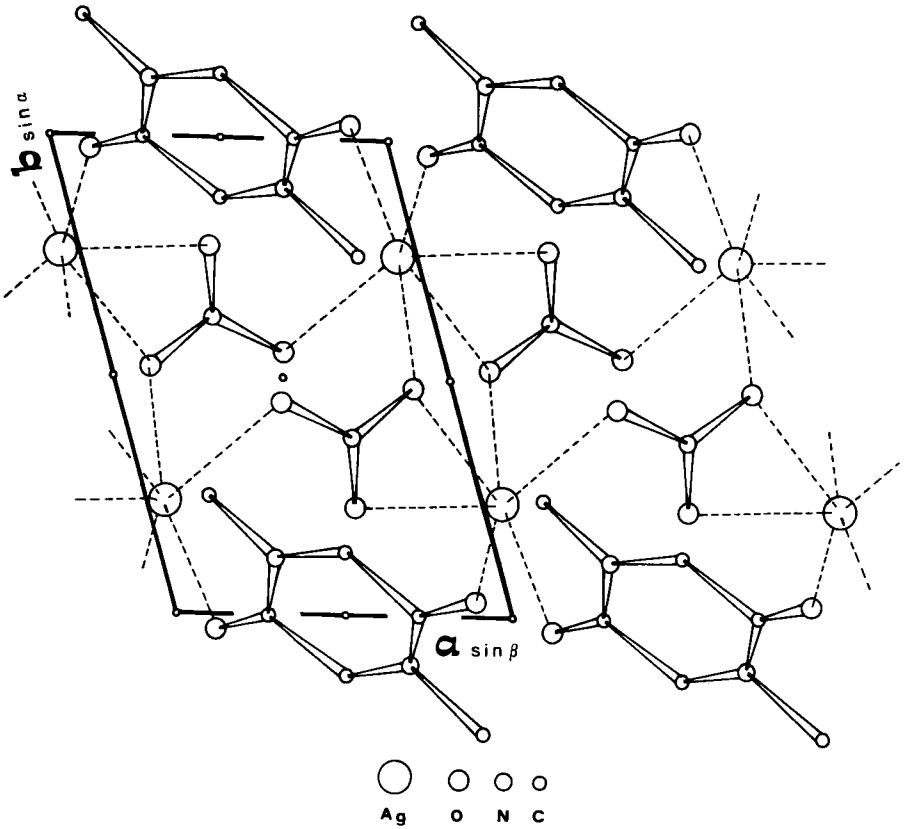

Fig. 2. Mode of packing as viewed along the c axis for the Ag(I)-complex.

The Ag(I) ion, as in the case of lithium, directly interacts with the peptide moiety while the Cu(II) is shielded from these interactions by the coordinated water molecules. The existence of a $Cu(ClO_4)_2 \cdot 2 \cdot (cyclo\text{-}Sar_2)_2 \cdot 2H_2O$ complex,[2] however, may be indicative of a possible direct interaction between Cu(II) and peptide molecules. In these three peptide-metal salts complexes the crystals are composed of parallel stackings of organic and inorganic layers, the alternation of which is reflected in the observed difference in stoichiometry. In all cases the cyclodisarcosyl ring may be described as nearly planar or a very flattened chair form.

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CONFORMATION OF THE CYCLIC HEXAPEPTIDE (D-PHE-PRO-VAL)$_2$:$C_{38}H_{50}N_6O_6$

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The structure of the synthetic cyclic hexapeptide (D-Phe-Pro-Val)$_2$ (Figure 1) was determined primarily to add to the compendium of information being assembled concerning the solid state conformation of cyclic polypeptides containing prolyl groups. In this case the cyclic backbone of the molecule is not stabilized by 4→1 intramolecular hydrogen bonds but rather by a water bridge between O_2 and O_5.

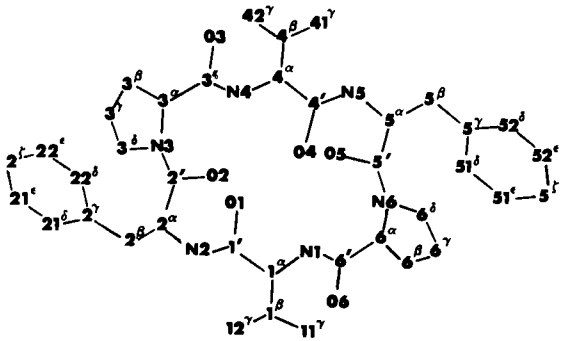

Fig. 1. (D-Phe-Pro-Val)$_2$

The peptide crystallizes from a DMSO:H_2O mixture in the monoclinic space group $P2_1$ with a = 14.389A, b = 11.928A, c = 13.511A, β =111.2°, $d_{calc.}$ = 1.22 g/cc and Z = 2. The structure was solved by direct methods.[1,2] During refinement one molecule of DMSO and 2 molecules of H_2O were found in difference maps. Hydrogen atoms were placed at ideal calculated positions and included in the refinement as constant parameters. The final R-factor (agreement between observed and calculated structure factors) was 0.099 for the full set of 3709 observed reflections. Atomic coordinates for the non-hydrogen atoms are given in Table I.

The molecule has approximate 2-fold symmetry which can be seen in the stereodiagram[3] in Figure 2. Bonds lengths and angles are all within normal ranges for a molecule of this type. All peptide bonds have the *trans* conformation. The torsion angles illustrated in Figure 4 indicate the

Table I. Fractional Coordinates

Atom	x	y	z	Atom	x	y	z
N1	0.2400	0.6495	0.1547	C41$^{\gamma}$	0.5407	1.0683	0.2005
C1$^{\alpha}$	0.2569	0.5879	0.0673	C42$^{\gamma}$	0.6943	1.0780	0.3674
C1$^{\beta}$	0.1869	0.6358	-.0403	C4'	0.4487	0.9221	0.3278
C11$^{\gamma}$	0.0790	0.6193	-.0578	O4	0.3954	0.8699	0.2472
C12$^{\gamma}$	0.2108	0.5860	-.1328	N5	0.4095	0.9690	0.3947
C1'	0.3648	0.6036	0.0795	C5$^{\alpha}$	0.3062	0.9529	0.3776
O1	0.4015	0.6954	0.0829	C5$^{\beta}$	0.2856	1.0036	0.4735
N2	0.4187	0.5100	0.0859	C5$^{\gamma}$	0.1790	0.9950	0.4646
C2$^{\alpha}$	0.5225	0.5197	0.0981	C51$^{\delta}$	0.1142	1.0846	0.4247
C2$^{\beta}$	0.5717	0.4064	0.1014	C52$^{\delta}$	0.1415	0.8991	0.4966
C2$^{\gamma}$	0.6772	0.4147	0.1097	C51$^{\epsilon}$	0.0168	1.0788	0.4149
C21$^{\delta}$	0.7020	0.4348	0.0182	C52$^{\epsilon}$	0.0418	0.8965	0.4866
C22$^{\delta}$	0.7571	0.4091	0.2048	C5$^{\zeta}$	-.0198	0.9829	0.4472
C21$^{\epsilon}$	0.7990	0.4479	0.0291	C5'	0.2817	0.8274	0.3670
C22$^{\epsilon}$	0.8524	0.4199	0.2134	O5	0.3393	0.7623	0.4281
C2$^{\zeta}$	0.8750	0.4413	0.1237	N6	0.1958	0.7966	0.2919
C2'	0.5795	0.5841	0.2029	C6$^{\alpha}$	0.1613	0.6800	0.2877
O2	0.5676	0.5519	0.2859	C6$^{\beta}$	0.0502	0.6927	0.2550
N3	0.6421	0.6638	0.2034	C6$^{\gamma}$	0.0268	0.7902	0.1869
C3$^{\alpha}$	0.7174	0.7017	0.3041	C6$^{\delta}$	0.1155	0.8665	0.2231
C3$^{\beta}$	0.7982	0.7489	0.2677	C6'	0.1859	0.6054	0.2077
C3$^{\gamma}$	0.7499	0.7785	0.1605	O6	0.1542	0.5103	0.1959
C3$^{\delta}$	0.6628	0.7090	0.1105	W1	0.4579	0.5801	0.4183
C3'	0.6807	0.7926	0.3630	W2	0.1089	0.3703	0.3533
O3	0.7343	0.8162	0.4520	S (DMSO)	0.3212	0.2291	0.1973
N4	0.5970	0.8453	0.3091	O (DMSO)	0.3426	0.2964	0.1144
C4$^{\alpha}$	0.5594	0.9378	0.3568	C (DMSO)	0.1926	0.2341	0.1661
C4$^{\beta}$	0.5843	1.0538	0.3234	C (DMSO)	0.3560	0.3111	0.3141

deviations from planarity for the amide bonds, particularly in Pro^3 where the ω torsion angle is $-161.6°$ corresponding to a twist of 18.4°. The overall conformation of the molecule appears to be the same in solution[4] as it is in the solid state.

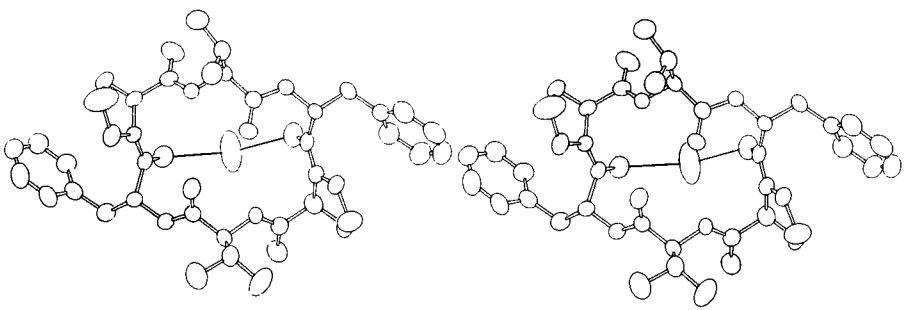

Fig. 2. Stereodiagram based on the experimentally determined coordinates.

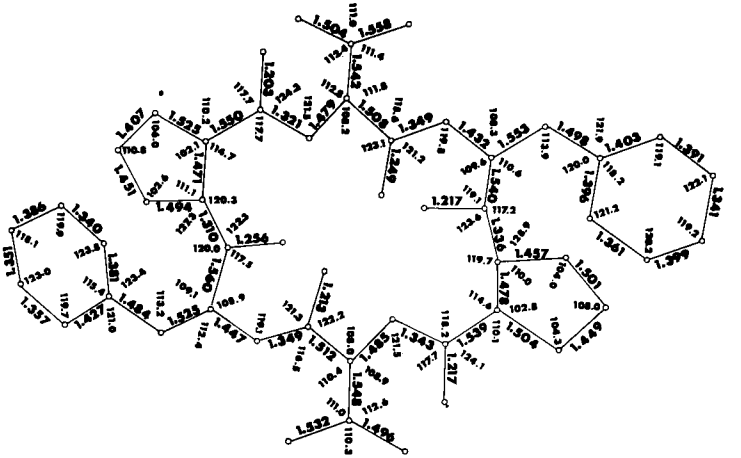

Fig. 3. Bond lengths and angles. In the backbone the esd for bond lengths is ~0.004A and for bond angles it is ~0.3°. Standard deviations are somewhat higher for the side groups.

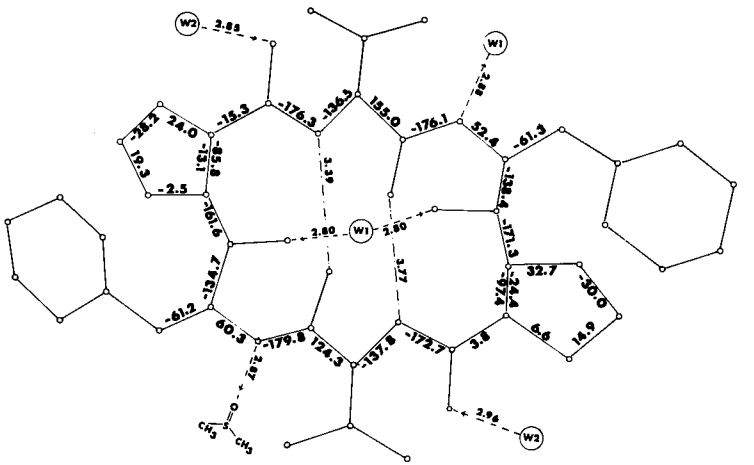

Fig. 4. Torsion angles, hydrogen bond lengths, and selected intramolecular approaches.

There is no intra- or intermolecular peptide-peptide hydrogen bonding in this crystal. In cyclic hexapeptides 4→1 intramolecular hydrogen bonding often occurs.[5,6,7] However, it has been noted that in cyclic hexapeptides containing prolyl residues, the N(1,4)···O(4,1) distances are usually quite long, ranging from 3.04-3.56A[8,9,10] while the O(1)···O(4) approach is very close (2.84-2.90A). In the present molecule the N(1)···O(4) and N(4)···O(1) distances are 3.39A and 3.77A,

respectively, precluding any hydrogen bond formation, while the O(1)···O(4) distance is only 3.02A. It is interesting to note that N(1), N(4), O(1) and O(4) do not participate in any hydrogen bonding (also predicted from solution studies[5]). However, the conformation of the backbone is stabilized in a rather unusual fashion by a water bridge forming hydrogen bonds to O(2) and O(5), Figure 4. Packing forces in the crystal are primarily hydrogen bonds between peptide and solvent molecules (DMSO and H_2O). The type of hydrogen bonding and the bond lengths are shown in Figure 4.

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A DIFFERENCE FOURIER ANALYSIS AT 4.0-A RESOLUTION OF THE SEMI SYNTHETIC COMPLEX FORMED BY RESIDUES 1-118 AND 111-124 OF BOVINE PANCREATIC RIBONUCLEASE

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Successive digestion by pepsin and by carboxypeptidase A of bovine pancreatic ribonuclease (RNase A) removes six residues from the COOH-terminus of the molecule to provide a shortened chain, RNase 1-118, which is devoid of enzymatic activity.[1] Complementation of this inactive segment with a chemically-synthesized tetradecapeptide comprising the COOH-terminal sequence of RNase A, *viz.*, residues 111-124, results in the formation of a semisynthetic noncovalent complex exhibiting 98% of the enzymatic activity of RNase A.[2,3] The preparation and characterization of several analogues of the complex which contain structural modifications within the tetradecapeptide segment and possess altered steady-state kinetics has been reported.[4,5,6]

Crystals of the complex of a size and order suitable for high resolution X-ray diffraction analysis now have been obtained under the following conditions: 9.5 x 10^{-4} M RNase 1-118 (1.2% by weight), 9.9 x 10^{-3} M RNase 111-124 (1.5% by weight), 3.0 M CsCl, 28% saturated $(NH_4)_2SO_4$, 0.1 M ammonium acetate, pH 5.7, and 5 x 10^{-3} M 1,10-phenanthroline.[7] The crystals, designated form D, belong to the trigonal space group $P3_221$ and have unit cell dimensions of a and b = 64.4 A, c = 64.5 A and $\gamma = 120°$. They are isomorphous with form M of RNase A[8] as well as form W[9] and form R[8] of RNase S. The crystals diffract sufficiently well to permit the determination of a structure to a resolution of at least 3.0 A.

An electron density map for the complex was determined to a resolution of 4.0 A by combining an appropriate set of diffraction intensity measurements with phase angles previously determined for crystal form M of RNase A.[10] Individual molecules could be delineated

with very little reference to known atomic coordinates for RNase A[10] while the remarkably large solvent channels first observed in form M of RNase A and anticipated for form D on the basis of its high solvent content were clearly evident. Regarding the structure at the COOH-terminal region of the molecule, it was found that the positions for the ten alpha carbon and alpha amino nitrogen atoms of residues 116 through 120, taken from the atomic coordinates calculated for RNase A, all fell within regions of significant electron density. The situation with respect to residues 111 through 115 was more complex. Three pertinent sets of coordinates are not available, *viz.*, those for the alpha carbon atoms of residues 112 and 113 and that for the alpha amino nitrogen atom of residue 113. Of the seven remaining atoms, only the alpha amino nitrogen of residue 115 fell outside a region of significant electron density. Coordinates are not available for any of the atoms of residues 121 through 124.

A difference map with respect to RNase A was then calculated to 4.0-A resolution. Several regions of significant positive and negative difference density were apparent; there was a predominance of positive difference density, which had been expected in view of the presence of eight redundant residues (6.5% of the total number) in RNase 1-118:111-124. The most prominent feature of the difference map was an extensive region of positive density located between and below the positions for the alpha carbon atoms of residues 111, 114, and 116. Directly adjacent to the most intense portion of this positive density, located between the positions of the alpha carbon atoms of residues 111 and 114, appeared the most intense negative density in the map. In contrast, no significant difference density was associated with the regions corresponding to the positions occupied by the alpha carbon atoms of residues 116 through 120.

Among the molecular structures which could result in the observed electron density distributions in both the absolute and difference maps, we presently favor the following one, primarily because it also correlates well with certain studies in solution. In this model residues 116 through 120 of the tetradecapeptide precisely occupy the positions assumed by these same residues in RNase A. This strict alignment from residue 120 through residue 116 corresponds very well with the observations of Gutte et al.[3], who studied the effect of varying the length of the complementation peptide between the heptapeptide comprising residues 118 through 124 and the tetradecapeptide comprising residues 111 through 124 and observed the critical role played by the valine-116

residue of the peptide moiety in both stabilizing the complex and inducing a catalytically efficient structure. Such a model necessarily excludes the possibility that residues 116 through 118 of polypeptide 1-118 occupy the positions assumed by these residues in RNase A. The absence of positive difference density in the nearby region suggests that residues 116 through 118 of polypeptide 1-118 may not be well-ordered in the crystal of the complex. The electron density distribution for the remaining redundant portion of the structure, i.e., residues 111 through 115, is too complex to interpret at a resolution of 4.0 A and will have to await the preparation of maps at a resolution of 3.0 A and possibly further crystallographic study of derivatives in which the structure of the tetradecapeptide is modified. In regard to this latter possibility it may be noted that crystals of RNase 1-118:(3-nitrotyrosine^{115})111-124[11] of a size suitable for X-ray diffraction analysis have now been grown in our laboratory.

Acknowledgement

The work reported here was supported in part by a grant from the National Science Foundation.

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TRI-HALOGENATED PHENYLALANINE FOR TRITIUM LABELING OF POLYPEPTIDE HORMONES AND FOR STRUCTURE-ACTIVITY STUDIES

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Introduction

One of the standard methods for studying hormone-receptor interactions is the binding assay with radioactively labeled ligands. Often the native hormone is iodinated with ^{125}I by various techniques,[1,2] but this method has the drawback of changing the physicochemical and pharmacological properties of the hormone and gives inhomogeneous products. These disadvantages have encouraged the increasing use of tritiated hormones, although scintillation counting and much lower specific activities have to be accepted. In many cases tritium has been introduced by catalytic tritiation of a halogenated[3,4] or unsaturated precursor.[5] For smaller peptides and binding studies at concentrations down to 10^{-10} M it is difficult to obtain the required specific activity, especially in peptides without tyrosine in which two ^{3}H are possible. We therefore undertook to develop a polyhalogenated phenylalanine. Phenylalanine is a frequent amino acid and the tritiated analogue would not give side-chain protection problems or synthetic restrictions except for hydrogenation.

Amino Acid Synthesis

We have recently described[6] the use of 4'-amino-3',5'-diiodo-L-phenylalanine as a precursor for radioactive photoaffinity labels. This derivative was very unreactive to further reactions on its aromatic amino group. The sterically less hindered 4'-amino-3',5'-dibromophenylalanine was therefore synthesized and a third halogen was introduced by the Sandmeyer reaction.

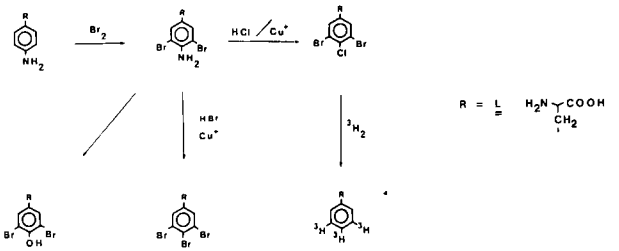

Fig. 1. Reaction scheme for the synthesis of tri-halogenated and tri-tritiated phenylalanine.

4′-Amino-L-phenylalanine was synthesized as previously reported[7] and brominated in 75% acetic acid. The crude product was extracted with a strongly acidic ion exchange resin and isolated in a yield of 50.4%. The product was dissolved in HCl (or HBr), diazotized and $Cu^{I}Cl$ (or CuBr) added. After evaporation of the solvent the product was isolated with the use of an ion exchange resin, and the amino group protected (t-Boc-dicarbonate). The Boc-derivative was chromatographed on silica gel with chloroform-methanol. Pure Boc-4′-chloro-3′,5′-dibromo-L-phenylalanine and Boc-3′,4′,5′-tribromo-L-phenylalanine were recrystallized from ethylacetate-petroleum ether; as a major by-product, Boc-3′,5′-dibromotyrosine was recovered. Boc-4′-chloro-3′,5′-dibromophenylalanine was prepared from 4′-amino-3′,5′-dibromophenylalanine in 33.5% yield (mp 155°C, $[\alpha]^{25}$ = 2.4). Boc-3′,4′,5′-tribromophenylalanine was isolated in a yield of 12.5% (mp 145°C). Both compounds had satisfactory analytical and spectroscopical data. Hydrogenation of Boc-4′-chloro-3′,5′-dibromophenylalanine gave identical spectra, Rf-values and $[\alpha]^{25}$ as Boc-phenylalanine. Boc-4′-chloro-3′,5′-dibromophenylalanine was chosen for peptide synthesis because of its higher yield in synthesis.

Peptide Synthesis

In order to test the usefulness of this amino acid (4′-chloro-3′,5′-dibromophenylalanine) (= (3X)Phe) as a precursor for tritiation and as a structural analogue of phenylalanine, angiotensin II (AT) and bradykinin (BK) derivatives were synthesized. The two peptides Sar-Arg-Val-Tyr-Val-His-Pro-(3X)Phe (= [Sar^{1},(3X)Phe^{8}]AT) and Arg-Pro-Pro-Gly-(3X)Phe-Ser-Pro-(3X)Phe (= [(3X)$Phe^{5,8}$,des-Arg^{9}]BK) were assembled by a slightly modified solid phase method,[8] cleaved with HF and purified by gel filtration and partition chromatography. Each peptide (5 mg) was catalytically hydrogenated and compared to the parent peptides

[Sar^1]AT and [des-Arg^9]BK, respectively. They were identical in tlc, amino acid analysis and biological activity.

Biological Test

The [Sar^1,(3X)Phe^8]AT, the [(3X)Phe^{5,8},des-Arg^9]BK and their hydrogenation products were tested on rabbit aorta strips. From previous studies on AT[8], we expected [Sar^1,(3X)Phe^8]AT to be an antagonist or at least a partial agonist with considerable retention of affinity due to the enlargement of the phenylalanine nucleus. The result was, as predicted, a half maximal dose ED_{50} of $2.6 \cdot 10^{-8}$ M (or 6.2% relative affinity) with intrinsic activity $\alpha_E = 0.47$ (See Figure 2). The BK-analog [(3X)Phe^{5,8},des-Arg^9]BK was completely inactive. The hydrogenation products of both (3X)Phe-peptides were absolutely identical on rabbit aorta strips with [Sar^1]AT and [des-Arg^9]BK, respectively.

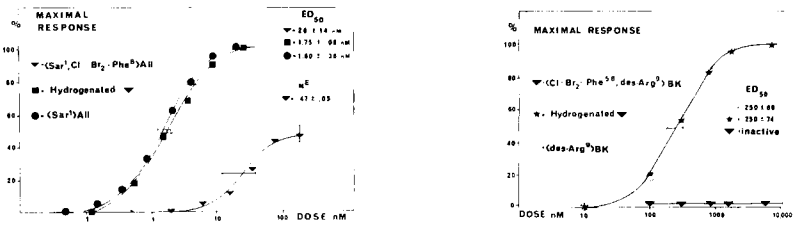

Fig. 2. Biological activities. Dose-response curves of the angiotensin II (AT) analogs (left) and of the bradykinin (BK) analogs (right). ED_{50} is the half maximal dose, α_E is the intrinsic activity. Every point is the mean of at least 3 determinations.

Radioactive Labeling

A 1 mg sample of [(3X)Phe^{5,8},des-Arg^9]BK was exposed to $^3H_2$ in dimethylacetamide in the presence of 2 mg of Pd/Rh on charcoal. After removal of labile ^3H and gel filtration. $\simeq$6.6 μg of [(3′,4′,5′-$^3H_3$)Phe^{5,8},des-Arg^9] with a specific activity of $\simeq$150 Ci/mmol resulted.

Acknolwedgements

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Conseil de la Recherche en Santé du Québec, and the Canadian Heart Foundation.

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A METHOD FOR DISTINGUISHING α- AND ω-DICARBOXYLIC AMINO ACID LINKAGES IN PEPTIDES

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Introduction

Dicarboxylic amino acids present a problem both in the synthesis and the structural determination of peptides since there are two carboxyl groups which can be involved in peptide bond formation. This problem is complicated by the fact that the two possible isomers can be interconverted through formation of an imide under a variety of conditions.[1] This paper describes a method for distinguishing between the two possible isomers based on the Lossen rearrangement of the free carboxylic acid. Both the rate of rearrangement and the products obtained are indicative of the type of linkage present at the dicarboxylic amino acid.

Procedure

The Lossen rearrangement as shown in Figure 1 results in the conversion of a carboxylic acid to an isocyanate. To accomplish this on a peptide, O-pivaloylhydroxylamine is coupled quantitatively to the free carboxylic acids using a water soluble carbodiimide.[2] The resulting *N,O*-diacylhydroxylamine undergoes a Lossen rearrangement under basic conditions. At pH = 8.5, 50°C, the reaction is over in 20 hours.

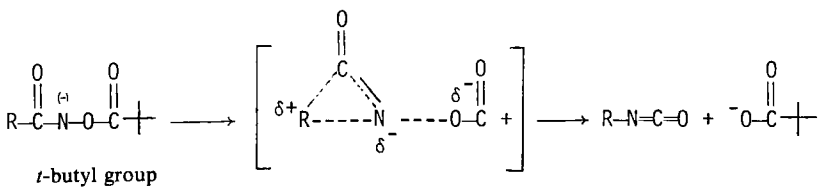

Fig. 1. Mechanism of Lossen Rearrangement

Results

Table I. Results of Lossen Degradation of Aspartyl Peptides

Peptide	% Loss of Asp	Peptide	% Loss of Asp
Ac-Gly-Asn	71	Phe-α-Asp-Ala-Ser-Val	0
A Chain of Insulin	75	Ac-α-Asp-$NHCH_3$	<2
(C-terminal Asn)		Ac-Phe-α-Asp-Val-NH_2	<5
Ac-Gly-β-Asp-$N(Et)_2$	74	Ac-Gly-α-Asp $N(Et)_2$	20
Ac-Phe-β-Asp-Val-NH_2	65	Ac-Phe-α-Asp-Gly-NH_2	13
Bzl-Gly-β-Asp-$NHCH_3$	55	Bzl-Gly-α-Asp-$NHCH_3$	14
PLRAIGPPAEPNGLVPLQYWPFSS-Ala-α-Asp-Leu-Tyr			<4

Table II. Results of Lossen Degradation of Glutamyl Peptides

Peptide	% Loss of ~~Asp~~Glu	Peptide	% Loss of ~~Asp~~Glu
Ac-γ-Glu-$Cys(SO_3H)$-Gly	95	Ac-Gly-α-Glu-Gly-Phe	27
Ac-γ-Glu-$N(Et)_2$	87	Ac-Gly-α-Glu-Val-Phe	22
Ac-γ-Glu-β-Ala	91	Ac-Gly-α-Glu-$N(Et)_2$	25
Ac-Gly-γ-Glu-Met	92	Ac-Phe-α-Glu-Ala	11
Ac-Gln	99	Phth-α-Glu-$N(Et)_2$	35
		Bzl-Gly-α-Glu-$NHCH_3$	27*
		Ac-α-Glu-$NHCH_3$	31

*Of the glutamic acid lost 20% was recovered as diaminobutyric acid after 6*N* HCl/110°C/22 hr hydrolysis. This increased to 80% after 6*N* $Ba(OH)_2$/110°C/9 hr hydrolysis.

Discussion

Earlier investigations into the mechanism of the Lossen rearrangement[3] have shown that electron donating substituents on the α carbon facilitate the rearrangement. Thus, when the α-carboxyl group is free as in β-linked aspartyl or γ-linked glutamyl peptides the rearrangement is fast due to the electron donating resonance effect of the α-amido group. This leads to loss of the amino acid on subsequent analysis (Figure 2). From Table I the loss of aspartic acid from β-aspartyl peptides is generally about 70%. The loss of glutamic acid from γ-glutamyl peptides is consistently $\geq 90\%$.

In the case of α-linked peptides the rearrangement is much slower. For α-aspartyl peptides the carboxamide groups in the β position inductively destabilize the transition state, so little rearrangement is seen. From Table I, the loss of aspartic acid from α-peptides is generally less than 5%. The α-Asp-$NHCH_3$ and the structurally similar α-Asp-Gly peptide as well as the α-Asp-$N(Et)_2$ peptide show higher loss of Asp than expected. Presumably these results are due to side reactions occurring during the rearrangement. Preliminary studies indicate imide formation during the coupling reaction is not occurring.

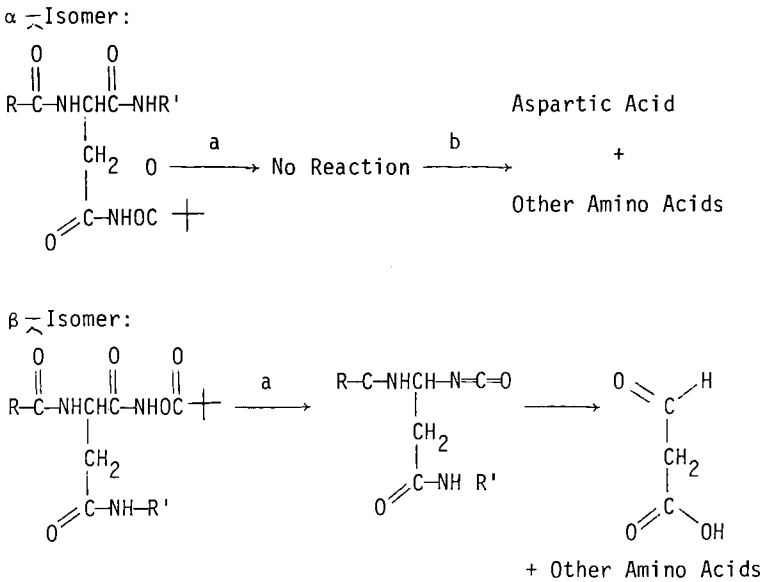

Fig. 2. Lossen Degradation of Aspartyl Peptides. a.) pH = 8.5, 50°C, 20 hr b.) 6*N* HCl/110°C/22 hr.

For α-linked glutamyl peptides the loss of glutamic acid is generally between 15% and 30%. Under these basic conditions some of the isocyanate initially formed upon rearrangement is trapped by the adjacent carboxamide group (Figure 3) to generate a hexahydropyrimidin-2-one derivative which is exceedingly stable to acid hydrolysis.[4] However, this can be hydrolyzed in 6N $Ba(OH)_2$/110°C/10 hours and the resulting diaminobutyric acid (DABA) quantitated on amino acid analysis (see Table II). The failure of earlier workers[5] to consider the possibility of 4-carboxyhexahydropyrimidin-2-one formation upon Lossen rearrangement of glutamyl residues in collagen may be the cause of the apparently erroneous[6] conclusion that there are γ-glutamyl linkages in collagen.

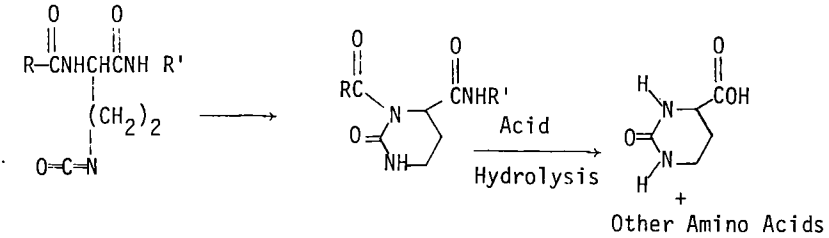

Fig. 3. Formation of 4-Carboxyhexahydropyrimidin-2-one

Conclusions

The Lossen rearrangement can thus be used to identify the type of linkage present at dicarboxylic amino acids. The α-isomers rearrange slowly resulting in recovery of the amino acid on subsequent analysis; any rearranged product is recovered as the diaminocarboxylic acid. The β (or γ) isomers rearrange rapidly with loss of the amino acid on subsequent analysis.

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ANALOGS OF THE PEPSIN INHIBITOR

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Introduction

Activation of porcine pepsinogen at pH 2 proceeds by the intramolecular removal of the first 16 amino acids, peptide (1-16) followed by further removal of residues 17 through 44[1]. Some of the peptide fragments released are inhibitors of pepsin function at pH 5.5. We have synthesized analogs of the strongest inhibitor, peptide (1-16), in which the arginine residues at positions 8 and 13 are replaced by threonine to examine the role of these positively charged side chains in binding of the peptide to pepsin. We have also modified the lysine side chain with cyanate to eliminate their positive charge. In addition, we have prepared peptide (1-12), a carboxyl truncated analog, and Gly^5-peptide-(1-12). The Gly analog was prepared to test the role of Pro^5 of the native peptide in possible rate limiting steps during association with pepsin.

Results

The peptides synthesized had the following structures:

syn-(1-16)	L V K V P L V R K K S L R Q N L
Thr^8-syn-(1-16)	——————T——————
Thr^{13}-syn-(1-16)	——————R———T———
$Thr^{8,13}$-syn-(1-16)	——————T———T———
syn-(1-12)	——————R———————
Gly^5-syn-(1-12)	———·—G———————

These were prepared by solid phase peptide synthesis using standard procedures.[2] One to three gram samples of protected peptidyl resin were

cleaved with anhydrous HF at 0° for 1.5 hours. The resulting crude peptides were passed through a Sephadex G-10 column to remove salts, small fragments, or free amino acids. The main peak was then purified on a 0.9 x 30 cm column of CM-Sepharose at pH 5.0 with a gradient from 0.01 to 1.0 *M* ammonium acetate. This purification takes advantage of the highly basic character of the peptides.

In addition to giving satisfactory amino acid compositions, the various peptides have been sequenced by solid phase methods using a Sequemat Mini-15 instrument.[3] PTH-amino acid products were identified by thin layer chromatography and HPLC. In all cases the expected amino acid sequence was observed.

These peptides were assayed for their ability to inhibit the pepsin catalyzed clotting of a dilute milk solution. This assay is a sensitive test for free pepsin at pH 5.5.[4] All peptides demonstrated significant inhibition in the μmolar range and gave inhibition curves that reached 100% in a hyperbolic fashion. Dissociation constants for the enzyme inhibitor complex were calculated by curve fitting and are presented in Table I.

Table I. Inhibition of Pepsin Catalysis by Synthetic Peptides

Peptide	K_I, μM
syn-(1-16)	0.07 - 0.2
syn-Thr^{8}-(1-16)	0.35
syn-Thr^{13}-(1-16)	0.26
syn-Thr^{8,13}-(1-16)	0.4
syn-Homocit^{3,9,10}-(1-16)	0.5
syn-Homocit^{3,9,10}-Thr^{8,13}-(1-16)	5.25
syn-(2-16)	0.25
syn-(1-12)	0.36
syn-Gly^{5}-(1-12)	1.54

In addition, we have reacted several of these peptides with potassium cyanate to convert the lysine residues to homocitrulline.[5] The modified peptide was purified by Sephadex G-25 chromatography and the

substitution was confirmed by amino acid analysis. The modified peptides were inhibitors of pepsin with K_I's as listed in Table I.

Discussion

We have replaced the arginine residues of inhibitory peptide (1-16) with Thr, since the conformationally directing parameters of these two residues are similar[6] (Arg P^{α} 0.98, Thr P^{α} 0.83; Arg $P\beta$ 0.93, Thr $P\beta$ 1.19) and this substitution will convert a charged locus to a neutral one. Despite this change, all three analogs are strongly active as inhibitors of pepsin catalysis at pH 5.5 with K_I's well below 1 μM (Table I). Thus, the arginine residues are not critical for interaction between peptide (1-16) and pepsin.

Further confirmation of the non-essential nature of Arg 13 is provided by the (1-12) peptide. This carboxyl-truncated analog is also fully active as an inhibitor. We have previously isolated native peptide (1-12) from an activation of pepsinogen and reported a K_I of 0.48 μM.[1]

Through reaction of cyanate with the lysine residues of peptide (1-16) we have neutralized the charges on these residues. The peptide with neutral homocitrulline at positions 3, 9, and 10 is a good inhibitor with K_I of 0.5 μM. The peptide with all positive groups replaced or modified, syn-Homocit^{3,9,10}-Thr^{8,13}-(1-16), is a weaker inhibitor, by a factor of 10-50, than the native sequence. However, the fact that this modified peptide is still a decent inhibitor with K_I of 5.25 μM suggests that electrostatic interactions are not of prime importance in this complex formation. This would imply that conformational factors may be important along with hydrophobic interactions involving the other, uncharged residues of peptide (1-16).

Preliminary studies have demonstrated a unique time dependence to this association process. We have observed a slow, rate limiting process that has the kinetic characteristics of a *cis-trans* proline isomerization. Since peptide (1-16) has Pro at position 5, we have replaced this with glycine which exhibits similar conformational preferences in peptide (1-12). The glycine analog is an inhibitor of pepsin with a K_I approximately 4 fold higher than the natural (1-12) sequence. We have also observed similar rates of inhibition. We conclude that isomerization of Pro^5 is unlikely to be responsible for the slow step we observe. This implies that the rate limiting process may be associated with a rearrangement of the pepsin structure.

Acknowledgement

This research is supported by NIH grant AM18865 and AM00303. We thank Joan Somerville for excellent technical assistance.

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SOLUBLE DERIVATIVES OF PEPSTATIN: NEW, POTENT, IN VIVO INHIBITORS OF RENIN

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Pepstatin, a pentapeptide isolated from culture broths of Streptomyces strains is an in vitro inhibitor of a variety of aspartyl proteases, such as pepsin, cathepsin D, and renin.[1] *In vivo* inhibition of renin, as measured by diminution of blood pressure, has also been demonstrated in the rat.[2,3,4] These results were opened to question, however, by the demonstration of a general hypotensive effect of pepstatin in nephrectomized animals.[5] Part of the problem in the unequivocal demonstration of inhibition of renal renin in vivo is the marked insolubility of pepstatin in physiologic buffers (145μM) and the resultant difficulty of administration of adequate doses of peptide. Clearly, pepstatin analogs with increased solubility are needed to unravel the complexities of the renin angiotensin system.

Our research was aimed at increasing the solubility of pepstatin by attaching charged groups to the C-terminus of the molecule. The coupling reaction was effected in high yield with "Le BOP" reagent (Figure 1).[6]

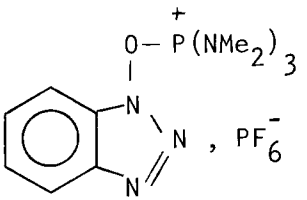

Fig. 1. "Le BOP" coupling reagent (Benzotriazolyloxytrisdimethylaminophosphonium hexafluorophosphate).

"Le BOP" is a preactivated form of N-hydroxybenzotriazole[7] which rapidly forms peptide bonds in a variety of solvents (DMF, CH_3CN, CH_2Cl_2, etc.) with minimum racemization. This reagent has been used to form the peptide bond[8] and for the synthesis of peptide phenyl esters.[9] In the synthesis reported here, pepstatin is allowed to react with 2

equivalents of both Arg-OMe·2HCl[10] and "le BOP" in the presence of 5 equivalents of triethylamine in DMF over a period of two days to yield 81% of the desired pepstatyl-arginine methyl ester as identified by amino acid analysis (Val, 2.08; Ala, 1.01; Arg, 0.94; mp, 262-263°C).

Derivatives of pepstatin containing C-terminal aspartic and glutamic acid were also prepared and characterized.

Table I. Properties of Pepstatin Derivatives.

Pepstatin derivative	Yield/Pepstatin	m.p. °C	$[\alpha]_D^{24^\circ}$
Pepstatyl-Arg-OMe, HCl	81%	262-263	-40.1(0.19 MeOH)
Pepstatyl-Glu	83%	229-230	-79.0(0.55 MeOH)
Pepstatyl-Asp	68%	238-240	-67.6(0.51 MeOH)

The solubility of pepstatin is increased thirty-fold upon attachment of the arginyl residue. Interestingly, addition of a negative charge to the molecule (pepstatyl-aspartic acid) did not increase solubility although the pepstatyl aspartic acid is a better inhibitor of renin than free pepstatin (Figure 2).

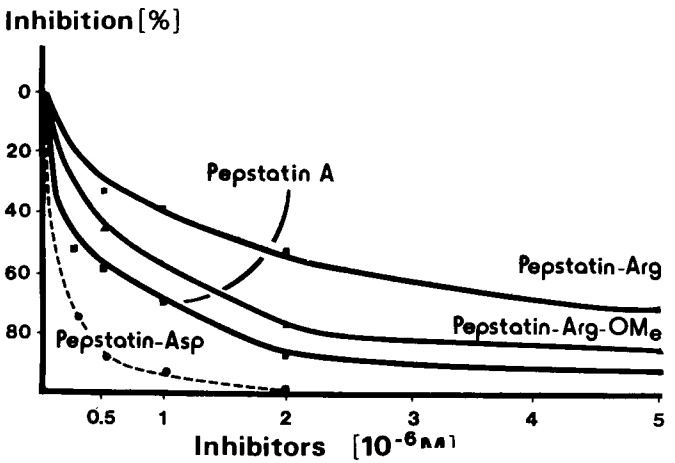

Fig. 2. Inhibition of renin by pepstatin and pepstatin derivatives

The pepstatin derivatives were tested in vitro as renin inhibitors. Activity of cathepsin free[11] hog renin (1100 GU/mg) is measured by the rate of cleavage of the leucyl-leucine peptide bond acetyltetradecapeptide. The extent of cleavage is quantitated by allowing the assay mixture to react with fluorescamine (Figure 2).[12]

Most derivatives inhibit renin with I_{50} values approximately equal to pepstatin. The aspartic acid derivative is somewhat more potent and able to inhibit quantitatively the enzyme substrate reaction at concentrations as low as 2μM.

In vivo inhibition of renin was demonstrated both with rats made hypertensive by ligature of the aorta between the two renal arteries[13] as well as binephrectomized rats in which an increase in blood pressure is induced by administration of exogenous renin. In the first model, injection of 350nMol of pepstatyl-arginine methyl ester causes a decrease in blood pressure (carotid artery) from 157±4.3 to 109±8.2 torr. The maximum decrease is obtained 6 minutes after administration of the pepstatin analog. After 10 minutes pressure returns to control values. Almost identical results are obtained with Saralasin.[14]

In the second model, intravenous administration of the pepstatyl-arginine methyl ester (1.2mg/kg) two minutes before hog renin injection causes an 85% inhibition in blood pressure rise. A linear log dose-response relationship is obtained for inhibition of blood pressure increases and in vivo half life of the pepstatin derivative is 20 minutes (Figure 3).

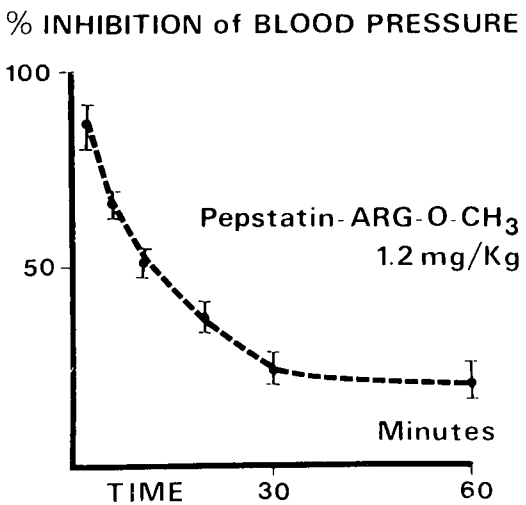

Fig. 3. The effect of Pepstatin-Arg-O-CH_3 on the rise in blood pressure

Solubility of pepstatin is increased 30-fold by attaching arginine methyl ester to the C-terminus. This derivative inhibits hog renin as effectively in vitro as pepstatin. It can be injected at sufficient concentrations to be a potent inhibitor of the renin angiotensin system in the rat.

Acknowledgement

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SYNTHETIC PEPTIDE SUBSTRATES FOR PROTEIN KINASES STRUCTURE AND BIOLOGICAL FUNCTION

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Introduction

A number of protein kinases have been found to catalyse the transfer of phosphate from ATP to small peptides corresponding to the local amino acid sequences around the phosphorylation sites in their natural protein substrates.[1] In the case of the cAMP-dependent protein kinase the heptapeptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly, corresponding to the phosphorylation site of liver pyruvate kinase[2,3] has been found to act as a particularly efficient substrate. The adjacent arginine residues in this peptide have been shown to act as important specificity determinants.[2,3] Synthetic peptide analogs of other natural substrates of this enzyme are, however, less efficient substrates. These include the peptide analogs for cardiac troponin, TN-I, and the phosphoprotein phosphatase inhibitor — 1 (Figure 1). Theoretical proposals of Small et al[4] and others[5] indicate that the cAMP-dependent protein kinase may recognize some preferred conformation for which the pyruvate kinase peptide evidently has the greatest propensity to assume.

We have undertaken CD and NMR studies of a number of peptide substrates of the cAMP-dependent protein kinase with the aim of identifying any structural features that may account for the differences in the kinetics of phosphorylation of these peptides.

Results and Discussion

The kinetics and CD spectra of a total of 16 peptide analogs of the pyruvate kinase peptide and the phosphoprotein inhibitor-1 have been examined (Figures 1 and 2). The dominant CD pattern observed for the pyruvate kinase peptide analogs in phosphate buffer was a strong negative extremun at 198-204nm, a positive extremun at 215-218nm and a shallow negative extremun at 225-230nm. The appearance of the second minimum at 225-230nm is indicative of β-turn conformations[6] whose

Peptide Sequence	Vmax (μmol. min^{-1}. mg^{-1})	Km^{app} (μM)
Leu - Arg - Arg - Ala - Ser - Leu - Gly - NH_2*	30	3
Leu - Arg - Arg - Ala - Ser - Leu - Gly-COOH*	20	16
Leu - Ala - Arg - Ala - Ser - Leu - Gly-COOH	9	5000
Arg - Pro - Ala - Pro - Ala - Val - Arg - Arg - Ser - Asp - Arg - Ala - NH_2**	0·23	2320
Arg - Arg - Arg - Arg - Pro - Thr - Pro - Ala - NH_2***	0·23	62
Arg - Arg - Pro - Thr - Pro - Ala - NH_2	0·10	1500
Arg - Pro - Thr - Pro - Ala - NH_2	0·006	12000
Pro - Thr - Pro - Ala - NH_2	0	-

Fig. 1. Kinetics of phosphorylation of synthetic peptide analogs of natural substrates of cAMP-dependent protein kinase. The synthesis, purification, characterization, and phosphorylation were as described previously.[2]

* pig liver pyruvate kinase[3]

** troponin TN-I, rabbit cardiac muscle[8]

*** Phosphoprotein phosphatase inhibitor-1, rabbit skeletal muscle.[9]

population is increased in nonpolar solvents and especially in trifluoroethanol. This is true for both the parent peptide (Figure 2A) and its kinetically inferior alanine analog (Figure 2B). The two phosphoprotein phosphatase inhibitor-1 peptide analogs of Figure 2C also show evidence of β-bend formation even in aqueous solution; in contrast the two peptides of Figure 2D show none. There is no clear correlation between the kinetic properties of these peptides and their CD spectra in the solvents tested.

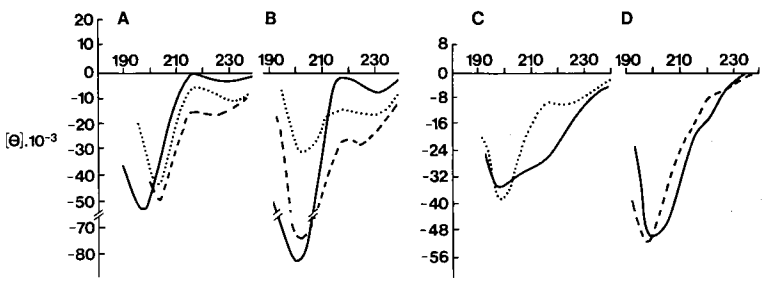
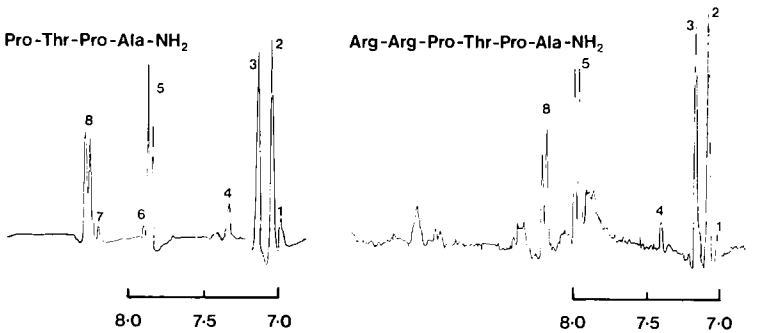


Fig. 2. CD spectra of synthetic peptides. Solvent effects on pyruvate kinase peptide A, Leu-Arg-Arg-Ala-Ser-Leu-Gly; B, Leu-Ala-Arg-Ala-Ser-Leu-Gly; ——— 0.02M phosphate pH 7.4, ——— Methanol, ... Trifluoroethanol.

Phosphoprotein phosphatase inhibitor peptides measured in phosphate. C, ..., Pro-Thr-Pro-Ala-NH_2, ——— Arg-Pro-Thr-Pro-Ala-NH_2; D, ——————— Arg-Arg-Pro-Thr-Pro-Ala-NH_2, ——— Arg-Arg-Arg-Arg-Pro-Thr-Pro-Ala-NH_2. Units $[\theta]$. 10^{-3} deg cm^2 · dmol^{-1}

The NMR spectra of the phosphoprotein phosphatase inhibitor-1 peptide analogs were determined in DMSO (Figure 3). Most of the ^{1}H and ^{13}C resonances were identified for the tetrapeptide, Pro-Thr-Pro-Ala-NH_2; however, the spectra for longer analogs containing arginine were not resolved. The tetrapeptide was found to be present in solution as two isomers, *cis* and *trans* about the Thr-Pro peptide bond in the ratio of 1:3, respectively. Evidence was obtained for a β-turn in which Thr was in position i, in Thr-Pro-Ala-NH_2 with NH_2 playing the role of residue i+3. A similar structure was previously observed for the peptide Gly-Pro-Ala-NH_2.[7]



Identification of Peaks

	Thr-Pro trans (major)	Thr-Pro cis (minor)
$CONH_2$ syn	2	1
anti	3	4
Ala NH	5	6
Thr NH	8	7

Fig. 3. Proton NMR Spectra of peptides in DMSO-d_6 (20-40 mg·ml^{-1} at 28°).

This would place the phosphorylatable threonine immediately before the β-turn in the peptide chain in contrast to the proposal by Matsuo et al[5] who have suggested that the phosphorylation site occupies the i+3 position of a β-turn. Owing to the complexity of the NMR spectra, it was not possible to obtain direct structural evidence to correlate with the kinetic properties of the arginine containing peptides (Figures 1 and 3).

Acknowledgement

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PITUITARY ENZYME WHICH SPLITS PROINSULIN

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Introduction

The proteases involved in post-signalase processing of secretory peptides have not been clearly characterized. Although several enzymes capable of specific processing *in vitro* have been isolated and purified, their role *in vivo* is uncertain.[1,2]

We have identified and isolated an enzyme from porcine pituitary glands which appears to be capable of proinsulin processing. The enzyme is distinct from previously described pituitary proteases.

Results and Discussion

The enzyme was purified from fresh anterior pituitaries (Oscar Mayer Co.) in a three step procedure. *Step I:* A 10% homogenate was prepared with a Polytron Homogenizer in 0.25M sucrose, 10mM HEPES, pH 7.5. Differential centrifugation resulted in microsomes and a particle-free supernatant, which contained 10% and 90% of the total activity, respectively. *Step II:* 10ml portions of the supernatant were fractionated on a 2.6x85cm Sephacryl 200 column in 100mM NaCl and 1mM HEPES, pH 7.5. Optical density was monitored at 280nm and enzyme activity of the eluted fractions was assayed on 0.1mM Arg-Arg-β-naphthylamide (AAβNA) and Leucyl-β-naphthylamide (LβNA). The absorption profile and arylamidase activities of a representative Sephacryl 200 run are shown in Figure 1. Although both activities eluted closely together, they were clearly distinct. Hog kidney leucine aminopeptidase (Sigma) eluted at the V_c of the LβNAase activity, whereas the AAβNAase activity coeluted with bovine serum albumin (BSA).

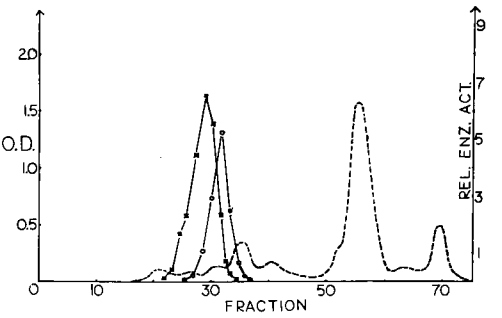

Fig. 1. Sephacryl 200 chromatography of 10ml supernatant (cf. Step II, purification). Optical density at 280nm (————), AAβNAase (—o—o—), LβNAase (—x—x—)

Step III: The Sephacryl 200 samples with the highest AAβNAase to LβNAase activity ratio were further fractionated on a DEAE-Sephadex column (1.6x10cm) equilibrated with 100mM NaCl and 10mM TRIS, pH 7.5. After elution of unretained material, a 0.1 - 1M linear NaCl gradient was started. Activity and absorption were monitored as above. Protein was determined with fluorescamine except when TRIS was the buffer, in which case absorption at 280nm was used. Figure 2 reveals loss of AAβNAase in the unretained material. However, the AAβNAase to LβNAase ratio changed significantly, indicating the presence of separate activities. SDS-Polyacrylamide gel electrophoresis of the most active DEAE fraction revealed one major and two nearly undetectable minor bands. The comigration of the major band with BSA suggests that the enzyme is homomeric. The specific activity of the AAβNAase had risen 500-fold after step III.

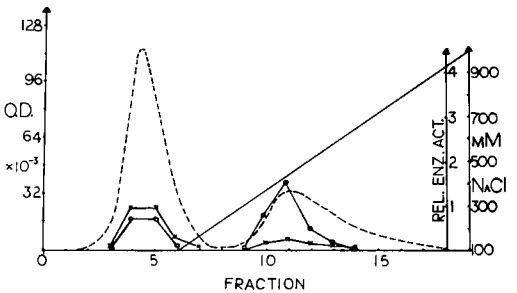

Fig. 2. DEAE-Fractionation of AAβNAase (—o—) and LβNAase (—x—). OD at 280nm.

The AAβNAase had a K_m of 4.6×10^{-6}M and a pH optimum of 8.2 on AAβNA. The MW was near 68 000 by SDS-PAGE and Sephacryl 200. The enzyme was stable for weeks in solution at 4°C but could not be lyophilized. Under similar conditions the LβNAase activity quickly decayed and could not be retrieved with mercaptoethanol.

Table I. Relative Rates of Arylamide Hydrolysis by AAβNAase.

LeuβNA, N-Bzl-argβNA	0
LysβNA, Lys-lysβNA	trace
ArgβNA, CBZ-gly-gly-argβNA	0.1
N-acetyl-gly-lysβNA	0.3
Arg-argβNA	3.0

AAβNAase was completely inhibited by 1mM o-phenanthroline, dithiothreitol, and 0.1mM p-chloromercuribenzoate. 10mM iodoacetamide gave 50%, and 10mM benzamidine 30% inhibition. No inhibition was observed with PMSF, SBTI, Aprotinin and puromycin.

AAβNAase activity was found in brain, liver, pancreas, heart, adrenals, and kidney. Specific activities of 10% homogenates were similar for all organs. *Candida albicans* contained AAβNA'ase.

Bovine proinsulin (0.5mg donated by R.E. Chance of Eli Lilly Co.) was incubated with 0.01mg of AAβNAase in 1ml of Clark and Lub's buffer (pH 9) at room temperature for 20 hours. Sephadex G-50 chromatography of a reaction mixture aliquot in 3N acetic acid yielded a single peak at the V_e of insulin. PAGE of this peak in a 10% gel is depicted in Figure 3:

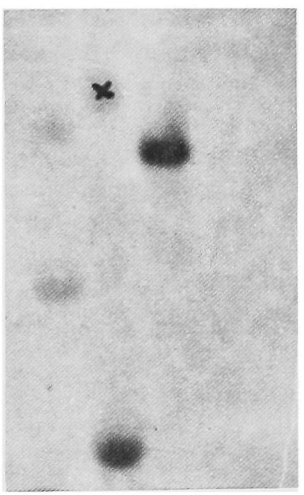

Fig. 3. PAGE of G-50 peak in 10% gel at pH 8.7.

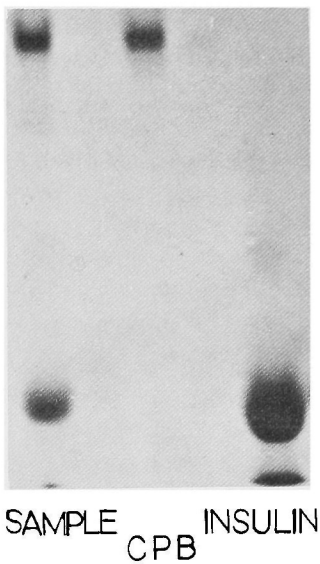

Fig. 4. PAGE of original incubation mixture after CPB

An aliquot of the original incubation mixture was incubated with TLCK-treated pancreatic carboxypeptidase B (CPB) and subjected to PAGE. The result is shown in Figure 4.

Comparison of AAβNAase with Aminopeptidase B (APB)[3] and Dipeptidyl-aminopeptidase III[4] suggests that it is distinct from both. APB is larger and Cl^- dependent, whereas DPAP III is not inactivated by EDTA and is inactive on N-protected substrates. The pancreatic proinsulinase isolated by Yip,[1] and pancreatic kallikrein, recently implicated as the human proinsulinase,[2] are both serine proteases.

In summary, a pituitary enzyme was purified 500-fold and shown to digest proinsulin. The specificity of proinsulin cleavage remains to be established and the role of the enzyme in the pituitary, i.e. in big-ACTH conversion awaits elucidation.

Acknowledgement

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DENATURATION STUDIES ON C-13 LABELED BASIC PANCREATIC TRYPSIN INHIBITOR (BPTI) BY C-13 NUCLEAR MAGNETIC RESONANCE (CMR)

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BPTI, a protein of 58 amino acid residues, which is stable under a wide range of conditions, serves as an excellent model for studying the solution conformational behavior of proteins. We report here CMR studies on the denaturation of modified BPTI molecules, methylated by $^{13}CH_3I$ on specific sulfur-containing functional groups. Under appropriate conditions other reactive functional groups ($-NH_2$, $-OH$) are not affected. Selective S-methylation of the single methionyl residue (Met-52) was achieved in 70% formic acid,[1] to give ^{13}C-S-methyl methionyl BPTI (SMM—BPTI). C-13-S-methylation of cysteine sulfhydryl groups produced by selective reduction of the 14,38 disulfide bridge[2] in dithioerythritol at pH 7 gave 14,38-S-methyl cysteinyl BPTI (SMC-BPTI). This disulfide bridge is close to the active site and at the opposite end of the protein molecule from Met-52 (Figure 1).

Results and Discussion

Trace A in Figure 2 is the CMR spectrum of ^{13}C-labeled SMM-BPTI. The doublet centered at 26.52 ppm arises from the non-equivalence of the S-methyl groups. Trace B is the spectrum of SMC-BPTI. The peaks at 16.78 ppm and 16.31 ppm are the two separate resonances from the 14 and 38 S-methyl groups. Both SMM-BPTI and SMC-BPTI were fully active at physiological conditions. Their conformational stabilities were monitored in solution by CMR as a function of pH, temperature or guanidine hydrochloride (Gu-Cl) concentration.

A particularly sensitive conformation dependent parameter

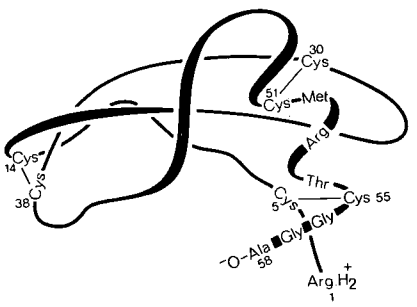

Fig. 1 (left): Backbone structure of BPTI.

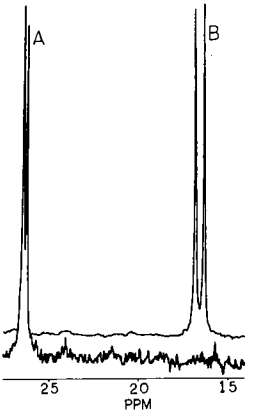

Fig. 2 (right): Proton-decoupled 25.16 MHz ^{13}C FT NMR spectra of (A) SMM-BPTI (B) SMC-BPTI. Each curve represents ca. 10,000 acquisitions on an 8 mM solution. PPM is relative to trimethyl silyl propionic acid.

associated with the S-methyl methionyl groups is the chemical shift separation between the S-methyl signals.[1] These signals reference one another and problems inherent in internal referencing are avoided. In the model compounds, S-methyl methionine and its N-substituted derivatives, the non-equivalence in the S-methyl signals varies between 0.6 to 2.2 Hz at 25.16 MHz and changes with titration of amino or carboxyl functional groups.[3] In SMM-BPTI, however, the observed non-equivalence was much larger (5 Hz), and independent of pH in the range where most functional groups titrate. It was, however, sensitive to environmental and conformational factors in the protein. In Curve 1, Figure 3A, the plot of the chemical shift separation ($\Delta\delta$) in the S-methyl groups as a function of pH shows that $\Delta\delta$ is invariant in the pH range 1-11 and the steep decline in $\Delta\delta$ between pH 11 and 13 correlates with the protein denaturation known to occur in this pH range.[4] At pH 13, the methyl signals coalesce beyond resolution, and the protein that was recovered from this solution was inactive.

A similar pattern was observed for SMC-BPTI. In this case the parameters which can be monitored are the chemical shifts (δ) of the S-methyl groups on cysteine residues 14 and 38. The high field methyl peak appears to be more sensitive to conformational changes in the protein than the low field peak and only the high field peak positions are reported here (Curve 2, Figure 3A). This signal shows a sharp downfield shift in δ between pH 12 and 13. At pH 13, the two SMC-BPTI methyl peaks coincide. The 0.2 ppm upfield shift in Curve 2 between pH 10 and 12 may

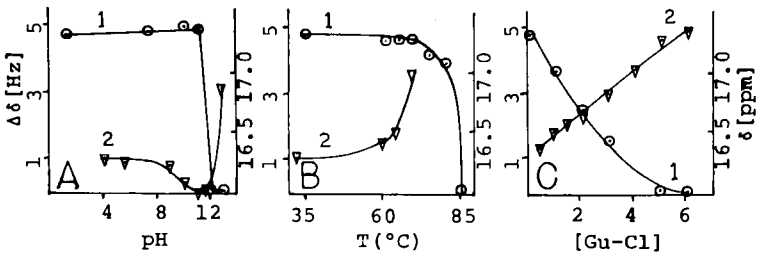

Fig. 3. Denaturation profiles for SMM-BPTI (Curve 1, left ordinate) and SMC-BPTI (Curve 2, right ordinate) with increasing pH (A), temperature (B) or Gu-Cl (C).

reflect the ionization of nearby functional groups or local conformational changes in the protein. The latter possibility is supported from T_1 measurements on the methyl peaks. A significant difference in the relative T_1 values between the low and high field peaks (0.52 and 0.47 ± 0.01 sec., respectively) was observed at pH 11, but not at pH 7 (0.58 and 0.56 ± 0.01 sec.).

Similarly sharp spectral changes were observed for both SMM-BPTI and SMC-BPTI as a function of temperature. Curve 1 in Figure 3B shows the sharp melting of SMM-BPTI between 80° and 85° as reflected in the steep decline in $\Delta\delta$. SMC-BPTI also melts sharply, but 15° lower than SMM-BPTI. This is seen in Curve 2, where δ for the high field peak moves steeply downfield between 60° and 70°. The lowered thermal stability of SMC-BPTI, which possesses one less disulfide bridge, seems entirely reasonable.

A different pattern of denaturation is observed in increasing concentrations of Gu-Cl. For both proteins this denaturation process appears to be a gradual loosening in protein structure. This is seen in the monotonic decrease in $\Delta\delta$ for SMM-BPTI (Curve 1, Figure 3C). In SMC-BPTI there is a similar steady downfield shift in δ for the high field methyl peak (Curve 2). The relative stabilities in the two proteins in Gu-Cl are different. Once again, SMM-BPTI was more stable than SMC-BPTI. The former recovered its full activity and the resolution of the S-methyl signals after removal of the Gu-Cl (5M) whereas the latter did not regain activity nor the resolution of the 14, 38 methyl signals. Apparently, the structural changes occurring in high Gu-Cl concentrations are reversible for SMM-BPTI (as they are for BPTI) but not for SMC-BPTI.

Despite the differences in the relative stabilities of the two modified inhibitors, their behavior in solution ran in parallel in the three sets of denaturing conditions. Both showed sharp denaturation profiles with rises of pH or temperature and more gradual changes with increasing Gu-Cl concentrations.

Acknowledgement

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CONFORMATION OF CYCLIC 5-PEPTIDES

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Calculations have shown, that cyclic 5-peptides with all-trans peptide bonds can adapt a C_5 symmetry[1], but also, that unsymmetric conformations with intramolecular hydrogen bonds are of even lower energy[2,3]. The ^{1}H NMR spectrum of *cyclo*[Phe$_5$] in DMSO exhibits only one set of signals at all temperatures[4]. The temperature dependence $\Delta\delta/T$ of the NH signal is of the medium value[5] of 3.8 ppb/K and we assume, that the apparent equivalence is due to rapid equilibration with respect to the NMR time scale.

Thus, a perturbation of the sequential symmetry by substitution of a phenylalanine by glycine (*cyclo*[Phe$_4$-Gly] (1)) leads to an immediate differentiation of the NMR spectrum, but the parameters in Table I are not extreme enough to assume predominance of one conformation.

Table I. DMSO Temperature Dependence and Maximal TFE Induced Shifts (at 25% TFE in DMSO) of the N*H* NMR Signals of 1.

1	Phe^1	Phe^2	Phe^3	Phe^4	Gly^5
$\Delta\delta/T$ [ppb/K]	3.5	5.7	4.2	4.3	5.4
$\Delta\delta_{TFE}$ [ppb]	24	5	11	28	11

A further variation of the amino acids, such as in *cyclo*[Phe-Gly-Aib-Val-Ala] (2) and *cyclo*[Phe-Gly-Leu-Val-Ala] (3), leads to a stronger differentiation of the ^{1}H NMR experimental results.

The results of Table II indicate, that the solvent interaction of the NH protons of Gly^2 and Val^4 in both compounds clearly differs from that of the other three amino acids (small $\Delta\delta/T$, positive solvent shifts). Also the large difference between the two Gly$^\alpha$H resonance positions (0.2 ppm in 2 and 0.4 ppm in 3)[13] gives some evidence for the population of a single conformation[6]. The relative indifference of the Gly^2 and Val^4 NH protons to changes of the environment can be explained by shielding from the solvent. Such internal orientation can be stabilized in cyclic 5-peptides by up to two β or γ type hydrogen bonds. Calculations[2,3] and experiments[4,6-10] also show, that these can lead to a $\beta\gamma$ or a $\gamma\gamma$ type

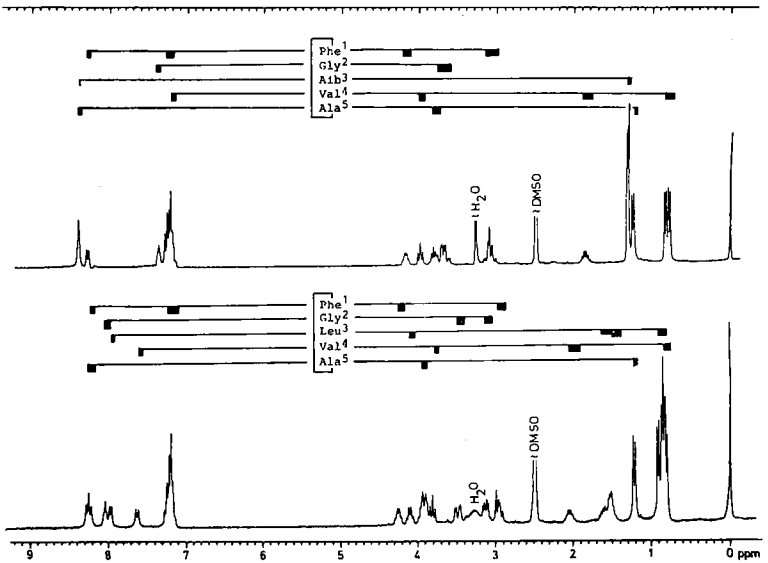

Fig. 1. 270 MHz spectra of 2 and 3 at 50°C in DMSO.

conformation. Thus, the internally orientated Gly^2 NH can form a β type turn to Val^4 CO or a γ type turn to Ala^5 CO, whereas the Val^4 NH can only be involved in a γ turn to Gly^2 CO, if the internal orientation is associated with an intramolecular hydrogen bond.

Table II. Relevant NMR Parameters of 2 and 3 in DMSO.[a]

2	Phe^1	Gly^2	Aib^3	Val^4	Ala^5
$\Delta\delta/T$ [ppb/K]	6.1	0.2	6.1	3.6	4.8
$\Delta\delta_{CDCl_3}$[b] [ppb]	-170	80	-220	90	-120
$\Delta\delta_{TFE}$ [ppb]	-160	140	-160	110	- 50
$^3J_{HNC^\alpha H}$ [Hz]	8.4	4.5,5.2[c]	-	8.7	7.2
3	Phe^1	Gly^2	Leu^3	Val^4	Ala^5
$\Delta\delta/T$ [ppb/K]	4.8	2.6	5.3	2.4	4.5
$\Delta\delta_{CDCl_3}$[b] [ppb]	-160	60	-270	30	-120
$\Delta\delta_{TFE}$ [ppb]	- 30	140	- 20	70	- 50
$^3J_{HNC^\alpha H}$ [Hz]	8.2	7.3,4.2[c]	7.1	8.6	7.6

[a]due to low solubility, investigations were only possible in DMSO solutions; [b]maximal shift values at 70% $CDCl_3$ and 40% TFE in DMSO; [c]AX and BX values, $^2J_{HC^\alpha H}$ = 14.9 (2) and 14.7 (3) Hz.

Furthermore a differentiation between type I or II β turns and γ^{I} or γ^{I} turns is necessary. (γ type turns can only be formed above or below the "plane" of the peptide backbone. We define γ^{I}, when the side chain of the L-amino acid in the central position of a γ turn is in an axial position of the 7 membered loop, contrarily γ^{I}, if it is in an equatorial one.) This can be obtained by evaluating the coupling constants $^{3}J_{HN C\alpha H}$ and $^{2}J_{HC\alpha H}$[11] under the assumption of a single, rigid solution conformation. We compared the sets of torsion angles so obtained with Dreiding-models of possible rigid conformations[12]. The vicinal coupling constant of Val^{4} correlates only with a γ^{I} type turn. No agreement is found between the other coupling constants and a βII or a γ^{I} type turn of the Gly^{2} NH, but βI or γ^{I} turns are allowed.

This leaves the two possible conformations shown in Figure 2, which are in agreement with all experimental results.

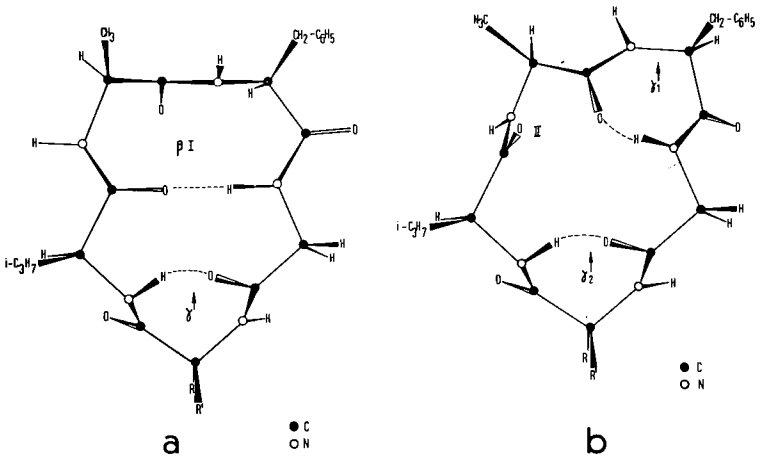

Fig. 2. βIγ^{I} and $\gamma^{I}\gamma^{I}$ conformation of 2 (R = R′ =CH$_3$) and 3 (R = H, R′ = i-C$_4$H$_9$). The experimental results do not allow an assignment of the conformation of the free Val^{4}-Ala^{5} amide bond. The "type II" structure of Figure 2b is therefore arbitrary.

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5. NH Protons with a $\Delta\delta/T > 5$ ppb are generally regarded to be DMSO exposed, those of below 2.5 ppb to be solvent shielded. See also Kumar, N. G., Izumiya, N., Miyoshi, M., Sugano, H., and Urry, D. W. (1975) *J. Amer. Chem. Soc.* **97**, 4105-4114.

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13. The chemical shift difference of both H^{α} signals in $\underline{\underline{2}}$ increases with increasing temperature in DMSO.

HIGH RESOLUTION NMR STUDIES ON OLIGOPEPTIDES

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We recently suggested a new strategy to assign nmr resonances in the NH and α-CH region for homologous oligopeptides — the inclusion of a "guest" amino acid into the sequence of a linear homo-oligopeptide chain[1]. This strategy was successfully applied to the assignment of the Boc-Met_n-OMe (n = 2-7) series in $CDCl_3$. This method, however, requires that no conformational differences be present in the "host" and "guest" oligopeptides that affect chemical shift assignments. In order to confirm the assignments using the new strategy, we synthesized the Boc-Met_n-OMe oligomers in which specific residues are α-deuterated. In this communication we verify the assignments previously reported by the "host-guest" procedure by using α-deuterated oligopeptides. This report represents the first complete assignment of a homo-oligopeptide series composed of chiral amino acids in which specific residues are α-deuterated.

The oligopeptides used in this study were synthesized using previously described procedures[2]. The amino acid L-α-deutero methionine was prepared by the method of Upson and Hruby[3]. The amino acid was approximately 90% α-deuterated as determined by high resolution nmr (220 MHz). All deuterated oligopeptides were subjected to HPLC analysis on a micro porasil silica column using cyclohexane-propan-2-ol as the eluent. Most oligomers were judged > 98% homogeneous by this procedure. The nmr studies were carried out on a Varian Associates 220 MHz spectrometer fitted with a Nicolet Instrument Co. TT 1010A Fourier Transform unit or on a modified Bruker-HXS-360 MHz spectrometer. Procedures employed were essentially the same as previously described[1].

Approximately 1000-2000 accumulations were necessary to obtain good signal to noise ratios.

Figure 1 illustrates the nmr spectra obtained on the methionine oligomers and Table I summarizes the complete assignments for the NH and α-CH regions from dimer through heptamer. It is evident from the figure that each resonance of the hexamer can readily be assigned by spectral inspection. All NH protons adjacent to the deuterated α-carbon appear as relatively sharp singlets in comparison to the doublets or broad resonances observed from non-deuterated residues. Although a number of α-CH protons overlap in the trimer and higher oligomers, α-deuteration gave unequivocal evidence for the specific contribution of individual residues to the envelope which is observed. For example, for Boc-Met_6-OMe in $CDCl_3$, six envelopes of equal intensity are found in the α-CH region. It is clear from the loss of intensity (indicated by the arrows) which resonance belongs to the specifically deuterated residues. Similar conclusions were made on all other envelopes in the α-CH regions except for the 3-proton envelope in the heptamer. The resonances in this envelope are not separable even at 360 MHz.

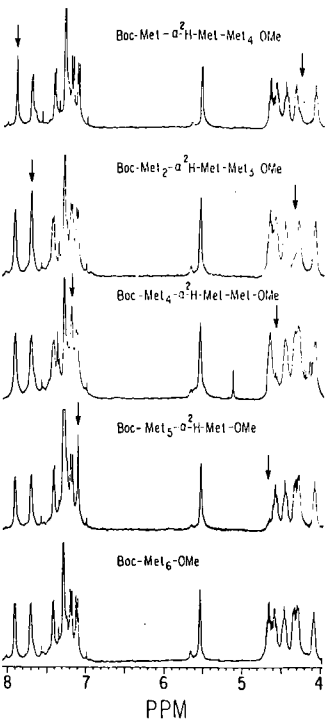

Fig. 1. Partial 360 MHz nmr spectrum of specifically α-deuterated hexamethionines in $CDCl_3$ at 23° C. Oligopeptide concentration 2 mg/ml. Arrow indicates region of NH or α-CH resonances which change on α-deuteration. Spectra represent average of 600 scans.

Table I. Chemical Shift Assignments for NH and α-CH Resonance of Boc-Met_n-OMe[a]

Oligomer	Methionine NH Region							Methionine α-CH Region						
Boc-Met_2-OMe	5.16	6.80[b]						4.28	4.74[b]					
Boc-Met_3-OMe	5.17	6.97[b]	6.90[b]					4.25	4.63[b]	4.69[b]				
Boc-Met_4-OMe	5.33	7.38	7.21[b]	7.03[b]				4.16	4.53	4.66[b]	4.68[b]			
Boc-Met_5-OMe	5.48	7.78[b]	7.51	7.29[b]	7.06[b]			4.09	4.37[b]	4.57	4.63[b]	4.67[b]		
Boc-Met_6-OMe	5.53	7.91[b]	7.70[b]	7.41	7.17[b]	7.11[b]		4.07	4.26[b]	4.31[b]	4.44	4.60[b]	4.66[b]	
Boc-Met_7-OMe	5.56	7.98	7.76[b]	7.55[b]	7.28[c]	7.20[b]	7.14[b]	4.06	4.24[d]	4.24[bd]	4.24[bd]	4.40	4.60[b]	4.67[b]
Residue Assignment	Met^1	Met^2	Met^3	Met^4	Met^5	Met^6	Met^7	Met^1	Met^2	Met^3	Met^4	Met^5	Met^6	Met^7

[a]Data for peptides at 2 mg/ml in $CDCl_3$ at 19° C. Most chemical shifts represent average values for all of the specifically deuterated oligomers at a given chain length. [b]These residues were specifically α-deuterated. [c]Partially under chloroform CH. [d]Three overlapping protons.

The assignments in this study confirm those made by the "host-guest" strategy[1]. In addition, since many more α-deuterated oligomers were prepared than the number of "guest" peptides used in the previous study, we believe that the present assignments are unequivocal. The agreement between the results of this study and the previous analysis using the "host-guest" strategy suggests that the latter method can be confidently employed to make nmr assignments when secondary structure is not a factor or when the "guest" residue does not perturb the secondary structure. The major advantage of α-deuterated oligopeptides is that assignments based on such compounds will be valid irrespective of the solvent or the secondary structure of the oligomers. We are currently comparing the results in $CDCl_3$ with those obtained under conditions where the oligomers are believed to form helices in solution.

Acknowledgment

The use of the 220 MHz nmr at Rockefeller University, which is supported in part by NSF grant PCM-74-1224Z, is acknowledged. We would also like to acknowledge the use of the HXS-360 spectrometer at the Stanford Magnetic Resonance Lab (GP-23633 and NIH Grant #RR-00711).

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AN EXPERIMENTAL APPROACH TO PEPTIDE-SOLVENT INTERACTIONS

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Local forces dominate in establishing the conformations of peptides and proteins. Thus diamides are valuable models for larger systems. We have obtained additional nuclear magnetic resonance and circular dichroism data on the well-studied diamides of proline and alanine. From these data, conformers in a range of solvents have been inferred. Through computations, we have tested a number of assumptions about alterations in conformation due to peptide solvation, including effects which may be unique to hydration.

For the *trans* isomer of AcProNHMe, a C_7 conformer (Φ, ψ) = (-70°, 70°) has been deduced from infrared[1] and CD[2] data in nonpolar solvents. Our ^{13}C NMR data ($\Delta\delta_{\beta\gamma}$) coupled with a published correlation[3] and the nuclear Overhouser enhancement (NOE) of the H^{α} when the N-H is irradiated, confirm this assignment. As the polarity of the solvent is increased, the increase in the ^{13}C chemical shift difference $\Delta\delta_{\beta\gamma}$ exactly parallels the decrease in n-π^* CD (Figure 1). Both observations signal the depopulation of the C_7 conformer, which is absent in water.

In dimethyl sulfoxide solution, upon irradiating the N-H, the proline H^{α} shows a NOE of 9% but the H^{δ}'s show none. $\Delta\delta_{\beta\gamma}$ indicates absence of the C_7 form. Thus exclusive population of conformers in the polyproline II region (P_{II}, Ψ = 160°) is indicated. By contrast in acetonitrile when the N-H is irradiated, all of the proline ring protons show comparable NOE's of *ca.* 6%. This observation plus ^{13}C NMR and CD data (Figure 1) indicate roughly equal populations of the P_{II}, C_7 and α_R (α-helical), Ψ = -60°) conformers in acetonitrile.

Intramolecular potential energies, which include the electrostatic stabilization of a hydrogen bond, predict nearly 90% of the population of the *trans* isomer to be in the C_7 conformer[4], in accord with observations in non-polar solvents. Neglecting electrostatic interactions, 75% of the

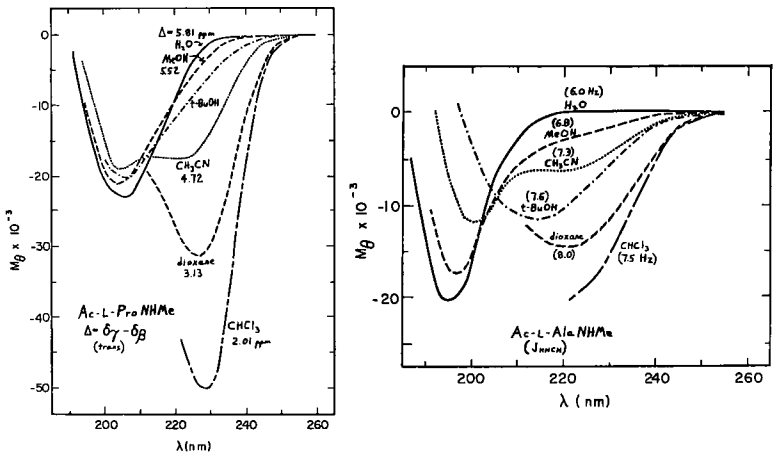

Fig. 1. CD Spectra and NMR parameters for two dipeptides. Solvents and numerical values of $\Delta\delta_{\beta\gamma}$ for *trans* AcProNHMe and $^3J_{HNC\alpha H}$ for AcAlaNHMe are indicated on the Figure.

population is expected in the P_{II} region. Solvation of the N-H, as modeled by the attachment of an oxygen at the hydrogen-bonded distance, places all of the molecules in this region, consonant with observations in dimethyl sulfoxide. Interactions of the peptide dipole with bulk solvent[5] and/or solvation of the carbonyl oxygens, modeled by increasing the oxygen van der Waals radii, increases the population of the α_R region as observed in acetonitrile and water.

The CD spectra of AcAlaNHMe are very similar to those of AcProNHMe in each of the solvents (Figure 1). The size of the n-π^* bands is reduced for AcAlaNHMe, but the magnitude of the band increases in the same solvent order for the two peptides except for exchange of acetonitrile and *tert*-butanol.

Precise $^3J_{HNC\alpha H}$ coupling constants were determined for ^{15}N-AcAlaNHMe (Figure 1). Our value of 7.5 Hz in chloroform, the CD spectrum and infrared data[6] are consistent with about 50% population of the C_7 conformer. This population and $\langle J \rangle$ = 7.4 Hz are predicted from the standard intramolecular potential energy with ϵ = 1 (Figure 2A).

The H-N-C$^\alpha$-H coupling constant is 6.8 Hz in methanol and 6.0 Hz in water, consistent with Φ values near -160° and/or -75°. Because of the parallelism of the CD spectra of AcAlaNHMe and AcProNHMe, we conclude that Φ values near -75° are preferred for the Ala peptide since Pro cannot assume the -160° values. Thus α_R and P_{II} conformers dominate for both compounds in polar solvents. For AcAlaNHMe curve-

fitting reveals a positive n-π^* band in water which indicates a substantial population of the P_{II} conformer, because only this region is predicted to give a positive contribution[7].

Our computations which model peptide solvation preferentially populate the α_R and P_{II} regions. The Solvated Dipole Model (Figure 2C) places 65% in these regions and gives $\langle J \rangle$ = 6.8 Hz as observed in methanol. The Big Oxygen Model (carbonyl solvation) places 98% of the population in the α_R and P_{II} regions ($\langle J \rangle$ = 6.2 Hz). Both models strongly favor α_R over P_{II}; they predict a negative n-π^* band rather than the positive band which is observed in water.

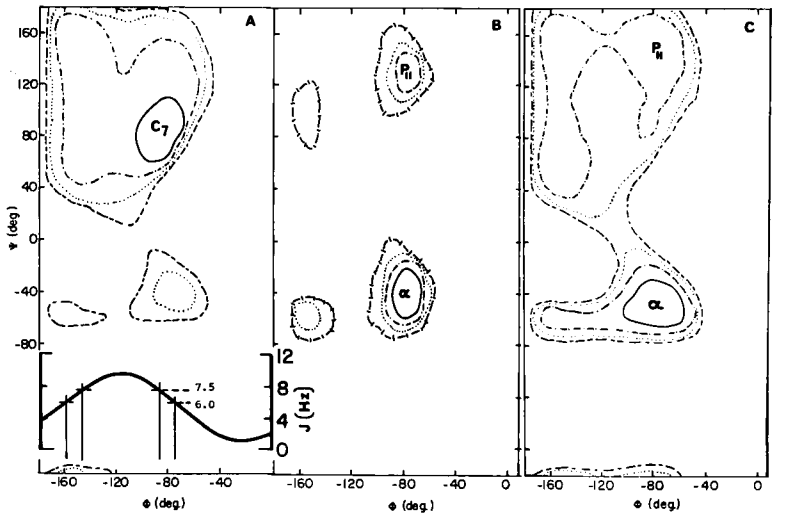

Fig. 2. Potential energy contours computed for AcAlaNHMe by the ECEPP method[8]. The height in kcal/mole above the minima is given by lines of different style: — 1; — · — · 2; ····· 3; -----4; - · - · -5. *A. Standard intramolecular potential* (ϵ = 1). Inset predicted $^3J_{HNC\alpha H}$[9]. *B. Big Oxygen Model* — steric energy ($\epsilon = \infty$) with oxygen radii increased by 1 A. *C. Solvated Dipole Model* — steric energy plus solvation energy computed as 14.4 kcal/mole x (μ^2/r^3) x $(\epsilon-1)/(2\epsilon+1)$ where μ (Debyes) is the peptide dipole moment and r(A) the radius of the sphere approximating the molecule[5].

In summary, for both *trans* AcProNHMe and AcAlaNHMe, the C_7 conformer dominates in inert solvents such as chloroform as predicted by intramolecular potential functions. In polar, hydroxylic solvents such as water and methanol, both dipeptides preferentially populate the α_R and P_{II} regions. This preference may reflect effects of solvent polarization and/or carbonyl oxygen solvation.

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CONFORMATIONAL STUDIES ON CYCLIC PEPTIDE ANALOGS OF THE REPEAT TETRAPEPTIDE OF TROPOELASTIN

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Introduction

Tropoelastin with sequential polypeptides of the type: Val_1-Pro_2-Gly_3-Gly_4; Val_1-Pro_2-Gly_3-Val_4-Gly_5; Ala-$_1$-Pro_2-Gly_3-Val_4-Gly_5-Val_6 is a soluble, non cross-linked precursor of elastin[1,2] isolated from the aortas of copper-deficient pigs. These monomeric peptide sequences, their oligomers and high polymers have been synthesized and their secondary and tertiary structures have been studied[4]. It is convenient to study the secondary structure of a cyclic peptide in solution since there is less freedom of movement of its backbone due to cyclization restraint. With small variation in the torsion angles of a cyclic peptide, it is possible to generate the conformation of a helical linear peptide, i.e. a helical peptide with a similar number of residues occurring per turn of helix as occurs in the cyclic structure[5].

Detailed spectral analysis of the monomeric tetrapeptide unit VPGG has led to the proposal of a Type II β turn involving Val_1-C-O···H-N-Gly_4 hydrogen bond[6,7] and an additional 14-membered Val_1-N-H···O-C-Gly_4 hydrogen bond under special circumstances[4]. Conformational energy calculations indicated these features in the minimum energy structure for the tetrapeptide[8]. The purpose of this study is to identify a cyclic peptide analog of the tetramer series which closely resembles that of poly (VPGG) and which in turn enables us to determine the conformational details of the polymer in solution. Cyclic peptides of the type $\overline{(VPGG)_n}$ (n = 2, 3, 4, 5, and 6) were synthesized by solution methods. Their PMR and CMR spectra are discussed here.

Materials and Methods

Intermediate compounds and final products from solution syntheses[9-12] were characterized by thin layer chromatography in three different solvent systems, elemental analyses and nuclear magnetic resonance spectra. Proton magnetic resonance spectra were obtained on a Varian HR-220 MHz continuous wave spectrometer operating at a probe temperature of 21° C (+ 2°) and equipped with an SS-100 computer system. Dimethyl-d_6-sulphoxide, 99.5% D (Me_2SO-d_6) was used as solvent, tetramethylsilane (Me_4Si) as internal reference. The carbon-13 magnetic resonance spectra were obtained on a JEOL-PFT-100 pulse spectrometer at 25.15 MHz utilizing an internal deuterium lock and proton noise decoupling. The spectrometer was operated by an EC-100 computer system in the Fourier transform mode with a Texas Instruments 980A computer containing 20K of memory. The ambient probe temperature ranged from 26 to 31° C. Deuterium oxide, (D_2O), 99.7% D was used as the solvent with p-dioxane as the internal reference at 67.4 ppm shift from external tetramethylsilane.

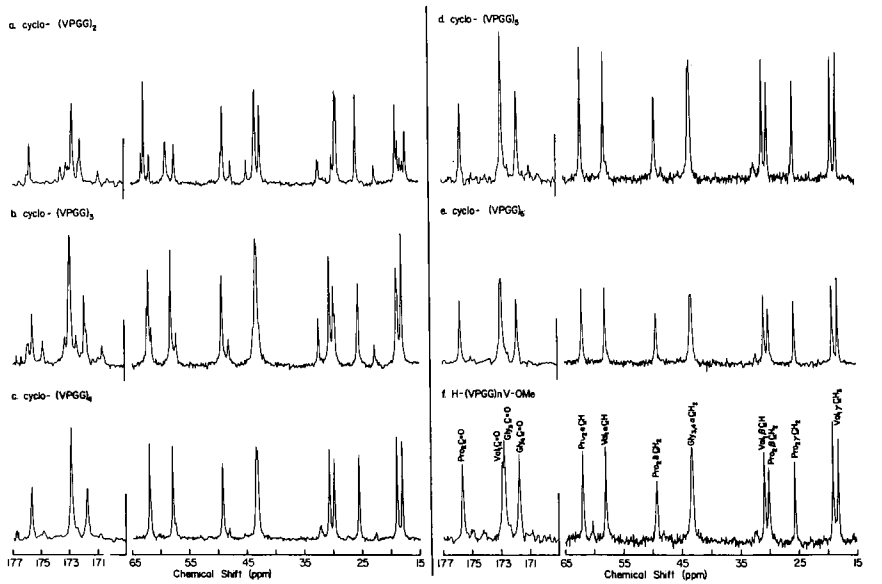

Fig. 1. ^{13}C Magnetic resonance spectra at 25 MHz of the elastin tetramer sequence, Val_1-Pro_2-Gly_3-Gly_4, in D_2O.

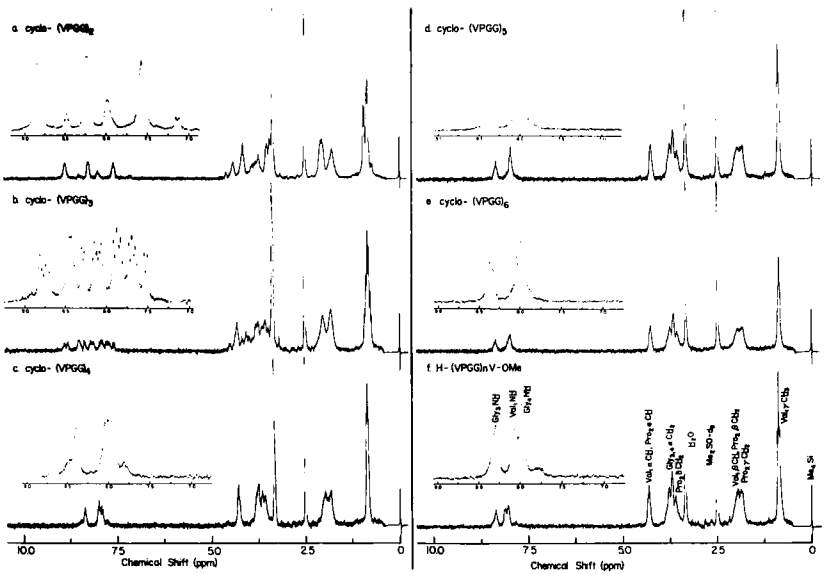

Fig. 2 ^{1}H Magnetic resonance spectra at 220 MHz of the elastin tetramer sequence, Val_1-Pro_2-Gly_3-Gly_4, in Me_2SO-d_6.

Discussion

The ^{13}C magnetic resonance spectra in D_2O of the elastin tetramer high polymer and of the cyclic tetramer analogues, where the number of repeats of the tetramer sequence are 2, 3, 4, 5 and 6, are given in Figure 1. Corresponding proton magnetic resonance spectra at 220 MHz in Me_2SO-d_6 of these molecules are given in Figure 2. Upon initial examination of the CMR spectra it may be seen that the spectral lines of the cyclic peptides where n = 4, 5 and 6 more closely correlate with those of the linear high polymer than those where n = 2 or 3. In fact, one may make tentative assignments of $\overline{(VPGG)_4}$, $\overline{(VPGG)_5}$, and $\overline{(VPGG)_6}$ for each ^{13}C resonance based on those (previously determined assignments) of the polymer[13].

As the higher observation frequency of 220 MHz in the PMR (compared to 25 MHz in the CMR) gives rise to greater resolution of spectral lines, it is possible to note more subtle differences and similarities between the linear tetramer and its cyclic oligomers. Again, the PMR spectra of $\overline{(VPGG)_2}$ and $\overline{(VPGG)_3}$ have little in common with the polytetrapeptide. Upon examination of the peptide-N*H* and αC*H* regions of the $\overline{(VPGG)_4}$ spectrum slight differences from the linear polymer are seen, thus limiting this oligomer as a cyclic correlate. The

spectra of both $\overline{\text{(VPGG)}_5}$ and $\overline{\text{(VPGG)}_6}$ correlate closely with the linear polymer; however, the expansions of the peptide N*H* regions of these peptides show the Val_1 N*H* doublet of the cyclic tetraeicosapeptide to be more downfield, as it is in the high polymer, than the Val_1 N*H* of the cyclic eicosapeptide. Also the Val_1, Gly_3 *C* = O resonance of VPG-G_6 appears to be broadened, as if, with higher resolution, the two *C*= O's might be split as in the polytetrapeptide.

Thus from the NMR spectra shown, the cyclo-$(\text{VPGG})_6$ appears to be the closest correlate to the linear polytetrapeptide. The question now arises as to what occurs as one studies higher repeats of the cyclic tetramer, that is, where n = 7 and 8 or higher. Further PMR and CMR studies, such as solvent dependence of peptide N*H* and *C*=O and temperature dependence of peptide N*H*, are being carried out in this laboratory on the cyclic oligomers already synthesized. Comparisons of these studies with similar secondary structure determinations of the linear polytetrapeptide should facilitate choosing the best cyclic correlate for the linear tetramer.

Acknowledgment

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PREFERRED HYDROGEN-BOND CONFORMATIONS OF CYCLIC PENTAPEPTIDES

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Introduction

Intramolecularly hydrogen-bonded conformations are adopted by a variety of cyclic pentapeptides[1-8]. The presence of both i←i+3 and i←i+2 hydrogen bonds in solution[1-6] and in crystals[7,8] makes these peptides useful models for β- and γ-turns[9]. We have synthesized and examined by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) several cyclic pentapeptides to study the relationship between sequence and preferred conformation. Three new analogues (5, 6, and 7 in Table I) are discussed in this paper and are compared with other proline-containing cyclic pentapeptides.

Table I. Hydrogen Bonding in Proline-Containing Cyclic Pentapeptides.

	Peptide[a]	Refs.
1	cyclo(Gly^1-Pro^2-Gly^3-D-Ala^4-Pro^5)	3(soln), 8(Xstal)
2	cyclo(Gly^1-Pro^2-Ser^3-D-Ala^4-Pro^5)	6(soln), 9(Xstal)
3	cyclo(Gly^1-Pro^2-Phe^3-Gly^4-Phe^5)	2
4	cyclo(Gly^1-Ala^2-Gly^3-Gly^4-Pro^5)[b]	1
5	cyclo(Gly^1-Pro^2-Ala^3-D-Phe^4-Pro^5)	This paper
6	cyclo(D-Phe^1-Pro^2-Gly^3-D-Ala^4-Pro^5)	This paper
7	cyclo(Gly^1-Pro^2-D-Phe^3-Gly^4-Ala^5)	This paper

[a]Hydrogen-bonded N-H's are underlined (Solution; Crystal). All optically active amino acids are of the L-configuration unless indicated otherwise. [b]Unambiguous assignment of Gly's not possible; the described conformation was independently suggested for 4 in Refs. 4 and 5 by comparison with related peptides.

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Results

^{1}H NMR data on the N-H resonances of seven proline-containing cyclic pentapeptides are presented in Table II. Data cited are for all-*trans* species, which are predominant in all cases ($\geq$ 90% for all but 4; 65% for 4)[2,3,5,10]. Note that the N-H of residue 1 appears to participate in hydrogen-bonding in all seven cases (low $\Delta\delta/\Delta T$ and small $\Delta\delta_{CDCl_3 \rightarrow (CD_3)_2SO}$), and that the N-H of residue 4 does so as well except in peptides 5 and 2. In these latter two peptides the data suggest that the N-H of residue 4 is nonetheless somewhat sequestered (*cf.* N-H of residue 3). Peptide 7 is probably undergoing some conformatioal averaging, as indicated by $\Delta\delta/\Delta T$ values and by the significant downfield shift of all four N-H resonances between $CDCl_3$ and $(CD_3)_2SO$ solutions.

Table II. N-H Data for Proline-Containing Cyclic Pentapeptides[a]

		Residue							
		1		3		4		5	
Pept.	Solv.	δ	Δδ/ΔT	δ	Δδ/ΔT	δ	Δδ/ΔT	δ	Δδ/ΔT
1	$CDCl_3$	7.81	1.6	6.60	13.9	7.81	3.2		
	$(CD_3)_2SO$	7.40	0	8.49	4.5	7.64	0.5		
2	CD_2Cl_2	7.71	0	6.57	11	7.45	15		
	$(CD_3)_2SO$	7.53	0.5	7.59	4.2	7.73	3.2		
3[b]	$CDCl_3$	7.58	*d*	6.97	*d*	7.37	*d*	6.61	*d*
	$(CD_3)_2SO$	7.74	1.7	8.26	7.3	7.68	1.7	7.56	4.3
4[c]	$(CD_3)_2SO$	7.70	1.7	8.51	4.5	7.63	0.4		
5	$CDCl_3$	8.26	3.8	6.77	7.2	7.50	5.6		
	$(CD_3)_2SO$	7.64	0.6	7.68	3.7	7.82	4.0		
6	$CDCl_3$	7.90	2.7	6.47	7.8	7.76	6.1		
	$(CD_3)_2SO$	7.72	0.7	8.81	5.6	7.68	4.0		
7[b]	$CDCl_3$[e]	7.58	3	6.43	6	6.68	3	6.25	4
	$(CD_3)_2SO$	8.20	3.2	8.72	5.8	7.51	4.0	8.30	7.1

[a] δ is ppm from TMS; $\Delta\delta/\Delta T$ is ppm/deg X 10^3. Peptides 5, 6 and 7: conc 20 mg/ml, temp 24°. For others, see Refs. in Table I. [b] Gly assignments not definitive, based on comparisons with other peptides. [c] Residue 2 is Ala: δ=8.57, $\Delta\delta/\Delta T$=5.8. Gly assignments are those given in Ref. 4. [d] Not reported. [e] Conc < 4 mg/ml; limit of solubility.

^{13}C NMR spectra of peptides 5 (Figure 1a) and 6 resemble those of previously studied pentapeptides containing two prolines (*viz.* 1 and 2)[3,5] in that the C^α and C^β signals of the two prolines are markedly different from one another. The upfield-shifted resonances correlate with the presence of a γ-turn (where Pro is in position i+1)[3,5]. The spectrum of

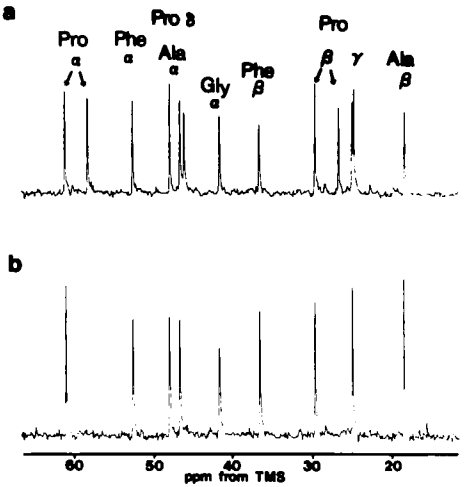
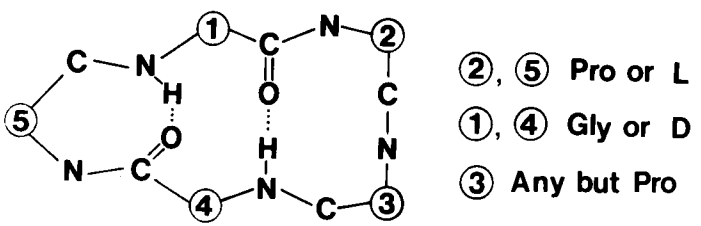


Fig. 1. 25.00 MHz ^{13}C NMR spectra (upfield region) of (a) *cyclo* (Gly-Pro-Ala-D-Phe-Pro), and (b) *cyclo* (Gly-Pro-Ala-D-Phe-Pro(d_7)). Conc 64 mg/ml; temp 25°; solv $CDCl_3$; ^1H noise decoupled.

peptide 5 synthesized with perdeuterioproline as residue 5 (Figure 1b) establishes clearly that Pro^5 is in the γ-turn. The close correspondence in results for peptides 5 and 2 supports the same Pro assignment for 2 (in contrast to that originally proposed[5]).

Conformational Interpretations

The results on proline-containing cyclic pentapeptides indicate a general preference for a solution conformation which contains a hydrogen-bonded γ-turn (see below). The rest of the molecule is composed of a β-turn (Type I or II) which may or may not be hydrogen-bonded. Alternative conformations containing Type II′ β-turns (such as that found in crystals of 2[8] and that proposed for 2 in solution[5]) are *not* observed, even though model building and sequence considerations suggest that they are possible.



The preferred solution conformation in these peptides is that which results in *Pro's in positions i+1 of γ- and/or β-turns*. A further conformational determinant appears to be the placement of *L-residues in corner positions of turns* (i+1 (preferably) or i+2 of β-turns, and i+1 of γ-turns).

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SOLVENT AND STRUCTURAL EFFECTS ON CYCLIC HEXAPEPTIDE CONFORMATION

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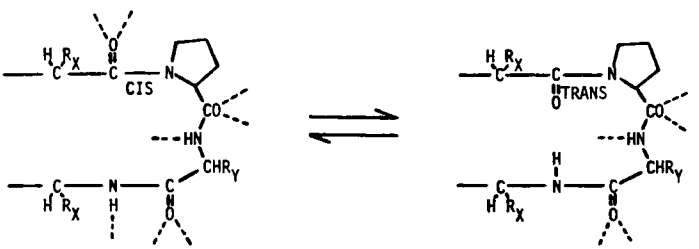
Cyclic hexapeptides of the type c-(L-Xxx-L-Pro-D-Yyy)$_2$ or c-(L-Xxx-L-Pro-Gly)$_2$ exist in solution predominantly in two forms of C_2 symmetry on the nmr time scale, one with all *trans* peptide links and the other with both Xxx-Pro bonds *cis*. These forms are in slow exchange below about 80°[1,2,3].

The all-*trans* form is well defined both by solution NMR work and by X-ray crystallographic analyses[4,5,6]. It is formed of two Type II Pro-D-Yyy or Pro-Gly β-turns, connected by two extended Xxx residues. The N-H and C=O bonds of the extended residues are directed to the interior of the peptide ring and are thereby sequestered. Several conformations are proposed for the two-*cis* backbone; although differing somewhat in proposed torsional angles, they agree in that all of the N-H and C=O units are accessible to solvent. The equilibrium between the two forms is represented in the equation below, where the dashed lines represent interaction with solvent.



We have been measuring the thermodynamic parameters of this equilibrium, using ^{13}C and 360 MHz proton NMR spectroscopy, as a means of identifying solvent influences on peptide conformation. This ratio of all-*trans* to two-*cis* form over a range of temperatures can be determined from the areas under the resonances of a given nucleus in the two forms, *e.g.*, proline β and/or γ carbons, or α or methyl protons. Pulse-FT NMR spectroscopy is used, and complete nuclear relaxation is allowed between pulses.

Table I presents the free energy change for the reaction as written above for c-(L-Xxx-L-Pro-Gly)$_2$ where Xxx is Ala, Leu or Val.

Table I

ΔG_{298} kcal/mol

Xxx	H_2O	CH_3OH	$(CH_3)_3COH$	CH_3SOCH_3
Ala	-1.2	-1.8	$\leqslant$-2	-1.0
Leu	-0.3	-0.9	$\sim$-1.5	-0.2
Val	1.6	0.8	$\sim$ 0	1.4

The values were obtained from plots of measurements made over a 40° or greater temperature range and are probably correct to ± 0.1 kcal/mole, except for the *t*-butyl alcohol data.

It will be noticed that the all-*trans* form is increasingly less stable in the order Ala, Leu, Val, and by about the same increments in each solvent. It is reasonable to ascribe these solvent-independent differences to the intrinsic properties of the peptides, and more specifically to non-bonded interaction between the side chain of Xxx and the δ-CH_2 of proline in the all-*trans* form. The effect of this interaction on the conformational distribution of an L-Ala-*(trans)*-L-Pro sequence has been described[7,8]. The effective bulk of the Xxx side chain increases as methyl, isobutyl, isopropyl.

Solvent dependent differences appear horizontally in Table I. The all-*trans* form is more stable in methanol than in water by about the same increment for all three peptides, and is still more stable in *t*-butyl alcohol. In contrast, ΔG in each case is about the same in dimethyl sulfoxide and water. The side chain-independent differences may be interpreted as reflecting solvation of the N-H and C=O groups that are internal in the all-*trans* form but external in the two-*cis* form. In the hydroxylic solvents, both the concentration and accessibility of hydroxyl groups increases in the order *t*-butyl alcohol, methanol, water; the resulting increasingly stable (in the sense of free energy) hydrogen bonding of the N-H and C=O groups will increase the relatively stability of the two-*cis* form.

There are two possible reasons for the dimethyl sulfoxide — water parallelism: Although DMSO can only hydrogen bond to N-H, its oxygen is probably an intrinsically better receptor than that of water, judging from infrared and calorimetric comparisons of DMSO with ethers in formation of hydrogen bonded complexes with proton donors[9,10]. Also, it may be that the C=O groups of Xxx in the all-*trans*

form are not entirely shielded from water in the first place. Crystals of c-(L-Ala-L-Pro-D-Phe)$_2$ in the all-*trans* form contain one water molecule, among the eight per peptide, that is associated with the two internally directed Ala carbonyls[4].

In Table II we show ΔH and ΔS values obtained for the three peptides and the additional one, c-(L-His-L-Pro-D-Phe)$_2$·2HCl, for which we have data in two solvents. Because the measurement of areas under nuclear resonances is not in general highly reproducible, and because the ΔH's characteristic of the equilibrium are small, the fractional error in the values of ΔH obtained from van't Hoff plots may be large. Table II gives values of ΔH and ΔS_{298} which we believe to be good to $\pm$ 0.3 kcal and $\pm$ 1 e.u., respectively.

Table II

Peptide		H_2O	CH_3OH	CH_3SOCH_3
c-(Ala-Pro-Gly)$_2$	ΔH	-0.3		0.0
	ΔS	3		3
c-(Leu-Pro-Gly)$_2$	ΔH	0.0	-2.5	-1.0
	ΔS	1	-5	-3
c-(Val-Pro-Gly)$_2$	ΔH	1.5	0.0	
	ΔS	0	-2.5	
c-(His-Pro-D-Phe)$_2$ ·2HCl	ΔH	1.0		3.8
	ΔS	0		11

A noteworthy point in these limited data is the generally larger magnitude of the entropy changes in the organic solvents. This is most apparent here for c-(His-Pro-D-Phe)$_2$·2HCl. Its large ΔH and ΔS values are not due to its charged side chain; there is no reason to believe that the side chain will be differently accessible to solvent in the two forms; in fact, the values of ΔH and ΔS for c-(Ala-Pro-D-Phe)$_2$ in dimethyl sulfoxide, communicated to us by Professor Giovanni Giacometti, of the University of Padua, are very similar, 3.8 kcal/mol and 12 e.u. Further good data are required before a firm explanatory hypothesis is reached, but we suggest now that an important factor will be that greater organization of the bulkier solvent is required to solvate the amide groups of the peptides with larger side chains.

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ARRANGEMENT OF THE AMINO ACID SIDE CHAINS IN ANGIOTENSIN II

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Introduction

The biological activity of peptide hormones such as Angiotensin II (Asp-Arg-Val-Tyr-Ile(Val)-His-Pro-Phe) is characterized by the high precision of the drug/receptor recognition process. All studies of structure-activity relationships using fragments and structural analogs bear witness to the fact that beyond the necessary presence of functional groups such as phenol, imidazole or the phenyl ring in the side chains of the peptide residues, it is the spatial orientation of all atoms in the molecules that ultimately determine the degree of their biological activity. Intense efforts to elucidate the conformation of the backbone of Angiotensin II have yielded a number of models having in common such features as a compact shape incorporating β- or γ-turns, and proximity of the N- and C-terminal groups. A precise description is given[1].

It has become clear though that the study of the backbone conformation is neither sufficient nor even dissociable from the study of amino acid side chain orientation if a better understanding of the structure-function relationships in peptides is to be achieved. In this paper we present the way we chose to solve the problem of amino acid side chain conformation in Angiotensin II, by associating ^{13}C enrichment (85%) of 6 residues one by one (Asp, Val, Tyr, Val, Pro, Phe) permitting their detailed ^{13}C-NMR study, with the use of high magnetic fields (360 MHz) for ^{1}H-NMR, making analysis of all ABX ($\alpha\beta$, $\alpha\beta'$) systems possible.

Results

^{13}C Spectra were recorded for each of the six labelled peptides at various pH values. As only the signal of the enriched amino acid is

observed, assignment and determination of chemical shifts become obvious. High resolution spectra also yield the ^{13}C-^{13}C coupling constants over 1, 2, and 3 bonds which are not obtainable at natural abundance of ^{13}C.

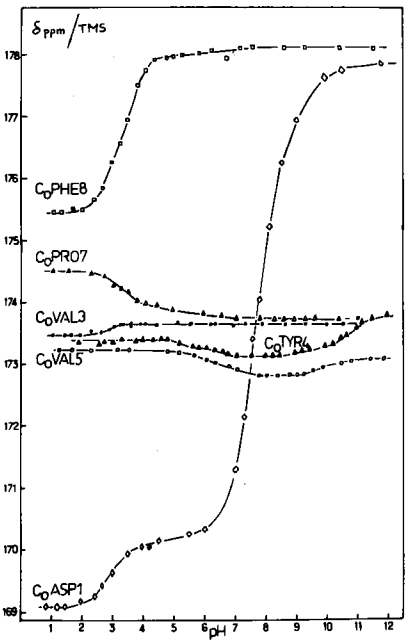

Fig. 1. ^{13}C Chemical shifts of carbonyl atoms in Angiotensin II vs. pH.

a) **Chemical shifts:** The chemical shift values of the carbon signals plotted as a function of pH yield titration curves an example of which (Co) is shown in Figure 1. The strongest titration effects are observed on the residues Asp^1 (β-COOH and α-NH_3^+ group) and Phe^8 (α-COOH) where the ionizable group is directly attached to the residue observed. Of consequence for the interpretation in conformational terms are the shapes of the Co and Cα titration curves of Tyr^4 and Val^3 and Val^5; Tyr^4 signals are shifted measurably during the His^6-titration, recalling to mind the results obtained by ^{1}H-NMR and Circular Dichroism studies demonstrating the mutual influence of the Tyr and His side chains [1]. The Co and Cα signals of Val^5 sandwiched between Tyr^4 and His^6 undergo the influence of the imidazole as well as the phenol titration, whereas Val^3 is less influenced by its neighbor Tyr^4 than by the Asp^1 side chain (COOH titration) 3 residues removed. Side chain-side chain (Tyr-His), side chain-

backbone (Asp-COOH - Val[3]), and backbone-backbone (Phe-COOH -Pro-Co) [2] interactions are probable explanations for these briefly described data.

b) **Coupling constants:** Direct ^{13}C-^{13}C couplings ^1J do not vary much from one residue to another nor during titration, except for the carbon atoms close to the ionization sites. They are comprised between 59 and 50 Hz for $^1J_{C_O\text{-}C_\alpha}$ couplings and 36 to 30 Hz for $^1J_{C_\alpha, C_\beta}$, $^1J_{C_\beta, C_\gamma}$ and similar couplings. Vicinal coupling constants of angular dependence ($^3J_{C_O\text{-}C_\gamma}$) are richer in information as they can be tied to the rotamer population of the amino acid side chains.

The usefulness of parallel studies by ^{13}C and ^1H-NMR is evident from the results presented in Table I. First, residues having two protons on the β-carbon (Asp, Arg, Tyr, His, Phe) show two $^3J_{H_\alpha H_\beta}$ coupling constants and only one $^3J_{C_O\text{-}C_\gamma}$ value, whereas residues with only one β-proton and two γ-carbons (Val, Ile) yield two values for $^3J_{C_O\text{-}C_\gamma}$ and only one $^3J_{H_\alpha H_\beta}$; secondly, where assignment of the β and β' protons or γ and γ' carbons is problematic, the comparison of rotamer I fractions in the former case and of rotamer II fractions in the latter (Table I) obtained from both ^{13}C and ^1H spectra resolves the question.

Table I. Coupling Constants and Rotamer Distribution in Antiotensin II.

	ASP	ARG	VAL	TYR	VAL	HIS	PRO	PHE
$^3J_{C_O\text{-}C_\gamma}$	3.0	-	2.25	2.5	2.25	-	0.0	2.2
			0.75		0.75			
Rotamer popul.	R^1=0.45	-	R^1=0.07 R^2=0.50	R^1=0.58	R^1=0.07 R^2=0.50	-	-	R^1=0.50
$^3J_{H_\alpha H_\beta}$	8.0	7.0	8.2	8.0	8.4	6.0	8.5	8.0
	5.4	7.0		6.6		5.5	5.0	6.0
Rotamer popul.	R^1=0.49 R^2=0.25	R^3=0.20	R^2=0.51	R^1=0.49 R^2=0.36	R^2=0.54	R^1=0.30 R^2=0.26	-	R^1=0.45 R^2=0.31

^1H coupling constants were obtained from the 360 MHz spectrum of Angiotensin II and refined by ITRCAL treatment[3]. Rotamer fractions (R^1:χ^1=-60°; R^2:χ^1=180°;R^3:χ^1=60°) were calculated throughout with J_g=2.6 Hz and J_t=13.56 Hz for $H_\alpha H_\beta$ couplings; the choice of J_g and J_t for the ^{13}C-^{13}C couplings was determined from our experience with amino acid models and modulated to take into account electronegativity effects of neighboring groups: for valine, tyrosine and phenylalanine J_g and J_t were 3.9 and 0.6 Hz; for aspartic acid, 5.9 Hz and 0.6 Hz were used.

As to the distribution of rotamers, it is evident that in aqueous solution, the side chains of Angiotensin II possess relative freedom of movement at all pH values, as all three rotamers appear populated. Nevertheless, individual residues have distinct distribution of rotamers: arginine[2], where the apparent equivalence of β and β' protons indicates high rotational freedom; valine[3] and valine[5] which in contrast to their strongly differing influence on biological activity both prefer rotamer II such as it is found for many valine residues in proteins; tyrosine[4] and phenylalanine[8] where rotamer I is slightly favored over rotamer II, and rotamer III is almost absent; finally histidine[6] with its unusually large proportion of rotamer III (~45%) that hints at some kind of interference with the more usual distribution $R^1 > R^2 > R^3$ which is based on steric factors.

Conclusion

The rotamer populations thus determined are to be considered characteristic of Angiotensin II in aqueous medium; in other words, analogs of Angiotensin II that are apt to modify the side chain spatial organization of, say, histidine or tyrosine, are certain to modify the affinity of the peptide to its receptor, if not the intrinsic activity as well. Examples definitely confirming this assertion are emerging from the work in progress in our laboratory: notable changes in tyrosine and histidine rotamer populations were observed for the N-methylated peptides (devoid of biological activity) such as [Sar[1]MeTyr[4]Ile[8]]AII, [Sar[1]-MeIle[5] Ile[8]]AII[4], as well as for the analogs [Ser[3]]AII, [Thr[3]]AII, [Leu[5]]AII with characteristically diminished potency.

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NMR STUDIES OF ^1H-^2H EXCHANGE IN GRAMICIDIN S

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Introduction

The H exchange process in peptide NH groups is both acid- and base-catalyzed:

$$RCONH^*R' + H_3O^+ \xrightarrow{k_a} RCONH^*HR' + H_2O \rightarrow RCONHR' + H^*H_2O^+$$

$$RCONH^*R + OH^- \xrightarrow{k_b} RCONR'^- + HOH^* \rightarrow RCONHR' + OH^{*-}$$

The first step in each reaction is expected to be rate limiting, and therefore the pseudo-first order rate constant for the reaction $RCONH^*R' + H_2O \xrightarrow{k} RCONHR' + HOH^*$ will be given by:

$$\begin{aligned} &\text{acid catalyzed : } k = k_a[H_3O^+] \\ &\text{base catalyzed : } k = k_b[OH^-] = k_b' / [H_3O^+] \end{aligned} \qquad (1)$$

Using data from model peptides, one may predict the influence of the primary structure on the rate constants[1]; any additional differences between individual peptide NH exchange rates may then be ascribed to secondary or tertiary structure, i.e. either intramolecular hydrogen bonds or hydrophobic regions with peptide bonds inaccessible to the solvent. The exchange under such conditions will depend on the dynamic equilibrium between "closed" and "open" forms of the peptide:

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I \xrightarrow{k_3} \text{exchange}$$

Where the peptide is mainly in a closed (non-exchanging) form N, the behavior of the exchange will depend on the relative size of k_2 and k_3; for $k_2 \gg k_3$ (EX_2 mechanism)[2] the observed rate of exchange is $k = (k_1/k_2)k_3 = K_{eq}k_3$. The first factor gives the degree to which the structure is open, while the second contains the pH dependence. If $k_2 \ll k_3$, on the other hand, $k = k_1$ (EX_1 mechanism), and the exchange will not exhibit the pH dependence of (1).

We have used ^{1}H NMR to measure the rate of amide H exchange in gramicidin S, the cyclic decapeptide (Val-Orn-Leu-D-Phe-Pro)$_2$. The accepted structure for gramicidin S has the valine and leucine NH's involved in hydrogen bonds. Because of the small size of the molecule, there are probably no buried regions, so differences in exchange rates for the different amide protons should reflect the hydrogen bond structure, and its lability. It is well known that the rates for leucine and valine are slower than for phenylalanine and ornithine[3]; our purpose here is to make this quantitative and to try to interpret it in terms of the "opening" of the molecule.

Methods

Gramicidin S (7-8 mg; Sigma Chemical Co.) was dissolved in 0.5 ml of the deuterated solvent, and the NMR spectrum taken immediately and at varying intervals (using the MPC-250 spectrometer). Peak heights as a function of time were fitted by least squares to a single exponential.

The solvent used was a mixture of $^{2}H_2O$ and dioxane (5:2, v/v). The $^{2}H_2O$ part contained varying proportions of 0.1 M KCl and 0.1 M HCl for the acid region, and 0.1 M KCl plus traces of NaOH for the base region. The pH reported is the uncorrected reading of a glass electrode in the final solution. All measurements were done at 25° C.

Results

Figure 1 shows the result of a set of measurements of the decay of the amide proton NMR signals. The dependence of pH is as expected for an EX_2 mechanism. The values of k_a and k_b' are given in Table I, along with their relative values. For several reasons (principally the nature of the solvent) only relative values have significance. The calculated influence of primary structure on the relative rates, based on the results of Molday *et al.*[1] is given in the third column of Table I, and the resultant secondary structure effects for gramicidin S in column 4.

In the acid-catalyzed region there is a clear separation into two groups, with the rates for the hydrogen-bonded amides leucine and valine ca. 30 times smaller than for the ornithine and phenylalanine. This is consistent with a model where a single opening reaction makes both the leucine and valine amide protons exchangeable.

The results are quite different for the base-catalyzed range, where all of the rates are substantially different. The reason for this is not at all clear. It would not be surprising if a different sort of opening were

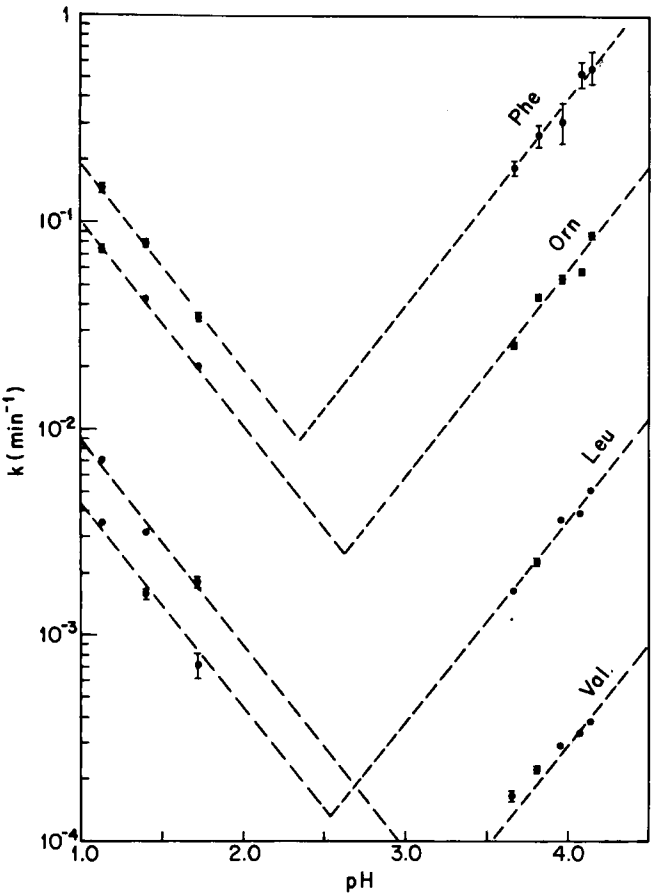

Fig. 1. Rate of disappearance of ^1H signals of gramicidin-S.

required for the acid and base catalysis, since the intermediate states in (1) are different. For acid catalysis the hydrogen bond might not need to be broken, while for the base catalysis it has to be. This cannot explain the difference between phenylalanine and ornithine, however, since they are supposedly free.

Conclusion

We have found that the exchange of amide protons in gramicidin S takes place through an EX_2 mechanism, and that the acid-catalyzed rates fall into two distinct groups — those involved in internal hydrogen bonds (leucine and valine) and those "free" (phenylalanine and ornithine). The rates for leucine and valine, when corrected for the influence of neighbor-

Table I.

Acid Catalyzed	$\log k_a$ [a]	$\Delta \log k_a$	S_1 [b]	S_2 [c]
Phe	0.284	-	-	-
Orn	0.020	- 0.26	- 0.35	0.09
Leu	- 1.35	- 1.63	- 0.2	-1.43
Val	- 1.04	- 1.33	0.2	-1.53
Base Catalyzed	$\log k_b'$ [a]	$\Delta \log k_b'$	S_1	S_2
Phe	- 4.390	-	-	-
Orn	- 5.232	- 0.84	0.2	-1.04
Leu	- 6.437	- 2.05	0.4	-2.45
Val	- 7.504	- 3.11	0.1	-3.21

[a] k in min^{-1}
[b] primary structure effect
[c] secondary structure effect

ing residues, are almost identical, suggesting a single opening reaction that makes these protons accessible. In the base-catalyzed range all four sets of amide protons exchange at different rates; we have at present no explanation for this. Whether this difference between acid and base catalysis will hold for other peptides remains to be seen.

This work was supported by NIH grants AM 16532 and RR 00292.

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A COMPARISON OF THE CONFORMATIONS OF ARGININE VASOPRESSIN, ARGININE VASOTOCIN, OXYTOCIN, AND OXYPRESSIN IN AQUEOUS SOLUTION BY ^{1}H NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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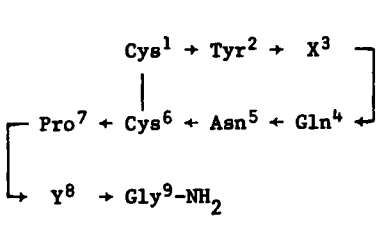
Introduction

The solution conformations of the neurohypophyseal hormones and their analogues have been studied by various forms of NMR spectroscopy[1,2]. We are particularly interested in studying the conformations of these peptides in aqueous solution by ^{1}H NMR. In this study, we explore some of the effects on ^{1}H NMR characteristics that result from changes at positions 3 and 8, the main sites of evolutionary modification in these hormones. Figure 1 shows the primary structures of the peptides used in this study.



	X^3	Y^8
Oxypressin (OP)	Phe^3	Leu^8
Arginine Vasopressin (AVP)	Phe^3	Arg^8
Arginine Vasotocin (AVT)	Ile^3	Arg^8
Oxytocin (OT)	Ile^3	Leu^8

Fig. 1. Primary structures of selected neurohypophyseal hormones and the synthetic analogue oxypressin.

Materials and Methods

All peptides were synthesized by the solid-phase method. Exchangeable protons of each peptide were replaced by deuterons, and 10 mg of

each pre-exchanged peptide was dissolved separately in 0.7 ml of D_2O. The pD of each sample was adjusted to 3.8.

^{1}H NMR spectra were obtained at 20° C on a Bruker 360-MHz spectrometer. Chemical shifts are reported downfield with respect to [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)propionate (TSP), which was used as an internal standard.

Results and Discussion

^{1}H NMR spectra of each peptide in D_2O at pD 3.8 and 20° C were obtained at 360 MHz and analyzed to yield chemical shifts and coupling constants. Justification of the assignments of chemical shifts to specific protons and of coupling constants to specific pairs of protons will be given in a later, expanded publication. The use of total spin decoupling to detect geminally and vicinally located pairs of protons aided the assignment process.

Figures 2-4 show the chemical shifts that fall in the three regions of primary interest in this study. The chemical shifts of corresponding protons generally change by 0.1 ppm or less in going from one peptide to another, except for the α and β protons of Tyr^2, which shift 0.13-0.24 ppm, and for the apparently equivalent γ protons of Gln^4, which shift 0.11 ppm, in going from one of the tocins (AVT and OT) to the corresponding pressin (AVP and AVT). The coupling constants between corresponding pairs of vicinal α and β protons appear to be equal within experimental error.

We conclude that the upfield shifts in the α and β protons of Tyr^2 observed in going from a tocin to the corresponding pressin are most likely related to ring-current shifts from the next residue inasmuch as an aromatic residue (Phe^3) replaces an aliphatic one (Ile^3) in going from one of the former to one of the latter peptides. (Also see Deslauriers and Smith[3] and Feeney *et al.*,[4] who compared OT and [8-lysine]vasopressin by ^{1}H NMR spectroscopy.)

The chemical shift of an α proton in a peptide is known to depend, in part, upon the backbone conformation[5]. The similarity of the chemical shifts of the corresponding α protons, with the aforementioned exception of those of Tyr^2, is compatible with a high degree of similarity in the basic backbone conformations of the four peptides in D_2O. Furthermore, the similarity of the chemical shifts of the corresponding β protons and of the coupling constants between corresponding pairs of α and β protons is compatible with similarity in the basic side-chain conformations — or rotamer populations — about the C^α-C^β bonds of the corresponding

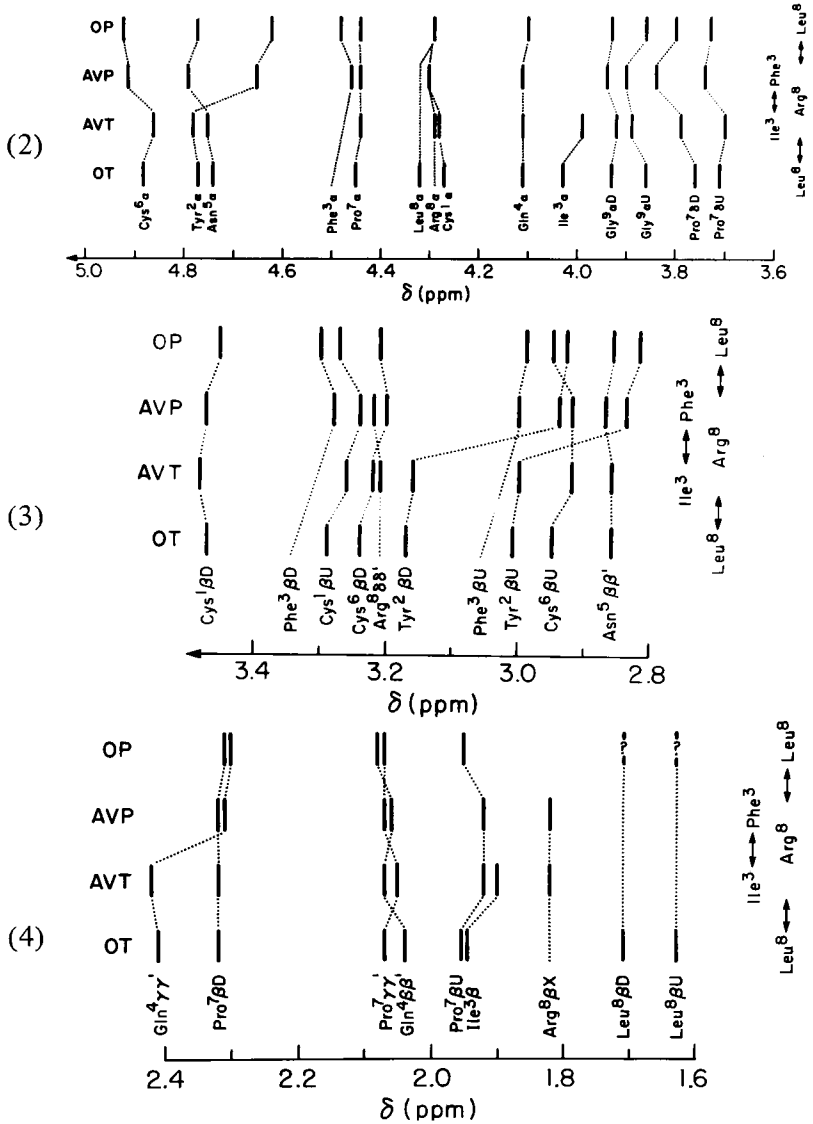


Fig. 2-4. Chemical shifts (δ) in three selected ranges. D and U denote downfield and upfield protons, respectively, which have not been defined stereochemically. X denotes a proton that has not been assigned to D or U. Protons that are apparently equivalent at 360 MHz are denoted by $\beta\beta'$, $\gamma\gamma'$ or $\delta\delta'$. The exact position of the β protons of Leu^8 in OP is not known.

residues. Nevertheless, an examination of our data (Figures 2-4) reveals small but consistent changes in chemical shift for certain protons that can be associated either with the replacement of an aliphatic by an aromatic residue in position 3 in going from a *tocin* (AVT or OT) to the corres-

ponding *pressin* (AVP or OP) or with the replacement of a residue with a neutral side chain by one with a positively charged one in position 8 in going from an *oxy* (OP or OT) to the corresponding *vaso* (AVP or AVT) compound.

In future studies, we plan to focus attention on the small differences in corresponding NMR characteristics and to determine whether they reflect subtle differences in conformation or merely differences in primary structure *per se*. We are particularly interested in pursuing the study of Gln^4 in the four peptides, for a moderate but significant change in chemical shift was observed for the γ protons in going from a tocin to the corresponding pressin. It should be noted that Deslauriers *et al.*[6] found evidence based on studies of ^{13}C spin-lattice relaxation time (T_1) for the side chain of this residue being more restricted in its mobility in [8-lysine]vasopressin (which, like AVP, has a basic residue in position 8) than in OT. From our studies, we would suspect that this difference is more likely related to the change at position 3 than to that at position 8 —i.e., that the restriction is related to the substitution of an aromatic for an aliphatic side chain at the neighboring position. Because we did not observe any consistent changes in the chemical shifts of the α and β protons of Gln^4, we also suspect that any differences in the conformational state of the side chain of this residue occurs about the C^β-C^γ or C^γ-C^δ bond and not about the C^α-C^β.

Acknowledgments

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2,5-DIHYDROPHENYLALANINE AS A PROBE FOR AROMATICITY IN STRUCTURE-ACTIVITY STUDIES IN PEPTIDES

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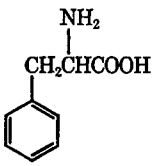
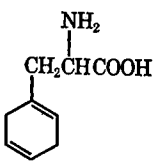
Introduction

2,5-Dihydrophenylalanine (DiHPhe) is an effective antimetabolite of phenylalanine for the rat[1]. It is also a naturally occurring antibiotic for a number of bacteria. These properties are based on the very similar molecular dimensions of Phe and DiHPhe. Like Phe, DiHPhe has a planar ring. Compared to Phe, however, DiHPhe has little resonance energy (probably only 5% of Phe as judged from heats of hydrogenation of cyclohexadiene rings). This suggested the use of DiHPhe to evaluate the importance of aromaticity in biologically active peptides that contain phenylalanine.



Phe



DiHPhe

8-Lysine vasopressin (LVP, Figure 1), the antidiuretic hormone of some species, has a Phe residue in position 3. This is one of the positions (3, 4, and 8) that varies naturally in the 9 known neurohypophysial peptides[2]. This locus is also thought to be important in the binding of the hormone to its receptor. Lysine vasotocin (LVT, Figure 1) has much diminished ADH activity (10% of LVP). Since DiHPhe is much closer sterically to Phe than to the Ile[3] of LVT, availability of [DiHPhe[3]]-LVP (Figure 1) would enable one to reexamine the relevance of aromaticity

and electron density at position 3 to activity, and possibly to binding if this is reflected accurately by the activity. Synthesis of [DiHPhe^3]-LVP (Figure 1) was therefore carried out, and this analog was compared with LVP and LVT in antidiuretic and pressor activity.

Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2	(LVP)
Cys-Tyr-DiHPhe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2	([DiHPhe^3]-LVP)
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys-Gly-NH_2	(LVT)

Fig. 1. Structures of 8-lysine vasotocin, 8-lysine vasopressin, and (DiHPhe^3)-8-lysine vasopressin.

The key intermediate, Z-Cys(Bzl)-Tyr-(Bzl)-DiHPhe-Gln-Asn-Cys(Bzl)-Pro-Lys(ε-Tos)-Gly-NH_2, was synthesized in stepwise fashion by the solid-phase procedure as modified for the posterior pituitary hormones[3]. DiHPhe was introduced using the Boc *p*-nitrophenyl ester[4]. Oxidative ring closure of the deprotected disulfhydryl nonapeptide was accomplished with I_2 in dilute AcOH. [DiHPhe^3]-LVP was purified by chromatography on Amberlite IRC-50, pH 6.38, in the manner described for LVP. In this system, as well as in tlc on silica gel H in 1-BuOH-AcOH-H_2O (4:1:5), [DiHPhe^3]-LVP and LVP tend to co-chromatograph. However, HPLC on the reverse phase μBondapak C_{18} column affords a means of distinguishing and separating [DiHPhe^3]-LVP and LVP (Figure 2).

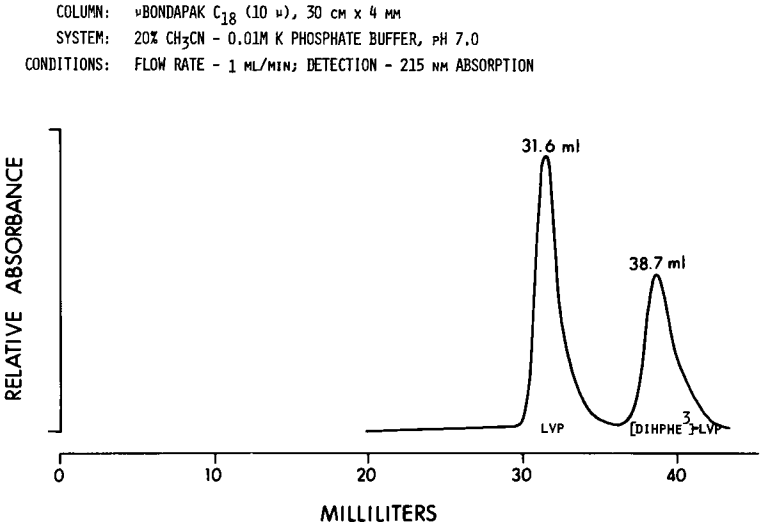


Fig. 2. Chromatographic separation by HPLC of [DiHPhe^3]-LVP and LVP

When present in the protected nonapeptide Z-Cys(Bzl)-Tyr-DiHPhe-Gln-Asn-Cys(Bzl)-Pro-Lys(ε-Tos)-Gly-NH_2, DiHPhe remained 95% stable for 5 years. When present in the free nonapeptide Cys-Tyr-DiHPhe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2, however, DiHPhe underwent approximately 45% conversion into Phe within 2 years. Figure 2 represents a chromatogram of such material.

Analysis of Free and Bound 2,5-Dihydrophenylalanine

DiHPhe is unstable under acidic hydrolytic conditions. However, it has been released from peptides without change by the action of a variety of proteolytic enzymes including Pronase and aminopeptidase M. A crude *Aspergillus oryzae* preparation, available as Sigma α-Amylase, is especially useful for liberating amino acid residue 3 from vasopressin and analogs. Yields of residue 3 are 75-100%. A blank is required to correct for Phe formed from DiHPhe and liberated from the enzyme. Alkaline hydrolysis of peptides with 3N NaOH at 107° C for 22 hr is also useful although more qualitative. Liberated DiHPhe can then be determined on the automatic amino acid analyzer[1]. On the Beckman-Spinco analyzer, model 120C, in the system 0.2N sodium citrate buffer, pH 3.25, followed by pH 4.25 at 56° C, DiHPhe is eluted 59 ml after the indicated pH change, at a position 17 ml after Phe.

Pharmacological Activities of (DiHPhe^3)-8-Lysine Vasopressin

Rat vasopressor activity of the analog was about half that of LVP and the same as that of LVT. Antidiuretic activity was about 62% of LVP and 7 times that of LVT. For very effective rat ADH or vasopressor activity, aromaticity in position 3 is not required. Electron density in residue 3 in LVP appears to enhance ADH activity but not vasopressor activity.

In conclusion, substitution of DiHPhe for Phe in a biologically active peptide constitutes a useful new technique for evaluating the importance to biological activity of the aromaticity of phenylalanine. This technique has been used with lysine vasopressin and it should be applicable to other peptides having phenylalanine in a position involved in the expression of biological activity. Treatment with *Aspergillus oryzae* is a useful procedure for degrading oxytocin, vasopressin and related structures especially to liberate an acid-labile residue in position 3.

Acknowledgment

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NITROGEN — 15 NMR INVESTIGATION OF 8-ARGININE VASOPRESSIN

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Introduction

^{15}N Nuclear magnetic resonance (NMR) is becoming increasingly recognized as a technique for studying peptides and proteins, particularly with the help of isotope enrichment of this rare nucleus (natural abundance 0.3%) and more sensitive spectrometers. Several reports have appeared recently on ^{15}N NMR of oxytocin and prolylleucylglycinamide[1], gramacidin S[2], α-lytic protease[3], and a synthetic peptide[4], as well as a general review of ^{15}N NMR[5]. The nitrogen nucleus is in an ideal position in the peptide linkage to probe peptide conformation and hydrogen bonding interactions. Its sensitivity to structural and electronic effects is indicated by its large (500 ppm) chemical shift range. The effect of hydrogen bonding to a peptide unit has been examined, and the resulting downfield shift for a hydrogen bond to the C = 0 or N — H components of the peptide link has been used to elucidate the solvent exposure of these groups in gramacidin S[2] and alumichrome[6]. This has been done by examining spectra in DMSO and trifluoroethanol. When studying peptides in water, as we wish to do, the situation in terms of hydrogen bonding can be more complicated, since this solvent can hydrogen bond to either end of the linkage. Conformational effects and possible sequence dependence provide a further challenge to a detailed understanding of the chemical shifts. On the basis of our data and that of others, we have proposed the use of ^{15}N shifts of *N*-acetyl-α-amino acids in DMSO as good indicators of the shift expected for the α-amino nitrogen of a residue within a peptide in aqueous solution when there is solvent exposure and a lack of conformational restriction in the region of the peptide linkage in question[1]. Deviation from these values may then be related to special hydrogen bonding or conformational effects. As part of the ongoing investigation of the structural characteristics of oxytocin and 8-arginine vasopressin (AVP) in our laboratory[7], we have carried out the ^{15}N NMR study of AVP reported here.

(A) H_2NCys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

(B) H_2NCys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

Fig. 1. Structures of (A) 8-arginine vasopressin (AVP) and (B) oxytocin.

Results and Discussion

The spectrum of AVP was recorded on a sample of 450 mg of the peptide, prepared in our laboratory[8], dissolved in 8.5 ml of water and adjusted to pH 4.0 in a 20 mm NMR tube. A Bruker Instruments CXP-200 spectrometer operating at 20.27 MHz was used. The spectrum of the amide region of AVP along with the previously reported spectrum of oxytocin[1] are shown in Figure 2. The shifts of the nitrogen nuclei are provided in Table I. Assignments of Cys[1], Tyr[2], Phe[3], Asn[5], Cys[6], and

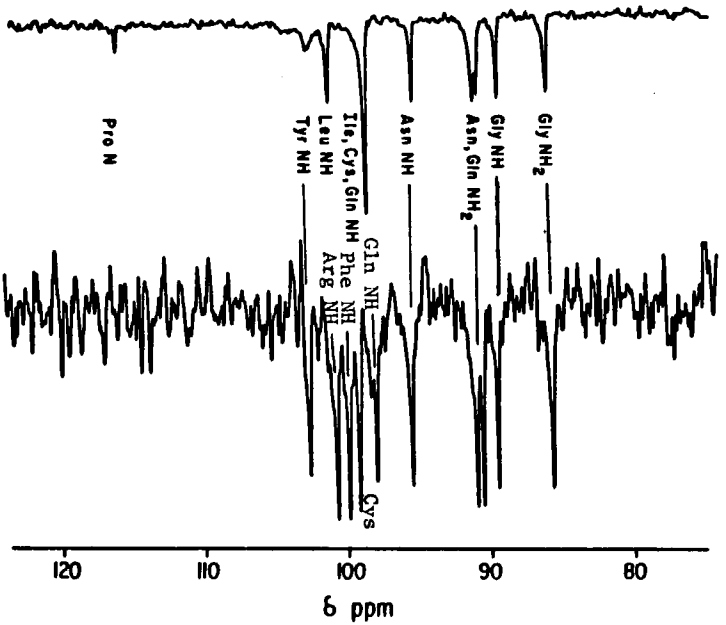

Fig. 2. ^1H Decoupled ^{15}N NMR spectra of 8-arginine vasopressin (52,000 accumulations) and Oxytocin (see Ref. 1 for details). Shifts are relative to 5M $^{15}NH_2NO_3$ in 2N HNO_3 (see footnote b in Table I).

Table I. ^{15}N Shifts of Oxytocin (OT) and 8-Arginine Vasopressin (AVP)

	AVP	OT		AVP	OT		AVP	OT
$Cys^1NH_3^+$	16.8	17.0	Asn^5 Gln^4NH_2	90.7 91.1	90.8 91.0	Phe^3NH $(Ile^3)^c$	99.8	98.7
Arg^8 N^η	50.9							
			Asn^5NH	95.5	95.3	Arg^8NH^b $(Leu^8)^c$	100.6	101.2
Arg^8 N^ϵ	64.6							
			Gln^4NH^b	97.9	98.7			
Gly^9NH_2	85.8	85.8				Tyr^2NH	102.5	102.6
			Cys^6NH	99.0	98.7			
Gly^9NH	89.7	89.3						

[a]Relative to $5M^{15}NH_4NO_3$ in 2N HNO_3 [b]Assignments of Gln and Arg peptide shift in AVP tentative [c]Corresponding residue in oxytocin.

Gly^9 peptide nitrogens were made by examining a series of individually ^{15}N labelled isotopic isomers of AVP from our laboratory. Assignments of the carboxamide nitrogens of Gln^4, Asn^5, and Gly^9 were made by analogy with the shifts of these functions in oxytocin and prolylleucylglycinamide[1]. The N^ϵ and N^η of the guanadino group of Arg^8 were assigned by comparison to their reported shifts in the free amino acid[5]. The proline nitrogen in AVP was not observed at this sensitivity. Two of the peptide nitrogens at 97.9 and 100.6 ppm which must arise from Arg^8 and Gln^4 have not been conclusively assigned. Although there is no data on the shift of the arginine α-amino nitrogen in a peptide, it would be expected to fall at about 102 ppm by extrapolation from the free amino acid data[5] which is essentially identical to the shift of Leu^8 in oxytocin. The Gln^4 peptide amide might also be expected around 102 ppm, however, we found in oxytocin that it was about 3.5 ppm upfield of this value[1]. The great similarity between the ^{15}N shifts of all the common residues of oxytocin and AVP leads us to assign the shift more upfield of the two resonances in question to Gln^4. A factor in support of this unusual shift is the particularly low (4.2 Hz) N-H to C^α-H proton coupling constant here[9] as in oxytocin[10] that suggests possible restricted conformation in the region of Gln^4.

The guanidino nitrogens of Arg^8 are shifted slightly downfield (2 ppm for N^α and 3 ppm for N^η) from their positions in the free amino acid[5]. This may arise from the absence of a free charged amino group when the residue is in a peptide, or some interaction of the Arg^8 side chain with another part of the molecule.

Conclusions

The close agreement of the shifts for the common residues of oxytocin and AVP, the observation of expected shifts for Ile^3 in oxytocin and Phe^3 in AVP, and the persistence of the anomalously high shift for Asn^5 and apparently Gln^4 N^{α}'s, suggest strongly that the peptide backbone conformations of these two hormones are quite similar.

Acknowledgments

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DYNAMIC CONFORMATIONS OF OXYTOCIN AND ARGININE-VASOPRESSIN (AVP) INVESTIGATED BY ^{1}H NMR COUPLINGS TO ^{13}C, ^{15}N, AND ^{1}H

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In seeking to understand the nature of the specific binding of a peptide hormone to its particular biological receptor and the possible subsequent conformational changes in both moieties, it is desirable to obtain as complete as possible a picture of the dynamic conformations of the hormone in aqueous solution, and of the energetics of the various interconversions among the conformations. One powerful technique for investigating this problem is the use of NMR vicinal coupling constants, both homo- and heteronuclear, to determine torsion angles and their time dependencies.

We have used this method in combinations with synthesis of a number of specifically tailored isotopic isomers to investigate oxytocin and AVP[1]. The solid-phase synthetic approach has enabled us to make[2] a series of such isomers rapidly, and in reproducibly high yield.

Table I. Constants Used in the Analysis of $C^{\alpha} - C^{\beta}$ Circumjacent Couplings for Staggered Rotamers and for Fixed Angles.

Coupling of H^{β} to	Values used in rotamer analysis			Values used in Karplus-like equations for fixed angles. (a)			
	gauche	trans		A	B	C	
$^{1}H^{\alpha}$	2.60	13.56	(b)	9.40	−1.40	1.60	(e)
$^{15}N'$	−2.04	−4.74	(c)	−3.75	0.26	−0.54	(f)
$^{13}C'$	1.3	9.8	(d)	7.20	−2.04	0.60	(f)

[a]Used in equation of the form $^{3}J(\tau) = A\cos^2\tau + B\cos\tau + C$, where τ is the dihedral angle between $H\beta$ and the α substituent. [b]Pachler, K. G. R. (1963) *Spectrochim. Acta* **19**, 2085. [c]Ref. 3 and reference therein. [d]Esperson, W. G. and Martin, R. B. (1976) *J. Phys. Chem.* **80**, 741. [e]Kopple, K. D., *et al.* (1973) *Biopolymers* **12**, 627. [f]Unpublished results from this laboratory using bicylcic model compounds.

We have extensively investigated the torsion angles around the C^{α} to C^{β} bonds, χ^1, in several of the residues of oxytocin and AVP. This angle determines by and large, the conformation of the side chain, and, by non-bonded interactions, strongly influences the backbone conformation in the vicinity of the residue. In Table I, the various calibration constants are presented for analysis of staggered rotamers and for Karplus-like relations for those situations where a fixed dihedral angle about the C^{α} — C^{β} bond exists.

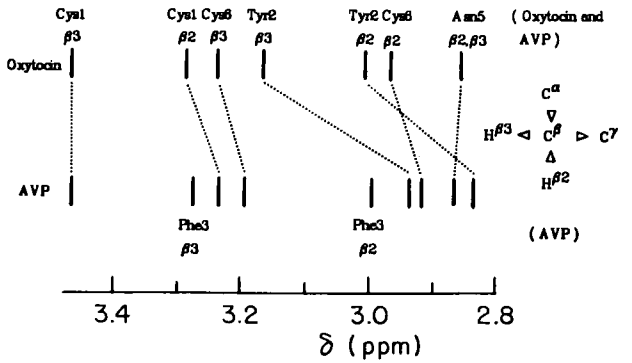

Fig. 1. Chemical shifts of individual β protons of half-cystyl, aromatic, and asparaginyl residues of oxytocin and AVP (22°, pD 3.8).

An important element in these investigations is the identification of the two prochiral β protons in the NMR spectra. We have described a technique for this identification using circumjacent homo-and heteronuclear coupling constants[3], and have recently confirmed these assignments by an independent method, using stereospecific deuteration, for all the β protons of the aromatic and half-cystyl residues of oxytocin and AVP (Figure 1). It will be noted that all $\beta 2$ protons are upfield of their respective $\beta 3$ protons. This may result from similarities in the spatial orientations of the α substituents to the $\beta 2$ protons of all these residues, in which the weighted averages of χ^1 fall in the semicircle, $\chi^1 = -60, -180, +120°$. Comparisons of couplings of the half-cystyls 1 and 6 and tyrosyl 2 in oxytocin and AVP reveal markedly similar conformations. The stacking of tyrosyl 2 and phenylalanyl 3, reported to occur in organic solvents and suggested for the receptor-bound conformations in vasopressins[4], is, therefore, not readily apparent in aqueous solution. The similarities of the circumjacent vicinal couplings for tyrosyl 2 in oxytocin and AVP are very substantial, and the differences in derived rotamer populations very small. It has been proposed that the residue has quite different conforma-

tions in the biologically active structures for the oxytocic[6] and antidiuretic[4] responses. No propensity for such different orientations is observed in aqueous solution. The energies for conversion of the conformation in solution to these two proposed biologically active structures may, however, be small[5] compared to the binding energies of the hormones to their receptors.

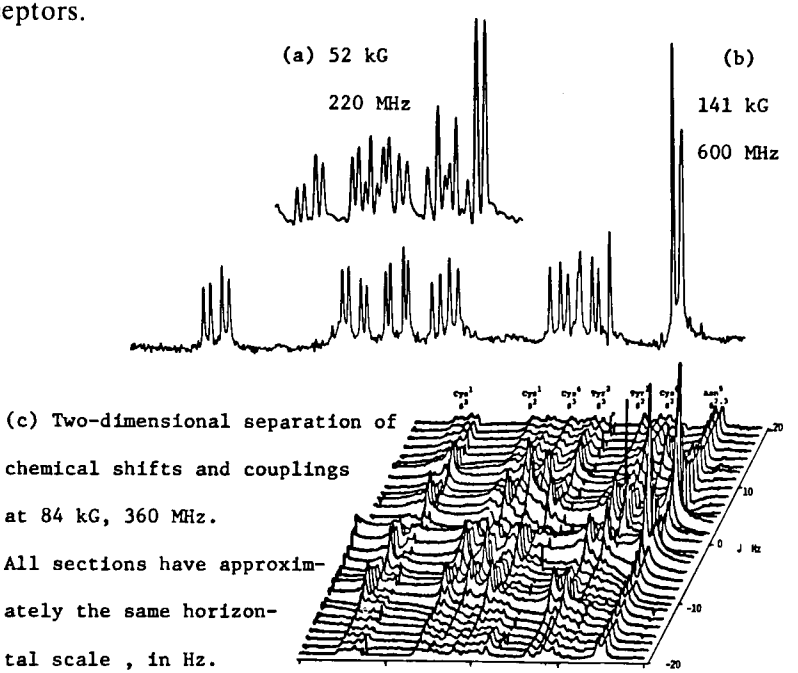

Fig. 2. Comparison of proton chemical shift range, 2.75-3.65 ppm downfield, of oxytocin at 52 kG(a), 141 kG(b), and 84 kG(c).

The couplings related to the backbone torsion angles which have been investigated so far in this study are consistent with extensive averaging of ϕ_2 and ϕ_3. No significant $^{15}N_{i+1}$—$^{1}H_i$ couplings ($>\pm 0.5$ Hz) have been observed. Theoretical calculations have shown that a fairly large coupling with a well defined dependence on ψ_i is expected[7].

Applications of circumjacent vicinal couplings is limited to some degree by problems of spectral overlap and sensitivity of NMR spectrometers. In the past we have overcome these obstacles through extensive synthesis of isotopic isomers. The availability of spectrometers of increased field strength (up to 141 kG) and use of advanced pulse sequences and data processing in two-dimensional NMR spectroscopy[8] can both significantly increase the molecular weight range in which complete spectral analysis is possible, and also reduce the number of synthetic

isotopic isomers required, particularly in regard to spectral overlap. To illustrate the spectral simplifications available, Figure 2 shows for comparison the region of the proton NMR spectrum of oxytocin containing resonances from the β protons of half-cystyls 1 and 6, tyrosyl 2 and asparaginyl 5 at 52 kG (220 MHz), 84 kG (360 MHz with two dimensional separation of couplings and chemical shifts) and at 141 kG (600 MHz). The application of these advanced NMR methods, in conjunction with preparation of isotopic isomers, seems increasingly practical for investigations of the conformations of other peptides up to the molecular weight range of about 6,000.

Acknowledgments

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REMOVAL OF THE DEGENERACIES IN THE [r(ϕ), r(ψ)] VS. (ϕ, ψ) AND ^{3}J VS. ϕ RELATIONSHIPS

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NMR evaluation of unique ϕ and ψ angles for each individual residue in a peptide is limited by the degeneracy in the relationships between the measured NMR parameters and dihedral angles, e.g. an experimental $^3J_{NHH_\alpha}$ corresponds to four ϕ angles and determination of both r(ϕ) and r(ψ) for individual residues only means that two ϕ and two ψ angles, and hence four (ϕ, ψ) combinations, are possible per residue. Further criteria are therefore needed to remove degeneracies and obtain a single (ϕ, ψ) pair for each residue. The magnitude of the problem can be seen for, if all r(ϕ), r(ψ) pairs are measured experimentally for a decapeptide, 4^{10} secondary conformations are in principle consistent with the experiment.

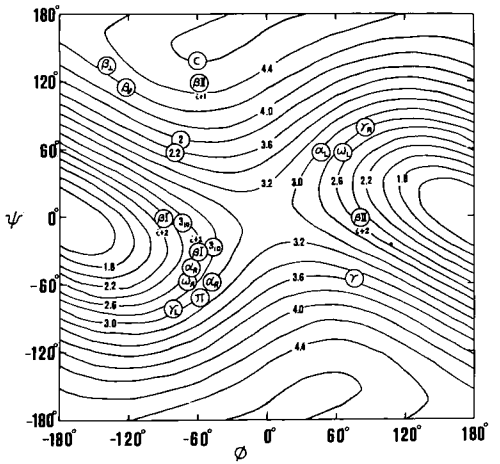

Fig. 1. The NH(i)—NH(i + 1) distance, r($\phi\psi$), plotted as a function of ϕ(i) and ψ(i). Contour lines connect points with equal values of r($\phi\psi$). α_R, α_L = right and left handed α helices; γ_R, γ_L = right and left-handed γ helices; $\beta_\perp$, $\beta_{//}$ = antiparallel and parallel β-pleated sheets; 2.2 = 2.2_7 helix, 2 = 2_7 ribbon; C = collagen coiled coil; βI, βII = β-I and β-II turns; 3_{10} = 3_{10} helix (β-III turn).

Removal of these degeneracies to yield unique ϕ or ψ angles has previously involved theoretical calculations[1,2,3], hydrogen bonding studies[4] or heteronuclear coupling constants[5]. Here we demonstrate that short range distances (< 4 A) between protons on contiguous or non-contiguous amino acid residues can remove this degeneracy and often

yield unique (φ, ψ) angles. Combined use of $^3J_{NHH_\alpha}$ vs. φ and r(φ) vs. φ partly removes the degeneracy in the former. The distance r(φψ), unlike r(φ) or r(ψ), depends on both φ and ψ. It represents the distances r[NH(i) — NH(i + 1)] which vary with φ and ψ according to the r(φψ) contour map in Figure 1; the r(φψ) distances for common conformations are indicated on this map. The intraresidue distance r(φ) and the two interresidue distances, r(ψ) and r(φψ), which, when experimentally evaluated, define a unique (φ, ψ) pair for a residue. At 270 MHz for peptides of MW = 1000 — 2000, r(φψ) values corresponding to < 3.5 A are readily detectable. Table I contains r(φ), r(ψ) and r(φψ) for β-turns and γ-turns.

A. Table I. r(φ), r(ψ), and r(φ,ψ). Values for β- and γ- Turns.[a]

B. Table II. Possible (φ,ψ) Combinations for Gln[9] and Tyr[10] in Tyrocidine A[a].

A.

β-turn	φ	r(φ) (Å)	ψ	r(ψ) (Å)	r(φψ) (Å)	φ	r(φ) (Å)	ψ	r(ψ) (Å)	r(φψ) (Å)
			$_{i+1}$					$_{i+2}$		
I (LL)	-60°	2.80	-30°	3.53	2.6	-90°	2.92	0°	3.30	2.4
I'(DD)	+60°	2.80	+30°	3.53	2.6	+90	2.92	0°	3.30	2.4
II (LL)	-60°	2.80	+120°	2.14	4.5	+80°	2.27	0°	3.30	2.5
(LD)	-60°	2.80	+120°	3.30	4.5	+80°	2.89	0°	3.30	2.5
II'(DD)	+60°	2.80	-120°	2.14	4.5	-80°	2.27	0°	3.30	2.5
(DL)	+60°	2.80	-120°	3.30	4.5	-80°	2.89	0°	3.30	2.5
III (LL)	-60°	2.80	-30°	3.53	2.6	-60°	2.80	-30°	3.53	2.6
III'(DD)	+60°	2.80	+30°	3.53	2.6	+60°	2.80	+30°	3.53	2.6

	ϕ_{i+1}	$r(\phi_{i+1})$ Å	ψ_{i+1}	$r(\psi_{i+1})$ Å	r(φψ) Å
γ-turn	+70°~+85°	2.25~2.28	-60°~-70°	3.60~3.31	3.7
Reverse γ-turn	-70°~-85°	2.85~2.91	+60°~+70°	2.48~2.59	3.7

B.

	r(φ)[b]	φ[c]	r(ψ)[d]	ψ[c]	r(φψ)[e] obs.	r(φψ)[f] calc.	
Gln[9] (i+1)	2.86Å	-167°(10°)	3.3Å	0°(20°)	2.7A	1.3Å	
		-167°(10°)		-120°(20°)		3.7Å	
		-73°(10°)		0°(20°)		2.6Å	
		-73°(10°)		-120°(20°)		3.7Å	Type I
Tyr[10] (i+2)	2.96Å	-108°(10°)	3.4Å	-10°(30°)	2.4Å	2.0Å	
		-108°(10°)		-110°(30°)		3.3Å	
		-132°(10°)		-10°(30°)		1.6Å	
		-132°(10°)		-110°(30°)		3.3Å	
D-Phe[4] (i+1)	2.83Å	+66°(10°)	2.2Å[g]	-130°(10°)	---	---	Type II'
Pro[5] (i+2)	---	-70°(10°)	> 3Å	-70°~-30°	---	---	
Residues 1,2,3,6,7, 8	2.95Å	-139°	2.18Å	+135°	---	4.3Å	
		-101°		+135°	---	4.5Å	
		-139°		+105°	---	3.9Å	
		-101°		+105°	---	4.2Å	

A. [a]The (φ,ψ) angles for β-turns see reference 6; the (φ,ψ) angles for γ-turns see references 7 and 8.

B. [a]The precision in converting r(φ) and r(ψ) to angles is in parenthesis. [b]Distances between NH(i)-Hα(i) which are obtained from $^3J_{NHH_\alpha}$, NOE, and relaxation time measurements. [c]There are two φ or ψ angles consistent with each experimental r(φ) or r(ψ). [d]Distances between Hα(i)—NH(i + 1) obtained from NOE and relaxation time studies. [e]Distances between NH(i)—NH(i + 1) obtained from NOE and relaxation time studies.

Example of Tyrocidine A. The crystal structure is unknown and, being an analog of gramicidin S, this molecule serves as a testing ground for the use of r(φ), r(ψ), r(φψ) and interchain distances r(IC) to determine unique (φ, ψ) pairs of angles for its residues. The NOEs among the backbone protons of tyrocidine A have been determined[9]; Figure 2 shows the conformation of tyrocidine A and the distances derived from these NOEs using the NOE ratio method[10] and cross-relaxation rates[11].

Many publications have reported NOEs and hence r(ψ) distances and claimed these proved or disproved the existence of specific conformations. This is not strictly correct since r(ψ) is consistent with two ψ

angles and a [r(ϕ), r(ψ)] pair determination is consistent with four pairs of (ϕ, ψ) per residue. To illustrate the importance of r($\phi\psi$) and interchain distance, r(IC), in removing the fourfold degeneracy between experimental [r(ϕ), r(ψ)] pairs and their corresponding (ϕ, ψ), we refer to Table II. There are four possible (ϕ, ψ) combinations for the Gln[9] and the Tyr[10] residues and therefore 16 possible dipeptide secondary conformations based on the r(ϕ) and r(ψ) measurements. The r($\phi\psi$) values corresponding to each combination were calculated and are listed in Table II. Compared to the experimental r($\phi\psi$) value of Gln[9], 2.7 A, only one combination, (-73°, 0°), which has r($\phi\psi$) = 2.6 A, can exist. For Tyr[10] the combination (-108°, -10°) has r($\phi\psi$) = 2.0 A which best matches the experimental r($\phi\psi$) value of 2.4 A; the deviation of 0.4 A is due to the inaccuracy of the measured r(ψ) = 3.4 A from a small NOE. The (ϕ, ψ) angles of these two residues correspond (Table II) to a type I β-turn rather than other β-turns or γ-turns. The major D-Phe[4] C^{α}-C^{β} rotamer is χ^1 = 180° (70%)[12]; with the β protons thus defined in space, the degeneracy in ϕ angle of D-Phe[4] was removed by the observation of the equal NOEs from NH(4) to both Hβ(4); this criterion eliminated the existence of ϕ = +174°. The ψ angle of D-Phe[4] residue was uniquely defined by the two Hα(4) — Hδ(5) distances (Figure 2)[9]. The ϕ angle of Pro[5] residue was derived from the spin-spin analysis of prolyl side chain protons[12] which lead to the conclusion that Pro[5] corresponds to the Ramachandran B conformation[12]. For these two residues, their (ϕ, ψ) values correspond to a type II β-turn (Table I, II). The measured r(ϕ) and r(ψ) distances of the other residues are shown in Figure 2. They are very close to the r(ϕ) and r(ψ) values for the antiparallel β-pleated sheet (Table II). However, the same r(ϕ) and r(ψ) distances could have 4 different (ϕ, ψ) combinations and their r($\phi\psi$) distances are too long to be determined from NOEs. Therefore the r($\phi\psi$) criterion cannot remove the (ϕ, ψ) degeneracy. From Figure 2, we predict, for an antiparallel β-pleated sheet, that the distances between Hα—Hα and NH—Hα across the chains is small and therefore strong NOEs should be observable. These distances as determined by NOE and/or the cross-relaxation method[9] are r[Hα(2)—Hα(7)] = 2.4 A, r[NH(3)—Hα(7)] = 3.1 A, r[NH(8)—Hα(2)] = 3.1 A and r[NH(8)—NH(1)] = 3.1 A.

These results uniquely delineate the backbone conformation of tyrocidine A consisting of a type I β-turn, a type II′ β-turn and an approximate antiparallel β-pleated sheet. Another method of removing degeneracy in (ϕ,ψ) angles per residue has been developed[2] involving the

performance of energy minimization calculations based upon r(φ), r(ψ), r(φψ) and r(IC) experimental distances. Applications to tyrocidine A confirmed the above conclusions.

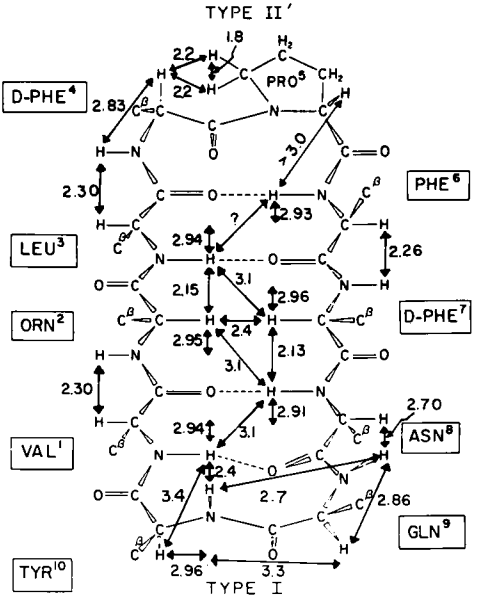

Fig. 2. Secondary conformation and interproton distances (in A) of tyrocidine A.

Acknowledgments

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PROTON NMR STUDY OF BLEOMYCIN BINDING TO POLY(dA-dT)[1]

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Introduction

The bleomycins (Figure 1) are glycopeptide antibiotics produced by *Streptomyces verticillus* which are employed in cancer chemotherapy and cancer detection[2]. The association of these agents with DNA and perhaps also with polyvalent metals is believed to play a crucial role in the mechanism of pharmacological activity of these antibiotics.

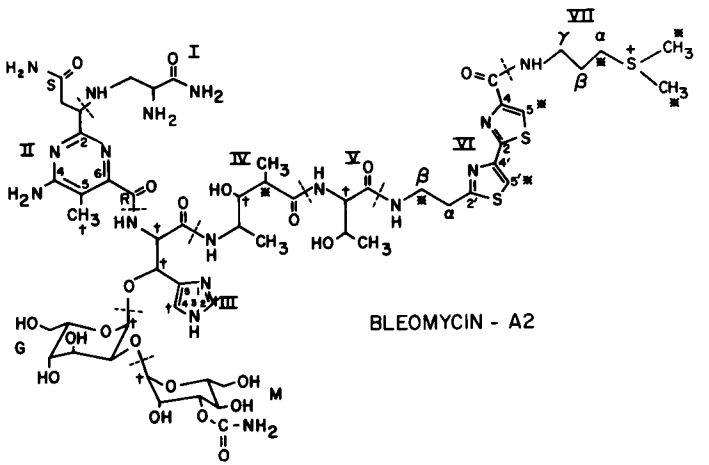

Fig. 1. The structure of bleomycin-A_2 indicating hydrogens whose resonances exhibit spectral shifts on binding to poly(dA-dT) (*) and those that don't (†).

Our laboratories have been engaged in a comprehensive program to characterize the conformation and dynamics of (1) the bleomycins in free solution[3,4], (2) their complexes with various polyvalent metals[5,6], and (3) their complexes with poly- and oligonucleotides.

The present investigation is directed at delineating the mode of binding of bleomycin-A_2, the most common natural congener of the bleomycins, to poly (dA-dT), a self-complementary deoxypolynucleotide which serves as a model of the A-T base pairs of DNA. Proton nmr spectroscopy provides a method of identifying specific sites of interaction on both the drug and the nucleic acid and for determining a number of structural and dynamic characteristics of the complexes which are formed[7].

Results and Conclusions

A single resonance is observed for each of the hydrogens of bleomycin-A_2 and poly(dA-dT) at all temperatures (0-75° C) and nucleic acid phosphate to drug ratios (P/D = 0.5-70), indicating that drug binding and helix-coil transitions occur at rates which are at least moderately rapid on the nmr time scale. The helix-coil transitions exhibited by the nucleic acid resonances and some of the bleomycin resonances *(vide infra)* are all highly cooperative and monophasic even at relatively high drug concentrations. At this ionic strength this drug has no effect on either the T_m (60 ± 1°C) or the width of the thermal transition. Temperature-induced spectral changes of all the resonances are completely reversible. No significant spectral shifts or line width changes are observed in resonances of either the drug or the nucleic acid at temperatures above T_m, indicating that at most nonspecific drug-nucleic acid interactions occur when the polynucleotide is denatured.

At temperatures below T_m all the nucleic acid resonances exhibit dipolar broadening. By contrast, the bithiazole VI-5 and VI-5′ resonances are the only peaks of the drug to exhibit dipolar broadening, indicating that the planar bithiazole group is preferentially immobilized through tight association with the slowly tumbling nucleic acid. Dipolar broadening of the bithiazole resonances of tripeptide S (residues V-VII) indicates that this fragment of bleomycin also binds to poly(dA-dT).

Spectral shifts exhibited by bleomycin-A_2 and poly(dA-dT) resonances are summarized in Figure 1 and Table I. It is noteworthy that the shifts of the drug resonances are all to high field and originate from hydrogens in close proximity to the bithiazole and dimethylsulfonium groups, supporting the conclusion that these groups are most intimately involved in binding this drug to nucleic acids. The fact that the bithiazole group exhibits the largest spectral shift and is preferentially broadened

Table I. Summary of Spectral Perturbations[a]

Resonance	30°C	50°C
	Bleomycin-A_2 Resonances	
	ppm	ppm
IV-$C^{\alpha}\underline{H}$	-0.041	-0.045
VI-$C^{\beta}\underline{H}_2$	-0.022	-0.053
VI-5	-0.042	-0.146±0.01
VI-5'	-0.039	-0.152±0.01
VII-$SC\underline{H}_3$	-0.013	-0.015
VII-$C^{\alpha}\underline{H}_2$	-0.021	-0.037
	Poly(dA-dT)	
A(H-2)	0.047(0.097)[b]	0.028(0.069)[b]
A(H-8)	(0.052±0.018)[b]	(0.019)[b]
A(H-2')[c]	0.064	∿0
A(H-2')[c]	0.022	∿0
T(H-6)	0.13±0.05	∿0

[a]Sample: 2mM bleomycin-A_2, 17mM poly(dA-dT) in 100mM sodium phosphate buffer, pH meter reading 6.8, 1mM EDTA. Assignments of resonances appear in references 3 & 7. Positive values correspond to low field shifts. The experimental error is ± 0.0014 ppm unless otherwise indicated. The following poly(dA-dT) resonances were not significantly perturbed: T(CH_3), A(*H*-1'), T(*H*-1'), A(*H*-3'), T(*H*-3'); [b]Sample: 5mM bleomycin-A_2, 10mM poly(dA-dT) in same buffer- [c]Higher field resonance listed first.

on binding to the nucleic acid indicates that this group plays a central role in the interaction of bleomycin with nucleic acids.

The small magnitudes of the spectral shifts argue against drawing any definitive conclusions about the mechanism of binding at this stage. However a few general preliminary conclusions can be drawn. The perturbation of the bithiazole resonances is maximal just below the T_m (near 50° C) and diminishes at lower temperatures (Table I). This diminution is not caused simply by dissociation of bleomycin-A_2 from the polymer since other resonances of both the drug and the nucleic acid remain perturbed at the lower temperatures. Indeed, some of the perturbations increase at lower temperatures. Consequently, there must be at

least two classes of complexes formed between bleomycin-A_2 and poly(dA-dT)-a type-I complex preferred near 50° C which is associated with a significant high field shift of the bithiazole resonances and a type-II complex preferred at lower temperatures which is associated with substantially smaller perturbations of the bithiazoles. Least squares analysis of the chemical shifts of the bithiazole shifts at various P/D values indicates that the maximal shift of these resonances is about -0.27 ppm at 50° C if one assumes a single type of complex (the type-I complex) with equivalent and noninteracting binding sites. This shift is substantially less than is normally observed with classical intercalating agents[7] but might be explained by a partial intercalation mechanism. The smaller magnitude of the bithiazole spectral shifts at 30° C makes an intercalative mechanism less likely for type-II complexes, but such a mechanism cannot yet be definitively ruled out.

References

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SYNTHESIS AND CONFORMATION OF CYCLO(THR-D-VAL-PRO-SAR-MeALA)

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Introduction

The title cyclopeptide is related via N,O-acyl shift to the peptide lactone structure present in the actinomycins. Its amino acid sequence resembles that of actinomycin D except that *N*-methylalanine replaced *N*-methylvaline; this replacement occurs naturally in the actinomycin Z series[1]. The present study permits conformational comparisons with other cyclic pentapeptides and with the actinomycin-related peptide lactones investigated by Lackner[2].

Results

The crystalline cyclic pentapeptide I, containing O-benzyl-L-threonine, was synthesized via cyclization of H-MeAla-Thr(OBz)-D-Val-Pro-Sar-OH with dicyclohexylcarbodiimide. The title cyclopeptide II was obtained by catalytic hydrogenation of I.

Proton NMR spectra of I and II in several solvents were obtained at 220 MHz. No conformational heterogeneity and no significant differences between I and II were observed. A spectrum of I in $CDCl_3$ is shown in Figure 1. From the behavior of the NH signals in respect of (a) solvent shifts, (b) temperature dependence of shifts, and (c) deuterium exchange rates, it was concluded that the Val NH was exposed whereas the Thr NH was involved in an intramolecular hydrogen bond. The large, equal benzene shifts of the two *N*-methyl singlets suggested *trans* configurations for the Pro-Sar and Sar-MeAla peptide bonds. Also, ^{13}C NMR data (Table I), in particular the Pro Cβ and Cγ shifts, indicated a *trans* Val-Pro peptide bond. From these conclusions a space-filling (CPK)

model was constructed having all peptide bonds *trans*, a β-turn and a 4 → 1 (Thr → Pro) hydrogen bond. This model was compatible with the ^3J(NH, CαH) of Thr and Val, and with the ^2J of Sar.

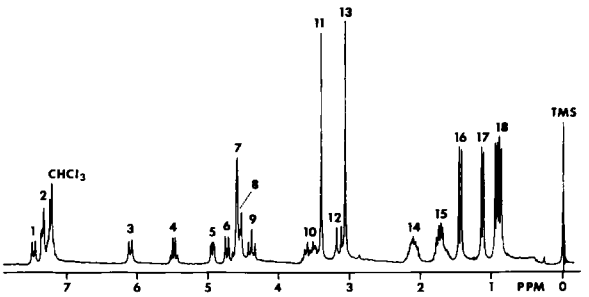

Fig. 1. ^{1}H NMR Spectrum of I in $CDCl_3$. 1: Thr NH; 2: ArH; 3: Val NH; 4: MeAla α-H; 5: Pro α-H; 6: Thr α-H; 7: Bz CH_2; 8: Sar α-H and Thr β-H; 9: Val α-H; 10: Pro δ-H; 11, 13: N-CH_3; 12: Sar α-H; 14, 15: Pro and Val β-H, Pro γ-H; 16: MeAla C-CH_3; 17: Thr CH_3; 18: Val CH_3.

Subsequently, X-ray crystallographic data were obtained for I (Figures 2 and 3). Two independent molecules, differing mainly in the orientation of the benzyl group, occupy the asymmetric unit. The geometry approximates that deduced for solution from NMR data (see Table II), although the weakness of the hydrogen bond (see N-O distances, Figure 3) and divergence of the Val-Pro peptide bond from planarity (ω = 156°) were unforeseen features of the structure.

Table I. ^{13}C Chemical Shifts for I in $CDCl_3$

Shift	Assignment	Shift	Assignment	Shift	Assignment
14.6	MeAla β	47.1	Pro δ	126.9	
17.7	Thr γ	50.7	Sar α	127.0	Ar
18.4	Val γ	52.8	MeAla α	127.8	Ar
19.2	Val γ	56.2	Pro α	139.4	
24.5	Pro γ	56.7	Thr α	170.1	
28.0	Pro β	58.0	Val α	170.1	C=O
29.2	Val β	71.3	Bz CH_2	170.8	C=O
31.1	N-CH_3	73.1	Thr β	171.8	C=O
38.8	N-CH_3			174.3	C=O

Table II. Dihedral Angles θ from NMR* and X-Ray Data

	$^3J(CDCl_3)$	θ (Soln.)	θ (Cryst. 1)	θ (Cryst. 2)
Val NH,C_αH	10.0 Hz	180°	161°	154°
Val C_αH,C_βH	10.7 Hz	156°	175°	172°
Thr NH,C_αH	9.8 Hz	180°	166°	178°
Thr C_αH,C_βH	1.2 Hz	90°	78°	65°

*For NH,C_αH[3]: $J = 7.9\cos^2\theta - 1.55\cos\theta + 1.35\sin^2\theta$;
for C_αH,C_βH[4]: $J = 11.0\cos^2\theta - 1.4\cos\theta + 1.6\sin^2\theta$.

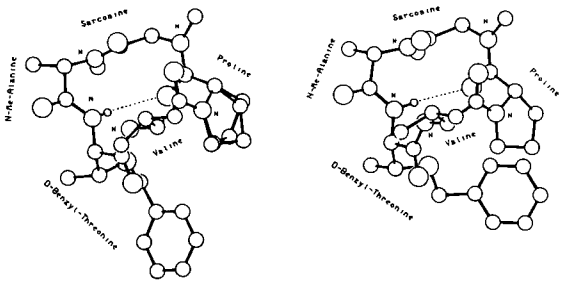

Fig. 2. The crystal conformations of the two independent molecules showing the different orientations of the benzyl groups and disorder of the proline ring of the first molecule. Space group: $P2_1$. a = 10.167(1) A, b = 28.047(2) A, c = 10.581(1) A, β = 106.39(1)A. Z = 4. 4992 observed reflections. Maximum sin θ/λ 0.61 A^{-1}.

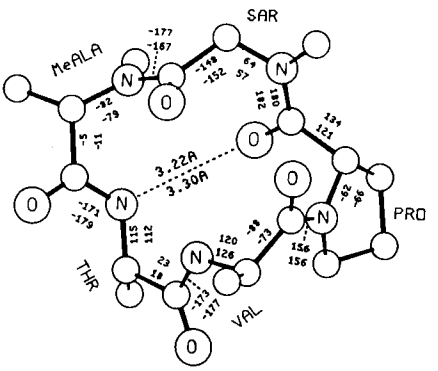

Fig. 3. Conformational angles ω, ϕ. ψ for the two independent molecules in the crystal. For the purpose of clarity, the O-benzyl group and the valine methyl groups are omitted.

Discussion

The crystal conformation of I is similar to, but more open than that described for the related *cyclo*(Ser-D-Ala-Pro-Gly-Pro)[5]. The latter adopts a different conformation in solution, with the Gly NH hydrogen bonded[6]. The latter conformation is impossible for I, which has Sar in place of Gly, and the NMR evidence presented here indicates a solution conformation for I (and II) approximating that found in the crystal.

Comparisons of the NMR data for I and II with those reported[7,8] for a pentapeptide lactone representing half of the actinomycin molecule are inconclusive. In $CDCl_3$, chemical shifts of corresponding protons and carbons are significantly similar, but the lactone was shown to exchange deuterium faster at the Thr NH than Val NH, in contrast to I. The cyclopeptide does not display solvent-dependent conformational duality as does the lactone.

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VIBRATIONAL ANALYSIS OF PEPTIDES, POLYPEPTIDES, AND PROTEINS. VII. NORMAL MODES AND VIBRATIONAL SPECTRA OF A TYPE I β-TURN TETRAPEPTIDE

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Recent work from our laboratory[1-3] has clearly indicated that the interpretation of vibrational spectra of proteins cannot be complete without incorporating the contributions arising from β-turns. Until now this has been difficult, however, because of the absence of model compounds suitable to the variety of such observed structures[4,5]. We have therefore used the force fields derived from our earlier studies of β-sheet[6,7] and α-helical[8] structures to calculate the normal vibration frequencies of β-turns[1,3], and have obtained good agreement with the observed spectra for a supposed tetrapeptide β-turn[1] and for the β-turns in insulin[3]. We present here some of the results of experimental infrared and Raman studies, and of normal vibration calculations, on a tetrapeptide system carbobenzoxyl-glycyl-prolyl-leucyl-glycine (*Z*-Gly-Pro-Leu-Gly-OH) that is known from crystal structure studies[9] to form a type I[4] β-turn. This good agreement between predicted and observed frequencies shows that normal vibration analysis is a powerful tool for studying the conformations of peptides.

Experimental and Calculations

Infrared and Raman spectra of *Z*-Gly-Pro-Leu-Gly-OH (Peptide Institute, Inc., Japan) were obtained at room and liquid N_2 temperatures. The tetrapeptide was deuterated by dissolving it in D_2O with 5% pyridine (by volume) for 60 hours, followed by freeze drying. Small traces of pyridine could not be completely removed from the sample.

The tetrapeptide was modeled by CH_3-O-CO-Gly-Ala-Ala-Gly-O-CH_3, with the dihedral angles of the observed structure[9]. External hydrogen bonds were included. The force field was that used in our previous studies[1,2]. The force constants of the ester and urethane C = O groups were chosen so as to reproduce the assigned frequencies for the acid (1743 cm^{-1}) and urethane (1686 cm^{-1}) groups of the tetrapeptide[10]. Transition

dipole coupling[11], which is crucial to explaining the amide I and amide II frequencies of β-turns[1,2], was included. Its effects on the peptide group amide I frequencies are not influenced by the presence of the urethane and carboxyl C = O groups. For the transition dipole moments we used $\Delta\mu_{eff}$ = 0.45D for amide I and $\Delta\mu_{eff}$ = 0.4D for amide II[1,2].

Results and Discussion

In this paper we present some results for the main characteristic amide modes.

In Table I we list the observed and calculated amide I and II mode frequencies for this tetrapeptide. No amide II Raman bands are observed (as usual), and the infrared band corresponding to the (2 + 1) mode is not expected to be seen since there is a prolyl group at the 2 position in the actual compound. The frequency agreement is quite good. Since the amide I frequencies lie within a ~2 cm^{-1} interval in the absence of transition dipole coupling[1,2], the ability to account for a ~35 cm^{-1} splitting demonstrates the importance of including transition dipole coupling. All three amide II bands are observed to disappear on deuteration, thus confirming their assignments. It is interesting that the 1568 cm^{-1} band is at a significantly higher frequency than the highest as-yet observed amide II mode, viz., 1555 cm^{-1} in β-poly(L-alanine)[7]. We have suggested that high amide II mode frequencies may serve to identify type I β-turns[2].

Seven vibrational modes of the model tetrapeptide containing NH in-plane bend components are predicted in the 1280-1390 cm^{-1} region, of which two (1291 and 1313 cm^{-1}) have substantial nonproline group CN stretch contributions and therefore could be labelled as amide III modes. The infrared spectrum exhibits a strong band at 1294 cm^{-1} that disappears on deuteration, which we believe is assignable to the calculated mode at 1291 cm^{-1}. As we observed previously[1,2], this value is consistent with the high amide III frequencies predicted for β-turns.

Our previous studies[2] show that the amide V (NH out-of-plane bend) should also be characteristic of β-turns. Modes with this internal coordinate contributing are predicted in the range of 360-610 cm^{-1}. We observe a strong band at 599 cm^{-1} in the infrared spectrum that disappears on deuteration. It can be assigned to calculated modes at 583 and 609 cm^{-1}. It is interesting to note that the observed band falls below the frequencies found for this mode in the β-sheet[7] (710 cm^{-1}) and the α-helix[8] (618 cm^{-1}).

Table I. Amide I and II Frequencies (in cm^{-1}) of CH_3-O-Gly-Ala-Ala-Gly-O-CH_3 with Dihedral Angles of Z-Gly-Pro-Leu-Gly-OH.

	Group	ν(calc)	ν(IR)	ν(R)
Amide I	5	1743	1741s	1741ms
	1	1688	1686s	1689s
	3+2	1681	1673w	1674w
	4	1659	1655vs	1656s
	2+3	1647	1639vs	1644mw
Amide II	2+1	1579	-	-
	3	1562	1568ms	-
	4	1544	1548ms	-
	1+2	1534	1525m	-

Conclusions

Our analysis: (1) confirms the validity of our force field[6-8] and the mechanism of transition dipole coupling[11] in predicting the characteristic amide modes of β-turns; and (2) suggests that normal vibration analysis can be an important new method for studying peptide conformation.

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VACUUM-UV CD AND SOLID-STATE IR OF PROTECTED HOMOOLIGOMERS DERIVED FROM L-NORLEUCINE

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As part of our study of peptide β-sheet formation[1], we have measured the CD and IR properties of *t*-Boc-(L-Nle)$_n$-OMe (n = 2-7). The synthesis of the series was reported earlier[2].

Circular Dichroism Measurements

The vacuum-UV CD measurements were carried out as previously described[1]. Data were converted to molar ellipticities based on total peptide molecular weight assuming that the molar absorption coefficient of the film is the same as that in a 3 x 10^{-4} M 2,2,2-trifluoroethanol solution[2]. Results are shown in Figure 1.

The Nle spectra resemble the Met spectra[1] indicating that the Met sulfur atom has little influence on the spectral properties of the peptide chromophore. The spectra of the longer chains indicate well developed β-conformations. Four peptide series are compared schematically in Figure 2, where the growth in the intensity of the 200 nm positive CD band is plotted as a function of chain length for each series, that intensity being measured relative to its intensity in the heptapeptide (assumed to be in a fully developed β-sheet). The peptide series having the longest, unbranched side chain (Nle) displays the CD characteristic of β-structure at the shortest chain length; replacement of a methylene group with a sulfur atom (Met) makes little difference once a critical chain length (5-mer) is reached. Shortening of the side chain (Nva) and branching, even at the γ-carbon position (Leu), inhibit β-sheet formation.

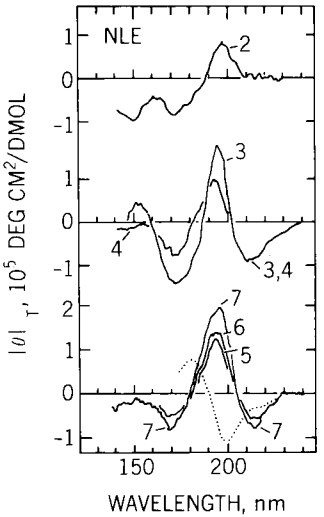

Fig. 1. Vacuum-UV CD of *t*-Boc-(L-Nle)$_n$OMe films, n = 2-7, and n = 7 in TFE.

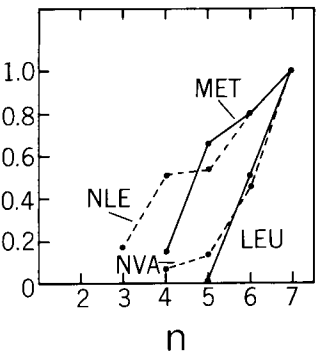

Fig. 2. Increase in 200 nm CD band as a function of chain length.

The solution CD spectrum of the heptapeptide in TFE (3 X 10^{-3} M) to 174 nm (Figure 1) shows a negative band at 200 nm with a negative shoulder near 220 nm, both characteristic of a disordered peptide chain, and also a positive band at approximately 180 nm. Generally, negative dichroism has been observed near 175 nm;[3,4] the positive band observed here may be an end effect arising from the urethane blocking group.

The position of the crossover of the Nle heptapeptide is 182 nm. Applying the criterion of wavelength position of the crossover in the 175-195 nm region[1], we draw the conclusion that the solid state conformation of *t*-Boc(L-Nle)$_7$-OMe contains substantial amounts of both parallel and antiparallel chains.

Infrared Absorption

The convergence of the positions of strong IR bands (Table I) near 3275 cm^{-1}, 1629 cm^{-1}, 1538 cm^{-1} and 708 cm^{-1} indicates that the penta-, hexa-, and heptapeptides form well developed β-conformations.

All of the longer chains display a band near 1690 cm^{-1}, usually assigned to the parallel component of a split amide I band in antiparallel β-sheets. Since the carbamate chromophore of the *tert*-butyloxycarbonyl N-protecting group also shows absorption near 1690 cm^{-1}, we removed

Table I. IR Absorption Data (cm^{-1}) of X(L-Nle)$_n$OMe in the Solid State*.

X	n	3500-3200	1800-1600	1600-1500	750-600
t-Boc	2	3347(sh), 3294	1755, 1710(sh), 1685, 1652	1551, 1530	730, 654(ℓ), 615(sh)
	3	3320, 3295	1758(sh), 1743, 1716(sh), 1658(sh), 1641	1547, 1526	732, 695, 666
	4	3382, 3281	1742, 1707, 1688, 1662, 1634	1546, 1516	732, 701, 650 606
	5	3380(sh), 3279	1745, 1717, 1692, 1655(sh) 1632	1546(ℓ), 1525(sh)	730, 708, 630 605
	6	3380, 3275	1741, 1710, 1691, 1650(sh), 1629(ℓ)	1539(ℓ), 1517(sh)	710(ℓ), 615
	7	3382, 3275	1743, 1713, 1692, 1629(ℓ)	1538(ℓ)	730, 708, 645 615
H_2^+	7	3380(sh), 3276	1741, 1691, 1629(ℓ)	1536(ℓ)	730, 710, 637 615

Legend: ---, weak band; ——, strong band, l, large band; sh, shoulder.
*KBr pellets.

the N-blocking group of the heptapeptide (with hydrochloric acid in anhydrous methanol) and recorded the IR spectrum of the salt form in the solid state. The observation of the 1691 cm^{-1} band (Table I) indicates that the β-sheet formed is, at least in part, of the antiparallel type. The conclusions derived from the IR measurements are therefore identical with the conclusions derived from the vacuum-UV CD measurements.

Acknowledgment

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VALUES OF HYDROPHOBIC PARAMETERS π FOR AMINO ACID SIDE CHAINS DERIVED FROM PARTITION AND CHROMATOGRAPHIC DATA

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Whereas the selective nature of a peptide-macromolecule interaction is largely assessed by their structural complementarity, the hydrophobicity of certain areas of the two moieties may play a significant role in the stability of their association[1]. In this respect, the "side-oriented" hydrophobicity belongs to the most important determinants of both intensity and duration of a biological response and a question arises as to the reliable way of its expression.

To these aims, hydrophobicity π-constants[2] of amino acid side chains may be considered. The π-constant of a substituent group R in a given series of compounds is defined as

$$\pi_R = \log P_R - \log P_H, \qquad (1)$$

where P_R and P_H are partition coefficients in n-octanol/water (O/W) of the derivative and of the parent compound, respectively. As to the amino acids, their modest solubility in lipophilic organic solvents is the major obstacle for direct measurements of P's. Therefore, a set of π's for side chains has been calculated[3] from the contributions of single substituent groups[4], using their additive properties. Clearly, these contributions originate from series of compounds different from amino acids and one feels rather uneasy about possible errors of such a procedure. In this contribution, we wish to describe our attempts to estimate π-values from partition data available in the literature and/or obtained in our laboratory.

The older literature[5] reports partition coefficients in n-butanol/water (B/W) for 7 amino acids with hydrophobic side chains. Newer collections of partition data[6] contain values for several others but only 6 of them have been measured in the reference system O/W. The two groups overlap only in 2 amino acids and the parameters γ, δ of the Collander equation[7] correlating partition coefficients P_1, P_2, for the same substance in two various solvent systems,

$$\log P_1 = \gamma + \delta \log P_2 \quad (2)$$

cannot be estimated with sufficient statistic significance. Nevertheless, we were able to employ these partition coefficients as comparative sets for conversion of amino acids R_f-values obtained by paper[8] and thin layer chromatography[9] into π-values. This has been carried out in the following steps:

(1) First, the R_f-values in a given solute system were converted into partition coefficients P_c for the same system using[8]

$$1/P_c = \rho(1/R_f - 1) \quad (3)$$

where ρ stands for the volume ratio of solvent and water phase in the chromatogram. Relation between P_c and partition coefficient in another solvent system, say P_1, is given by (2). Combining (2) and (3),

$$\log P_1 = \alpha + \beta \log (1/R_f - 1) \quad (4)$$

where α, β are simple functions of parameters involved in (2) and (3). They can, in our case, be estimated for B/W and O/W since the P_1 and R_f values for the two sets mentioned above are known. In this way, R_f values can be transformed into P (O/W) and π-values can be derived from (1), using glycine as parent compounds. π-Values are summarized in Table I. Let us mention in addition that, using (4) and constants α, β for the two systems, Collander parameters for O/W (subscript 1) vs. B/W (subscript 2) valid for the group of amino acids can be estimated:

$$\delta = \beta_1/\beta_2 \quad (5)$$

$$\gamma = \alpha_1 - \alpha_2\delta; \quad (6)$$

for 16 systems used here, $\gamma = -0.59 \pm .29$ and $\delta = 1.46 \pm 0.22$ (mean $\pm$s.d.). The constants are not likely to be significantly different from the literature values[7] for substances different from amino acids.

(2) π_R values for each side chain were then expressed as a sum of group contributions π_g to π_R,

$$\pi_R = \sum_{g=1}^{n} p_{Rg}\, \pi_g. \quad (7)$$

When group g is present m times in the side chain R, $p_{Rg} = m$, otherwise $p_{Rg} = 0$. Discarding values outside the acceptable range, we have obtained 277 equations for 24 unknown π_g's (cf. Table II). Multiple regression analysis carried out with these data yielded the group contributions π_g (Table II) and "corrected" values of π_R (Table I, last column). The multiple regression coefficient is highly significant (0.980), proving the additive nature of π-values for amino acid side chains. However, as assumed, the π_g's are somewhat different from those for other aliphatic series[4].

Table I. Hydrophobicity π-Constants for Amino Acid Side Chains

	Amino acid	π-values		
		mean	s.d.	reg.[A]
Abu	α-Aminobutyric acid	0.58	0.14	0.82
Acy	α-Aminooctanoic acid	2.66	0.31	2.62
Aib	α-Aminoisobutyric acid	0.62	0.10	
Ala	Alanine	0.38	0.08	0.37
Arg	Arginine (noncharged)	1.58	0.28	1.58
	Arginine (charged)	-1.23	0.31	-1.23
Asn	Asparagine	-0.27	0.18	-0.40
Asp	Aspartic acid (noncharged)	-0.16	0.06	-0.17
	Aspartic acid (charged)	-1.23	0.27	-1.42
Cys	Cysteine (noncharged)	0.69	0.09	0.69
Gln	Glutamine	-0.09	0.21	0.05
Glu	Glutamic acid (noncharged)	0.28	0.20	0.28
	Glutamic acid (charged)	-1.20	0.35	-1.30
Gly	Glycine	0		0
His	Histidine (noncharged)	0.94	0.06	0.94
	Histidine (charged)	-1.30	0.35	-1.30
Hpr	Hydroxyproline (acidic soln.)	0.24	0.16	0.24
	Hydroxyproline (alkaline soln.)	0.77	0.14	0.77
Ile	Isoleucine	1.56	0.09	1.58
Leu	Leucine	1.66	0.07	1.58
Lys	Lysine (noncharged)	1.03	0.34	1.20
	Lysine (charged)	-0.93	0.48	-0.87
Met	Methionine	1.39	0.15	1.39
Nle	Norleucine	1.77	0.09	1.72
Nva	Norvaline	1.24	0.10	1.27
Orn	Ornithine (noncharged)	0.92	0.22	0.75
	Ornithine (charged)	-1.25	0.61	-1.32
Phe	Phenylalanine	1.80	0.13	1.83
Pro	Proline (acidic soln.)	0.56	0.11	0.56
	Proline (alkaline soln.)	1.69	0.05	1.69
Ser	Serine (acidic soln.)	0.04	0.13	0.05
	Serine (alkaline soln.)	-0.26	0.10	-0.25
Thr	Threonine (acidic soln.)	0.33	0.10	0.39
	Threonine (alkaline soln.)	0.10	0.07	0.09
Trp	Tryptophan	1.87	0.30	1.87
Tyr	Tyrosine (noncharged)	1.70	0.24	1.70
	Tyrosine (charged)	0.77	0.26	0.67
Val	Valine	1.06	0.11	1.13

[A] Expected values obtained by multiple regression analysis

In conclusion, we suggest that π-values in the last column of Table I be tentatively used as a measure of side chains hydrophobicity. If π_R constants for additional amino acids are to be calculated, group contributions π_g given in Table II should be employed.

Table II. Group Contributions to π-Values of Amino Acid Side Chains

	Group	State/conditions	Contribution to π
CH_3	Methyl		0.340
CH_2	Methylene		0.450
CH	Methine		0.421
$C_6H_5-CH_2$	Benzyl		1.808
OH	Hydroxyl/aliphatic	acidic soln.	-0.429
		alkaline soln.	-0.728
OH	Hydroxyl/pyrrolidine	acidic soln.	-0.325
		alkaline soln.	-0.919
OH	Hydroxyl/aromatic	noncharged (acidic soln.)	-0.129
O^-	Hydroxyl/aromatic	charged (alkaline soln.)	-1.163
SH	Thiol	noncharged (acidic soln.)	0.218
NH_2	Amino	noncharged (alkaline soln.)	-0.626
NH_3^+	Ammonium	charged (acidic soln.)	-2.701
$NH=C(NH_2)NH$	Guanidino	noncharged (alkaline soln.)	0.208
$[NH=C(NH_3)NH]^+$	Guanidino	charged (acidic soln.)	-2.609
$C_3H_3N_2-CH_2$	Imidazole	noncharged (alkaline soln.)	0.913
$[C_3H_4N_2-CH_2]^+$	Imidazole	charged (acidic soln.)	-1.132
COOH	Carboxyl	noncharged (acidic soln.)	-0.643
COO^-	Carboxyl	charged (alkaline soln.)	-1.896
$CONH_2$	Carboxamido		-0.879
SCH_3	Methylthio		0.466
C_3H_6	Pyrrolidine	acidic soln.	0.536
		alkaline soln.	1.665
$C_8H_6N-CH_2$	Indole		1.839

Acknowledgment

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APPLICATION OF AN EVOLUTIONARY STRATEGY TO THE CALCULATION OF PEPTIDE CONFORMATIONS

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Introduction

To predict a polypeptide structure requires a good approach to the multiple-minimum problem[1]. So far, several assumptions have been necessary to limit the number of initial conformations[2,3]. However, low-energy conformations resulting from these rather arbitrary and intuitive assumptions afford further examination.

In this paper we are applying an evolutionary strategy to the search for a minimum in energy of a polypeptide conformation. Previously, the evolutionary strategy was invented and applied in the field of bionics[4,5]. Several parts of the strategy were adopted to the special problem, but no restrictions concerning initial conditions are made. We tested the new method by calculating the structure of the hypothetical central pentapeptide of Luteinizing Hormone Releasing Hormone (LHRH), Ac-Ser-Tyr-Gly-Leu-Arg-NH_2.

Description of the Method (Figure 1)

(A) Choice of initial vectors: A set of initial conformations (= initial vectors) is chosen in such a way that no preference exists. The number of vectors remains constant throughout the calculation.
(B) Control of the stochastic variations: the varying component is defined by equation (1)

$$\xi_i := \xi_i + Z(\sigma) \quad (1)$$

where Z is a normally distributed random number with the variance σ and the mean value zero. The variance of the random number Z is controlled by trial and error. Initially σ has relatively high values (e.g. 90°). As soon as the energy drops beyond a certain threshold, control starts. The control is governed by the value w (Equation 2)

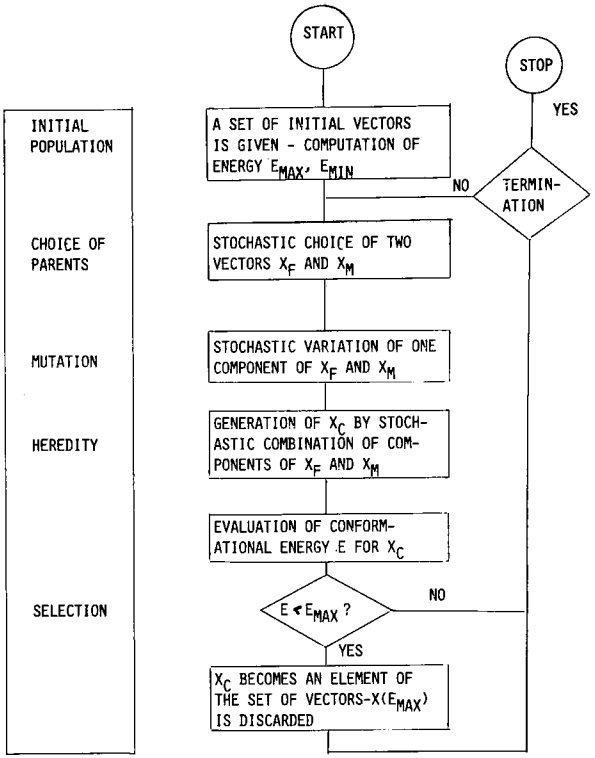

Fig. 1. Flow chart of the minimization program.

$$w = \frac{\text{number of successful steps}}{\text{number of steps}} \qquad (2)$$

w is recalculated after 5 n times of the generation of a new vector (n = the number of components of the vector). If the energy of a new vector is lower than all energies calculated before, this step is regarded as a successful one. σ varies as follows:

$$w = \begin{cases} < 0,2 & \sigma := \sigma \cdot g \\ \approx 0,2 & \sigma := \sigma \\ > 0,2 & \sigma := \sigma \cdot f \end{cases}$$

where g and f are chosen in such a way, that σ scans maximally about one order of magnitude after ten calculations of w.

(C) Heredity: The principle of heredity applied are shown graphically:

$\vec{x}_f$ $[\varphi_1\ \psi_1\ \chi_1^1\ \chi_1^2]$ $[\varphi_2\ \psi_2\ \chi_2^1\ \chi_2^2]$ $[\varphi_3\ \psi_3]$ $[\varphi_4\ \psi_4\ \chi_4^1\ \chi_4^2]$ $[\varphi_5\ \psi_5\ \chi_5^4]$

$\vec{x}_m$ $(\varphi_1\ \psi_1\ \chi_1^1\ \chi_1^2)$ $(\varphi_2\ \psi_2\ \chi_2^1\ \chi_2^2)$ $(\varphi_3\ \psi_3)$ $(\varphi_4\ \psi_4\ \chi_4^1\ \chi_4^2)$ $(\varphi_5\ \psi_5\ \chi_5^4)$

$\vec{x}_c$ $(\varphi_1\ \psi_1\ \chi_1^1\ \chi_1^2)$ $[\varphi_2\ \psi_2\ \chi_2^1\ \chi_2^2]$ $(\varphi_3\ \psi_3)$ $(\varphi_4\ \psi_4\ \chi_4^1\ \chi_4^2)$ $[\varphi_5\ \psi_5\ \chi_5^4]$

The choice is governed by equally distributed random numbers.

(D) Energy evaluation: The conformation energy calculations are carried out using the program ECEPP[6].

(E) Termination: The program is stopped after a given number of iterations or if there is no decisive gain in energy after several iterations. The calculation was carried out on a UNIVAC 90/80-3. The program is written in FORTRAN IV. Symbolic cards: 300.-

Table I. Ten Low-Energy Conformations of Ac-Ser-Tyr-Gly-Leu-Arg-NH_2

		Dihedral angles [deg.]									
Residue	*)	1	2	3	4	5	6	7	8	9	10
Ser	φ_1	-64	-64	-75	-70	-77	-80	-67	-73	-159	-86
	ψ_1	-54	-56	110	112	102	82	-38	116	151	65
Tyr	φ_2	-76	-70	-64	-64	-60	-72	-63	-64	-81	-72
	ψ_2	-33	-31	-40	-41	-41	-145	-41	-40	-24	145
Gly	φ_3	-63	-62	-96	-64	-94	-80	-70	-94	-166	-80
	ψ_3	-45	-47	169	168	164	85	-57	150	-149	85
Leu	φ_4	-71	-71	-73	-79	-77	-80	-92	-151	-80	-78
	ψ_4	-48	-55	85	83	91	86	71	132	87	92
Arg	φ_5	-157	-93	-78	-78	-78	-158	-81	-75	-78	-75
	ψ_5	106	92	102	103	104	117	101	106	105	106
Calc. energy using **) [kcal/mol]		-31,6	-31,2	-28,4	-28,4	-28,3	-27,9	-27,7	-26,6	-26,5	-26,2
Minimized energy using ***) [kcal/mol]		-32,2	-31,5	-28,8	-28,7	-28,7	-28,1	-28,0	-27,2	-27,0	-26,7
Rel.stat. weight [%] ***)		82,9	12,2	0,97	0,98	0,98	0,53	0,60	0,42	0,12	0,07

*) Only the backbone dihedral angles (φ, ψ) are listed.

**) The calculation was done by the evolutionary minimization procedure described; the results were obtained after about 1200-1400 function calls.

***) A further minimization was achieved by a Newton-Raphson method, which is an integral part of the program STATWT (from S. S. Zimmerman, modified version by Shipman). Another 1000-1500 function calls were needed. This program provides the statistical weight of the different conformations.

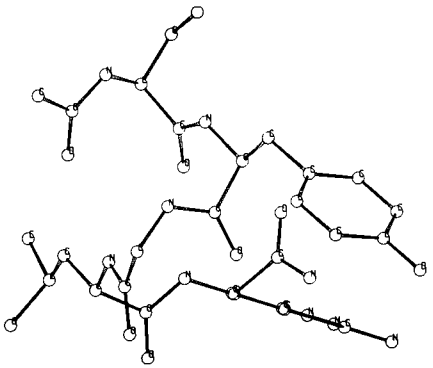

Fig. 2. Conformation No. 1 of Ac-Ser-Tyr-Gly-Leu-Arg-NH_2.

Test Case

An example was computed with 17 variable dihedral angles. A position near a local minimum is reached after approximately 1200 energy evaluations (Table I, third line from bottom) without any input information. The final energy values (Table I, penultimate line) result from Newton-Raphson minimization requiring even more energy evaluations (1000-1500). A comparison of both methods reflects the efficiency of the evolutionary strategy. Table I shows ten of the lowest conformations, ordered according to their energy. The first and second are of high probability (95% together) and differ only in one angle from one another (φ_5). They represent a slightly distorted α-helix (Figure 2). Conformation 3, 4 and 5 are identical (bend type structures), indicating the algorithm to find a definite conformation several times though starting from different initial positions.

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COMPUTER SIMULATIONS OF THE CONFORMATIONAL PROPERTIES OF OLIGOPEPTIDES

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The solution conformation of oligopeptides has been the subject of much interest recently[1,2]. This interest stems from the desire to understand the factors influencing molecular conformation in solution, from the fact that oligopeptides are composed of the same structural units as proteins, and from the conviction that their biological activity is related to their low energy conformations[1,3].

We have developed an integrated approach to study the conformational and statistical thermodynamic properties of oligopeptides. This approach includes flexible geometry empirical force field calculations[4,5], Monte Carlo simulations[6] and *ab initio* molecular orbital calculations[7].

The flexible geometry calculations include calculation of the relative vibrational energies of different minimum energy conformations. The results of these calculations for "host-guest" hexapeptides of methionine with glycine are summarized in Table I[4].

The Monte Carlo method[6] is used to better simulate the ensemble of conformations present in solution. It generates random peptide chains from which average energies, average end-to-end distances and probability maps of the conformational states of a given residue as a function of its position in the chain are obtained. The results of the Monte Carlo

Table I. Energy, Entropy, and Free Energy (kcal/mole) of Co-Oligopeptides of Methionine with Glycine as a Function of Position of Guest Residue.

Oligopeptide	$E_\alpha - E_{C_7}$	$E_\alpha^\nu - E_{C_7}^\nu$	$T \cdot (S_\alpha - S_{C_7})$	$A_\alpha - A_{C_7}$
Boc-Met_6-OMe	-5.09	0.05	-3.99	-1.05
Boc-Gly-Met_5-OMe	-7.56	-0.04	-5.16	-2.45
Boc-Met_3-Gly-Met_2-OMe	-2.35	0.05	-4.13	1.83
Boc-Met_5-Gly-OMe	-6.22	-0.09	-3.79	-2.53

The vibrational energy, S^ν and entropy, S^ν, are derived from the calculated vibrational frequencies[4,8]. The importance of comparing the free energy A, of different molecular conformations is underlined in the result for Boc-Met_3-Gly-Met_2-OMe where the energetically most stable conformation does not correspond to the free energy minimum.

chain simulation of hexaalanine and "host-guest" hexapeptides of alanine and glycine are presented in Table II.

Table II. Monte Carlo Simulation (>500,000 Chains) of Hexapeptides .

Oligopeptide	<E>	<R>	<A>	<T·S>
Ac-Ala_6-NMe	16.68	20.84	-10.12	26.79
Ac-Gly-Ala_5-NMe	15.44	19.15	-11.62	27.06
Ac-Ala_5-Gly-NMe	15.31	19.12	-11.69	27.00
Ac-Ala_3-Gly-Ala_2-NMe	11.50	7.20	-13.66	25.17

Units: E, A and T·S in kcal/mole, R in A; T = 298° K.

Analysis of the results shows that the markedly different behavior of Ac-Ala_3-Gly-Ala_2-NMe arises from the fact that a relatively small number of conformations, with high statistical weight, accounts for most of the energy contribution[4]. In contrast, all the other hexamers have a more evenly distributed set of conformations, a fact reflected in their higher entropy. The "folded" conformation of Ac-Ala_3-Gly-Ala_2-NMe corresponding to the highest statistical weight is shown in stereo in Figure 1.

Ab initio calculations are used to achieve a better understanding of the intramolecular interactions in model systems. *Ab initio* extended basis set calculations[7,9] have been used along with rigid and flexible geometry calculations to study the conformational properties of *gem*-diaminoalkyl and 2-substituted malonyl analogs of residues[10] occurring in retro-inverso-peptides such as modified enkephalins[11]. These calcula-

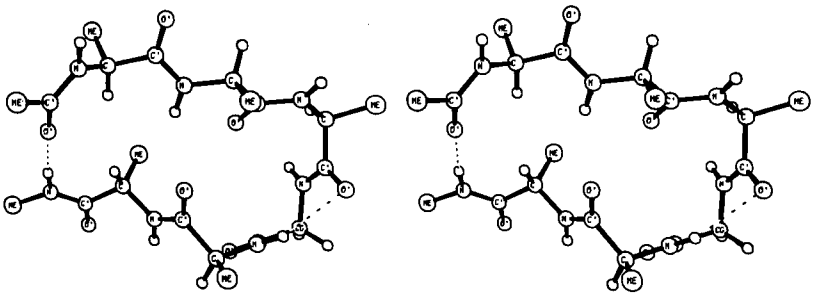

Fig. 1. The "folded" conformation of Ac-Ala$_3$-Gly-Ala$_2$-NMe corresponding to the highest statistical weight as generated in the Monte Carlo simulation. Broken lines indicate hydrogen bonds.

tions serve as an index of reliability for the force field calculations and provide information about the charge distribution and electron density in the molecules as exemplified in Figure 2.

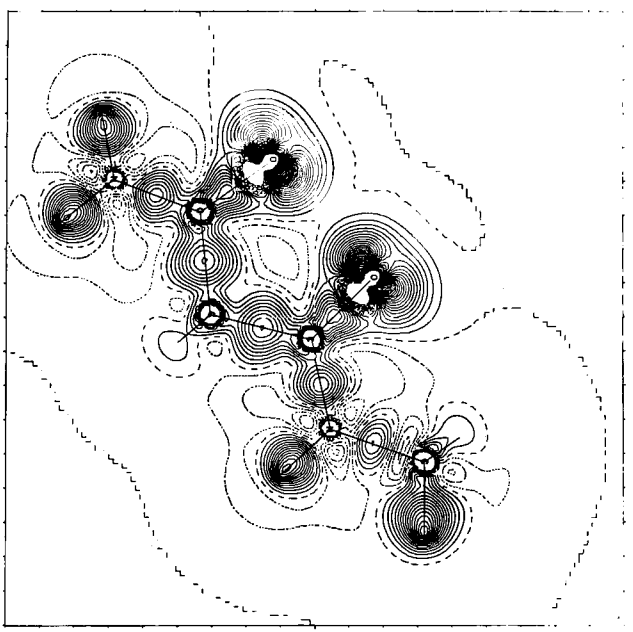

Fig. 2. Difference map of the electron density in the plane of the malonyl group of *N*-methylmalonamide (ϕ = 180°, ψ = 180°) minus the electron density of the spherical atoms, as calculated with an extended 4-31G basis set. Solid, dashed, and dotted lines represent positive, zero, and negative difference densities, respectively. (The density is contoured at 0.05 electron/A^3).

The preference of the malonamide (CH_3-NH-CO-CH_2-CO-NH_2) for an α-helical conformation vis-a-vis the preference of the glycyl amide (HCO-NH-CH_2-CO-NH-CH_3) for the extended conformation, is evident from the electron density map.

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THE OXAZOLONE AS A NEW STABLE INTERMEDIATE IN THE COUPLING OF BOC-AMINO ACIDS USING CARBODIIMIDES UNDER SELECTED CONDITIONS

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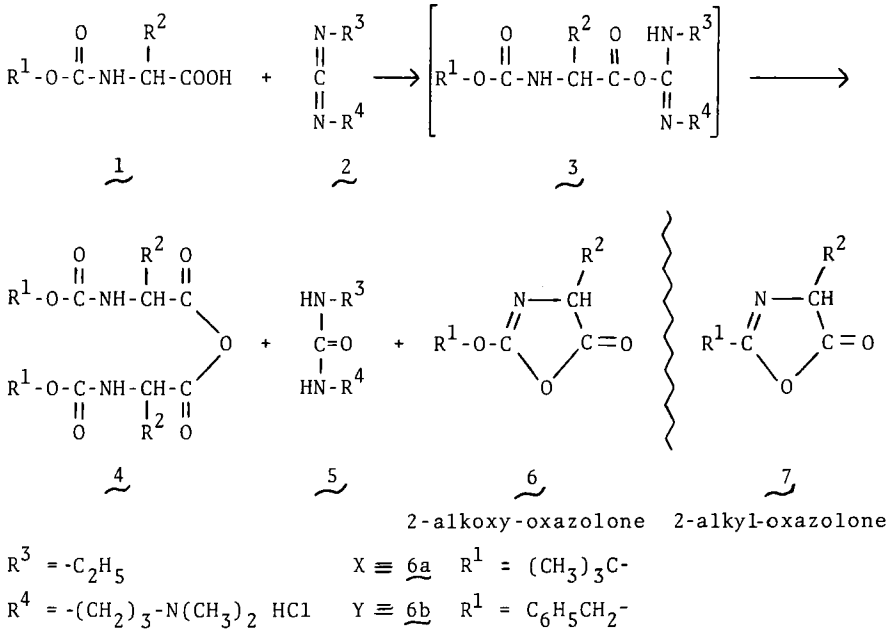
We have recently described the preparation of the symmetrical anhydrides 4 of Boc-amino and Z-amino acids 1 using the soluble carbodiimide *N*-ethyl-*N'*-(γ-dimethylaminopropyl)-carbodiimide 2 (EDC) (0.5 molar equiv.) and the acid 1 in dichloromethane at 0°C followed by removal of all side-products by washing with dil. acid and aq. $NaHCO_3$.[1] Noticeable among the results was that yields obtained from Boc-Val-OH (amino-acid symbols represent the L-isomer) and Boc-Ile-OH (50-55%) were lower than those obtained from Boc-Leu-OH (83%) and most other starting materials. An attempt to increase the yield of $(Boc\text{-}Val)_20$ by using more EDC (1 mol equiv.) resulted in the formation of a second product X'. Removal of the crystalline $(Boc\text{-}Val)_20$ (50% yd) after addition of petroleum ether and evaporation of the solvents left a clear oil (20% by wt; $[\alpha]_D$ –49.6° (*c* 2, $CHCl_3$)) which seemed pure on the basis of ^{1}H-nmr spectroscopy (60 MHz). Its spectrum showed one $(CH_3)_3C$– group per valine isopropyl side-chain at δ = 1.60 ppm ($CDCl_3$ *vs* Me_4Si) which is 0.13 ppm downfield from a $(CH_3)_3C\text{-}O\text{-}C'{=}O$ group, and a sharp one-proton doublet at 4.13 ppm. The ir spectrum showed strong absorption at 1845 and 1700 cm^{-1} not unlike that of the known 2-alkyl-oxazolones 7. Elemental analysis indicated the formula $C_{10}H_{17}NO_3$ which corresponds to Boc-Val-OH minus $1H_2O$. X' reacted with CH_3NH_2 to give Boc-Val-$NHCH_3$, and gave rise to a 90% yield of optically pure Boc-Val-Lys(Z)-OMe in the presence of H-Lys(Z)-OMe·HCl (N-methyl-morpholine, NMM).[2] X' was converted quantitatively to the crystalline L-valine *N*-carboxyanhydride (NCA) after standing in *tert*-butanol, or by the action of HCl gas. X' is assigned the structure 6a ($R^2 = (CH_3)_2CH$–), *(S)*-2-*tert*-butoxy-4-isopropyl-5-oxo-4,5-dihydro-1,3-oxazole, or the oxazolone from the Boc-amino acid, which is consistent with all the acquired data.



2-alkoxy-oxazolone 2-alkyl-oxazolone

R^3 = $-C_2H_5$ X ≡ 6a R^1 = $(CH_3)_3C-$

R^4 = $-(CH_2)_3-N(CH_3)_2$ HCl Y ≡ 6b R^1 = $C_6H_5CH_2-$

Using the characteristic nmr peak of the *tert*-butoxy group of X, it is easy to detect and quantitate the 2-butoxy-oxazolone 6a in a mixture. Under the conditions described, Boc-Ile-OH and Boc-Leu-OH gave 40 and 5% respectively of oxazolone, but these could not be isolated in the pure state.

In identical manner, a 20% yd of Y′ (oil, $[\alpha]_D$ −55.8° (*c* 1, $CHCl_3$)) was obtained from Z-Val-OH. Y′ gave the optically pure Z-Val-Lys(Z)-OMe after reaction with the pertinent ester, and Val-NCA after catalytic hydrogenation. Elemental analysis and ir and nmr spectroscopy pointed to the same type of structure. Mass spectrometry gave the molecular ion (M^+ 233) thus confirming 6b (R^2 = $(CH_3)_2CH-$) as the structure of the product. Here again, the characteristic nmr peak for the alkoxy protons ($-CH_2-O-C'=N-$) at 5.30 ppm which is 0.20 ppm downfield from the methyleneoxy protons of a Z-group can serve as the diagnostic peak. Crystalline Y″ (R^2 = $C_6H_5CH_2-$) with mp 65-66°, $[\alpha]_D$ −36.8° (*c* 1.5, THF) was similarly obtained in 4.5% yield from Z-Phe-OH.

Dicyclohexylcarbodiimide (DCC) gave much less product (6% from Boc-Val-OH) than did EDC, suggesting promotion of the reaction by the $-PrN(Me_2)\cdot HCl$ group of EDC. The yield increased from 6 to 14% in the

presence of 1 molar equiv. of $Et_3N{\cdot}HCl$, and increased further by decreasing the reactant concentrations.

Systematic studies showed that the highest yields of 6 could be obtained by adding the acid 1 (1 mmol/10 ml CH_2Cl_2) to a stirred solution of EDC and $Et_3N{\cdot}HCl$ (1.5 mmol each) in 40 ml of CH_2Cl_2 over 15-20 min at 23°C. This procedure allowed preparation of the pure oxazolones from the compounds with yields indicated: Boc-Val-OH, 50%; Z-Val, 55%; Z-Phe, 25%; Z-Gly, 5%; Z-Cys (Bzl), 3%.

X′ or Y′ reacted with an equiv. of 1 to give a quantitative yield of anhydride 4′, and 4′ remained unchanged in the presence of $Et_3N{\cdot}HCl$ (The hitherto undescribed type of anhydride Boc-Val-O-Val-Z, mp 80-82°, was obtained from either X′ and Z-Val-OH or Y′ and Boc-Val-OH). We therefore suggest that the immediate precursor of 6 is the *O*-acylisourea 3, and moreover that part of the symmetrical anhydride 4 in the scheme could have arisen from the reaction of 6 with 1.

X′ could also be detected (2% yd) in a solution of equiv. amounts of Boc-Val-OH, EtOCOCl, and NMM kept at 0°C for 5 min. Therefore it is not unlikely that 6 is formed in small amounts whenever 1 is activated in the absence of nucleophile (amine or phenol). A particular and common example of this is during solid-phase synthesis by the Merrifield method where a large excess of DCC and 1 are used.[3] The solution remaining after acylation of the peptide chain resembles that used here to prepare the oxazolones.

When reacted with H-Lys(Z)-OBzl·HCl (NMM)[2] in the presence of 0.20 molar equiv. of Et_3N (free base), X′ gave rise to 3% of epimeric peptide Boc-D-Val-L-Lys(Z)-OBzl, and Y′ gave rise to 7.5% of epimeric peptide Z-D-Val-L-Lys(Z)-OBzl, corresponding to 6 and 15% racemization, respectively. One molar equiv. of Et_3N led to 50% racemization in the coupling of Y′. No racemization could be detected for couplings where Et_3N was not added.

It is generally accepted that the intermediate primarily responsible for the racemization which occurs when acylamino acids or protected peptides are coupled with nucleophiles is the 2-alkyl-oxazolone 7 which is chirally unstable.[4] The oxazolone is formed as a result of nucleophilic attack by the amide (R^1-CO-NH-) carbonyl oxygen on the activated carbonyl carbon. Racemization is not observed when the activated component is an alkoxycarbonyl-amino acid 1, as is the case in stepwise synthesis, and the accepted reason for this has been that the oxazolone does not form because of the nature of the urethane (R^1-O-CO-NH-)

group.[4] Our results show that these 2-alkoxy-oxazolones 6 are indeed formed under some normal peptide coupling conditions, but they are much more chirally stable than the 2-alkyl-oxazolones 7. They are obtained and they couple without racemization even in the presence of salts; but they do undergo racemization when coupled in the presence of a tertiary amine base.

Oxazolones of type 6 have not previously been described except for the one report by Jones and Witty who concluded on the basis of analysis by nmr that the cyclodehydration of Z-Phe-OH using PCl_5 followed by Et_3N gave 6b ($R^2 = C_6H_5CH_2-$).[5] Allusion to the possibility of their chiral stability has been made.[5]

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APPLICATION OF *O*-NITROPHENYLSULFENYL-*N*-CARBOXYANHYDRIDES FOR THE SYNTHESIS OF *N*-HYDROXYPEPTIDES

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A large number of natural *N*-hydroxyamides have to date been described. Details of their structure are discussed in a review.[1] It has recently become the accepted view that some of these natural hydroxamic acids play a decisive role as iron transport agents.[2] Considering the need for syntheses of their peptide analogues, we have for some time been involved in developing a method for the synthesis of *N*-hydroxypeptides.[3]

Selective *N*-acylation of *N*-hydroxyamino acid esters is described in this report.

Hydroxamic acids can either be prepared by acylating hydroxylamine with acid chlorides, anhydrides, mixed anhydrides[4] or N-carboxyanhydrides.[5] Hydroxylamine *O*-acyl derivatives may also be formed. Acylation of hydroxylamine derivatives is more complicated.[6] Generally, mixtures of *N*- and *O*-acyl or *N*-, *O*-diacyl derivatives are formed. There is prevalence of *O*-acyl derivatives when the N-substituent introduces a steric hindrance or is itself a strongly electron-accepting group.

Unambiguous *N*-acylation can be brought about by using acid chlorides. A reaction involving N-hydroxyglycine sodium salt has been employed in the synthesis of *N*-hydroxypeptides.[7] This method of activation has, however, its disadvantages which are well-known in peptide chemistry and discourage its use with optically active compounds.

On the other hand, the use of the *p*-nitrophenyl ester of

$$Z\text{–DL–Phe–ONp} + \text{HO–Gly–OMe} \longrightarrow Z\text{–DL–Phe–O–Gly–OMe}$$

75%, m.p. 125-7°

$$Z\text{–Gly–OPht} + \text{DL–HO–Ala–OBu}^t \longrightarrow Z\text{–Gly–DL–O–Ala–OBu}^t$$

86%, m.p. 67-9°

where: HO–Gly–OMe denotes HO–NH–CH_2–$COOCH_3$

benzyloxycarbonyl-DL-phenylalanine or the *N*-hydroxyphathalimide ester of benzyloxycarbonylglycine yielded solely the corresponding *O*-acyl derivatives of the N-hydroxyamino acid esters.

We isolated these compounds to be able to know their properties and to rule out the formation of that kind of *O*-acyl derivatives when other methods are to be used. The R_f values for these derivatives differ somewhat from those for N-hydroxypeptide derivatives, and the Cα protons of the *N*-hydroxyamino acid residue are considerably more deshielded in the *N*-hydroxypeptide (δ 4.3) than they are in the *O*-acyl-*N*-hydroxyamino acid ester (δ 3.6).

Zvilichovsky and Heller[8] were among the first to attempt a synthesis of *N*-hydroxypeptides by acylating *N*-hydroxyglycine with amino acid *N*-carboxyanhydrides. These compounds have, however, a number of disadvantages as instability and tendency to polymerize. The *N-o-*

$$\text{NPS–NCA–DL–Phe} + \text{HO–Gly–OMe} \longrightarrow \text{NPS–DL–Phe(OH)–Gly–OMe}$$

75%, m.p. 152-4°

$$\text{NPS–NCA–D–Ala} + \text{HO–Phe–OMe} \longrightarrow \text{NPS–D–Ala(OH)–Phe–OMe}$$

73%, m.p. 130°

$$\text{NPS–NCA–D–Ala} + \text{HO–Gly–OBzl} \longrightarrow \text{NPS–D–Ala(OH)–Gly–OBzl}$$

85%, oil

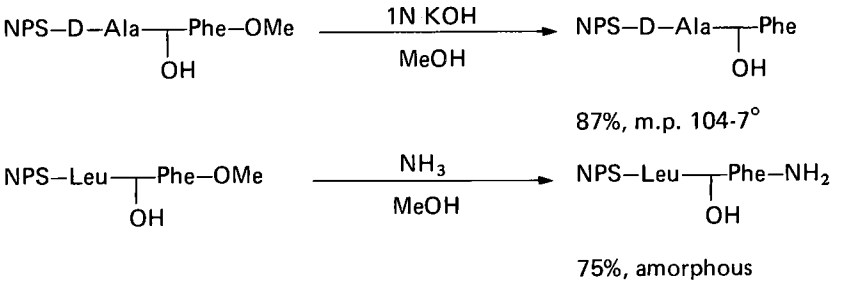
$$\text{NPS–NCA–Leu} + \text{HO–Phe–OMe} \longrightarrow \text{NPS–Leu(OH)–Phe–OMe}$$

86%, oil

nitrophenylsulfenyl-*N*-carboxyanhydrides recently introduced in peptide synthesis are free from such inconveniences. These compounds can easily be prepared by reaction of *o*-nitrophenylsulfenyl chloride with amino acid *N*-carboxyanhydrides.[9] We have successfully used the *o*-nitrophenylsulfenyl-*N*-carboxyanhydrides of phenylalanine, leucine and alanine to acylate the methyl esters of *N*-hydroxyglycine or *N*-hydroxyphenylalanine and the benzyl ester of *N*-hydroxyglycine.

The protected esters of the N-hydroxypeptides were formed in 70% yield; chromatography revealed no formation of the corresponding *O*-acyl derivatives. The compounds obtained as hydroxamic acids gave a positive test with $FeCl_3$, and their structure was confirmed by spectroscopy.

Earlier data having been inconsistent, we also checked the stability of the *N*-hydroxyamide bond to the action of the reagents used in peptide chemistry to remove the protecting groups. Earlier we had found this bond to be stable to acidolytic conditions, and had reported conditions of catalytic hydrogenation designed to leave the N-O bond intact.[3] We now report that hydrolysis or ammonolysis of the *N*-hydroxypeptide esters are also possible, as the N-hydroxyamide bond remains intact in either of



these processes, and the acid or amide are formed respectively. Thus, the method described herein is effective for synthesizing peptides containing one or several *N*-hydroxypeptide bonds. Further syntheses of such compounds will be carried out.

Acknowledgement

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ON THE FORMATION AND METHYLATION OF LYSINOALANINE-L-LYSINE POLYPEPTIDES

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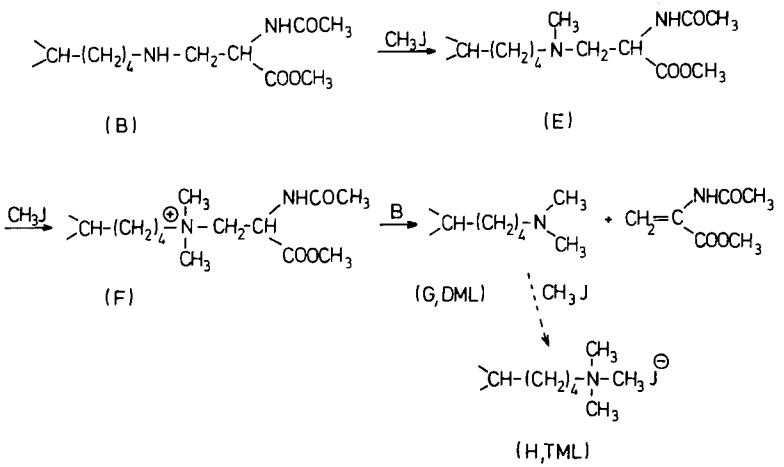
Introduction

Lysinoalanine (Lal) is formed by the addition of the N^{ϵ}-amino group of lysine (Lys) to the double bond of dehydroalanine (Dha) which is obtained from cysteine or serine residues by alkaline treatment[1,2] or by methylation of the cysteine SH-group and β-elimination.

Lal-Residues in keratine fibers are cleaved via methylation to give N^{ϵ}-dimethyllysine (Dml) and Dha according to the following reaction[4,5]. For studying this reaction in detail a copolymer of L-Lys and Lal ($\simeq$1:1) was used[6]. This copoly-(Lal-L-Lys) was prepared from poly-L-lysine (PLL) and acetyldehydroalanine methyl ester. In this way it should be possible to introduce N^{ϵ}-dimethyl groups in PLL.



Results

Because of the pH-dependence of the nucleophilic Michael-addition of PLL to the Dha double bond the reaction of PLL with N^{ϵ}-

acetyldehydroalanine methyl ester was studied at various pH values using a pH stat as well depending on temperature and molar ratios of the reactants. The reaction time in all cases was 8 hours and the amino acid composition of the reaction product was determined by ion exchange amino acid analysis.

Table I shows the amount of Lal formed at 20° C depending on pH and illustrates that the maximum occurs at pH 9.5.

Table I. Amount of Lal Formed by Treating PLL with Acetyldehydroalanine Methyl Ester at 20° C (Mole Ratio 1:1).

pH	6,0	7,0	8,0	9,0	9,5	10,0	10,5	11,0	12,1
Lal (%)	0	2,0	6,3	28,7	36,9	32,7	30,1	18,2	2,3

Investigating the temperature dependence of this reaction at pH 9.5, it was found that the reaction optimum occurs at 30° C as one can see from Table II.

Table II. Amount of Lal on PLL at pH 9.5 Depending on Temperature (Mole Ratio 1:1).

Temperature (°C)	20,0	30,0	40,0	50,0
Lal (%)	36	39,7	31,9	13,2

The conversion obtained is relatively low. By using a 2-fold excess of the Dha-derivative the conversion could be increased to 67%, which agrees to some extent with the results of Friedman[7].

To investigate the cleavage of Lal by methylation, 0.1 mM copoly-[Lal-Lys], containing 50 and 64.5% Lal, was treated with an excess of CH_3J at constant pH and temperature for 8 hours. The reaction product was hydrolyzed by 6N HCl and the amino acid composition was determined by ion exchange chromatography. Figure 1 shows amino acid chromatograms of some of these reaction products. With increasing pH Dml is converted to N^{ϵ}-trimethyllysine. An unknown peak at 90 minutes was identified as N^{ϵ}-monomethyllysinoalanine (N^{ϵ}-methyl-N^{ϵ}-(2-amino-2-carboxyethyl)-L-lysine (Mlal). This amino acid was synthesized according to the lysinoalanine synthesis used by Finley and Snow (Scheme 1)[6,8]. From the reaction mixture of D,L-alanine and N^{ϵ}-methyllysine, Mlal was isolated by Dowex 50W-X8 chromatography in a yield of 52%, m.p. 165-167° C.

Table III. Amino Acid Composition of Copoly-[Lal-Lys] Treated with CH_3J at Various pH and Temperatures.

at 20° C on Copoly-LAL (64.5)-Lys (35.5)

pH	MLAL (%)	LAL (%)	Lys (%)	MML (%)	DML (%)	TML (%)
6	0	64.5 (100)	35.5 (100)	0	0	0
7	7.3	55.7 (86.4)	34.6 (97.5)	1.5	1.0	0
8	11.0	33.3 (51.7)	30.4 (84.6)	5.5	20.2	0
9	3.9	4.9 (7.6)	24.0 (67.6)	4.4	61.6	1.7
10	0	0.7 (1.2)	11.3 (31.8)	4.6	69.0	14.4
11	0	1.0 (1.4)	4.6 (13.0)	2.3	46.4	45.7
12	0	0.8 (1.2)	2.2 (6.2)	1.2	3.5	92.3

at 30° C on Copoly-LAL (50.6)-Lys(49.4)

MLAL (%)	LAL (%)	Lys (%)	MML (%)	DML (%)	TML (%)
1.0	49.8 (98.5)	49.0 (99.0)	0.2	0	0
9.7	21.8 (43.1)	48.0 (97.1)	3.6	16.9	0
0.8	2.2 (4.3)	39.4 (79.7)	4.0	50.7	3.0
0	0.9 (1.8)	17.3 (35.1)	3.8	51.0	27.0
0	1.2 (2.3)	6.3 (12.8)	0.3	39.7	52.5
0	0.7 (1.3)	5.1 (10.3)	0	0.3	94.0
0	1.2 (2.3)	1.8 (3.6)	0	0	97.0

at 40° C on Copoly-LAL (64.5)-Lys (35.5)

MLAL (%)	LAL (%)	Lys (%)	MML (%)	DML (%)	TML (%)
6.6	52.9 (82.0)	34.8 (98.0)	3.1	2.6	0
7.5	15.0 (23.3)	31.0 (87.3)	6.5	39.7	0.4
0	1.1 (1.8)	24.5 (69.0)	2.7	54.9	16.8
0	1.3 (2.0)	2.4 (6.8)	0	45.3	50.9
0	1.1 (1.8)	2.2 (6.2)	0	22.6	74.1
0	0.5 (0.8)	1.1 (3.1)	0	0	98.4
-	-	-	-	-	-

* Lal and Lys content of the copolymer in percent in parentheses. Mlal = N^{ϵ}-Methyl-lysinoalanine (N^{ϵ}-Methyl-N^{ϵ}-(2-Amino-2-carboxyethyl)-L-lysine), Lal = Lysinoalanine, Lys = Lysine, MML = N^{ϵ}-Methyllysine, Dml = N^{ϵ} Dimethyllysine, Tml = N^{ϵ}-Trimethyllysine

Table IV. Amino Acid Composition of PLL Treated with CH_3J at Various pH and Temperatures.

	at 20°C				at 30°C				at 40°C			
pH	Lys	MML	DML	TML	Lys	MML	DML	TML	Lys	MML	DML	TML
6	-	-	-	-	100.0	0	0	0	99.4	0.6	0	0
7	100.0	0	0	0	99.3	0.7	0	0	64.8	6.8	1.1	27.4
8	98.4	1.6	0	0	86.7	9.3	0.8	3.2	49.3	17.0	2.1	31.6
9	63.4	6.6	16.3	13.4	9.2	3.3	0.3	87.3	0.4	0	0	99.6
10	34.5	0.8	0.3	64.3	2.8	0	0.3	96.9	0.3	0	0	99.7
11	1.3	0	0	98.7	0	0	0	100.0	-	-	-	-

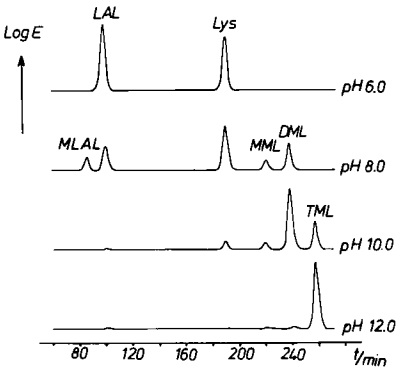
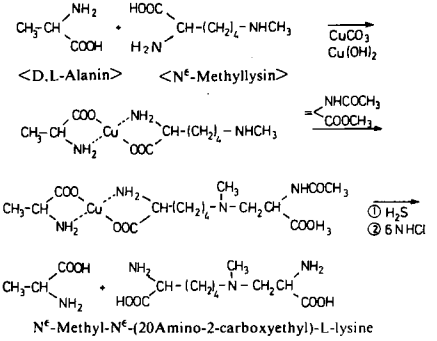


Fig. 1. Amino Acid Chromatograph of CH_3J-Treated Copoly-[Lal-Lys] (20° C) Depending on pH.



Scheme 1. The Synthesis of N^{ϵ} monomethyllysinoalanine.

According to Table III at moderate pH-values the formation of Dml predominates and maximum yields occurred at 20° C and pH 10 (69%) and at 30° C and pH 9 (51%) and at 40° C and pH 8 (54.9%). However with increasing temperature already at low pH remarkable amounts of Tml are formed. At higher pH values (11-12) Tml only was obtained.

For a comparison Table IV shows the results of the direct methylation of PLL by CH_3J between pH 6 and 11 and at 20, 30, 40° C. The yield of Dml also under weak alkaline pH conditions and at 20° C is much lower than that obtained by the cleavage reaction of Lal.

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PSEUDODIPEPTIDES: SYNTHESIS AND INCORPORATION WITHIN ANALOGUES OF LH-RH

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Introduction

Interest in peptide analogues in which amide linkages are replaced by enzymatically stable units has led to the development of new synthetic routes toward such structures, *via* dipeptide analogues or pseudodipeptides. We reported the synthesis of GlyψX analogs where X = Leu, Ile and ψ refers to the thiomethylene ether replacement of the amide bond [-CH_2S-] for [-CONH-].[1] New routes have since been devised for the synthesis of analogues of the type XψGly[2] and XψY (where X, Y $\neq$ Gly). The use of thiomethylene ether pseudodipeptides offers several advantages: 1) synthetic accessibility from amino acid precursors; 2) retention of chirality at both α-carbons; and 3) steric compatibility with the amide linkage. In view of their demonstrated resistance to enzymatic degradation,[1] the introduction of these structures into peptide hormones is clearly of interest.

The pseudodipeptides can be incorporated into peptides *via* solid phase synthesis. Preparation and bioactivity of several LH-RH analogues containing the GlyψLeu linkage are discussed herein.

Results

Pseudodipeptides can be synthesized by the routes shown in Scheme 1, with overall yields ranging from 40 to 80%. Physical properties of several different pseudodipeptides are summarized in Table I. The compounds were also characterized by elemental analyses and by their proton and carbon magnetic resonance spectra. In addition, the lipophilic nature of the free pseudodipeptides *vis a vis* their dipeptide analogues is readily discerned by their behavior on HPLC and by their longer retention times on amino acid analysis (Table I). The

*Deceased, April 6, 1979

stereochemical integrity of the chiral centers in the various amino acids was established by the known stereospecificity of the reactions involved and, in several cases, by preparing the identical compound by two routes. In the case of SerψLeu, formation of the diastereomeric D-SerψLeu and subsequent baseline resolutions of these compounds on analytical HPLC unequivocally established the optical purity of each of the pseudodipeptides at both chiral centers.

Table I. Physical Properties of Pseudodipeptides.

PSEUDODIPEPTIDE	M.P.	$[\alpha]_D$	PSEUDODIPEPTIDE AAA RETENTION TIME (MIN) [VS. DIPEPTIDE]
GLY ψ LEU	112-13°	-26.7 (C2,H_2O)	164 [157]
GLY ψ ILE	218-20°	-57.2 (C2,H_2O)	166 [157]
SER ψ LEU	189-81°	-5.4 (C1.5,H_2O)	160 [154]
D-SER ψ LEU	173-74°	-53.7 (C2.5,H_2O)	160
BOC PHE ψ GLY DCHA	140-41°	+5.6 (C17,MEOH)	238 [214]
BOC TYR (OBzl) ψ GLY DCHA	74-75°	+3.2 (C28,MEOH)	164 [157]
BOC PRO ψ GLY	79.5-80°		158 [152]

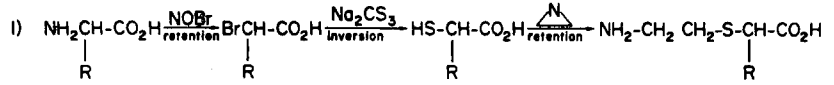
As a first test for the utility of the pseudodipeptide linkage, analogues of LH-RH were prepared in which the most vulnerable linkage, the Gly-Leu^{6,7} bond[3] was replaced. An expected agonist, [GlyψLeu^{6,7}] LH-RH, and an antagonist, [D-pGlu^1, D-Phe^2, D-Trp^3, GlyψLeu^{6,7}] LH-RH, were synthesized by the solid phase method on a 1% cross-linked benzhydrylamine resin using DCC-mediated coupling and N-α-Boc protection for all residues including GlyψLeu. No special precautions were necessary during the synthesis, although scavengers were used during subsequent HF cleavage and during acid hydrolysis in order to preserve the pseudodipeptide unit. The peptides were purified to >95% homogeneity by Sephadex gel filtration, partition chromatography and/or semi-preparative HPLC, and gave acceptable amino acid analyses for all residues including GlyψLeu.

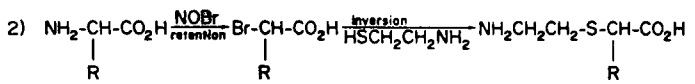
The biological activities are shown in Table II. In the case of [GlyψLeu^{6,7}]LH-RH, the potential agonist was found to have activity at the .01μg/ml level, but not at the 0.1, 1 or 10 μg/ml level. The antagonist [D-pGlu^1, D-Phe^2, D-Trp^3, GlyψLeu^{6,7}]LH-RH was determined to inhibit the release of LH at 10 μg only slightly.

Table II. Biological Activities of LH-RH, Analogues, and LH-RH Pseudopeptides.

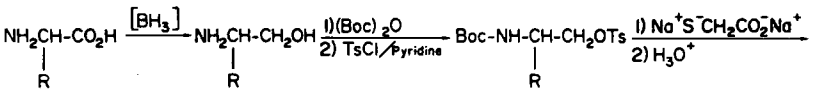
POLYPEPTIDES	LH-RH AGONIST	LH-RH ANTAGONIST
LH-RH	100	-
[GLY ψ $LEU^{6,7}$] LH-RH	0.1	VERY SLIGHT
[D-PHE^{2}] LH-RH	-	40
[DES-HIS^{2},D-ALA^{6}] LH-RH	-	100
[D-pGLU^{1},D-PHE^{2},D-TRP^{3},D-TRP^{6}] LH-RH	-	7500
[D-pGLU^{1},D-PHE^{2},D-TRP^{3},GLY ψ $LEU^{6,7}$] LH-RH	-	10

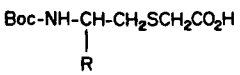
a) GlyΨX (Yankeelov et al., J. Org. Chem., 43, 1623 (1978))





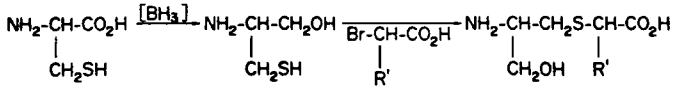
b) XΨGly (This work)



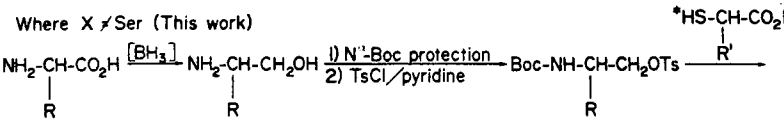


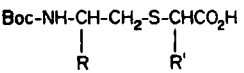
c) XΨY

1) Where X = Ser (Bettag and Spatola, Manuscript in prep.)

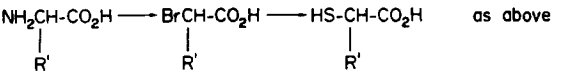


2) Where X ≠ Ser (This work)





*Via



Scheme I. General Schemes Leading to Pseudodipeptides.

Discussion

The utility of the pseudodipeptide linkage should reside first in its established resistance to peptidase action and also in its increased lipophilic character in appropriate applications. However, the anticipated increase in rotational mobility could result in unacceptable flexibility when in or near critical binding or active elements of a peptide chain.

Both agonist and antagonist analogues exhibited drastically reduced activities as a result of the pseudodipeptide modification. Since the amide bond itself is probably not an active element, it might be expected that conformational rather than electronic effects are being substantially altered by this substitution. In LH-RH analogues, the 6-7 position has proven critical for affecting activity; D^6 substitutions are good examples of this. Residues at 5 and 8 are demonstrably vital for full activity and there continues to be residual support for the presence of a β-turn in LH-RH. Thus, substitution of a flexible linkage at this site has apparently resulted in unacceptable flexibility in a portion of the molecule where conformational rigidity may be essential.

Acknowledgements

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FORMATION OF IMIDES IN STRONGLY ACIDIC MEDIA AND PARTICIPATION OF THE IMIDE IN TRANSFORMATION TO PIPERAZINE-2, 5-DIONE DERIVATIVES IN NEUTRAL MEDIA

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Synthesis of aspartyl peptides is generally accompanied by imide formation in acidic, neutral and basic media. Unfortunately, it is often the very application of protective ester groups that contributes to this undesired side reaction[1-7]. For the elongation of peptide chains containing an aspartyl residue, use of the unprotected β-carboxyl was proposed; however, imide formation is not completely eliminated during active ester coupling reactions[8-10].

In the course of a recent synthesis of pentagastrin analogs[11-12] we have observed the formation of aminosuccinyl peptides in strongly acidic media (Figure 1). It was clearly demonstrated that they are directly formed from the aspartic acid residue with a free β-carboxyl group. This conversion has been examined under different reaction conditions (Table I). Within the time interval necessary for removal of the β-*tert*-butyl ester group with 4N HCl in acetic acid the imide formation cannot be completely eliminated. This intramolecular ring closure significantly increased in HF and 4N HBr in acetic acid but has not been detected in trifluoroacetic acid.

The examination of imide formation has been extended to other tripeptides containing an aspartic acid residue in the middle position (Table II). The influence of the C-terminal residue was greater than the N-terminal on the rate of the formation of the imide[7].

In the course of treatment of different protected derivatives of L-aspartyl-L-phenylalaninamide (BOC-Asp(O-^{t}Bu)-Phe-NH_2, H-Asp (O-^{t}Bu)-Phe-NH_2, Z-Asp(O-^{t}Bu)-Phe-NH_2, Z-Asp-Phe-NH_2, H-Asp-Phe-NH_2·HCl) by 4N HCl in acetic acid and 4N HBr in acetic acid, a series of simultaneous and consecutive reactions leading to aminosuccinyl derivatives has been observed. The unacylated L-aminosuccinyl-L-

phenylalaninamide is more sensitive to hydrolysis as compared to its protected and aminoacylated derivatives.

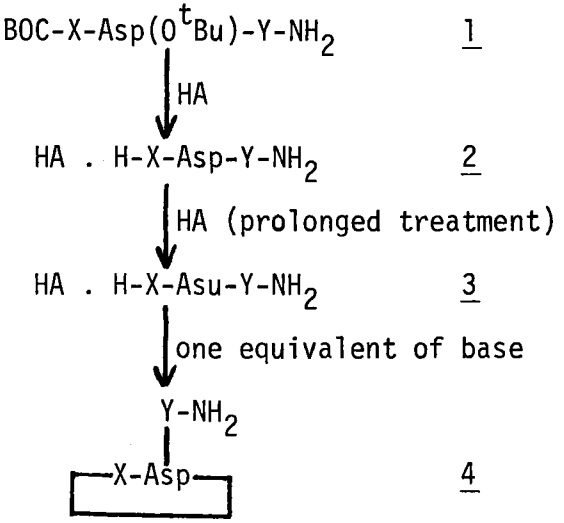

Fig. 1. Treatment of Boc-X-Asp(O^{t}Bu)-Y-NH$_2$ (1) with strong acids resulted in formation of the desired deprotected 2 that partially further reacted to imide derivative 3 as a by-product. The isolated 3 slowly transformed to the piperazine-2,5-dione derivative 4 in neutral medium (X: Leu,Pro; Y: Phe, Phg, Gly, Ala, Val; HA: HCl, HBr, HF; Asu L-aminosuccinyl, Phg L-phenylglycyl).

Table I. Deprotection of Boc-Leu-Asp(OBu^t)-Phe-NH$_2$ (1) to the Appropriate Salt of H-Leu-Asp-Phe-NH$_2$ (2) Followed by Conversion of 2 to the Appropriate Salt of H-Leu-Asu-Phe-NH$_2$ (3) in Different Acidic Media (R.T., room temperature; Asu, aminosuccinyl).

medium	time h	temperature °C	amounts estimated by TLC(%) 2	3	others
4N HCl/AcOH	1	R.T.	>95	>5	0
4N HCl/AcOH	24	R.T.	50	50	0
4N HCl/AcOH	48	R.T.	20	80	0
4N HCl/AcOH	96	R.T.	>5	>95	0
4-5N HBr/AcOH	1	R.T.	60	40	0
TFA	2	R.T.	100	0	0
HF + anisole	1	0	80-85	15-20	0
HF	1	0	50-60	15-20	20-25(3 compounds)

Table II. The Effect of the Neighboring Amino Acids in X-Asp-Y-NH_2 on the Imide Formation in 4N HCl/AcOH at Room Temperature (1. estimated by TLC; 2. refers to the isolated material)

X	Y	time of treatment day	conversion[1] %	yield[2] %
Leu	Phe	4	>95	74.2
Leu	Gly	4	100	92.5
Pro	Gly	3	100	89.5
Leu	Ala	7	100	88.9
Leu	Phg	3	>95	not isolated
Leu	Val	14	>95	74.5
Pro	Val	16	>95	88.5

Table III. The Effect of Neighboring Amino Acids in X-Asu-Y-NH_2 on Formation of the Piperazine-2, 5-dione Derivatives (Asu; aminosuccinyl, Phg; phenylglycyl, 1. %estimated by TLC, 2. EtOAc/pyridine/AcOH/water = 60 : 20 : 6 : 11 (v/v))

X	Y	medium	temperature	time	conversion[1]
Leu	Phe	pyridine	reflux	20 hours	95
Leu	Phe	DMF	R.T.	7 days	60
				22 days	80
Leu	Phg	mixture[2]	R.T.	2 days	100
Leu	Gly	90% EtOH	R.T.	3 days	95
Pro	Gly	90% EtOH	R.T.	2 days	95
Leu	Ala	90% EtOH	R.T.	3 days	80
Leu	Val	90% EtOH	R.T.	7 days	70
Pro	Val	90% EtOH	R.T.	7 days	80

Unexpected transformations to piperazine-2,5-dione derivatives have been observed during purification of L-leucyl-L-aminosuccinyl-L-phenylalaninamide hydrochloride and L-leucyl-L-aminosuccinyl-L-phenylglycinamide hydrochloride on a silica gel column using a buffered solvent mixture of ethyl acetate/pyridine/acetic acid/water = 60 : 20 : 6 : 11, i.e. in neutral medium (Figure 1).

As addition of benzylamine to L-leucyl-L-aminosuccinyl-L-phenylalaninamide did not result in formation of α- and β-benzylamides at all, it

is reasonable to suggest that the favorable molecular conformation is the driving force towards these one step intramolecular opening of the succinimide ring and closure of the piperazine-2,5-dione ring. The examination of this conversion has been extended to different reaction conditions as well as to other tripeptides. The greater effect of the C-terminal amino acid rather than N-terminal was again observed (Table III).

The possibilities of these reactions must be kept in mind in the synthesis and purification of aspartyl peptides in order to prevent or decrease the undesired consequences.

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SYNTHESIS OF 2-HYDROXY-3-AMINO ACIDS SEPARATION OF DIASTEREOMERS AT THE PROTECTED PEPTIDE STAGE

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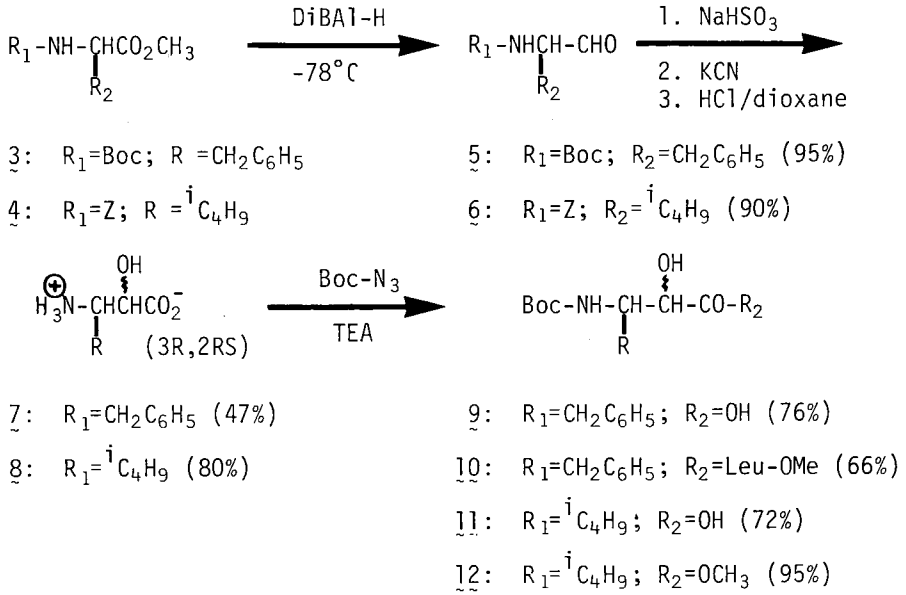
Introduction

Bestatin (1a) an inhibitor of leucine aminopeptidase (LAP) and aminopeptidase B (AP-B)[1] modifies the functions of immune-response participating cells.[2] Amastatin 2a, a LAP inhibitor structurally related to bestatin inhibits aminopeptidase A (AP-A) but does not inhibit AP-B. Both bestatin and amastatin contain the unusual 2-(S)-hydroxy-3(R)-amino acids 7 and 8. While both 7 and 8 have been synthesized[4] the isolation of the (2S,3R) diastereomer found in the natural inhibitors has been reported only for 7. We report here two methods for obtaining optically pure derivatives of 2-hydroxy-3-amino acids.

Synthesis of Amino Acids

The synthesis of amino acids 7 and 8 was carried out as shown in Scheme 1. The *tert*-butyloxycarbonyl (Boc) or benzyloxycarbonyl (Z) amino acid methyl esters 3, 4 were reduced with diisobutylaluminum hydride at -78°C to give the protected aldehydes in excellent yield.[5] Because amino aldehydes are easily racemized the optical purity of aldehyde was checked by reducing each aldehyde to the corresponding alcohol with $NaBH_4$.[6] Comparisons with authentic samples showed that the aldehyde was 99% optically pure. Without purification aldehydes 5 and 6 were converted to the cyanohydrins and hydrolyzed following the reported procedure.[4] Reaction with Boc-azide in aqueous dioxane for 24 hours gave the N-protected derivatives 9 and 11 as mixtures of diastereomers (2S,3R and 2R,3R).



3: R_1=Boc; R =$CH_2C_6H_5$

4: R_1=Z; R =$^iC_4H_9$

5: R_1=Boc; R_2=$CH_2C_6H_5$ (95%)

6: R_1=Z; R_2=$^iC_4H_9$ (90%)

7: R_1=$CH_2C_6H_5$ (47%)

8: R_1=$^iC_4H_9$ (80%)

9: R_1=$CH_2C_6H_5$; R_2=OH (76%)

10: R_1=$CH_2C_6H_5$; R_2=Leu-OMe (66%)

11: R_1=$^iC_4H_9$; R_2=OH (72%)

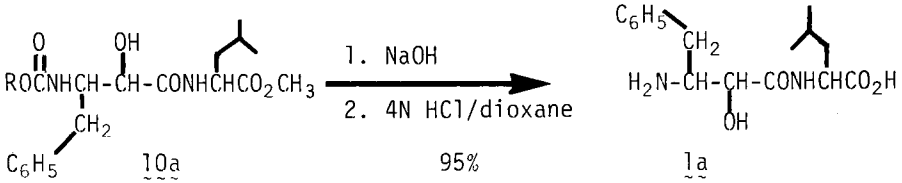
12: R_1=$^iC_4H_9$; R_2=OCH_3 (95%)

Scheme 1. Synthesis of (3R)-Amino-(2RS)-Hydroxy Acids from D-Amino Acid Precursors.

Separation of Diastereomers

Separation of diastereomers was achieved by converting the amino acid to a neutral derivative and chromatographing over silica gel. Thus reaction of Boc-acid 11 with diazomethane gave a nearly quantitative yield of methyl ester 12. Chromatography of 12 over silica gel eluting with 10% ethyl acetate in benzene gave pure 2S,3R (R_f = 0.22) and 2R,3R (R_f = 0.28) diastereomers.

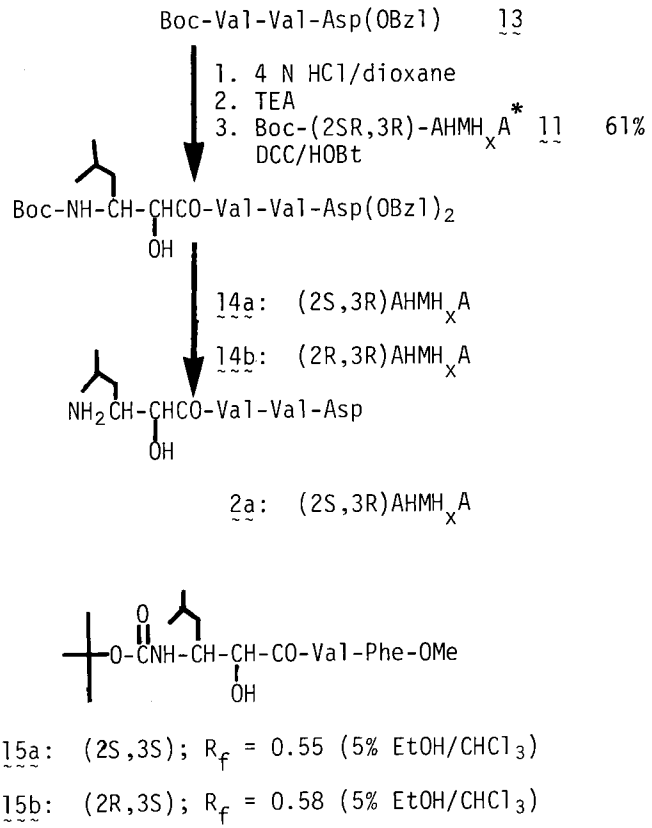
Alternatively the Boc-acid 9 could be coupled to a C-protected amino acid, eg. H-Leu-OMe, and the amino acid diastereomers separated at the dipeptide stage. Dipeptide 10 was synthesized from amino acid 9. Chromatography of 10 over silica gel eluting with ethyl acetate in benzene (1:2 v/v) afforded (2R,3R,αS)-10a (R_f = .32) and (2S,3R,αS)-10b (R_f = .20) in 66% overall yield from 9. The chirality of each diastereomer was established by converting 10a and 10b into bestatin 1a and (2R,3R) bestatin (1b).



10a

1a

Separation of amino acid diastereomers also is possible when the amino acid is part of a larger peptide. This is illustrated by the synthesis of amastatin (Scheme 2). Chromatography over silica gel eluting with 1%



15a: (2S,3S); R_f = 0.55 (5% EtOH/$CHCl_3$)

15b: (2R,3S); R_f = 0.58 (5% EtOH/$CHCl_3$)

Scheme 2. Synthesis of Amastatin and Epiamastatin
*AHMH$_x$A, amino-hydroxyl-methylehexanoic acid

methanol in chloroform gave pure 14a (R_f = 0.33) and 14b (R_f = 0.38). The optical configuration of each diastereomer was established by converting 14a and 14b to amastatin 2a and epiamastatin 2b and determining their respective inhibition constants on LAP.

The methods reported here provide efficient routes to optically pure peptides containing 2-hydroxy-3-amino acid residues. It should be emphasized that while separation of diastereomers can be achieved by column chromatography, selection of the proper eluting solvent system is not routine. Diastereomers 15a and 15b could be resolved by chromatography over silica gel eluting with ethanol but not methanol in chloroform.

Acknowledgements

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AZACYCLOLS AND CYCLOTRIPEPTIDES

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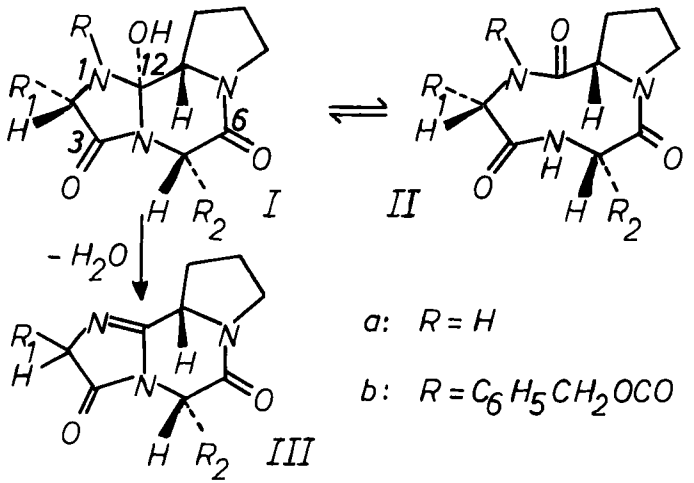
In all carbonyl reactions in peptide chemistry, tetrahedral adducts (e.g. cyclols) are formed which are usually unstable and cannot be isolated. They possess the structures of ortho acid amide derivatives and are involved both in peptide synthesis and rearrangements. Among the various stable types of cyclols the oxygen analogs[1,2] occur in the ergot alkaloids.

Cyclols are bicyclic isomers of medium-sized cyclopeptides, cyclodepsipeptides,[2] and cyclothiodepsipeptides.[3] Our interest in cyclic tripeptides[4] led to the study of the nitrogen analogs (azacyclols, Ia,b). These compounds should exist in equilibrium with both open-chain aminoacyl diketopiperazines and cyclic tripeptides formed by cleavage of the central bond of the bicyclus. Hence, they have been postulated as intermediates in aminoacyl incorporation into peptide bonds.[4] So far, only *N*-alkyl and *N*-acyl azacyclols (Ib, R = CH_3,[5] $C_6H_5CH_2OCO$[6]) have been obtained.

For the synthesis of unsubstituted azacyclols (Ia) we first studied the aminoacyl incorporation reaction of aminoacyl diketopiperazines in which the ring amide is activated as a diacylimide structure. *N*-(Aminoacyl)diketopiperazines were prepared from trimethylsilyl diketopiperazines and N-protected amino acid chlorides at 0°C. In this way, we have obtained the monobenzyloxycarbonyl glycyl, β-alanyl, sarcosyl, and L-prolyl derivatives of *cyclo*-diglycyl, *cyclo*-(glycyl-sarcosyl), and *cyclo*-(glycyl-L-prolyl). After removal of the N-protecting group by HBr/acetic acid or catalytic hydrogenation the ensuing aminoacyl diketopiperazines reacted intramolecularly via cyclolization. In each of these cases, however, no cyclolic peptide could be isolated. Unfortunately, neither could the cyclotripeptide be obtained because the azacyclols are stabilized by splitting off water rather than by transannular isomerization which would yield a very strained 9-membered ring. In the case of the glycyl derivatives of the diketopiperazines mentioned above, imidazolinones (III) having the structures of acylamidines were isolated as crystalline hydrobromides. On the other hand, in the case of *N*-

alkylamino acid side chains the cyclolic hydroxy group and a vicinal α-hydrogen of the dipeptide ring are cleaved to give unstable ketene aminals (anhydrocyclols).[4] All the products mentioned could be identified by IR, UV, NMR, and mass spectroscopy.



It follows from earlier work[1,2,3,6] that the formation of stable cyclols is strongly dependent on electronic and steric factors in the amide ring as well as in the side chain. Azacyclols (Ia,b) should be favored in the equilibrium relative to the tautomeric aminoacyl diketopiperazines if branched amino acid side chains are present due to the gem. dialkyl effect.[6] We made use of these considerations in our latest approach to obtain 9-membered cyclotripeptides containing CONH groups via N-unsubstituted azacyclols (Ia). These hitherto unknown compounds can be regarded both as ortho acid diamides and acid aminals. They were obtained from their benzyloxycarbonyl (Z) derivatives which were in turn prepared via Z-aminoacyl diketopiperazines as mentioned above or by treatment of tripeptide *p*-nitrophenyl esters with carbonate/bicarbonate buffer in aqueous dioxane.[6] In this reaction Z-aminoacyl side chains containing alkyl substituents cyclize to give Z-azacyclols (Ib) in spite of the low nucleophilicity of the benzyloxycarbonylamino group.

Subsequently, we succeeded in converting these compounds to the free azacyclols by catalytic hydrogenation in the presence of tertiary amine under particularly mild conditions (0°C). In this way, the azacyclols derived from the sequences Ala-Phe-Pro (m.p. 151°C) and Val-Leu-Pro (m.p. 127°C) were prepared. At higher temperatures or when less mild conditions were used for deprotection (transfer hydrogenation with cyclohexene/Pd) the azacyclols (Ia) formed were spontaneously dehydrated to yield the corresponding acylamidines (III). In this reaction racemization of the L-alanyl residue in the imidazolinone ring occurred (as indicated by NMR) due to a transient formation of an aromatic imidazole system similarly to a keto-enol tautomerism.

These new azacyclols represent the first examples of acid aminals which are isomers of the corresponding cyclotripeptides. Their structures have been unequivocally determined by spectroscopic means (C-13 NMR, IR, MS). As an example, characteristic data of the cyclol Ia derived from Ala-Phe-Pro are given: *m/e* 315 (M^+), 297 (M^+-H_2O); C-13 NMR 95.4 (C-12), 167.0 (C-6), 173.6 (C-3) ppm; IR (KBr) 3305, 3290 (NH), 3190 (OH), 1705, 1625 (CO) cm^{-1}. For the corresponding acylamidine (III), the following data were obtained: *m/e* 297 (M^+); C-13 NMR 159.1 (C-12), 164.6 (C-6), 182.0 (C-3) ppm; IR (KBr) 1730, 1658/1650 (CO) cm^{-1}; m.p. 110-112°C.

On the other hand, cyclopeptides should be preferred relative to the tautomeric azacyclols if the former contain more than 9 or 10 ring atoms. In the case of cyclotripeptides, however, the increase in stability resulting from the resonance-stabilization of the macrocyclic amide is more than counterbalanced by the high strain in these 9-membered rings. If these cyclotripeptides were stable they should have been formed under the conditions employed in our aminoacyl incorporation experiments. This is shown by the ready formation of 10-membered cyclotripeptides via incorporation of a β-alanyl residue into diketopiperazines. *c*-β-Ala-Gly-Gly, *c*-β-Ala-Gly-Sar, *c*-β-Ala-Gly-Pro, and *c*-β-MeAla-Gly-Pro were obtained in yields up to 40%. In these cases, however, the existence of an equilibrium between the cyclotripeptide and the azacylol follows from treatment of the former with strong acids which protonate the cyclolic tertiary hydroxy group yielding the imidazolinone salts by dehydration.

During the normal conditions used for peptide cyclization (high dilution, room temperature, DMF/tertiary amine) the *p*-nitrophenyl ester trifluoroacetate of Ala-Phe-Pro yielded the acylamidine (III) which was separated from cyclotripeptide oligomers by GPC and identified with an authentic sample obtained as mentioned above. The presence of the

acylamidine can only be explained by dehydration of an azacyclol intermediate. It represents the first proof of formation of a cyclol as an intermediate during peptide cyclizations at high dilution as was postulated by us previously.[4] It remains to be established whether the cyclols are formed from active tripeptide derivatives either via aminoacyl diketopiperazines or cyclotripeptides.

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STUDIES ON THE SYNTHESIS OF PEPTIDES CONTAINING γ-CARBOXYGLUTAMIC ACID

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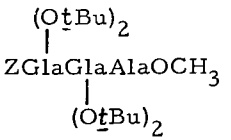
This report concerns our studies on the synthesis of several peptides containing γ-carboxyglutamic acid (Gla) residues. The long range goal of this portion of our program involves the chemical synthesis of sequences of the 1-39 region of bovine prothrombin (I).[1] In this report we describe our current progress on synthesis of the 18-34 region, specifically fragments 29-34 (Figure 1), 30-34 (Figure 2), and 25-28. In addition, we are interested in fragment 1-10 and have included the synthesis of an analog of pentapeptide 5-9, having replaced the C-terminal valine with leucine.

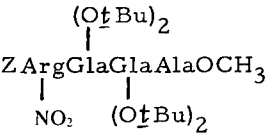
```
                    5                   10
Ala-Asn-Lys-Gly-Phe-Leu-Gla-Gla-Val-Arg-Lys-Gly-Asn-
    15              ┌──── 20 ─────────┐      25
Leu-Gla-Arg-Gla-Cys-Leu-Gla-Gla-Pro-Cys-Ser-Arg-Gla-
            30                  35
Gla-Ala-Phe-Gla-Ala-Leu-Gla-Ser-Leu-Ser-Ala-Thr-Asp...
```

The synthesis of the hexapeptide derivative VII shown in Figure 1 represents a portion of a three part fragment approach to the decapeptide 25-34. Of these peptides only II resisted purification, requiring three chromatographic separations to give 70% of an oil which still contained a small amount of impurity. Nevertheless, this material was reduced and used in the fragment coupling step. Azide coupling of reduced II by the modified Honzl-Rudinger method[2] gave the hexapeptide derivative III in 35% yield overall from XI.

The final portion of this fragment approach involves the proposed coupling of the hydrazide derived from V with the appropriate derivative of III. Both IV and V have been prepared on a small scale (water soluble carbodiimide, hydroxysuccinimide, dimethylformamide) in yields of 47% and 32% respectively overall from XI.



IV



V

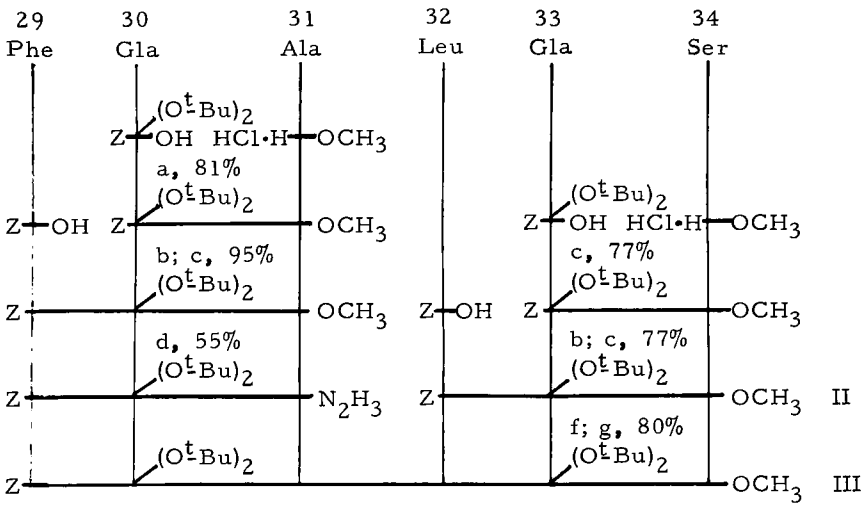


Fig. 1. Synthesis of Hexapeptide 29-34 Derivative III

[a] HOBt, DCC, MeCN, DCHA; [b] 10% Pd·C, DMF; [c] HONSu, WSCDI, DMF; [d] MeOH, $N_2H_4 \cdot H_2O$ (x7 excess); [e] HONSu, *N*-methylmorpholine, WSCDI, DMF; [f] 10% Pd·C, MeOH; [g] *n*-BuONO, HCl/EtOAc, DMF.

The linear approach to decapeptide 25-34 has proceeded as far as the pentapeptide VIII (Figure 2). Coupling to form tetrapeptide derivative VI gave a mixture with three components. Separation of the major product (VI, 135 mg, 70%) from the other two (15-20 mg, ~ 10%) could be accomplished by preparative hplc[3]. Recrystallization of the bulk of the reaction mixture from ethyl acetate afforded pure VI in 31% yield.

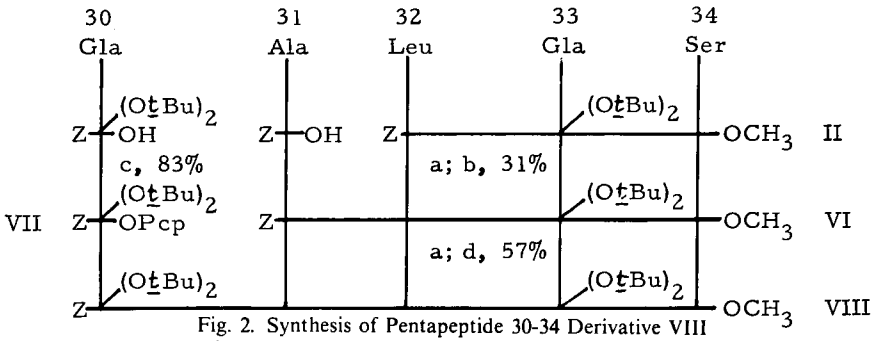


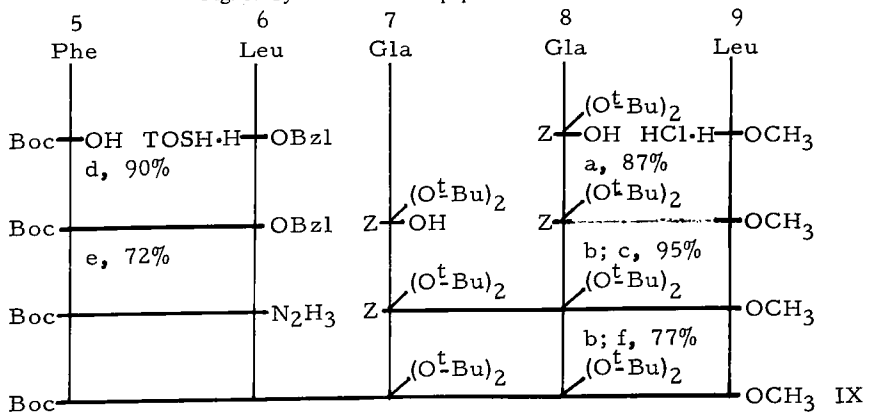
Fig. 2. Synthesis of Pentapeptide 30-34 Derivative VIII

[a] 10% Pd·C, EtOAc; [b] HOBt, DCC, MeCN; [c] C_6Cl_5OH, DCC, EtOAc; [d] VIII, 2, 4-dinitrophenol, DMF.

Although the coupling of *N*-deprotected VI with the pentachlorophenyl ester VII required seven days at room temperature, the pentapeptide derivative VIII was produced in 57% yield. One equivalent of 2, 4-dinitrophenol was added to avoid serine *O*-acylation.[4]

Interest generated by the enzyme carboxylation studies of Suttie *et al.*[5] led us to synthesize the pentapeptide IX (Figure 3). Since pentapeptide 5-9 from bovine prothrombin has C-terminal valine in place of leucine, this scheme is a model for a fragment approach to decapeptide 1-10. The overall yield of IX from *N-tert*-butoxycarbonyl-phenylalanine was 50%.

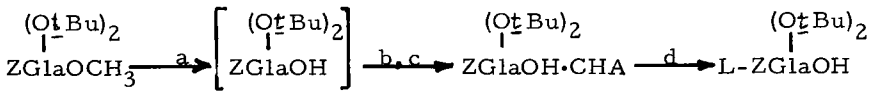
Fig. 3. Synthesis of Pentapeptide 5-9 Derivative IX



[a] HOBt, DCC, NMM, DMF, 0°C; [b] 10% Pd·C, MeCN; [c] HOBt, DCC, MeCN, 0°C; [d] HOBt, DCC, NMM, MeCN, 0°C; [e] $N_2H_4·H_2O$, CH_3OH; [f] *n*-BuONO, HCl/EtOAc.

Finally, shown in Figure 4 are two changes in our reported[6] procedure for isolation of XI. First, a bicarbonate wash of an ether solution of the sodium salt from a crude saponification mixture, followed by regeneration of the acid by 20% citric acid removed persistent low R_f (95/5, chloroform/methanol) material. The acid is then precipitated as the cyclohexylamine salt (X) from anhydrous ether, thus eliminating the need for tyrosine hydrazide.

These two changes have facilitated processing large scale syntheses and have increased our overall yield of XI from 10-12% to 18-20%.



X XI

Fig. 4. Isolation Procedure Variation

[a] 0.47 *N* KOH, MeOH; [b] $NaHCO_3$ wash of crude saponification mixture in ether; [c] cyclohexylamine in ether; [d] resolution procedure.

Acknowledgement

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SYNTHESIS AND PROPERTIES OF γ-CARBOXYGLUTAMYL PEPTIDES

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γ-Carboxyglutamic acid (Gla) is found in various vitamin K dependent proteins involved in blood clotting (Suttie et al., 1977) and in the structure of calcified tissue (Hauschka et al., 1978), as well as in the human placenta or in ribosomal proteins. Gla is synthesized by the vitamin K dependent, enzymatic carboxylation of L-glutamic residues contained in the precursors of these proteins and localized in their N-terminal part. It has been assumed that Gla-Gla dipeptide units ("tandems") might be responsible for the calcium complexation displayed by such proteins.

Once we had prepared for the first time derivatives of L-Gla that are suitable for peptide synthesis (Märki et al., 1977), we pursued this line of research. We now report our continuing studies and describe the chemical synthesis of several short γ-carboxyglutamic acid containing peptides: Ac-Gla-Gla-NH_2; Factor IX_{6-9}-tetrapeptide (Figure 1); descarboxy Factor IX_{6-9}-tetrapeptide; Factor VII_{5-9}-pentapeptide (H-Phe-Leu-Gla-Gla-Leu-OH) and Prothrombin$_{26-35}$-decapeptide (Figure 2).

Chemical Synthesis

The idea was to produce protected peptides from which the α-amino-protecting group could be selectively removed without affecting side-chain protection. The strategy was that of Schwyzer and Kappeler (1963), who had introduced side-chain carboxyl protection as *t*-butyl esters. The *t*-butyl groups were cleaved from the side-chain protected peptides by trifluoroacetic acid. Figure 1 demonstrates this for bovine Factor IX_{6-9}-tetrapeptide. The pathway follows fragment condensation of the current

dipeptides, which have been synthesized by active ester couplings for the Gla residues or condensation with DCC and *N*-hydroxybenzotriazole. All compounds were crystalline except the free peptide, which was obtained as an amorphous solid.

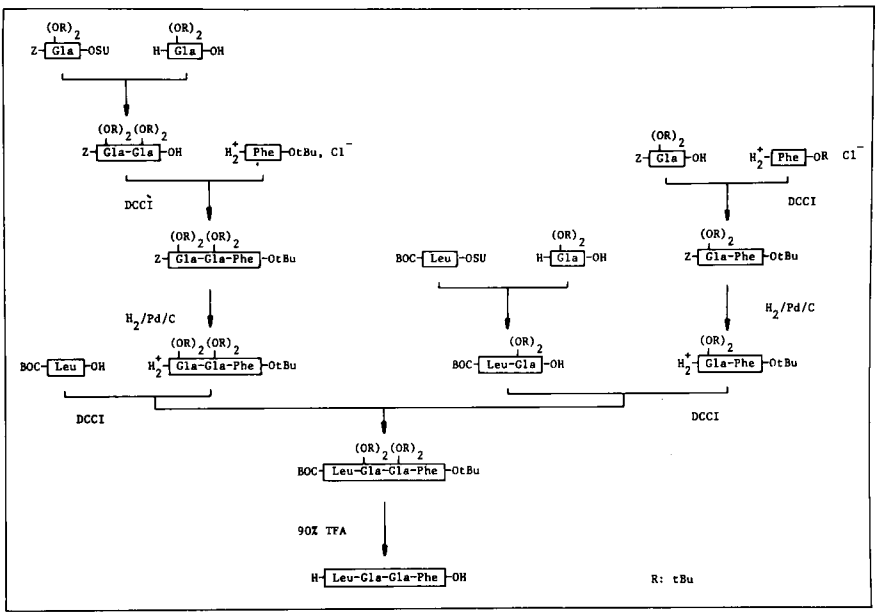

Fig. 1. Synthesis of bovine Factor IX_{6-9}-tetrapeptide

Table I. Physical Data of Synthetic Gla-peptides

Compound	M.p. (dec)	$[\alpha]_D^{20}$ C=1/MeOH	^{M}ASP pH 6.4	R_f[a]	Ca pK_{ass}
Ac-Gla-Gla-NH_2	110°	−24.6°	1.7	0.5[b]	1.4
H-Leu-Gla-Gla-Phe-OH	193°	− 7.5°	1.1	0.36	1.6
H-Leu-Glu-Glu-Phe-OH	190°	+ 7.6° (1/HMPT)	0.6	0.43	0.8
H-Phe-Leu-Gla-Gla-Leu-OH	110°	−15.2° (1/DMF)	0.9	0.51	1.9
H-Leu-Gla-Ser-Leu-OH	130°	−17.5°	0.7	0.30	——
Z-Prothrombin$_{26-35}$	140°	−50.4°	0.9	0.41	——
Z-Gly-Gly-Gla-Ala-OMe	110°	−18.4°	0.7	0.48	——

[a] *n*-Butanol-Acetic Acid-Water 5:2:3; [b] 2-Propanol-Water-Pyridine 7:6:6

Figure 2 shows the path adopted for the synthesis of bovine prothrombin$_{26-35}$-decapeptide. Condensations were routinely made in DMF as the solvent with DCC and HOBT. The reactions were followed by thin layer chromatography and all intermediates were characterized.

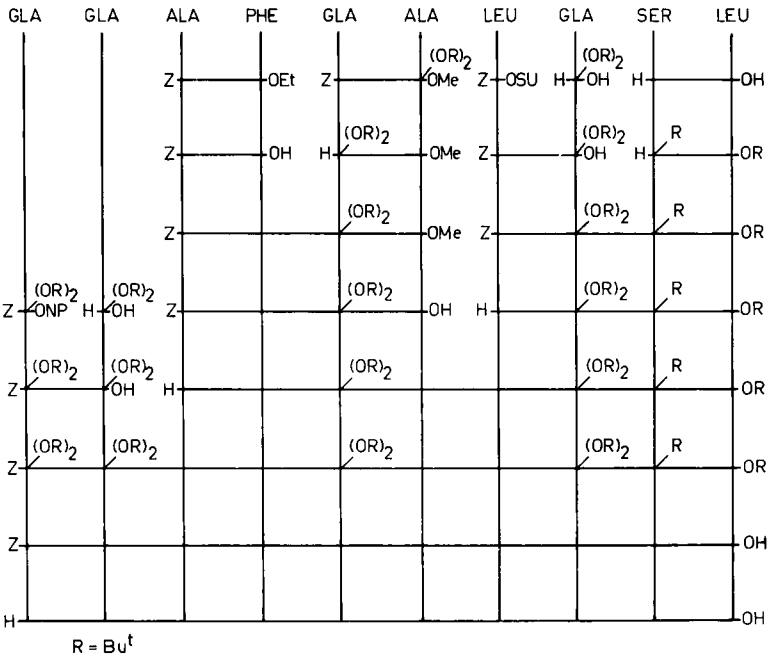

Fig. 2. Synthesis of bovine prothrombin$_{26-35}$-decapeptide

Table II. ^{1}H-NMR Parameters at 360 MHz of Gla in the Linear Tetrapeptide Z-Gly-Gly-Gla-Ala-OMe in D_2O at 35° with TSP as Internal Standard. The Chemical Shifts δ are in ppm; the Spin-spin Coupling Constants, J, in Hz. The Assignments were made with Decoupling Experiments. Additional data: δ(α) Gly(1) 3.96; Gly(2) 3.91; Ala(4) 4.37; δ(β) Ala(4) 1.30; -OCH_3 3.73; C_6H_5- 7.43; -CH_2- 5.14.

Resonance		pD 1.2	pD 7.0	pD 9.6
δ (α)		4.47 dd	4.27	4.27
δ (β)	A	2.26 t	2.17	2.10
	B	2.44 q	2.32	2.28
$^3J_{\alpha\beta}$	A	8.1	—	9.9
	B	8.5	—	4.0
$^2J_{\beta A\beta B}$		14.3	—	14.3

In Table I the physical characteristics of the synthesized Gla-peptides are summarized.

^{1}H-NMR- and ^{13}C-NMR-spectroscopy have been used to confirm the different structures and revealed the exchangeability of the gamma Proton of Gla against a Deuteron in D_2O below pH 5.0 (Märki et al., 1977). Table II presents the "random coil" proton magnetic resonance parameters of γ-carboxyglutamic acid in a linear tetrapeptide.

From this data, as well as from other NMR-experiments (unpublished results) a restricted side chain rotation around the Cα-Cβ-bond in the Gla residues can be assumed.

Metal binding properties — In preliminary studies, the metal binding properties of such Gla-peptides have been investigated. The titration curves for different Gla-peptides indicate relatively weak, but significant calcium binding above pH 4.0 (lowered proton dissociation constants in the presence of calcium). The calculated data are assembled in Table I.

It was also shown that these peptides form insoluble terbium-(III)-complexes in aqueous solutions above pH 2.5; however, the descarboxypeptide was soluble at this pH up to pH 7. These observations indicate relatively non-specific calcium anion interactions with these smaller peptides, reflecting surface carboxylic acid groups titration behavior in the protein. Similar findings have been reported by Robertson and coworkers (1979) for model peptides, studying magnesium binding.

Acknowledgements

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NEW COUPLING REAGENTS FOR PEPTIDE SYNTHESIS

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Introduction

In this communication we describe our initial experiments with sulfonates of *N*-hydroxysuccinimides as activating and coupling agents in peptide synthesis. Related work seems to be under way in two other laboratories.[1,2] Horiki and Murakami recently reported the synthesis of the *p*-chlorobenzenesulfonate of *N*-hydroxysuccinimide and the negative results of their attempt to apply this compound as a coupling agent for the synthesis of a dipeptide. Sahni *et al.*, on the other hand, prepared *p*-toluenesulfonate and methanesulfonate of copoly(ethylene-*N*-hydroxymaleimide) and used them successfully for the preparation of some simple dipeptides and amides. Although we have been working with a related polymer, a cross-linked macrolattice copoly(styrene-*N*-hydroxymaleimide),[3] our results and conclusions do not agree very well with those of the latter authors. A short note on some of our results has recently appeared.[4]

Experiments

Methanesulfonate, *p*-toluenesulfonate and *p*-nitrobenzenesulfonate derivatives of copoly(styrene-*N*-hydroxymaleimide) were prepared by essentially the same method as follows: 10.0 g (approx. 40 mmol) of resin was suspended in a solution of 6.22 g (58.6 mmol) of anhydrous sodium carbonate in 75 ml of water. Visible swelling of the resin occurred. Under vigorous stirring a solution of 13.0 g (58.6 mmol) of *p*-nitrobenzenesulfonyl chloride in 80 ml of acetone, cooled to 0°C, was added in one portion. A further 25-ml portion of acetone-water (1:1) was added to improve stirring. After stirring for 1 hour the resin was filtered off and was carefully washed on the filter with water, acetone, water, acetone,

methanol, and finally with ether. The weight after drying was 16.1 g. The weight increase of the resin corresponded to an 83% conversion and this value was confirmed by elementary analysis for sulfur. The estimated reaction capacity was 2.0 mmol/g. In two other preparations products with capacities of 2.0 mmol/g were obtained. Our methanesulfonate resin and *p*-toluenesulfonate resin had capacities of 2.6 and 2.0 mmol/g, respectively.

A large number of small-scale experiments were performed to determine the influence of different reaction parameters. A typical experiment was as follows: 5.0 ml of DMF (or other solvent) containing 0.85 mmol of Boc-L-Leu-OH, dried over P_2O_5, was added to slightly more than an equivalent amount of base and 0.60 g (1.2 mmol) of *p*-nitrobenzenesulfonate resin and the reaction was allowed to procede for 1 hour. Aminolysis was initiated by addition of 0.118 g (0.68 mmol) of H-L-Val-O*t*Bu and allowed to procede for 5 hours. The resin was filtered off and washed carefully on the filter with small portions of methanol (3 times), acetone (once), methanol again (twice) and ether (twice). The mixed filtrate was taken to dryness *in vacuo*, generally leaving a yellow residue.

In most cases our primary interest was to find out to what extent reaction had taken place and the extent of racemization, if any. This could be tested very nicely at the same time in this model peptide on an amino acid analyzer.[5] Therefore, a small, constant aliquot (3%) of the evaporation residue was deprotected with 1 ml of TFA for 15 minutes. After evaporation, the product was taken up in the pH 2.2 application buffer and submitted to analysis using a 0.185 M sodium citrate buffer, pH 4.20. Peaks appeared in the order Val, Leu, D-Leu-L-Val and L-Leu-L-Val.

Results

So far the following solvents have been tried in the activation procedure above: DMF, pyridine, tetrahydrofuran, dichloromethane and ethyl acetate. Of these DMF was the most promising with respect to the yield of product. Poor swelling of the resin could be an explanation in some cases, but not in others. Very good swelling was observed in DMF and pyridine. The yield of product was also influenced by the nature of the base used.

Already in the first experiments, when we used triethylamine as a

base, we noted that a considerable amount of D-Leu-L-Val was formed (D-Leu-L-Val:L-Leu-L-Val ratio up to 15%). As a first step in our further work we decided to investigate the influence of different bases on this ratio. The following bases were checked: dicyclohexylamine, ethyldiisopropylamine, *N*-methylmorpholine, 2,4,6-trimethylpyridine and pyridine. Since we obtained a value of about 8% with the first one, we had to abandon our idea to use DCHA-salts directly. The following two gave ratios of a few percent, 2,4,6-trimethylpyridine below 1% and pyridine below 0.1%. Judging from the amount of visible starting material in the chromatograms, reactions were fastest with triethylamine. In an attempt to raise the yield in one experiment we added 10 equiv. of pyridine as a base, but to our surprise the yield dropped instead of increased. When pyridine was used as a solvent, the yield dropped even more. Other experiments on the contrary seemed to indicate that less than one equiv. of pyridine was optimal.

We only obtained trace of product when methanesulfonate resin or *p*-toluenesulfonate resin was used instead of *p*-nitrobenzenesulfonate resin in the activation procedure described above.

Discussion

Conditions have been established under which coupling takes place in acceptable yield without sacrificing the sterical purity of the product. It should be possible to vary the reactivity of the resin within wide limits according to one's need by variation of its sulfonic acid component. Whether racemization will ultimately become a problem in this case we have not yet investigated.

Not only polymeric sulfonates of the type discussed here may be useful for formation of amide and peptide bonds but also sulfonates of *N*-hydroxysuccinimide itself. Synthesis of and work with such compounds are now in progress.

Acknowledgements

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PEPTIDE SYNTHESIS WITH A NEW TYPE OF ACTIVATED ESTER: 4-Acyloxy-3-oxo-2,5-diphenyl-2,3-dihydrothiophene 1,1-dioxides

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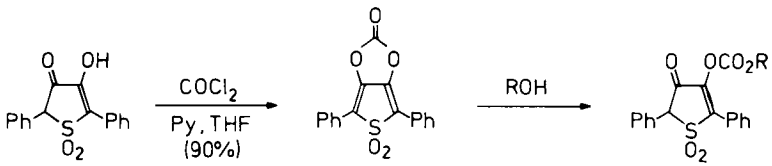
Recently we introduced the cyclic carbonate 4,6-diphenylthieno [3,4-d] [1,3] dioxol-2-one 5,5-dioxide 2 as a new reagent for the preparation of activated esters in peptide synthesis[1]. It is easily prepared from the hydroxysulfone 4-hydroxy-3-oxo-2,5-diphenyl-2,3-dihydrothiophene 1,1-dioxide 1[2] by treatment with $COCl_2$/pyridine. On reaction with alcohols, 2 undergoes ring opening to give the activated carbonates 3 [3,4].

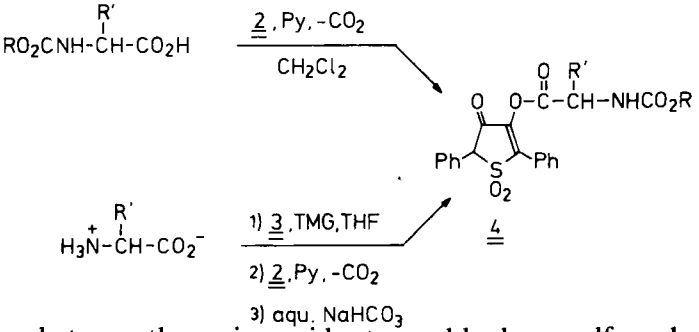


1 2 3

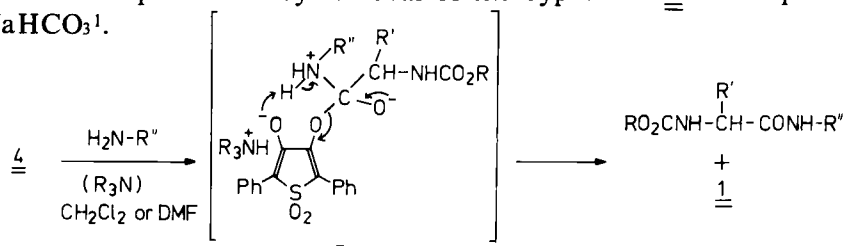
N-Protected amino acid TDO* esters 4 (4-acyloxy-3-oxo-2,5-diphenyl-2,3-dihydrothiophene 1,1-dioxides) may be quantitatively obtained by reaction of the N-protected amino acid with 2 in the presence of pyridine[1]. They may be used for peptide coupling without prior isolation. Alternatively amino acids can be directly converted into urethane protected TDO esters 4 by refluxing with 3 in THF in the presence of *N,N,N',N'*-tetramethylguanidine (TMG), followed by treatment with one equivalent of 2. The activated esters 4 are obtained in high yield after removal of 1 with aqueous $NaHCO_3$[4]. Attempts to prepare Boc-Asn-OTDO were unsuccessful.

The high reactivity of TDO esters 4 toward amines makes them very attractive for peptide bond formation. The reactions are performed in CH_2Cl_2 or DMF in the presence of one equivalent of NEt_3 to avoid salt

*TDO = Thiophene Dioxide



formation between the amino acid ester and hydroxysulfone 1 released during the reaction. The end of the coupling is indicated by a color change from red to yellow. The N-protected peptide esters are easily obtained in pure form by removal of the byproduct 1 with aqueous $NaHCO_3$[1].



The yields of some selected dipeptide derivatives are given in Table I. Even sterically hindered dipeptides like Z-Val-Val-OMe are formed in high yield within 1 h. The presence of free hydroxy groups in the amino acid ester presents no problems, whereas activation of Boc amino acids with unprotected OH groups leads to diminished yields. During synthesis of the sequences Boc-Phe-Ser(OBzl)-Pro-Gly-OMe, Boc-Ala-Val-Phe-Gly-OMe, and Boc-Phe-Pro-Pro-Phe-Phe-Val-Pro-Pro-Ala-Phe-OMe the yields in the coupling steps were in most cases higher than 90%.

Table I. Dipeptide Derivatives Prepared from *N*-Acyl Amino Acid TDO Esters.

Dipeptide	Yield [%]	Method*	Dipeptide	Yield [%]	Method*
Z-Val-Val-OMe	97	A	Boc-Pro-Tyr-OMe	82	B
Boc-Met-Ala-OMe	99	A	Boc-Phe-Thr-OMe	91	A
Boc-Pro-Val-OMe	80	A	Boc-Thr-Tyr-OMe	70	B
Boc-Val-Trp-OMe	96	B	Boc-Cys(SBzl)-Gly-OMe	97	B
Boc-Phe-Asn-OMe	87	B	Boc-Ser-Phe-OMe	54	B
Boc-Phe-Ser-OMe	83	B	Boc-Lys(Boc)-Dopa-OMe	78	B

*A: With, and B: without isolation of the activated ester 4.

Table II. Competitive Amide Formation Studies.

Acylation Reagents	Amine	Products	Ratio
C_2H_5CO-OTDO	H_2N-Bzl	C_2H_5CONH-Bzl	49
$(CH_3CO)_2O$		CH_3CONH-Bzl	51
C_2H_5CO-OTDO	H_2N-Bzl	C_2H_5CONH-Bzl	74
CH_3CO_2H/DCC		CH_3CONH-Bzl	26
$(CH_3)_3CCO$-OTDO	H_2N-Bzl	$(CH_3)_3CCONH$-Bzl	97
CH_3CO-ONp		CH_3CONH-Bzl	3
Boc-Val-OTDO	H-Phe-OMe	Boc-Val-Phe-OMe	95
Boc-Leu-ONp		Boc-Leu-Phe-OMe	5
Boc-Leu-OTDO	H-Phe-OMe	Boc-Leu-Phe-OMe	100
Boc-Val-ONp		Boc-Val-Phe-OMe	0

Reaction conditions: TDO ester 4, second acylation reagent, amine, and NEt_3 (1 mmol of each; concentration: 0.1 mol/l) were stirred for 1 h in CH_2Cl_2 at 25°C. The ratio of products was determined by GLC.

The exceptionally high reactivity of the TDO esters is demonstrated by several competitive amide formation studies (Table II). The aminolysis rates of 4 are comparable to those of carboxylic acid anhydrides and exceed those of DCC/RCO_2H and other activated esters. This may be explained by intramolecular base catalysis exhibited by the neighboring enolate ion in intermediate 5.

It was found in several experiments that preparation and coupling of Boc amino acids proceed without any detectable racemization. To test the possible application of TDO esters 4 for fragment condensations, we performed several racemization tests (Table III). The results obtained indicate that the esters 4 in general show a low tendency for racemization if the fragment coupling is performed at 0°C in the presence of *N*-ethyl morpholine instead of NEt_3. In some cases racemization may be further depressed by addition of *N*-hydroxybenzotriazole (HOBt)[5]. The optical purity of the products in the latter case is comparable or even better than that of the corresponding peptides obtained via the well established DCC/HOBt procedure. The ease of purification in the case of 4/HOBt offers advantages which should make this reagent combination preferable.

Table III. Racemization Tests for Fragment Coupling.

Test	Additional Reagent	Solvent	Ratio
1st Weygand Test	-	CH_2Cl_2	0
(Tfa-Val-Val-OMe)	HOBt	DMF	0
Tfa-Leu-Phe-OMe	-	CH_2Cl_2	3.3
	HOBt	CH_2Cl_2	0.8*
		DMF	5.8
3rd Weygand Test	HOBt	CH_2Cl_2/DMF (10:1)	0
(Tfa-Pro-Val-Pro-OMe)	HOBt	DMF	5.9
2nd Young Test	-	CH_2Cl_2	12
(Bz-Leu-Gly-OEt)	HOBt	CH_2Cl_2/DMF (10:1)	4.5
Anderson Test	-	CH_2Cl_2	0
(Z-Gly-Phe-Gly-OEt)			

*With DCC/HOBt 13.6%.

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NOVEL PROTECTING GROUP ACTIVATED BY TERTIARY PHOSPHINE FOR PEPTIDE SYNTHESIS

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Recently the synthesis of 3-nitro-2-pyridinesulfenyl (Npys) halide has been reported and found to be unusually stable among nitrogen-containing heterocyclic sulfenyl halides.[1] Mild and efficient methods for the esterification of cephalosporanic acids have been explored by the use of this reagent.[2]

First, the use of the Npys moiety as a new protecting function for amino and hydroxyl groups of amino acids and peptides is described. Second, it is demonstrated that the Npys group may not only serve as a protecting group, but moreover, that the S-N and S-O bonds can be activated by Ph_3P to allow in the presence of R'COOH the formation of amide and ester bonds, respectively. In this method of peptide and glycopeptide synthesis the formal N- or O-deprotection step is omitted.

Preparation of Npys-Derivatives of Amino Acids and Peptides

Npys halide reacts readily and smoothly in the presence of a base with primary and secondary amino and hydroxyl groups to afford the respective sulfenamides and sulfenates. *N*-Npys amino acids were synthesized by the Schotten-Baumann type reaction and *O*-Npys, *S*-Npys amino acids or *N*-Npys amino acid esters were obtained under anhydrous conditions using tertiary amine as a base. Some of the derivatives prepared are shown in Table I.

Table I. Npys Derivatives of Amino Acids and Peptides

Derivatives	Yield,%	Mp,°C	$[\alpha]_D^{22}$,° (c, Solvent)[a]
Npys-Gly-OEt	74	70-1	-
Npys-Gly-Gly-OEt	72	121-2	-
Fmoc-Lys(Npys)-Gly-OEt	81	137-9(dec)	-26.3(c1,DMF)
Npys-Ile-Gly-OMe	69	121-2	-64.3(c1,MeOH)
Z-Ser-(Npys)-Gly-OMe	78	113-5	-38.3(c1,MeOH)
Npys-Ile-OH	65	114-6	+45.8(c1,MeOH)
Npys-Leu-OH	76	104-6	-15.3(c1,MeOH)
Npys-Met-OH·DCHA	60	173-5	+50.1(c1,MeOH)
Npys-Pro-OH	71	133-5	-135.9(c1,MeOH)
Npys-Trp-OH·DCHA	52	128-31	-55.9(c1,MeOH)
Npys-Asp(NH_2)-OH	74	175-6	-11.0(c1,DMF)
Npys-Tyr(Bu^t)-OH	64	96-8	+99.2(c1,MeOH)
Fmoc-Lys(Npys)-OH	67	112-5(dec)	+32.1(c1,MeOH)
Npys-Ser-OH	44	132-4	+8.1(c1,MeOH)
Z-Ser(Npys)-OH·DCHA	62	151-3	-17.4(c1,MeOH)
Boc-Cys(Npys)-OH·DCHA	66	150-2	-86.5(c1,MeOH)
Z-Thr(Npys)-OH·DCHA	58	92-4	-47.9(c1,MeOH)

[a] Optical rotations were measured by Baron Consulting Co., Orange, Connecticut.

Stability of Npys-Derivatives

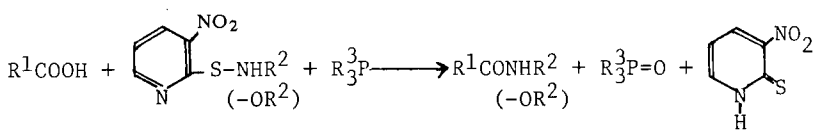
Npys-derivatives were found stable enough to be stored in the COOH-free form at room temperature; no decomposition has been observed during a 6-month period. This is in contrast to weak acid labile Bpoc-amino acids or Nps-amino acids which usually have to be stored as dicyclohexylamine salts.

The Npys group is easily removed by highly dilute HCl such as 0.1~0.2 N HCl/dioxane, but it is resistant to 88% HCOOH and to trifluoroacetic acid as well. To cleave the S-N or S-O bond of Npys groups, nucleophilic attack of the sulfur atom, following protonation, is necessary: soft base[3] such as chloride anion is effective, but hard base[3] such as trifluoroacetate anion is not. The resistance of the *N*- and *O*-Npys to trifluoroacetic acid is significantly different from such acid labile protecting groups as Nps, Bpoc and Boc. This may be due to the protonation of the nitrogen of the Npys pyridine ring by trifluoroacetic acid to form a salt which results in the stabilization of the S-N or S-O bond.

The degree of selectivity that exists to remove one or more groups from a peptide protected with Bpoc, Boc, Nps and Npys groups is noteworthy: Bpoc, Boc and Nps are removed by trifluoroacetic acid in the presence of Npys; Bpoc is removed by 88% HCOOH in the presence of Boc and Npys; Bpoc, Nps and Npys are removed by 0.1~0.2 N HCl/dioxane in the presence of Boc; Nps and Npys are removed by the neutral cleavage technique mentioned below in the presence of Boc and Bpoc.

Activation of Npys Protecting Group or Removal Under Neutral Conditions

The neutral cleavage technique[4], which was recently shown for the Nps protecting group by the use of Ph_3P in the presence of a proton source, is also effective for the deprotection of the Npys group without affecting such groups as Z, Boc, Bpoc, OBzl, OBu^t, Fmoc, and also the indole ring of tryptophan. Most important is the fact that the S-N or S-O bond of an Npys substituted amino acid or peptide can be activated by Ph_3P to form in the presence of an N-substituted amino acid, a peptide or an ester linkage via the oxidation-reduction condensation:[5]



For example, the tripeptide Fmoc-Lys(Boc-Pro)-Gly-OEt was prepared without the use of a formal N-deprotection step: Fmoc-Lys(Npys)-OH + H-Gly-OEt $\xrightarrow{\text{Mixed Anhydride}}$ [Fmoc-Lys(Npys)-Gly-OEt] $\xrightarrow{\text{Boc-Pro-OH + Ph3P}}$ Fmoc-Lys(Boc-Pro)-Gly-OEt (65% yield).

Sometimes, the reaction proceeds slowly depending on the strength of the S-N bond which varies with amino acids; however, addition of HOSu, HOBt or 2-pyridinethiol 1-oxide (Py(O)SH) promotes the reaction. Among these additives, Py(O)SH is particularly recommendable since it is highly soluble in various kinds of solvents and easily removed by washing with sodium bicarbonate. Py(O)SH, a commercially available non-smelling solid, is a very soft nucleophile[3] for the S-N and S-O bonds; therefore it cleaves well the Npys and Nps protecting groups at room temperature.

With respect to racemization, the method of peptide synthesis by activation of the S-N bond via the oxidation-reduction condensation gave favorable results. When the procedure was applied to the synthesis of Boc-Leu-Ile-Asp (NH_2)-Leu-OBu^t, which was used for the detection of racemizations during the azide and DCC plus HOB^t coupling methods,[6] we obtained less than 0.8% racemization. The resistance to racemization of the Npys protecting group was confirmed by a comparison of the αIle content of Npys-Ile-Gly-OMe and Boc-Ile-Gly-OMe prepared by the mixed anhydride method: the amounts of αIle found after acid hydrolysis of both peptides were the same and not greater than would be expected to be generated during acid hydrolysis conditions per se (i.e.$<$0.9%).

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ISOBUTYLOXYCARBONYL, A USEFUL N^{im}-HISTIDINE PROTECTING GROUP

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Racemization of histidine in peptide synthesis is prevented by protection of the imidazole nucleus with urethane type protecting groups[1,2]. The usefulness of imidazole protection with the isobutyloxycarbonyl group (Ioc) has been investigated both with respect to its inhibition of racemization and with respect to its stability during coupling and deprotection reactions.

The Ioc group is introduced into the imidazole mucleus by addition of 1.1 equivalent isobutyl chloroformate to suspensions of α-amino protected histidine derivatives in dioxane/water (1:1) at pH 7.0. The yield of Z-His(Ioc)-OH and Boc-His(Ioc)-OH was 80% and 74% respectively.

The stability of the Ioc protection and the racemization of histidine has been investigated in the reaction,

R-His(Ioc)-OH + H-Phe-OMe → R-His(Ioc)-Phe-OMe (Table I).

The amount of D-isomer in the isolated compounds was estimated in Z-His-Phe-OMe by HPLC[3], using a 30 cm μ-Porasil column. The eluent was chloroform/*tert*-butanol (90:12.5) saturated with an aqueous solution of 0.05 molar CH_3COONH_4. The flow rate was 0.5 ml per

Table 1. Yield of isolated compounds and determination of racemization in His in different coupling reactions. [a] Reaction time was 24 hours to simulate fragment coupling conditions. [b] The coupling was complete within 3 hours. [c] HOBt and HONSu give rise to deprotection of the Ioc group.

	R	Ioc R-His-Phe-OMe	%D-isomer	R-His-Phe-OMe	%D-isomer
DCC a)	Z	66%	< 0.2	trace	< 0.2
DCC/HONSu a)b)	Z	58%		6.2%[c]	
DCC/HOBt a)b)	Z	77%		9.6%[c]	
MA	Z	90%			
ONSu-ester	Boc	75%		trace	

minute. The retention time was for the D-His isomer 15.2 and for the L-His isomer 17.2 minutes, respectively. Z-His(Ioc)-Phe-OMe was converted to Z-His-Phe-OMe by pyridine HCl in MeOH before analysis.

The α-amino protection in Boc-/Z-His(Ioc)-Phe-OMe could be removed selectively by acidolysis (TFA/CH_2Cl_2 (1:1), 15 minutes; yield 82%) or by hydrogenolysis (10% Pd/C, MeOH, 4.5; yield 89%).

From H-His(Ioc)-Phe-OMe, the tripeptide Boc-Ala-His(Ioc)-Phe-OMe was prepared by the MA and ONSu-ester procedure in yields of 85% and 93%, respectively.

H-Ala-His(Ioc)-Phe-OMe was obtained in 90% yield from Boc-Ala-His(Ioc)-Phe-OMe by treatment with TFA/CH_2Cl_2 (1:1) during 15 minutes.

The N^{im}-Ioc group was removed selectively from di- and tripeptides by treatment at 50°C with MeOH containing pyridine HCl (4% w/v) or TEA (1% v/v). Reaction time was 2 hours and 1 hour, and the yield of isolated compounds was 90% and 89%, respectively. By saponification in aqueous solution at pH 10.5 the Ioc group as well as the methyl ester are removed after 6 hours. The yield of isolated compound was 78%.

The stability of the N^{im}-Ioc group in Boc-Ala-His(Ioc)-Phe-OMe dissolved in different solvents was examined (see Table II), and in methanol especially in presence of different bases (see Table III).

Table II. Stability of the N^{im}-Ioc group in Boc-Ala-His(Ioc)-Phe-OMe, estimated by TLC as % deprotection of the Ioc group after 24 hours treatment at 25° and 50°C.

	CH_2Cl_2	EtOAc	DMF	Propanol -2	H_2O/EtOAc pH 2.5	H_2O/EtOAc pH 8.5
25°C	stable	<1	stable	stable	<1	<1
50°C		<1	stable	<1		

Table III. Stability of the N^{im}-Ioc group in Boc-Ala-His(Ioc)-Phe-OMe, estimated by TLC as % deprotection of the Ioc group after 5 hours treatment with MeOH containing equimolar amounts of different bases. [a] after 1 hour treatment.

	MeOH HCl, H-Phe-OBu^t	MeOH	MeOH H-Phe-OBu^t	MeOH NMM	MeOH TEA
25°C	2	5	40	50	100[a]
50°C	15	40	10[a]	50[a]	100[a]

In Boc-Ala-His(Ioc)-Phe-OMe the N^{im}-Ioc group showed a rather good stability. When methanol is used as solvent care must be taken, since methanolysis occurs to a rather high extent, when bases are present. Acceptable stability was found when HCl·H-Phe-OBu^t was added. pH 4.5 was determined in that solution.

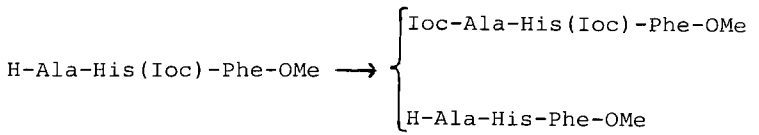
The stability of the N^{im}-Ioc protection in H-Ala-His(Ioc)-Phe-OMe dissolved in different solvents has been examined too, both with free α-amino group and with its HCl-salt (see Table IV and V).

Table IV. Stability of the N^{im}-Ioc protection in H-Ala-His(Ioc)-Phe-OMe, estimated by TLC as % deprotection of the Ioc group after 24 hours treatment at 25° and 50° C. [a] Rearrangement of the Ioc group, see below. [b] After 5 hours treatment. [c] After 1 hour treatment.

		CH_2Cl_2	EtOAc	DMF	Propanol-2	MeOH
25°C	NH_2-	stable[a]	stable	5[a]	5[a]	80[b]
	HCl,NH_2-	stable	stable	stable	2	5
50°C	NH_2-		10[a]	5[a]	20[a]	90[c]
	HCl,NH_2-		stable	30	5	40

As seen from Table IV a free α-amino group gives rise to pronounced methanolysis of the N^{im}-Ioc group, whereas a protonation of the α-amino group increases the stability.

In the α-amino unprotected peptide H-Ala-His(Ioc)-Phe-OMe a rearrangement occurs in which the Ioc group is transferred from the imidazole nucleus to the α-amino group.



Estimated by TLC, a few % rearrangement occur after 24 hours treatment in most of the examined solvents (see Table IV).

The stability of the N^{im}-Ioc protection in H-Ala-His(Ioc)-Phe-OMe in methanol at different pH has been examined (see Table V). The pH was kept constant by the addition of either NaOH or HCl in methanol.

Table V. Stability of the N^{im}-Ioc group in H-Ala-His(Ioc)-Phe-OMe, estimated by TLC as % deprotection of the Ioc group at different pH after 24 hours treatment with MeOH. [a] pH of the solution of HCl, H-Ala-His(Ioc)-Phe-OMe in MeOH. [b] After 4 hours treatment. [c] After 1 hour treatment.

pH	3.5	4.5[a)]	5.5	6.5	8.5
25°C	2	<1	10	25[b)]	90[c)]

In conclusion the N^{im}-histidine isobutyloxycarbonyl protection is easily introduced and removed. Use of the N^{im}-Ioc group results in an efficient protection against racemization in histidine during coupling reactions. Less than 0.2% racemization was detected. But the lack of stability of the N^{im}-Ioc group require observation of carefully selected reaction conditions.

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THE MECHANISM OF THIOLYTIC REMOVAL OF THE DITHIASUCCINOYL AMINO PROTECTING GROUP

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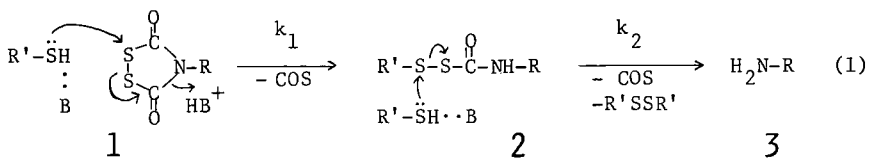
The dithiasuccinoyl (Dts) amino protecting group 1 was developed for eventual application to *orthogonal* schemes[1] of peptide synthesis. On the one hand, the Dts group is stable under the acidolytic conditions used to remove *tert*-butyl and benzyl-based protecting groups, and it is also resistant to photolysis as applied to cleave *o*-nitrobenzyl and α-methylphenacyl esters. Conversely, the Dts function is rapidly and quantitatively removed under mild and specific conditions[1,2] using thiols



[Eq.(1)], borohydrides, and trialkylphosphines. The present paper provides practical details for effectively carrying out the reaction of Eq.(1); such information is a prerequisite for optimizing the conditions of solid-phase peptide synthesis with Dts-amino acids. All of the reported rates were derived for Dts-glycine; earlier work[2] established that rates with other Dts-amino acids never differed by a factor of more than three.

The kinetic studies were facilitated by the rapid, convenient, and quantitative chromatographic method outlined in the legend to Figure 1. Dts-compounds are detected[1,2] with the standard ninhydrin-hydrindantin reagent of automated amino acid analyzers because the hydrindantin acts as a reducing agent and the released amino acid reacts *in situ* with the ninhydrin to give a purple color.

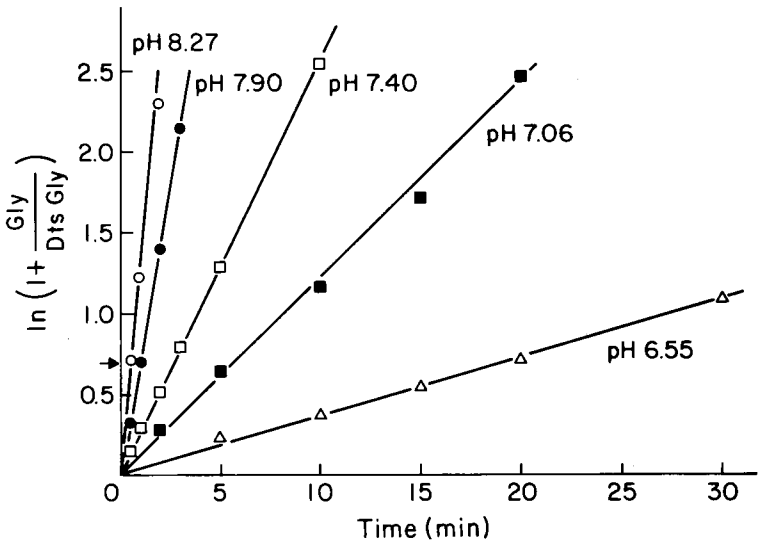


Fig. 1. Typical first-order rate plots for reductive deprotection of Dts-glycine (1 m*M*) by β-mercaptoethanol (20 m*M*) in phosphate buffers at different pH values. The arrow at ln 2 marks 50% conversion. Plots were linear past 90% conversion, and air-oxidation of the thiol was negligible (~0.2%) during the time periods examined.

Reactions were analyzed as follows: Aliquots (0.5 ml) were quenched at the indicated times into 0.2 *N* sodium citrate buffer, pH 2.2 (2.5 ml). There followed eight serial injections, at 12-min. intervals, of 1-ml samples onto the long column of a standard amino acid analyzer, developed with 0.2 *N* sodium citrate buffer, pH 3.20. Dts-glycine eluted at 37 min. (integration constant 0.3 relative to glycine), and glycine eluted at 140 min. In these experiments conducted in aqueous solutions, β-hydroxyethyldithiocarbonylglycine (elution time 42 min., relative integration 1.0) was never observed, meaning that k_1 in the reaction mechanism [Eq.(1)] was rate-limiting.

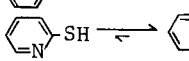
The rates obtained in *aqueous* solution (Figure 1) were independent of buffer composition and varied directly with hydroxide concentration at pH values below the pK_a of the thiol. The observed rates reached a saturation level with full ionization of the thiol; hence, true rates due to the thiol anion could be calculated from the rates at pH 7 (Table I, column 4) according to $k_{S^-} = k_{pH7}\,[1 + \text{antilog}(pK_a - 7)]$. For the monofunctional thiols 4-7 and 9, a good Brønsted correlation [Eq.(2)] was observed, but the bifunctional thiols 11 and 12 reacted 3- and 30-fold faster than

$$\log k_{S^-} = \beta pK_a + G;\ \beta = 0.9,\ G = -7.1 \qquad (2)$$

units of k are liter-mol^{-1}-sec^{-1}

expected. A positive deviation from Eq.(2) was also seen with *N*-methylmercaptoacetamide 8; whereas 2-mercaptopyridine 10 was found to react negligibly because of tautomerization.

Table I. Relative Rates of Reductive Deprotection of Dts-Glycine Mediated by Various Thiols

No.	Thiol	pK_a	at pH 7[a]	in 0.1 M Et_3N-dioxane			
			$k_{1,rel}$	$k_{1,rel}$	k_{rel}^{env}	κ	
4	CH_3CH_2SH	10.5	1.8	0.2	0.2	0.3	
5	$HOCH_2CH_2SH$	9.6	1.0	1.0	1.0[b]	0.3	
6	$CH_3CONHCH_2CH_2SH$	9.4	2.2	4.7	17	5	
7	$CH_3O_2CCH_2CH_2SH$	9.3	1.7	0.4	1.0	2	
8	$CH_3NHCOCH_2SH$	8.0	10	4.7	17	5	
9		6.8	1.5	0.2	0.7	$\sim 10^6$	c,d
10		10[e]	$\leq 10^{-3}$	10^{-3}	—	$\geq 10^9$	c,d
11	$HSCH_2CH_2OCH_2CH_2SH$[f]	9.5[f]	6.1	0.2	1	>10	d
12	$HSCH_2CHOHCHOHCH_2SH$[f]	8.6[f]	67	2.1	9	>100	d

[a] $\kappa \sim 375$, determined as in note c; [b] The half-time of this reaction, based on k^{env}, was 5.6 min; [c] κ was estimated by independently measuring the rate of reduction of an isolated sample of β-hydroxyethyldithiocarbonylglycine; [d] No carbamoyl disulfide intermediate was ever observed; [e] This ionization constant refers to removal of a proton from the nitrogen of the predominating pyridine thione tautomer; [f] Both the rate constants and pK_a values of bifunctional thiols have been corrected by the symmetry factor of 2.

In *anhydrous* solutions, the open-chain carbamoyl disulfide intermediates 2 were demonstrated directly by chromatography[2]. On a molar basis, 1, 2, and 3 quantitatively accounted for all of the amino acid residue at all stages of the reductive deprotection. The observed kinetic patterns were in exact agreement with curves calculated from experimental rate constants for consecutive pseudo-first order reactions, hence providing strong evidence for the mechanistic sequence of Eq.(1). Both steps were first order in thiol. Whereas k_2 varied directly with tertiary amine concentration, plots of log k_1 against log [base] had a slope of 0.7-0.8. Among eight thiols tested, the rates k_1 differed over a ten-fold range (Table I, column 5; exclude 10). The fastest rates were observed with *N*-acetyl-β-mercaptoethylamine 6, *N*-methylmercaptoacetamide 8, and dithiothreitol 12. The pK_a values of these thiols are intermediate to the pK_a values of the two most slowly reacting thiols (4 and 9). The ratio of rates, $\kappa = k_2/k_1$, increased steadily (Table I, column 7) with increasing acidity (lower pK_a) of the attacking thiol. Thus, the first step of the mechanism was rate-limiting and carbamoyl disulfide intermediates 2

were never observed for thiophenol 9 and 2-mercaptopyridine 10. The same was found to hold for bifunctional thiols 11 and 12 because there the second step of Eq.(1) can proceed intramolecularly.

The quantity $k^{env}=k_1k_2/(k_1+k_2)$ defines a good "envelope" approximation for production of 3 [see Eq.(1)] according to the simple law $100\%[1-\exp(-k^{env}t)]$. By this useful criterion (k^{env}), overall rates under varying conditions of thiol, base, and solvent compositions can be readily compared despite differing values of κ (see Table I, column 6). With β-mercaptoethanol as thiol and Et_3N or *N*-methylmorpholine as bases, the rate ratios in dioxane, benzene, dichloromethane, and acetonitrile were approximately 1 : 20 : 30 : 100. In 1:1 (v/v) mixtures of pyridine with respectively dioxane, ethyl acetate, chloroform, dichloromethane, benzene, acetone, pyridine, methanol, acetonitrile, and *N,N*-dimethylformamide, κ ranged from 0.1 to 0.5 and the rates were in the order 1 : 1.5 : 2 : 7 : 9 : 25 : 45 : 50 : 280 : 1100.

In conclusion, the two steps in the mechanism of thiolytic deprotection [Eq.(1)] have strikingly different electronic requirements. In aqueous solution (large κ), the reactive species is a thiol anion. In anhydrous solutions, an association complex of a thiol and a base is involved which is preferentially formed in polar aprotic media of high dielectric constant. In the transition state of the first step, the attacking sulfur has an effective charge of $-0.1[(1-\beta)$ from Eq.(2); transition state resembles products]; for the second step, the nucleophilicity of sulfur is essentially independent of its basicity (low β in Brønsted-type correlation].

Acknowledgement

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SPECIFIC, REVERSIBLE ESTERIFICATION OF CARBOXYL GROUPS WITH NITROPHENYLDIAZOMETHANE

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Diazoalkanes have been the most useful reagents for reversible esterification of carboxyl groups in preformed peptides[1,2]. The diazoalkanes thus far applied to this purpose have certain limitations: the reagents are not specific for carboxyl groups and require prior protection of amino and imidazole functions; the esters obtained are reversible only under conditions which would be considered harsh by the standards of enzymology and of protein chemistry. α-Diazocarbonyl compounds have been used as reagents for specific, but not quantitative, esterification of carboxyl groups of proteins[3].

p-Nitrophenyldiazomethane (NPD) has chemical properties intermediate between those of typical diazoalkanes and those of α-diazocarbonyl compounds[4]. The nitrobenzyl esters expected from esterifications with NPD are subject to cleavage by a variety of methods including catalytic hydrogenolysis and reduction with sodium dithionite or titanium trichloride in neutral aqueous solutions[5]. A simple preparation of NPD was developed by modifications of published methods[6,7]. At an apparent pH of 3.8-4.2 in 16% ethanol (glass electrode), NPD reacted slowly with α-aminomoncarboxylic acids, but more rapidly with isoglutamine and with glycylglycine. By treatment with a large molar excess of reagent in several portions over two hours more than 95% of the isoglutamine and glycylglycine were esterified. There was little, if any, alkylation of histidine under the same reaction conditions. Bovine pancreatic ribonuclease A was esterfied extensively, but not quantitatively, under these conditions. There was no extensive alkylation of the histidine residues of the enzyme. Attempts to esterify bovine insulin were unsuccessful because of rapid precipitation of incompletely esterified protein upon treatment with NPD. Quite possibly it will be

necessary to control the solubilities of some peptides by reversibly attaching them to solubilizing groups through other amino acid side chains[7] before esterification of the carboxyl groups.

Experimental

***p*-Nitrophenyldiazomethane** — The tosylhydrazone of nitrobenzaldehyde[7] (1.6 g) was dissolved in a mixture of 5 ml 2 M NaOH and 5 ml methanol. After 12-15 hours 20 ml water was added to the reaction mixture. The crude product was collected by filtration and dried under reduced pressure. Crystalline nitrophenyldiazomethane[7] (345 mg) was obtained from toluene/petroleum ether.

Esterification of Amino Acids and Glycylglycine with NPD — Histidine, isoglutamine, and glycylglycine were dissolved at 10 μmol/ml in water. Ethanol (0.4 ml) was added to 2 ml aliquots of each stock solution and the pH was adjusted to 3.8, as indicated by a glass electrode. NPD (3 x 50 mg) was added to the stirred solutions over 1½ hours. The pH of the reaction mixtures was kept in the range 3.8-4.2 during this time and for ½ hour after the last addition of reagent. Afer removal of solids from the reaction mixtures these were analyzed quantitatively for residual amino acids or peptide. Samples of NPD-treated isoglutamine and histidine were hydrolyzed in 6 M HCl and the hydrolysates were analyzed for glutamic acid and histidine, respectively. An aliquot of the esterfied glycylglycine solution was evaporated and analyzed by TLC. A second sample of the esterified glycylglycine solution (1 ml) was treated with hydrogen gas in the presence of 2 mg 10% Pd on charcoal for 1 hour. The solution was diluted to 5 ml with 0.2 M sodium citrate buffer, pH 2.2, centrifuged to remove the catalyst, and analyzed for glycylglycine.

Esterification of Bovine Ribonuclease A — Ribonuclease A (13 mg) was dissolved in 2 ml water and 0.4 ml ethanol. The protein was treated with NPD at pH 3.8-4.2 as described above. The reaction mixture was diluted to about 5 ml with water and washed with ether. The clear solution was dialyzed against water and adjusted to a final volume of 10 ml. To 2 ml of the esterfied enzyme solution was added 1 g urea and 2 mmoles norleucine methyl ester hydrochloride. The pH of the solution was adjusted to 4.75 and maintained at that value with 1 M HCl for 2 hours after the addition of 100 mg 1-ethyl-3-dimethylaminopropylcarbodiimide[9]. The reaction mixture was dialyzed against water, lyophilized, and hydrolyzed for amino acid analysis. A sample of ribonuclease A,

coupled with norleucine methyl ester in the presence of water soluble diimide using the same experimental format was also hydrolyzed for amino acid analysis.

Results

Treatment of isoglutamine and glycylglycine with NPD left less than 5% of the free peptide or amino acid in solution. The histidine concentration was decreased by about 30%. Acid hydrolysis of the esterified isoglutamine and histidine yielded 86% and 83%, respectively of the expected amounts of glutamic acid and histidine. TLC of the NPD-treated glycylglycine showed one strong ninhydrin reactive spot corresponding to the expected nitrobenzyl ester. A second spot visible on heavily loaded plates corresponded to free glycylglycine. Glycylglycine was recovered quantitatively by catalytic hydrogenolysis of the NPD-treated peptide.

Ribonuclease A coupled to norleucine methyl ester without prior esterification of the enzyme yielded 7 moles of norleucine per mole of enzyme derivative on acid hydrolysis. Ribonuclease esterified with NPD before coupling to norleucine methyl ester incorporated only 1.6 residues of norleucine per mole of protein derivative. Within the error of the experimental methods neither histidine nor methionine contents of ribonuclease samples were changed by treatment with NPD.

Acknowledgements

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SOME OBSERVATIONS CONCERNING *S*-ACETAMIDOMETHYL-CYSTEINE AND RELATED COMPOUNDS

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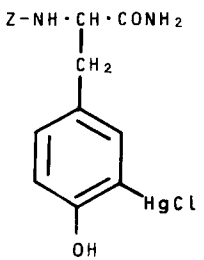
In view of the importance of *S*-acetamidomethyl for the protection of the thiol group of cysteine[1] we have investigated certain aspects of the removal procedures and we report here some points of interest.

It is a common and not unexpected experience that protection which is readily removed from simple peptides may require prolonged reaction times for removal from large peptides. It has been reported[2] that the *S*-p-methoxybenzyl group, when incorporated in an insulin A-chain (having 4 cysteine residues), gave no more than 30% of the fully deprotected A-chain. A striking example was given by J. Meienhofer[3] who reported that *t*-butyl ester groups on a protected β-endorphin resisted the action of trifluoroacetic acid. It was also reported that only 4 of the 6 *S*-acetamidomethyl groups could be removed from protected pancreatic trypsin inhibitor II (Kazal) by mercury (II) acetate.[4] The possibility that protection, readily removable from model compounds, might resist cleavage from the final protected peptide is one of the major fears when planning the synthesis of a large peptide.

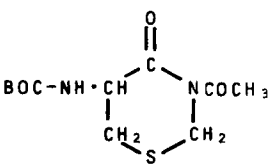
The rate of removal of *S*-acetamidomethyl from simple derivatives is extremely fast. The reaction of *S*-acetamidomethylcysteine itself (4.1 x 10^{-4} M solution in water) with mercury(II) acetate (2.3 molar proportions) at 21.5°C was complete within 1 minute, as shown by immediate precipitation of the mercury with hydrogen sulphide, evaporation of the latter and estimation of the remaining thiol by Ellman's spectrophotometric method.[5] The reaction of *N*-*t*-butoxycarbonyl-*S*-acetamidomethyl-L-cysteinyl-L-tyrosine methyl ester with mercury(II) acetate (1 molar proportion) in 50% acetic acid (1.0 x 10^{-4} M solution) at room temperature was complete within 3 minutes (t.l.c. evidence, confirmed by isolation of authentic thiol derivative in 75% yield). It seemed possible that when two cysteine residues are adjacent the attack on the second *S*-acetamidomethyl group might be hindered by the acetoxymercuri already present on the first residue, but again liberation

of both thiol groups from *N*-benzyloxycarbonyl-*S*-acetamidomethyl-L-cysteinyl-*S*-acetamidomethyl-L-cysteine methyl ester was complete within 2 minutes under similar conditions.

If an extended reaction time with mercury(II) acetate is required for the deprotection of large peptides, then there would appear to be a danger that more readily available nucleophilic centres might be mercurated. For example, *p*-cresol reacted with mercury(II) acetate (5 molar proportions) in 50% acetic acid during 1 hour at room temperature; addition of sodium chloride precipitated a mixture of mono- and di-chloromercuri derivatives, from which 2-chloromercuri-4-methylphenol (m.p. 167-168°C) was isolated, in 24% yield after recrystallization from ethanol-water. We therefore examined the action of mercury(II) acetate on *N*-benzyloxycarbonyl-L-tyrosine-amide. With 1 molar proportion of mercury(II) acetate in 50% aqueous acetic acid at room temperature mercuration of the tyrosine nucleus occurred slowly; after 30 hours, sodium chloride was added and the chloromercuri derivative, assumed to be the 3′-derivative (I), was precipitated in 29% crude yield. Recrystallization from ethanol-water gave (I) of m.p. 211°C. The same reaction, in 75% acetic acid at 24°C, was followed by HPLC (μ Bondapak) C18; solvent, 0.01 M ammonium acetate with 0-90% acetonitrile, with phenol as internal standard). With 0.05 M substrate and 0.1 M mercury(II) acetate, the substitution was half complete after 45 hours; for the corresponding methyl ester the half-time was 41 hours. It is therefore possible that acetoxymercuri groups may be introduced into peptides during prolonged reaction with mercury(II) acetate.



(I)

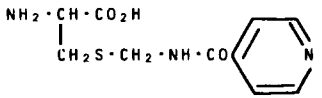


(II)

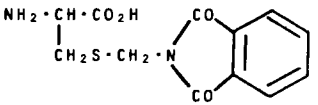
We encountered a reaction not, as far as we are aware, previously reported. *N*-*t*-Butoxycarbonyl-*S*-acetamidomethyl-L-cysteine was activated with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole in dichloromethane. After 16.5 hours at room temperature the ether-soluble products were separated on silica columns, giving 3-acetyl-5-*t*-

butoxycarbonylamido-tetrahydro-1,3-thiazin-4-one (II) of m.p. 98.5-100°, in 55% yield.

It seemed possible that an analogue which would be relatively stable to mercury(II) in acid conditions might result from the substitution of isonicotinamido in place of acetamido, as in (III). If so, an *S*-acetamidomethyl group could be removed selectively in acid conditions, and the isonicotinamido analogue would be removed subsequently at a higher pH. *N*-Hydroxymethylisonicotinamide[6] reacted with L-cysteine hydrochloride in trifluoroacetic acid at room temperature giving *S*-isonicotinamidomethyl-L-cysteine (III), m.p. 207-210°C. This compound was cleaved rapidly by mercury(II) acetate, even in trifluoroacetic acid solution; apparently protonation of the pyridyl-*N* is not sufficient to discourage the participation of the NH group in the second stage of the cleavage reaction.



(III)



(IV)

We have now prepared the hydrochloride of *S*-phthalimidomethyl-cysteine (IV) (m.p. 195-199°C) by the reaction of hydroxymethylphthalimide[7] with L-cysteine hydrochloride in boiling trifluoroacetic acid. It was stable to mercury(II) acetate in 50% acetic acid for 2 hours at room temperature. After opening the phthalimido ring by brief treatment (10 minutes at room temperature) with 0.2M sodium hydroxide, the thiol protection was rapidly cleaved by mercury(II) acetate. There is the possibility here of selective thiol protection.

Acknowledgement

This work was carried out in collaboration with Dr. J.S. Morley (I.C.I. Pharmaceuticals Division), as part of an SRC-CASE programme.

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1-(1-ADAMANTYL)-1-METHYLETHOXYCARBONYL (ADPOC): A NEW GROUP FOR AMINO PROTECTION IN PEPTIDE SYNTHESIS WITH ADVANTAGEOUS PROPERTIES

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In connection with our synthetic studies on hypothalamic releasing and inhibiting hormones (analogues) an amino protecting group cleavable under very mild acidolytic conditions was needed. Although the Bpoc (1-*p*-biphenylyl-1-methylethoxycarbonyl), Ddz(3,5-dimethoxy(α,α-dimethyl)benzyloxycarbonyl), and Azoc(α,α-dimethyl-4-phenylazobenzyloxycarbonyl) groups are cleaved under the desired conditions Bpoc, Ddz and Azoc amino acids may decompose during storage and e.g. Bpoc-Trp-OH can be synthesized with difficulties only. An intermediate in the synthesis of Adpoc amino acids is the tertiary alcohol 2-(1-adamantyl)-2-propanol(3) which can be obtained from 1-adamantanecarboxylic acid(1) via its ethyl ester(2) [Figure 1].

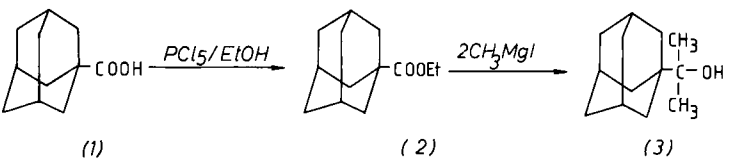

Fig. 1. Synthesis of 2-(1-adamantyl)-2-propanol.

As is demonstrated by the syntheses of several peptides with the natural sequence of somatostatin,[1] Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys, the new protecting group has the following advantages: The Adpoc residue can be incorporated easily by means of crystalline Adpoc-OPh,Adpoc-F or Adpoc-O-N=C(Ph,CN) [Figure 2].

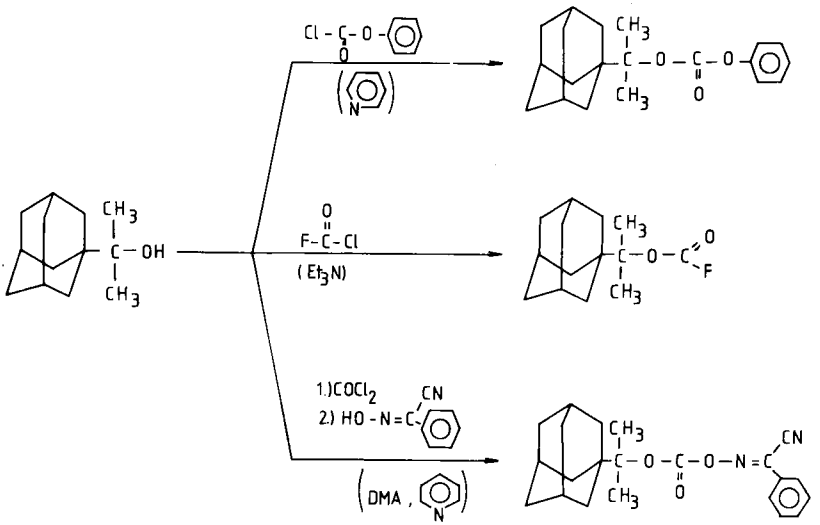

Fig. 2. Synthesis of reagents for the incorporation of the Adpoc group.

In Table I the physical data of some Adpoc derivatives are collected. Also the yields of pure isolated material are given.

Table I. Yields and Physical Data of Selected Adpoc Derivatives

Compound	Yield [%]	M.P. [°C]	$[\alpha]_D^{20}$ [°]
Adpoc-L-Ala.DCHA	75	155	+6.22 (c=0.65 ETOH)
Adpoc-Gly-OH	73	178	-12.8 (c=1.2 MeOH)
Adpoc-L-Trp-OH	72	116	-6.8 (c=1.04 (MeOH)
Adpoc-L-Thr(Bzl)-OH	68	51-52	+19.8 (c=1.02 MeOH)
Adpoc-L-Trp-L-Lys(Boc)-OH	73	134 (dec.)	-9.83 (c=0.5 MeOH)

Adpoc amino acids and peptides are stable for months at room temperature. The Adpoc group is removed 10^3 times faster than the Boc group under very mild acidolytic conditions (3% CF_3COOH in CH_2Cl_2, 0° C, 8-10 minutes) and therefore allows selective cleavage. The acidolytic cleavage of the Adpoc group can be easily followed by high-performance liquid chromatography (HPLC) as shown in Figures 3 and 4, using Adpoc-Trp-OH as an example.

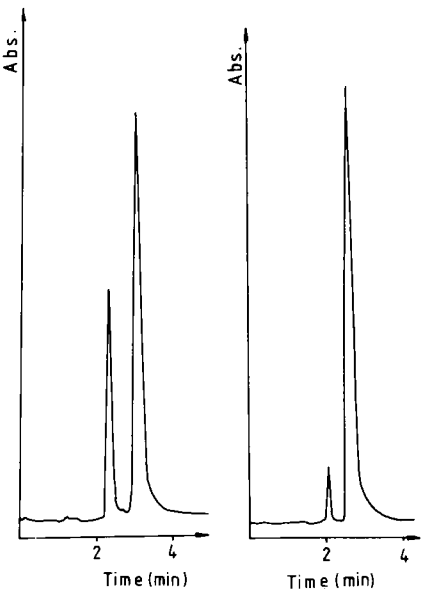

Fig. 3. HPLC chromatogram of the acidolytic cleavage of the Adpoc group from Adpoc-Trp-OH; left: 20 sec., right: 40 sec. after treatment with 3% CF_3COOH/CH_2Cl_2 at 25°C. Column: RP8 (25 x 0.4 cm, 10 μm particle size); eluent: Methanol (90%)/H_2O (brought to pH 5.2 with dilute H_2SO_4); flow rate: 2.5 ml/min.; pressure at column inlet: 120 atm; UV detection: 280 nm.

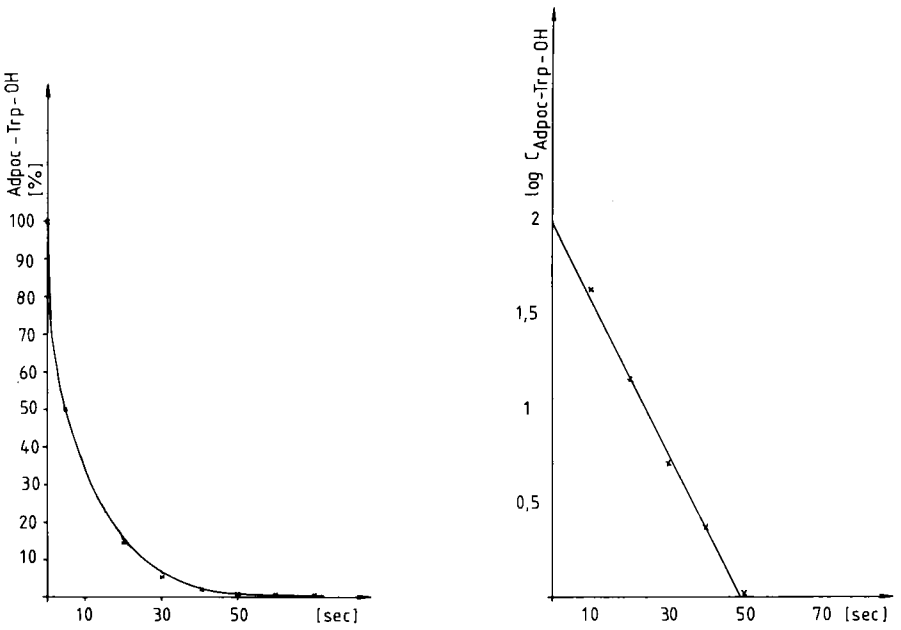

Fig. 4. Cleavage of Adpoc from Adpoc-Trp-OH in the presence of 3% TFA / CH_2Cl_2 at 25°C. Left: Decrease of Adpoc-Trp-OH in [%] and right: in log C as a function of time.

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A NEW AMINO PROTECTING GROUP THE AZO-TAC UNIT

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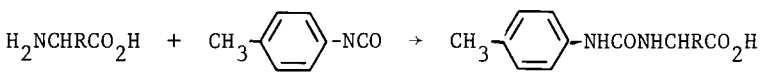
Introduction

In a search for new peptide coupling agents *N*-acetyl-L-phenylalanine and methyl L-alaninate were treated with phenyl isocyanate in the hope of obtaining the model racemization peptide methyl *N*-acetyl-L-phenylalanyl-L-alaninate[1] and phenylsulfonamide. Instead, the reaction gave in moderate yield alanyl phenylhydantoin. This result suggested that the treatment of an amino acid (I) with *p*-toluyl isocyanate (II) would yield the corresponding *p*-toluylaminocarbonyl derivative (III) or Tac-amino acid.



I II III

A variety of other such protected Tac-amino acids were made in yields of 20-80%. Most importantly, the deblocking of these compounds was found to proceed in hot 95% ethanol or *n*-propanol to form the free amino acid, plus some ethyl *p*-toluylcarbamate. The optical activities of the recovered amino acids were essentially identical to the beginning rotations, which implied that little or no racemization had occurred in the overall transformation. Moreover, these derivatives are stable to dilute base, hydrogen bromide-acetic acid, cold trifluoroacetic acid, hydrazine, and hydrogen gas (10% palladium-charcoal). Thus, it should be possible to use these compounds in a highly selective fashion for the preparation of peptides.

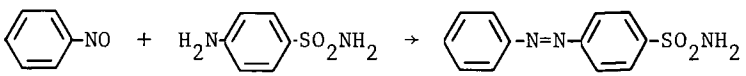
Along these lines, the Tac-amino acids can be coupled to other amino acid methyl esters by use of *N,N'*-dicyclohexylcarbodiimide or mixed anhydride procedures. Attempts to isolate *p*-nitrophenyl or 2,4,5-

trichlorophenyl esters failed due to an intramolecular condensation reaction that produced the hydantoin derivative. This work was done several years ago,[2] but apparently much interest has not been aroused by this approach.

Accordingly, a variation on this idea is now presented in the form of the *p*-phenylazophenylsulfonylaminocarbonyl group, which may be called the Azo-Tac amino acid derivatives.

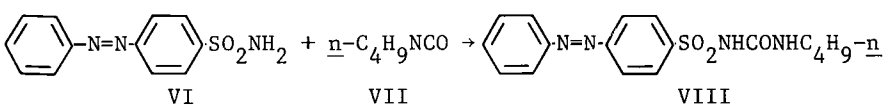
Results

To begin, nitrosobenzene (IV) is condensed with *p*-aminobenzenesulfonamide (V) in hot acetic acid to produce *p*-phenylazobenzenesulfonamide (VI).

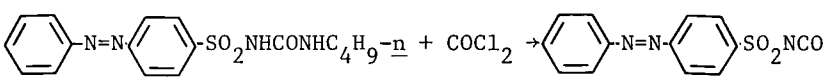


IV V VI

This latter compound on treatment with *n*-butyl isocyanate (VII) yields *n*-butyl-*p*-phenylazophenylsulfonylurea (VIII). Finally, the urea VIII is converted by phosgene into the desired *p*-phenylazophenylsulfonyl isocyanate (IX).



VI VII VIII



VIII IX

The brown isocyanate IX can be reacted with a variety of amino acids to give the desired derivatives. It is of interest that these N-protected compounds are yellow-brown to orange-brown in color and highly crystalline. In terms of deprotection, the Azo-Tac group can be removed

by warming with 95% ethanol or ethyl acetate containing 1% acetic acid. The free amino acid usually precipitates within a matter of several minutes and is optically pure. Currently, a number of these compounds are on hand and more shall be made in the near future. Some trial couplings have been undertaken with Azo-Tac-Gly-OH and the preparation of active esters is being studied.

Conclusions

It is important to note that these derivatives have potential wide applications in both solution and solid phase synthetic procedures. The idea of using water, aqueous organic solvents, or even 1% acetic acid to remove amino blocking groups is a useful concept and might be applied to enzyme chemistry also. Many variations on the azo unit are possible, both in terms of visual color and in stability (or instability). These and other related ideas are currently being actively explored.

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SYNTHESIS OF METHIONINE-CONTAINING PEPTIDES VIA THEIR SULFOXIDES

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Problems in the synthesis of Met-containing peptides are oxidation by air to diastereoisomeric sulfoxides and alkylation involving sulfonium salts during acidolysis of some protecting groups. We therefore made a virtue of necessity by protecting Met — by analogy with *Iselin*[1] — as its sulfoxides. We started with the synthesis of substance P (SP), an 11-peptide with C-terminal Met. The sequence was elucidated in 1971[2] and since then several syntheses have been described[3]. The strategy and tactics of our synthesis are outlined in Figure 1. Coupling proceeded *via* the repetitive excess mixed anhydride (REMA) method[4], a method for sequential peptide synthesis which has repeatedly been applied here[5]. Starting from Boc-Met(O)-NH_2 which had been prepared by oxidation of Boc-Met-NH_2 with hydrogen peroxide, we synthesized protected [5-11]-SP. Consecutive mixed anhydride coupling of the dipeptides Boc-Lys(Z)-Pro-OH and Z-Arg(NO_2)-Pro-OH yielded protected SP(O). We used this strategy to avoid coupling on the side of the auxiliary acid (isobutyl carbonate) which is to be expected when coupling to proline *via* a mixed anhydride[4]. After deprotection with liquid HF (plus anisole), SP(O) was purified on CM-Sephadex C-25 (gradient elution). Since the reduction of SP(O) by familiar methods[1] proceeded sluggishly, we investigated the reduction with I^- in aqueous TFA. The reduction proceeds as follows[6]:

$$R\text{-}S(O)\text{-}CH_3 + 2\ I^- + 2\ H_3O^+ \rightarrow R\text{-}S\text{-}CH_3 + 3\ H_2O + I_2$$

The iodine formed is removed by extraction with carbon tetrachloride or (preferably) reduced with an additive such as 2-mercaptoacetic acid. Substance P was purified by gelfiltration (Sephadex G 25). Besides amino acid analysis and TLC, homogeneity was confirmed by HPLC (Figure 3). Both *in vitro* and *in vivo* REMA-synthesized substance P had the same potency as the standard SP (Beckman) used.

*Taken in part from the Doctoral Thesis to be submitted by E.I.

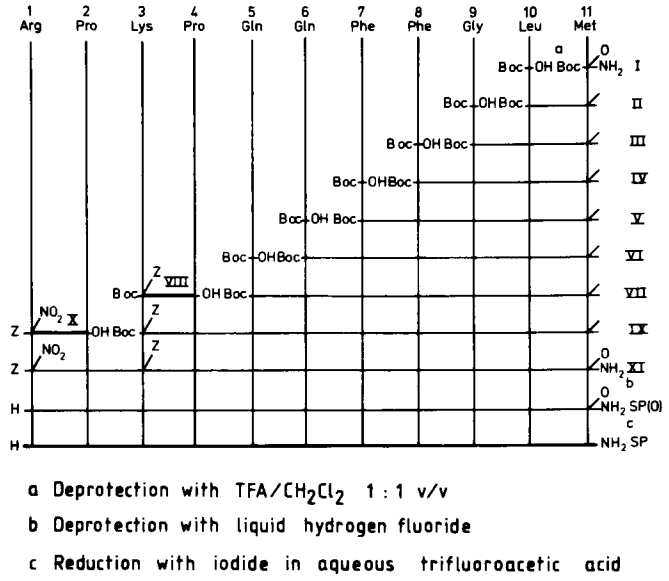

Fig. 1. REMA synthesis of substance P

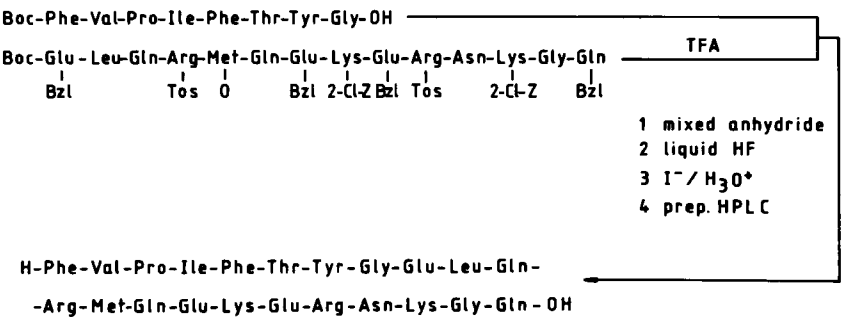

Fig. 2. Synthesis of porcine motilin

We next synthesized motilin (M), a 22-peptide with Met[13] (Figure 2). As M contains Gly[8] we chose the strategy: first, synthesis of |1-8|- and |9-22|-M, followed by coupling of the segments. Starting from Boc-Gln-Bzl, we synthesized protected |9-22|-M. All amino acids, except Asn[19] where side-reactions such as dehydration to β-cyanoalanine or imide-formation are to be expected, were coupled *via* mixed anhydrides. After REMA-synthesis of N-protected |1-8|-M, mixed anhydride coupling of this segment to N^{α}-deprotected |9-22|-M yielded protected motilin sulfoxide. After deprotection, M(O) was reduced by our method. Motilin was purified semi-preparatively by reverse-phase HPLC. Amino acid analysis showed that no iodination of tyrosine had occurred. The biological potency of our motilin still has to be determined.** Besides for purifying the end product, reverse-phase HPLC proved to be invaluable in monitoring both the REMA-syntheses[7] and the sulfoxide reductions. Chromatograms of substance P, motilin, and motilin sulfoxide are shown in Figure 3.

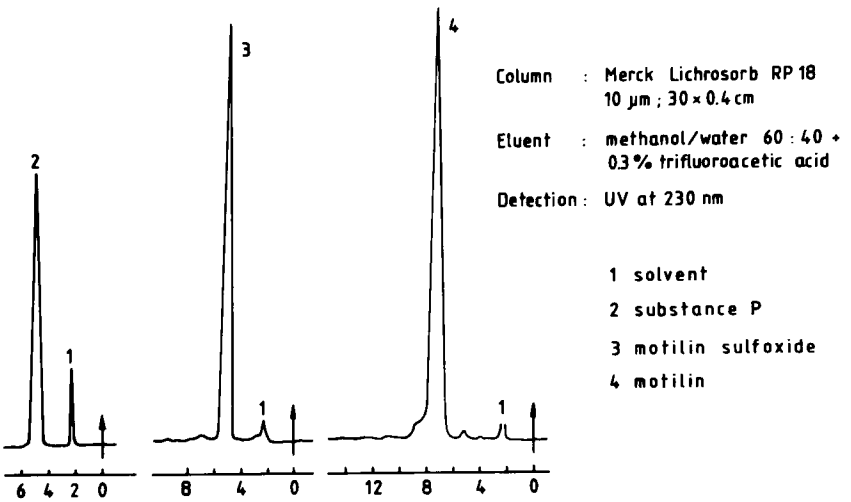

Fig. 3. HPLC of substance P, motilin sulfoxide, and motilin

**Added in proof: REMA-motilin was found to possess high biological activity.

For further evaluation we reduced human calcitonin sulfoxide (Figure 4), which had been prepared by mild oxidation of human calcitonin[8] by hydrogen peroxide in aqueous acetic acid.

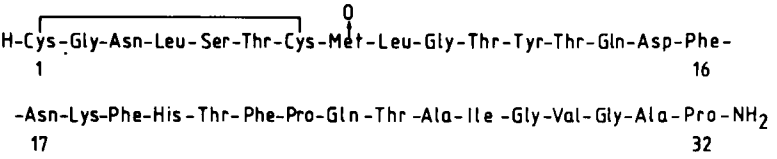

Fig. 4. The amino acid sequence of human calcitonin sulfoxide

Amino acid analysis indicated that neither Tyr, His, and Cys-Cys caused problems. An investigation into the reduction of Trp-containing peptide sulfoxides showed that Trp gives side-reactions.

Acknowledgement

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POLY-N-ACRYLYLPYRROLIDINE RESIN FOR PEPTIDE SYNTHESIS

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Only major advances in chemical synthesis of proteins will keep this approach of preparing biomolecules competitive with procedures in which chemically synthesized DNA or DNA copies of mRNA are cloned in bacteria for expression.[1] In this regard it is becoming increasingly more apparent that the full potential of the solid-phase method of peptide synthesis[2] cannot be realized for the facilitated synthesis of large polypeptides with the exclusive use of stepwise elongation of the peptide chain attached via its COOH-terminus. In our view a promising approach is one in which the sidechain of an amino acid residue is attached to a support which is compatible with both aqueous and nonaqueous environments and the polypeptide is assembled "bidirectionally"[4] utilizing a scheme of fragment condensation. Such an approach offers the following advantages: 1) the fragments can be prepared by any convenient scheme and can be purified prior to use 2) any deletion or truncation sequences should be sufficiently differentiated from the target molecule so that they can be easily separated 3) the bidirectional capability provides almost limitless latitude in the design of analogs and 4) the compatibility of the peptide-polymer matrix with aqueous environment offers the potential of integrating solid-phase methodology into a semisynthetic scheme.

Certainly any approach that depends on the coupling of fragments to polymer-bound peptide has special requirements in terms of protecting groups, anchoring links, and coupling agents. Moreover, the solubility of the fragment is critical to the success of the synthesis and is complicated by the fact that the fragments must penetrate a peptide-polymer matrix and remain solvated in this environment. In practice, syntheses using fragment couplings on a polystyrene-divinylbenzene resin have been limited to small molecules using even smaller fragments of only two to five residues.[5] As fragments get larger coupling yields become much less than quantitative.[6] Part of the difficulties with solid-phase fragment

couplings must be attributed to the use of polystyrene based supports. Liquids that solvate the polymer (with an insoluble polymer, swelling is a measure of solvation) may not be useful for dissolving peptide fragments. Even when dissolved the fragments may be partially excluded from the polymer matrix by partitioning effects.[7]

In a first step toward a system of polymer-assisted peptide synthesis via bidirectional fragment condensation, we recently reported the synthesis by reverse-phase suspension polymerization of a new resin based on *N*-acrylylpyrrolidine (PAP) in an entirely beaded form.[8] PAP in either its acetylated or protonated form exhibits swelling properties about the same as polystyrene-1%-divinylbenzene in solvents commonly employed in the Merrifield method (Figure 1A), but far surpasses polystyrene resin in polar media (including aqueous) more suited for dissolving fragments (Figure 1B).

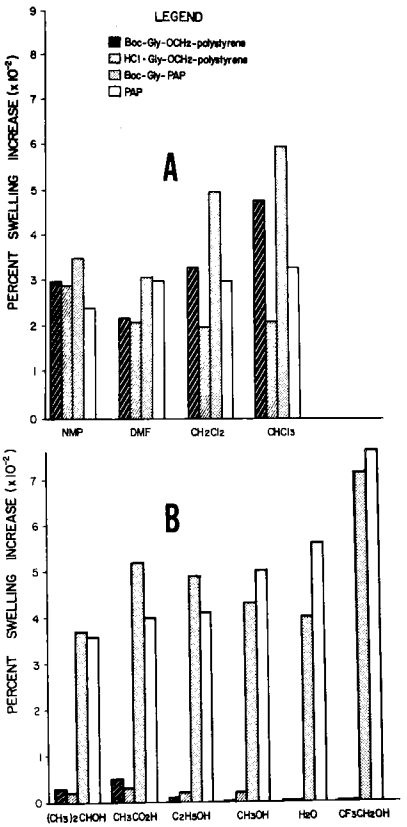

Fig. 1. A) Comparison of the swelling properties of PAP with polystrene-1%-divinylbenzene in solvents commonly employed in the solid-phase method. B) Demonstration of the potential for solid-phase synthesis with solvents commonly used in solution chemistry on PAP—but not on polystyrene.

In our first communication we also reported an S-carbamoyl peptide-to-polymer linking group to the sidechain of cysteine and used it to prepare fully biologically active deamino-oxytocin[9] in high yield via a scheme that used both fragment condensation and stepwise elongation.[8] Now we report here the successful solid-phase bidirectional synthesis of thyrotropin releasing hormone (TRH, <Glu-His-Pro-NH_2) through the application of the dinitrophenylene linking group[4] to PAP and using a scheme similar to that described previously.[4,10] <Glu-His-Pro-NH_2 was detached from the resin by treatment with mercaptoethanol and purified to full biological activity by a single chromatographic step on silica gel[11] using EtOH-H_2O(7:3 v/v). Furthermore, we report the solid-phase bidirectional synthesis of the model tripeptides Z-Pro-Glu-Gly-NH_2 and Z-Pro-Gln-Gly-NH_2 using a benzyl ester linking group to PAP via the glutamyl sidechain carboxyl group (Figure 2). Avoiding harsh treatment

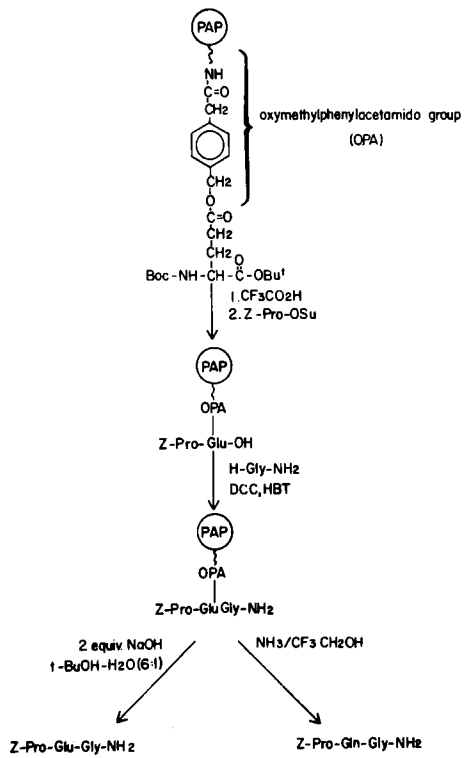

Fig. 2. Bidirectional synthesis ot glutamine or glutamic acid containing peptides.

with strong acid, 92% of the glutamyl containing tripeptide was released from the resin by brief exposure to 2 equivalents of NaOH in *t*-BuOH-H_2O(6:1, v/v) and the glutaminyl containing tripeptide was released quantitatively by ammonolysis in CF_3CH_2OH. Thus, the sidechains of Cys, His, Glu or Gln have been revealed as viable points of attachment for bidirectional polymer assisted peptide synthesis.

Acknowledgements

The authors thank Dr. C. Y. Bowers and Ms. G. Reynolds for kindly performing bioassays of TRH. We also thank Mrs. S. Hu, Mrs. E. Skala and Mr. G. Skala of this laboratory for their skillful assistance. This work was supported in part by Grant AM-20314 from the U.S. Public Health Science, by the donors of the Petroleum Research Fund administered by the American Chemical Society, and the American Philosophical Society, Grant 8156 of the Penrose Fund.

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MULTI-DETACHABLE RESIN SUPPORTS

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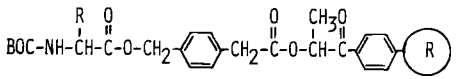
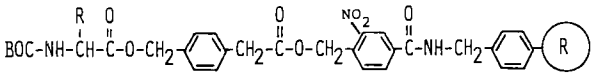
Introduction

Most peptide-resin attachments for solid phase synthesis to date have been designed for a single specific purpose. They each contain only one labile anchoring bond, are generally cleavable by one or a few related methods, and are intended for use in only one synthetic strategy. We report here the design and synthesis of two new multi-purpose and multi-detachable resin supports, 5 & 6, based on a new concept that provides much more flexibility in synthetic design than has been possible before.



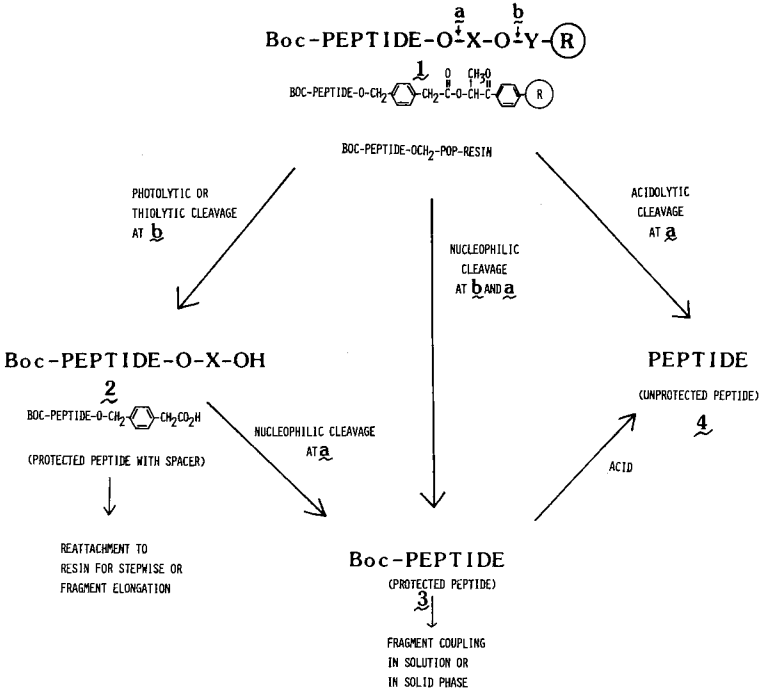
BOC-AMINOACYL-OCH_2-**POP**-RESIN 5



BOC-AMINOACYL-OCH_2-**PON**-RESIN 6

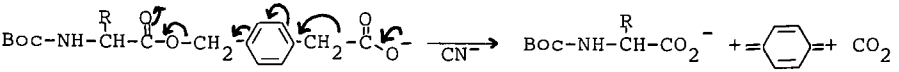
Concept and Development

The basic idea makes use of an attachment, peptide-O-X-O-Y-resin, with two orthogonal ester bonds, *a* (peptide-O-X) and *b* (X-O-Y-resin), separated by a suitable spacer (X), (Scheme 1). Thus, depending on the cleavage reaction, one or the other of the esters can be cleaved selectively under conditions where the second bond is stable, and the peptide can be obtained in a deprotected form 4, in a protected form 3, which is suitable for fragment synthesis, or in an intermediate form 2, which can be reattached to another resin support (e.g. an aminomethyl-resin) for stepwise or fragment elongation. Furthermore, the system is designed so that a mild interconversion from one form to the other can be effected.



Scheme 1. A design of Multi-detachable Resin

Two examples of the multi-detachable resin support are Boc-aminoacyl-OCH_2-Pop-resin 5, (Boc-aminoacyl-2-[4-(oxymethyl)phenylacetoxy]propionyl-resin) and Boc-aminoacyl-OCH_2-Pon-resin 6, (Boc-aminoacyl -4- [4-(oxymethyl)phenylacetoxymethyl] -3- nitrobenzamidomethyl-resin). Each resin contains an acid labile benzyl ester at *a* and a photolabile α-methylphenacyl ester[1] or o-nitrobenzyl ester[2] at *b*, with an oxymethylphenylacetic acid (OMPA) as spacer X. The flexibility of the multi-detachable resin system lies in the ability of the intermediate, Boc-peptide-OMPA 2 (or the starting material phenacyl ester 5) to be rapidly and selectively converted to Boc-peptide 3, by a decarboxylative 1,6-elimination in the presence of cyanide ion (Scheme 2). This reaction is 6,000 to 15,000 times faster than normal S_N2 cyanolytic cleavage of simple unsubstituted benzyl esters.[3] Thus, by selecting two orthogonal esters and a novel "collapsible" spacer, the multi-detachable resins such as Pop- and Pon-resins worked as designed.



Scheme 2. Decarboxylative 1,6-Elimination of Boc-peptide-OMPA

Synthesis and Properties

Boc-aminoacyl-OCH_2-Pop- or Pon-resin is best prepared by the esterification of Boc-aminoacyl-OMPA to either the bromo(α-methyl)-phenacyl or bromo(o-nitro)benzyl resin by using potassium fluoride in N-methylpyrrolidone as solvent at ambient or slightly elevated temperature (50°C) for 8-24 hours.[4] Under these conditions, total esterification was obtained in a short time and at a low temperature without discoloration of the resin. The preparation of the OMPA of the Boc-amino acids have been described earlier.[5]

Cleavage of Boc-amino acid or peptide from Pop- and Pon-resins could be achieved by many reagents as shown in Table I. For example,

Table I. ORTHOGONAL CLEAVAGES OF MULTI-DETACHABLE PEPTIDE-RESINS

CLEAVAGE POINT	CLEAVAGE CONDITION	PRODUCT
A	STRONG ACID (HF, $MeSO_3H$)	PEPTIDE **4**
A	HYDROGENOLYSIS	PEPTIDE **4**
B + A	CYANOLYSIS, BASE, HYDRAZINOLYSIS	BOC-PEPTIDE **3**
B	PHOTOLYSIS, THIOLYSIS	BOC-PEPTIDE-OMPA **2** (BOC-PEPTIDE-SPACER)

Boc-Leu-OCH_2-Pop-resin was cleaved by HF or $MeSO_3H$ to give Leu, by tetrabutylammonium cyanide, aqueous NaOH or hydrogenolysis to Boc-Leu-OH, by hydrazine to Boc-Leu-$NHNH_2$, and by photolysis or thiolysis to Boc-Leu-OMPA which was converted to Boc-Leu-OH by brief exposure to cyanide ion. All cleavage reactions gave satisfactory yields (75-89%).

Furthermore, the multi-detachable resins, 5 and 6, contain new properties that are not found in either normal benzyl or photolabile ester resin. For example, an electron-withdrawing 4-carboxymethyl substitution on the O-X benzyl ester bond (Scheme 1) of Pop resin (5) provides 35-200 times more acid stability than the ordinary benzyl ester resin. Thus, acidolytic loss during peptide synthesis is minimized with the multi-detachable resin. Also, by putting a spacer, OMPA, between the C-terminal amino acid and the phenacyl ester resin, we found increased photolytic cleavage even with anchoring amino acids other than Gly[1,2] and minimized loss of growing peptide chains (<3%) during the first two cycles of synthesis, a side reaction associated with phenacyl ester resins. Moreover, purified Boc-peptide-OMPA provided convenient reattachment to aminomethyl resin via dicyclohexylcarbondiimide coupling for either segment or stepwise elognation.

Application in Peptide Synthesis

Leu-Ala-Gly-Val, [Leu]enkephalin and [Val[5]]angiotensin II were synthesized on Pop- or Pon-resin 5-6. The cleavage yields from resins and more importantly, the homogeniety of peptide in the crude products after HF cleavage were used as two criteria to evaluate the efficacies of these multi-detachable resins in stepwise peptide synthesis. Leu-Ala-Gly-Val (synthesized on Pop- and Pon-resin) and [Leu]enkephalin (on Pop-resin) in which the Tyr residue was incorporated with an unprotected side chain, were obtained in >98.5% purity and eluted as a large single peak in either ion-exchange or C-18 reverse-phase liquid chromatography. Similarly, 88% of [Val[5]]angiotensin II (on Pop-resin) was found as a major peak. The decrease in homogeneity was largely attributed to the rearranged alkylated Tyr. All cleavage reactions (Table I) gave satisfactory yields.

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TRIFLUOROACETYLATION IN SOLID PHASE PEPTIDE SYNTHESIS

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Introduction

Solid phase peptide synthesis[1] involves the stepwise addition of Boc-amino acids to the polymer-bound peptide chain. In most of these syntheses the Boc group is removed by acidolysis using trifluoroacetic acid (TFA) in methylene chloride. It has been reported that *N*-trifluoroacetylated peptides are formed during synthesis[2-4]. This results in lower yields of the desired peptide and more complex product mixtures. The formation of Tfa-peptides has been attributed to carryover of TFA into the coupling step, where it is activated and reacts with the free α-amino group of the growing peptide, and for this reason coupling using preformed Boc-amino acid symmetric anhydrides has been used to minimize the activation of the TFA[4]. We have found that another, novel mechanism also exists for the formation of Tfa-peptides. This occurs during the neutralization step and is independent of the coupling.

Mechanism

The existence of this mechanism of trifluoroacetylation was first detected using aminomethyl-resin as a model for the resin-bound peptide chain[5]. IR studies on such a resin showed a band at 1785 cm^{-1} after TFA treatment, apparently due to an activated resin-bound TFA species responsible for the trifluoroacetylation. This was identified as the Tfa-OCH_2-resin ester. On neutralization, the Tfa-group was transferred to the aminomethyl group by an intersite nucleophilic reaction within a single resin bead. This *nucleophilic transfer mechanism* is shown in Figure 1.

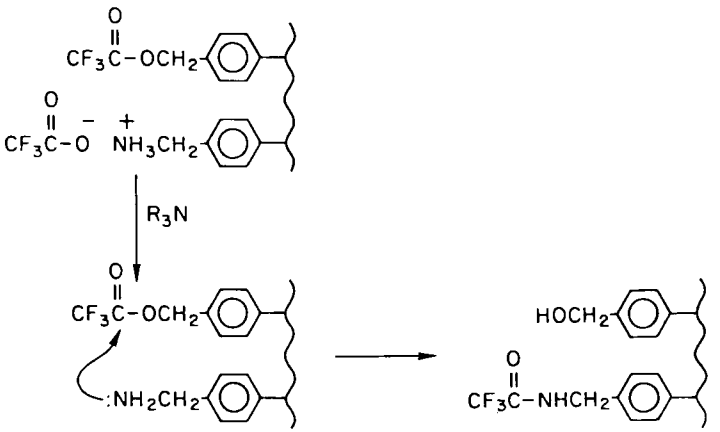

Fig. 1. Nucleophilic transfer mechanism of trifluoroacetylation.

Occurrence

The Tfa-OCH_2- sites responsible for trifluoroacetylation by this mechanism are formed in two principal ways. Pre-existing $HOCH_2$- sites on the resin react with 50% TFA-CH_2Cl_2 to form Tfa-OCH_2- resin esters, with a half-time of reaction of about 15-20 minutes. Commercially available resins may contain large amounts of $HOCH_2$- sites. For example, chloromethylated Biobeads S-X1 (0.7 mmol/g, lots #13668, #14391) was found to contain 0.35 mmol/g $HOCH_2$- sites. The other major route to Tfa-OCH_2- sites is the acidolysis, by TFA, of the peptide-resin benzyl ester bond. This happens to the extent of about 0.7% per 30 minute deprotection with 50% TFA-CH_2Cl_2. The acidolysis results in the formation of an equivalent amount of Tfa-OCH_2-resin sites, as shown by IR.

The extent of trifluoroacetylation was determined using (N^{ϵ}-protected)lysine-resins, prepared from $ClCH_2$-resins by the cesium salt procedure and subjected to cycles of simulated solid phase synthesis without the coupling step. After HF cleavage, amino acid analysis on a Beckman 121 analyzer gave N^{α}-Tfa-lysine at the position of leucine, with 22% the color yield. The results are shown in Table I. The rate of reaction of H-Lys(Z)-OBzl hydrochloride with Tfa-OCH_2-resin in 10% DIEA-CH_2Cl_2, to give N^{α}-Tfa-lysine, was determined: $k_2 = 6 \times 10^{-4}$ M^{-1} sec^{-1}. This was 250-fold slower than the reaction of benzylamine under the same conditions. Thus, for the poorly nucleophilic α-amino group

trifluoroacetylation under the conditions of solid phase peptide synthesis is *rate-limited* and the extent will therefore depend on the amount of time spent in the neutralized state.

Prevention

In order to prevent trifluoroacetylation by this nucleophilic transfer mechanism, resins must be initially free of $HOCH_2$- sites and must not generate Tfa-OCH_2- sites during the course of the synthesis. Levels of $HOCH_2$- sites >0.02 mmol/g can be readily detected by IR after conversion to the Tfa-OCH_2 ester and levels of $HOCH_2$- sites below this can be removed by treatment of the swollen unsubstituted polystyrene resin with strong Lewis acid when internal scavenging gives resins with inert additional crosslinks replacing the extraneous functionalities originally present.

Table I. Levels of trifluoroacetylation of (N^{ε}-protected)Lys-resins

Resin	Conditions	Extent (% per cycle)[a]
Lys(2ClZ)-OCH_2-resin[b]	5 cycles	0.6%
	5h TFA pretreatment[c], 5 cycles	1.4%
Lys(3ClZ)-OCH_2-resin[d]	10 cycles	1.3%
Lys(Z)-OCH_2-Pam-resin[d]	10 cycles	1.7%
	10 cycles, no neutralization	0.02%
Lys(Z)-OCH_2-Pam-resin[e]	10 cycles	0.02%
Lys(2ClZ)-OCH_2-Pam-resin[e]	22h TFA pretreatment[c], 5 cycles	0.12%

[a] %Trifluoroacetylation = (N^{α}Tfa-Lys)/(total Lys), x 100.
[b] From Pierce $ClCH_2$-resin (0.7 mmol/g), no hydroxymethyl sites.
[c] Pretreatment with 50% TFA-CH_2Cl_2 at room temperature.
[d] From Biorad $ClCH_2$-resin (0.7 mmol/g) containing 0.33 mmol/g hydroxymethyl sites.
[e] From NH_2CH_2-resin made from unsubstituted Biobeads S-X1 by direct amidoalkylation[8].

The standard peptide-resin benzyl ester bond is too labile to 50% TFA-CH_2Cl_2, generating Tfa-OCH_2- sites which lead to trifluoroacetylation. A peptide-resin bond which is stable to repeated exposure to TFA must be used. One such resin is the aminoacyl-OCH_2-Pam-resin[o]. Over the course of 50 deprotections with 50% TFA-CH_2Cl_2 (20 minutes each), a total of less than 0.5% acidolysis will occur, yet the final cleavage yields in HF are excellent[6,7]. The (N^{ϵ}-protected)lysine-resin model was used to determine the levels of Tfa-peptide formation on the Pam-resin. The results are shown in Table I. The virtual elimination of trifluoroacetylation from the nucleophilic transfer mechanism is due to use of resin initially free from detectable $HOCH_2$- sites, the strong Lewis acid treatment during the amidoalkylation[8] which effects the internal scavenging previously discussed, loading the resin by an unambiguous route[7], and the stability of the peptide-OCH_2-Pam-resin benzyl ester bond to TFA[6,7].

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CONTINUOUS-FLOW SOLID-PHASE PEPTIDE SYNTHESIS USING PAM-RESINS

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The chemical synthesis of peptides was greatly simplified by Merrifield's introduction of the solid-phase method.[1] Solid-phase peptide synthesis is normally performed by adding each reagent solution and wash solvent to the solid support in discrete portions.[2] The alternative mode of adding the solutions in a continuous flow was introduced by Scott and coworkers in 1971.[3,4] We have recently shown that solid-phase synthesis in the continuous-flow mode is easily performed using the efficient pump of a high-pressure liquid chromatographic (HPLC) system.[5,6] This paper describes the continuous-flow solid-phase synthesis of the model peptide Leu-Ala-Gly-Val using three types of phenylacetamidomethyl-resin (Pam-resin).[7]

General procedures have been described for continuous-flow synthesis using a Waters Associates HPLC system[5] and preparation of Pam-resins.[8] Boc-Val-OCH_2-Pam-resin was prepared from three types of polystyrene-based supports: [1] *copoly*(styrene-1% divinylbenzene) (Bio-Beads S-X1; Bio-Rad Lab.), [2] a highly crosslinked resin (Styragel 10^3 Å ; Waters Assoc.), and [3] a macroreticular resin (Resin B; Dionex Corp.).

Each Boc-Val-OCH_2-Pam-resin was packed into a stainless steel column and was converted into Leu-Ala-Gly-Val-OCH_2-Pam-resin by repeating the synthetic cycle: [1] deprotecting with 0.01 M methanesulfonic acid plus 0.10 M trifluoroacetic acid in dichloromethane,[9] [2] neutralizing with 0.3 M triethylamine, and [3] coupling with 0.1 M Boc-amino acid anhydride by recycling through the column, pump, and injector for 15 or 30 minutes. Each step was followed by a dichloromethane wash and the peptide products were cleaved from the resin with 9:1 (v/v) anhydrous HF/anisole (0°C, 1 hour).

The crude synthetic peptides were separated by reverse-phase HPLC and detected by ultraviolet absorbance at 220 nm (Figure 1). As little as

0.2% of a by-product was readily measured by injecting 100 nmol of crude peptides and simultaneously measuring the absorbance on scales of 0.08 and 0.64 absorbance units. This analytical HPLC system requires only 5% of the peptide sample and takes only 5% of the analysis time needed for conventional ion-exchange chromatographic analysis using ninhydrin detection.[5,10]

The relative peptide yields for six continuous-flow syntheses are shown on Table I. Double-coupling synthesis *1* (total syntheses time: 4.5 hours) and *2* (2.9 hours) provided an average of 1% detectable by-products per synthetic cycle. The single-coupling syntheses *3-5* (1.8 hours) were less efficient and produced more by-products. Synthesis *3* was the best single-coupling synthesis using Pam-*copoly*(styrene-1% divinylbenzene). Other syntheses with this resin under similar conditions provided Leu-Ala-Gly-Val in 90-95% yield. Even though the macroreticular Resin B and the highly crosslinked Styragel 10^3 Å were designed for reverse-phase HPLC chromatography, syntheses *4* and *6*

Table I. Reverse-phase HPLC analysis of Leu-Ala-Gly-Val and four by-products

Synthesis number	Polystyrene support[a]	Coupling conditions[b]	Peptide[c] (rel mol%) LAGV	AGV	LAV	LGV	LV
1	Bio-Beads S-X1	2 x 30 min	97.1	0.9	1.5	0.5	<0.1
2	Bio-Beads S-X1	2 x 15 min	96.5	1.2	1.4	0.9	<0.1
3	Bio-Beads S-X1	1 x 15 min	95.1	1.6	1.9	1.1	0.3
4	Resin B	1 x 15 min	87.6	8.1	1.7	1.8	0.8
5	Styragel 10^3Å	1 x 15 min	69.8	17.0	9.0	1.9	2.3
6	Styragel 10^3Å	1 x 15 min[d]	93.5	3.9	0.7	1.0	0.9

[a] Resin loadings (mmol Val/g dry polystyrene) were 0.13 for Bio-Beads S-X1, 0.18 for Resin B, and 0.40 for Styragel 10^4 Å.

[b] Concentration of Boc-amino acid anhydride used for each 4-ml coupling was 0.10 *M*. Single (1x) or double (2x) couplings were used.

[c] Code: L. leucine; A, alanine; G, glycine; V, valine.

[d] Two 15-min. couplings were used for leucine.

show that these polystyrene supports are nearly as efficient as the conventional Bio-Beads S-X1 for solid-phase peptide synthesis. We are currently examining these resins under various conditions to maximize their usefulness as synthetic supports.

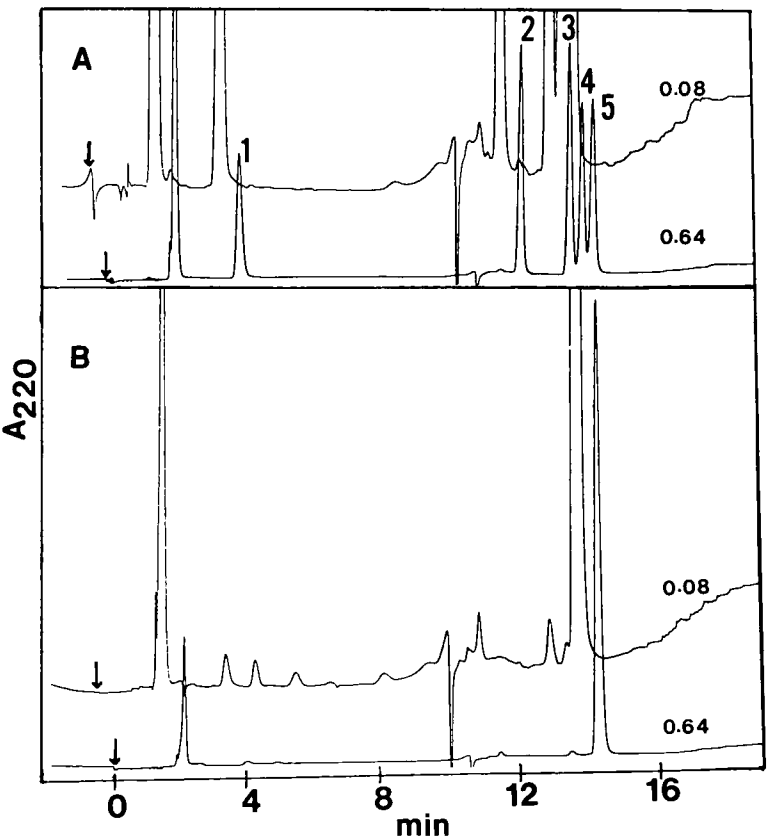

Fig. 1. Reverse-phase high-pressure liquid chromatography of Leu-Ala-Gly-Val and four deletion peptides. Peptides (1, AGV; 2, LV; 3, LAV; 4, LGV; and 5, LAGV) were eluted from a μBondapak C_{18} column with a linear gradient of 0-16% isopropanol in aqueous 5 m*M* triethylammonium phosphate using a Waters Associates HPLC system and were monitored at 220 nm with a Hitachi dual-wavelength ultraviolet detector (full scale: 0.08 and 0.64). A, synthetic peptide standards (50-70 nmol); B, crude peptide mixture from synthesis *1*. Arrows indicate point of injection and start of gradient.

In conclusion, the model tertrapeptide Leu-Ala-Gly-Val was assembled in 96.5% yield in less than 3 hours under continuous-flow conditions. A new reverse-phase HPLC analytical system was employed for measuring the peptide by-products from this synthesis. This HPLC system using ultraviolet detection is more efficient and more sensitive than the conventional ion-exchange system using ninhydrin detection. The same HPLC system can be used in both synthetic and analytical modes. The continuous-flow HPLC synthesizer is currently being converted into an automated solid-phase peptide synthesizer.

Acknowledgements

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THE SOLID-PHASE SYNTHESIS OF PEPTIDE α-CARBOXAMIDES: THE SYNTHESIS OF HANDLES AND THEIR CHARACTERIZATION BY THE USE OF INTERNAL REFERENCE AMINO ACIDS

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Introduction

Our continuing interest in the solid-phase synthesis (SPS) of peptide α-carboxamides has led to the synthesis of a series of suitable "handles." In SPS a handle provides a well-defined chemical linkage between the peptide being synthesized and the resin support. Rich and Gurawa have introduced the handle, 4-aminomethyl-3-nitrobenzoic acid, for the SPS of peptide α-carboxamides after cleavage by photolysis.[1] We have designed handles for the SPS of peptide α-carboxamides after cleavage by HF treatment.

In order to evaluate these handles, resins containing internal reference amino acids (IRA) have been employed. An IRA is stable to conditions of synthesis, but is readily quantifiable by amino acid analysis after propionic acid:HCl hydrolysis. The ratios of amino acids to IRA can be used advantageously to evaluate the rate and extent of peptide synthesis and cleavage.

Results

Synthesis of Handles — The Williamson synthesis reaction between N-protected phenols and halo acids was used to prepare four handles. For the synthesis of handle *IV*, the Na salt of *N*-Boc-*p*(α-phenyl)amino-methylphenol was reacted with an excess of ICH_2COO^- Na+ in EtOH to yield *N*-Boc-*p*(α-phenyl)aminomethylphenoxyacetic acid (handle *IV*) in 82% yield. The corresponding free amino acid (mp162-163°) was homogeneous by TLC. Similarly the Na salt of *N*-Boc-*p*(aminomethyl)-

phenol was reacted separately with the Na salts of a) ICH_2COOH, b) $BrCH_2C_6H_4CH_2COOH$ and c) $BrCH_2C_6H_4COOH$. After TLC, Boc-$NHCH_2C_6H_4OCH_2COOH$(handle *I*) and Boc-$NHCH_2C_6H_4OCH_2C_6H_4$-CH_2COOH(handle *III*) were judged homogeneous, but Boc-$NHCH_2C_6$-$H_4OCH_2C_6H_4COOH$(handle *II*) contained a minor contaminant. All four handles could be completely deprotected after treatment with: TFA for 10 minutes; 50% TFA in CH_2Cl_2 for 20 minutes; and 25% TFA for 30 minutes.

The Use of Internal Reference Amino Acids (IRA) — IRA are chosen because of their stability during acid hydrolysis and unique elution positions during amino acid analysis. Aminobutyric acid (Abu) norleucine (Nle), and β-alanine (β-Ala) satisfy these criteria. After IRA are coupled directly to aminomethylresin, they can serve as internal references.[2] The following experiments have demonstrated that IRA are sufficiently stable during SPS. 1. Prolonged acid treatment (92h) with 50% TFA in CH_2Cl_2 cleaved a negligible quantity (0.8%) of IRA as determined by amino acid analysis. 2. HF cleavage of IRA-resin failed to cleave any IRA (detection limit 0.2%). In order to quantify IRA and other amino acids simultaneously, the time for the maximum recovery after propionic acid:HCl hydrolysis[3] was determined. After 4 hours at 130° maximum values were obtained. These values were used to calculate the ratios of amino acid:IRA. Any additions or losses of amino acids from the resin during SPS are reflected by changes in the amino acid:IRA ratios.

Preparation of Handle(IRA) resins — Handle-resin containing IRA was prepared in the following way. Starting with NH_2CH_2 resin prepared by using trifluoromethanesulfonic acid as catalyst according to Mitchel *et al.*[4], a tripeptide was synthesized using Boc-Abu-OH, Boc-Nle-OH, and Boc-β-Ala-OH. Amino acid analysis of this product after hydrolysis yielded the following substitutions (mmol/g): Abu, 0.41; Nle, 0.42; and β-Ala, 0.41. Subsequently, each Boc-handle was coupled at 2x excess with equimolar DCC to the tripeptide IRA-resin. The unreacted amino groups were blocked with acetic anhydride: TEA (1:1) in CH_2Cl_2 before deprotection of the Boc-handle(IRA) resin with TFA:$CHCl_2$ (1:3) for 30 minutes.

Preparation of Gly-Handle(IRA) resins — To each deprotected handle(IRA) resin, Boc-Gly-OH was coupled with DCC until the ninhydrin test indicated complete coupling. After deprotection, the Gly substitutions were determined by amino acid analysis as shown in Table I.

Preparation of Z-Glu-His(Tos)-Pro-Handle(IRA) resins — TRH was synthesized on two IRA-resins: using handle III and handle IV. The amino acid substitutions of the peptidyl-resins as determined by internal reference amino acids are shown in the Table I.

Comparative TFA Acidolysis and HF Cleavage of Gly-Handle(IRA) Resins and TRH-Handle(IRA) resins — For the TFA acidolysis experiments, 200-300mg of resin was suspended in 10ml TFA:CH_2Cl_2 (1:1) for 24 hours at 0°C. Losses from the resin were measured by amino acid analysis of resin and TFA solution hydrolysates (See Table I). For the comparative HF cleavage experiments, 100-300mg of resin was treated with HF:anisole (9:1) at 0°C. The amino acid substitutions determined using IRA values are shown in the Table along with the percent of TRH cleaved.

Table I. Characterization and Comparative Chemistry of Handle(IRA)Resin Products

	Gly-			TRH-				
	nmol/mg	% cleaved		nmol/mg			% cleaved	
Handle Used	Gly	TFA[a]	HF[b]	Glu	His	Pro	TFA	HF
I	320	·5	11			-N.D.-		
II	11	N.D.	38			-N.D.-		
III	79	4	77	334	299	297	1	78
IV	224	<1	92	205	175	201	<1	86

[a] TFA:CH_2Cl_2(1:1) for 10 hr. at 25°C.
[b] HF:anisole (9:1) for 30 min. at 0°C.

Analysis of HF-products — TLC(100μg samples) of the putative $GlyNH_2$ and TRH products indicated that as side products Gly and TRH acid could not be detected. When handle III was used, the putative TRH sample (Glu:His:Pro/1.06:0.97:0.98) contained an alternate product, not TRH acid. By TLC (propanol/ammonia), this by-product migrated with a higher R_f than authentic TRH. This was consistent with the HF cleavage of the benzyl ether linkage in handle *III* and the partial cleavage of the benzyl amide bond between the peptide and the handle. On the other hand, the use of handle *IV* (Glu:His:Pro/1.05:0.97:0.98) provided essentially homogeneous TRH.

Conclusions

The use of handles permits the purification of the functional groups used during SPS before attachment of the handle to the solid support. Assuming a single batch of aminomethyl resin is used, a direct comparison of the chemistry of handles is possible. The comparison presented here was facilitated by the use of IRA.

Of the four handles synthesized, handle *IV* seems best suited for the SPS of peptide α-carboxamides: 1) handle *IV* can be prepared easily in large quantities. 2) The losses by TFA acidolysis of Gly and TRH are small. 3) The HF product corresponding to TRH is obtained in good yield (85%) and co-chromatographs with authentic TRH.

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SOLID-PHASE SYNTHESIS OF THE 32-AMINO ACID CARBOXYL-TERMINAL FRAGMENT OF HUMAN PARATHYROID HORMONE hPTH(53-84): COMPARISON OF STRATEGIES

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Introduction

In preparation for the eventual synthesis of the 84-peptide human parathyroid hormone by the solid-phase procedure a comparison of strategies and an investigation of the side-products generated during synthesis of the carboxyl-terminal fragment 53-84 was undertaken.

Two main approaches were employed in separate syntheses of hPTH(53-84). The standard dicyclohexycarbodiimide (DCC) coupling method was compared to amino acid incorporation using preformed symmetrical anhydrides of Boc-amino acids. Also, protection of the γ-carboxyl function of glutamic acid by the benzyl group was compared to protection by the chlorobenzyl group. The products of these synthetic strategies were chemically evaluated.

Results

The 32-peptide fragment hPTH(53-84) was assembled on a 1% cross-linked polystyrene benzhydrylamine resin using the protected amino acids shown in Figure 1. Linkage to the resin was made through the side chain carboxyl group of Glu at position 84, yielding Gln as the carboxyl terminus following HF cleavage. In Synthesis A the standard carbodiimide-promoted method of coupling with 2.5 excess of Boc-AA and 2.5 excess of DCC was used. In a separate synthesis (B) the coupling reaction was carried out by either the *in situ* symmetrical anhydride

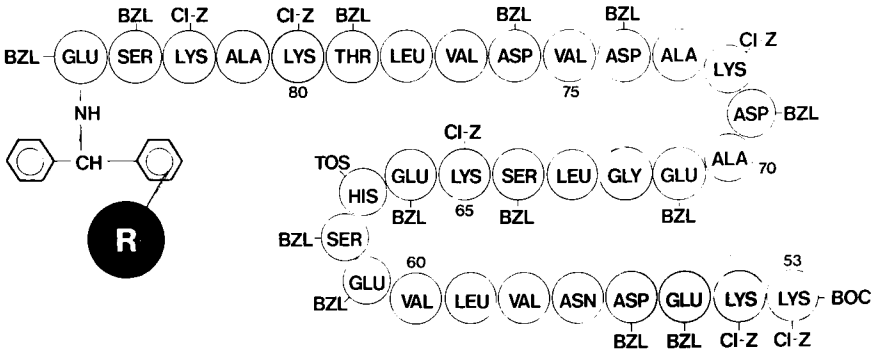

Fig. 1

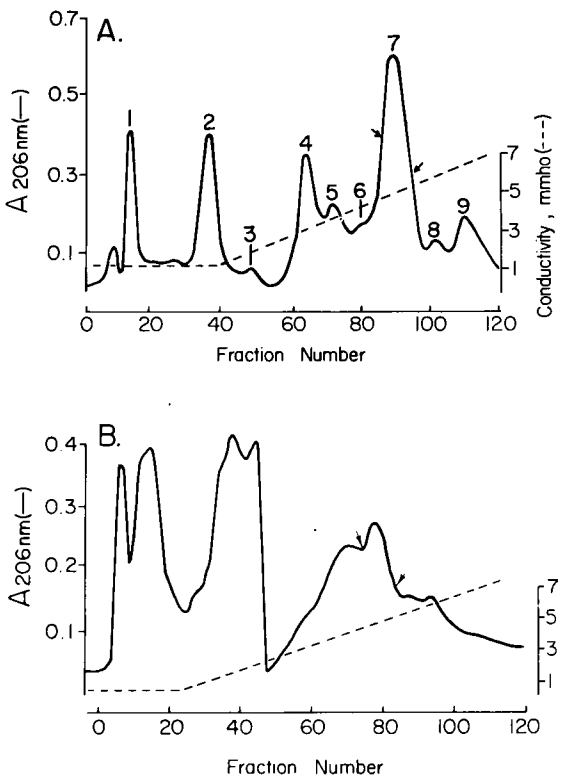

Fig. 2

method of Rebek & Feitler[1] using 3.0 excess of Boc-AA and 1.5 excess of DCC (up to Glu at position 69) or the preformed symmetrical anhydride procedure of Hagenmaier & Frank[2] using 5.0 excess of Boc-AA with 2.0 excess of DCC. Fluorescamine was used to monitor the completion of the coupling reactions. Synthesis A required 6 repeat couplings (at residues 79,77, 76,71,59 and 57) whereas during the symmetrical anhydride procedure 11 repeat couplings were required (at 79,78,74,71, 72,69,64,61,63,62 and 57). Following HF-anisole cleavage (1 hour at 0°C) the crude synthetic product from each of the syntheses was purified by

gel-chromatography on P6/1M acetic acid then by ion exchange chromatography on DEAE cellulose (see Figure 2).

Amino acid analysis of the purified peptide from Syntheses A and B were indistinguishable and within ±5% of the theoretical ratios. Each peptide appeared homogeneous as assessed by TLE at pH 3.5 and pH 6.5. However, differences in the purity of the peptide preparations were clearly seen on gel isoelectric focusing. Heterogeneity was detected for the product of Synthesis B, but none for Synthesis A. The isoelectric point was 4.8. Tryptic digestion followed by TLC gave an identical pattern for a sample of purified native hPTH (53-84) and Synthesis A. The digest of the Synthesis B peptide contained at least one additional band indicating the probable presence of side chain heterogeneity. Automated Edman sequence analysis of the purified peptides confirmed that the correct sequence had been assembled and that the side chain protecting groups had been removed. The level of deletion peptides seen as preview during sequence analysis amounted to less than 6% for A and 10% for B. The differences in homogeneity of the purified peptides was most apparent on HPLC (see Figure 3).

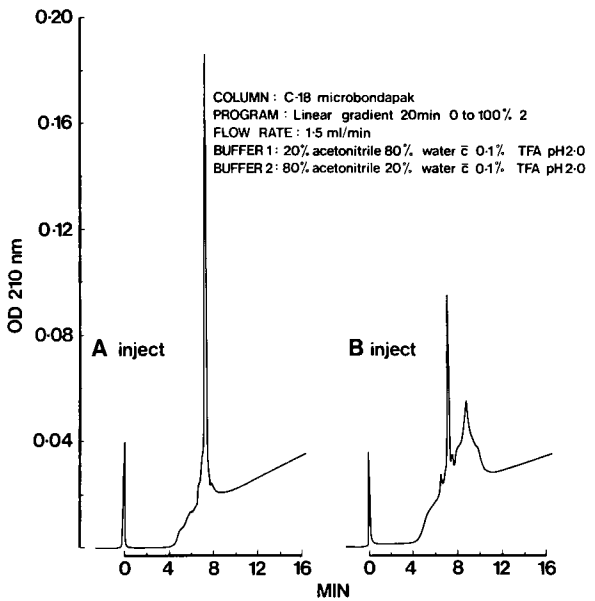

Fig. 3

The side products separated by ion-exchange chromatography of Synthesis A peptide were identified by compositional and Edman sequence analysis. Peak 1 was identified as fragment (72-84); peak 2 was found to be a mixture of (61-84), (62-84), (63-84) and (64-84); peak 3 (64-84); peak 4 was a mixture of the des-His^{63} analogue of (57-84) (58-84) and (59-84); peak 5 was found to be the Glu-anisole adduct of (53-84); peak 8 a mixture of (53-84), (54-84) and (55-84) and peak 9 predominantly (54-84). Peak 7 corresponded to authentic hPTH(53-84) which was recovered in 5% yield (relative to crude). A majority of the impurities isolated and identified are consistent with cyclization and chain termination at the Glu residues.

Conclusion

The use of standard carbodiimide-promoted coupling was found to be preferable to the symmetrical anhydride technique for the preparation of the peptide hPTH(53-84). The purified hormone fragment prepared by the standard method was found to be homogeneous and identical to native carboxyl-terminal hormone by multiple analytical criteria. The synthetic product of the symmetrical anhydride method was heterogeneous. Several practical advantages were noted for the standard method which were related to the increased efficiency of the coupling reaction. Since the use of symmetrical anhydride coupling has been an extremely successful approach to the synthesis of other peptides[3] this report indicates that the choice of symmetrical anhydride versus standard techniques may in large part be determined by the sequence selected for synthesis.

The major side product encountered, irrespective of coupling procedure, was cyclization and chain termination at glutamic acid. No significant improvement was obtained using the chlorobenzyl protecting group for Glu. Further investigation directed at minimizing side product formation will be required prior to undertaking syntheses of larger carboxyl-terminal fragments of PTH or the entire hormone molecule by the solid-phase stepwise elongation procedure.

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FURTHER APPLICATION OF FLUORENYLMETHOXYCARBONYLAMINO-ACIDS TO SOLID PHASE SYNTHESIS ON POLYAMIDE SUPPORTS

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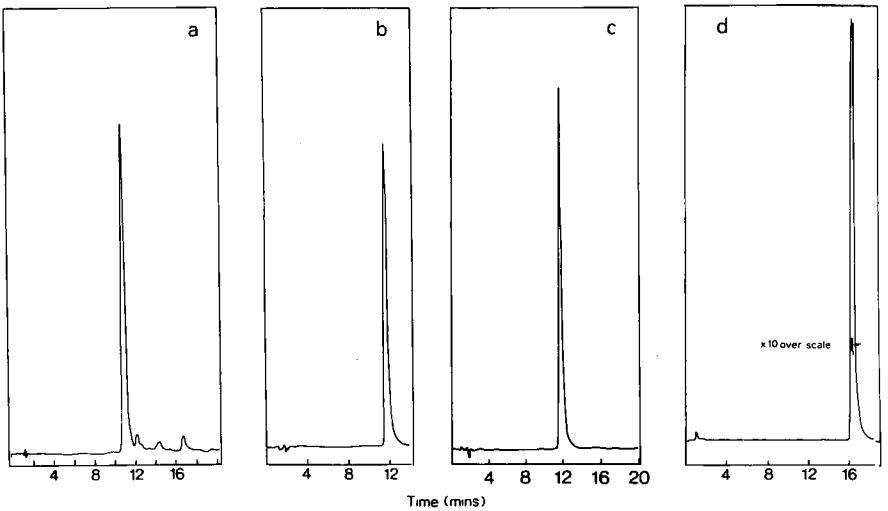
At the 5th Symposium we described[1] a synthesis of human β-endorphin using our polar, polydimethylacrylamide-based solid phase system.[2] Substantial progress has since been made in the further development of this method. A new, more easily prepared and finely beaded resin has been prepared[3] by suspension copolymerisation of the readily available monomers dimethylacrylamide, ethylene bisacrylamide, and acryloylsarcosine methyl ester. Carbomethoxy groups are converted to functional amine by reaction with ethylene diamine after polymerisation. Use of Fmoc-amino-acids has permitted synthesis under much milder reaction conditions than formerly, raising the yield of our standard test decapeptide to 74%[4] and of β-endorphin to 40%.[5] We now report further investigations regarding use of Fmoc-amino-acids in polyamide based syntheses with particular regard to (i) efficiency and product purity exemplified by new syntheses in the gastrin series; (ii) development of orthogonal protecting group combinations for fragment condensation strategies; and (iii) observations on use of the trifluoroacetyl group for the side chain protection of Fmoc-lysine.

(i) Syntheses of 12-leucine human minigastrin I and its des-Trp^{1}*-analogue* — The unfavorable amino acid composition of the gastrins has impeded application of conventional solid-phase methods involving strongly acidic conditions. Acidic treatments are minimised in the Fmoc-procedure[4] and we elected to investigate the stepwise synthesis of the 14-residue 12-leucine human minigastrin I (I), and also of its des-Trp^{1}-analogue in view of a recent comparative classical synthesis of the latter.[6] The new polydimethylacrylamide resin[3] was used to which was attached first an internal reference and spacer norleucine residue, then the *p*-hydroxymethylbenzoic acid linkage agent and the first amino-acid

residue of the sequence (Boc-phenylalanine). Subsequent *t*-butyl side chain protected Fmoc-amino-acids were added and Fmoc-groups cleaved using essentially the procedures described in our recent synthesis of substance P.[3] After the final glutamic acid, the resin was divided and one part terminated with Boc-leucine. The remainder was further extended with Fmoc-leucine and then terminated with Boc-tryptophan. Incorporation was stoichiometric throughout within the limits of resin-bound amino-acid analysis.



H.(Trp).Leu.Glu.Glu.Glu.Glu.Ala.Tyr.Gly.Trp.Leu.Asp.Phe.NH_2 (I)

Fig. 1. [a] Hplc of total crude 12-Leu-des-Trp-1-minigastrin I; [b] Purified product; [c] Classically synthesised 12-Leu-des-Trp-1-minigastrin I;[6] [d] Purified 12-Leu-minigastrin I.

All the *t*-butyl based protecting groups were removed from the resin-bound tridecapeptide by 90% trifluoroacetic acid. Ammonolysis of the unprotected, resin-bound peptide with methanolic ammonia was extended from 3 hours (when detachment was complete) to 16 hours to eliminate methyl ester formation. Hplc of the total crude product is shown in Figure 1a. Anion exchange chromatography gave 98% of the material in a single peak which was purified further by preparative hplc. The final product (Figure 1b) was obtained in 59% overall yield and was seemingly identical with classically synthesised material by hplc (Figure 1c), analytical ion-exchange chromatography, and other criteria. Excellent analytical data were obtained and the product was equiactive with the same molar quantity of natural human gastrin (heptadecapeptide amide) in stimulating gastric secretion in dogs equipped with gastric cannulae. 12-Leucine human minigastrin (I) was similarly obtained

except that the ion-exchange step was omitted. The yield of fully active tetradecapeptide amide (Figure 1d) was 51%.

(ii) Orthogonal group combinations utilising Fmoc-derivatives. — *t*-Butyl side chain and Nα-Fmoc protection provide a basis for true solid phase fragment condensation strategies. For the third element — the resin linkage — we envisaged use of benzyl ester derivatives specifically cleavable by hydrogenolysis.[7] Exploration of this system showed surprisingly that Fmoc groups are incompletely stable to catalytic hydrogenation.[8] We have therefore examined photolysis for detachment of protected peptides from polyamide resins.

The potential of the photo-labile 2-nitrobenzyl ester resin linkage[9] in polystyrene-based orthogonal systems was discussed at the last Symposium.[10] We felt that it might be particularly applicable to polyamide resins because of the formal absence of uv-absorbing groups in the polymer matrix. The new linking agent, 3-nitro-4-hydroxymethyl-benzoic acid was readily synthesised from 4-bromomethyl-benzonitrile and was incorporated into the polymer as its trichlorophenyl ester. Formation of the 2-nitrobenzyl ester bond to the functionalised polymer was straightforward using the acylamino-acid anhydride in the presence of dimethylaminopyridine. Irradiation of Boc-glycyl-nitrobenzylpolymer as a suspension in methanol using a medium pressure mercury lamp and $CuSO_4$ filter cleaved 83% of Boc-glycine after 29 hours. Similar irradiation of Fmoc-Leu-Ala-Gly-Val-polymer liberated the Fmoc-

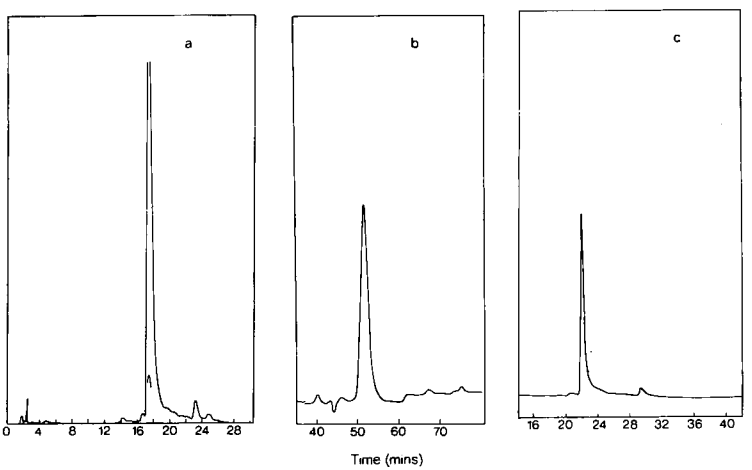


Fig. 2. [a] Hplc of crude Fmoc-Leu-Ala-Gly-Val-OH. [b] Ion-exchange chromatogram of Leu-Ala-Gly-Val from [a]. [c] Hplc of Fmoc-Glu(OBu^t)-Ala-Tyr(Bu^t)-Gly-OH.

tetrapeptide which was characterised by hplc (Figure 2a) and by base cleavage to the free tetrapeptide (Figure 2b) and dibenzofulvene, both identified with authentic samples. The yield in the photolysis step was 64-66% after 24 hours, not raised significantly on further irradiation. Similar irradiation of Fmoc-Glu(OBu^t)-Ala-Tyr(Bu^t)-Gly-polymer cleaved 53% of the more complex tetrapeptide (Figure 1c). These yields are only modest, but most importantly the byproducts of the photolysis reaction are almost completely retained on the resin, and the liberated protected peptides are obtained directly in a remarkably pure state (Figure 2a,c). The method would seem to have considerable potential and further work is in progress.

(iii) Use of the trifluoroacetyl group for the side chain protection of Fmoc-lysine. — On several occasions difficulties were experienced in the use of Fmoc-Lys(Boc)-OH for introduction of lysine residues.[1,3] Although we believe these difficulties have been due to the presence of hard-to-remove contaminants in some preparations of the protected amino-acid, we have also investigated the readily prepared Fmoc-Lys(Tfa)-OH as an alternative. The side chain trifluoroacetyl group [in Boc-Lys(Tfa)-OH] was completely unaffected on treatment with 10% piperidine in DMF over 24 hours, equivalent to 144 deprotection cycles. Fmoc-Lys(Tfa) was used without difficulty in two new syntheses of substance P giving results comparable to those obtained previously.[3]

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THE USE OF N-CARBOXYANHYDRIDES OF α-METHYLAMINO ACIDS IN THE SOLID PHASE METHOD: THE SYNTHESIS OF [α-(^{13}C)METHYL-PHENYLALANINE^4]-ANGIOTENSIN II

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An indirect approach to the elucidation of receptor-bound conformation of small and medium size peptides has been proposed[1] based on the introduction of α-methyl analogs of amino acids which result in dramatic restriction of the conformational freedom within the peptide at that residue. If such an analog displays biological activity comparable with the parent compound, the biologically relevant conformation is partially defined.

The synthesis of peptides containing α-methylamino acids is not easy, however, because the α-methyl group diminishes dramatically the intrinsic reactivity of the amino acid moiety due to additional steric constraints.[2] Consequently, yields of the particular synthetic steps involving α-methylamino acids are usually low.

This communication presents an approach which, at least in part, overcomes the difficulties mentioned above. The method employs the N-carboxyanhydride (NCA) of α-methylphenylalanine as an active reagent and the preparation and utilization of *D,L*(α-^{13}C-methyl)phenylalanine, *D,L*-Phe(α-Me-^{13}C), in peptide synthesis.

The principal goal was the synthesis of [α-(^{13}C)methyl-phenylalanine^4]-angiotensin II, [Phe(α-Me-^{13}C)^4]-AII, for the purpose of elucidation of the backbone conformation within the Phe(α-Me) moiety by exploitation of normally not available ^{13}C-^{1}H vicinal coupling constants. Consequently, the most efficient way of incorporation of the

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precious Phe(α-Me-^{13}C) unit into the peptide sequence was desired. *D,L*-Phe(α-Me^{13}C) was prepared from phenylalanine (Fluka) and (^{13}C)methyliodide (Prochem, 90% ^{13}C-enrichment) by a modification of the procedure described[3] in 50% overall yield. As an active form of an α-methylamino acid, we decided to use its NCA. *D,L*-Phe(α-Me-^{13}C)-NCA was obtained by reaction with phosgene in 80% yield. NCAs of amino acids have been long used in the synthesis of homopolypeptides due to their high tendency to polymerize.[4] Only during the past decade, however, the successful general use of them in the peptide synthesis had been reported.[5] The procedures described require relatively rigorous restrictions regarding such conditions as solvents, pH, temperature and time.

We assumed that the chain propagation might not occur when using an NCA of an α-methylamino acid. Indeed, the model synthesis of a dipeptide Phe(α-Me)-Val-OBu^t, which was to be further incorporated into the sequence of [Phe(α-Me)^4]-AII, showed that the assumption was right. Phe(α-Me)-NCA and H-Val-OBu^t × HCl were reacted in the presence of 2.1 mol excess of N-methylmorpholine in THF or CH_2Cl_2 at –40°C allowing the reaction mixture to warm up slowly during the next 3-4 hours. The homogeneous dipeptide was obtained repeatedly with about 80% yield. Moreover, when using *D,L*-(Phe(α-Me)-NCA we isolated homogenous *L*-Phe(α-Me)-Val-OBu^t from the diastereoisomer mixture by simple crystallization. However, further attempts to incorporate the Phe(α-Me)-Val-OBu^t into the desired sequence [Phe(α-Me)^4]-AII failed due to low yields of the consecutive reactions although we obtained Z-Val-Phe(α-Me)-Val-OBu^t as well as Val-Phe(α-Me)-Val-OBu^t with fairly good yields.

Consequently, we opted for the solid phase synthesis as an alternative. The C-terminal tetrapeptide BOC-Val-His(Tos)-Pro-Phe- P was prepared by the established procedure using DCC/HOBt coupling method in CH_2Cl_2-DMF (3:1) and the customary cycle of operations.[6] Next, the deprotected tetrapeptide-polymer was reacted with the 1.5 molar excess of Phe(α-Me)-NCA in CH_2Cl_2 as described above. Following thorough washing with CH_2Cl_2, we continued solid phase synthesis but now used 6-fold excesses of the two next Boc-amino acids and DCC, and 9-fold excesses of HOBt in the following steps. Each coupling was repeated twice. The incorporations of Boc-Asp(Bzl)-OH was carried out in regular way. The peptide ws simultaneously cleaved from the polymer and deprotected by the HF method. The crude peptide

was purified by 200 transfers in BAW (4:1:5) (K=0.41) countercurrent distribution (CCD) system yielding 41% of pure material based on the Phe attached to the polymer. The obtained peptide showed the same characteristics (amino acid analysis, tlc, NMR, CD) (Table I) as the one

Table I. Properties of Angiotensin Analogs

	CCD; K[b]	TLC[c]	CD Band[d] λ[nm]/[Θ] $\frac{deg \cdot cm^2}{dmol}$
[Phe(α-CH_3)^4] AII[a]	0.41	0.36	228/-2200
[Phe(α-CH_3)^4] AII	0.40	0.36	227/-2100
[Phe(α-$^{13}CH_3$)^4] AII	0.41	0.36	227/-2000
[D-Phe(α-$^{13}CH_3$)^4] AII	0.77	0.51	e

[a]Prepared by J. Turk in this laboratory (J. Turk, P. Needleman, G.R. Marshall, *Molecular Pharmacology*, **12**, 217-224 [1976]).
[b]n-Butanol:acetic acid:water, 4:1:5
[c]n-Butanol:pyridine:acetic acid:water, 15:10:3:12
[d][Θ] per residue, accuracy $\pm 150 \frac{deg \cdot cm^2}{dmol}$
[e]not inspected

from the previous preparation in our laboratory.[2a] In its ^{1}H NMR spectrum the ratio of CH_3 proton signal of Phe(α-Me) to the CH_3 proton signal of Val corresponded to 1:2 ratio of Phe(α-Me) to Val residues. The synthesis of [Phe(α-Me-^{13}C)^4]-AII was accomplished as described above except that the *D,L*-Phe(α-Me-^{13}C)-NCA was used rather than *L*-Phe(α-ME-^{13}C)-NCA. After 360 transfers in BAW (4:1:5) CCD system, the contents of 77-100 tubes was collected giving 36% of the theoretical yield of [*L*-Phe(α-ME-^{13}C)^4]-AII. The [*D*-Phe(α-Me-^{13}C)^4]-AII was also collected (tubes 141-173, K=0.77) with the yield equal 48%.

As a conclusion we suggest that N-carboxyanhydrides of α-methylamino acids may be of general utility in both solid phase and solution syntheses as acylating agents.

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PEPTIDE SYNTHESIS IN WATER AND THE USE OF IMMOBILIZED CARBOXYPEPTIDASE Y FOR DEBLOCKING

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Introduction

We have developed a method for peptide synthesis in aqueous medium using a water soluble handle, carboxymethyl poly(ethylene glycol)(CM-PEG). Chain elongation and deprotection steps are carried out in water at pH 6.0 and 8.5 respectively. Amino acid ethyl esters are coupled to the C-terminus of the growing peptide chain using ethyldimethylaminopropyl carbodiimide (EDC). Carboxpeptidase Y (CPY) immobilized on Sepharose is used for deblocking of the ethyl ester group at pH 8.5. Peptide elongation is carried out on CM-PEG-Gly-Met or CM-PEG-Phe-Met and the finished peptide is released from the handle by treatment with cyanogen bromide.[1]

CPY is a metal-free carboxypeptidase from yeast with broad specificity.[2] In addition to exopeptidase activity at acid pH, the enzyme is an effective esterase at alkaline pH.[3] *N*-α-Acetyl-L-tryosine ethyl ester is hydrolyzed faster by CPY than by chymotrypsin. Using immobilized CPY(I-CPY), N-blocked L-amino acid or peptide ethyl esters can be deblocked at the C-terminus without any side reactions as shown in Table I.[4] Further, as indicated by the results in Table I, D-amino acid at the C-terminus prevents ester hydrolysis by I-CPY. This assures optical purity of the final product. Use of I-CPY for ester deblocking also prevents many side reactions previously reported in the literature.[5]

Table I. Deblocking of Peptide Ester by I-CPY

Substrate	Deblocking conditions	Yield (%) [a]	[b]	Peptidase activity (%)[c]
Z-Leu-Phe-Leu-OEt	20 mg in 200 ml 25% Dioxane	100	(87)	0.45
Z-Asn-Phe-OEt	20 mg in 160 ml 25% MeOH	100	(95)	0.44
Boc-Asp(OBzl)-Phe-OEt	10 mg in 15 ml 20% MeOH	97	(84.3)	0
Z-Phe-D-Ala-OEt	10 mg in 48 ml 25% DMF	No base consumption, no reaction		

[a] Calculated from base consumption. [b] Calculated from isolated product. [c] As seen on the amino acid analyzer.

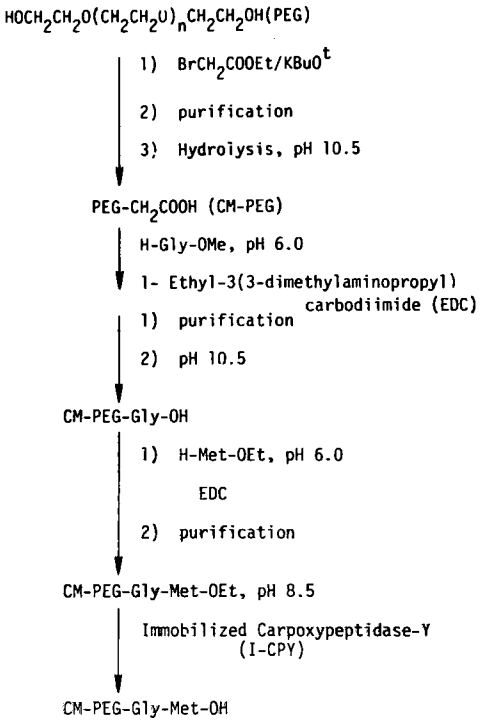

Fig. 1. Preparation of the Handle

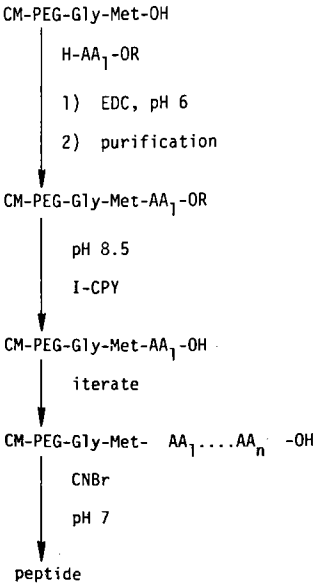

Fig. 2. Chain Elongation and Release

The advantages of a polymeric handle in peptide synthesis have been previously reported.[6] For our purpose, PEG is derivatized and used for peptide synthesis as shown in Figure 1. Steps involved in peptide synthesis and the release of the peptide from the handle are shown in Figure 2. Derivatization of PEG, condensation of Phe-OEt to CM-PEG and use of I-CPY for deblocking are described below:

PEG (M W 6000-7000, MCB, 14g) and potassium *tert*-butoxide (10g) were dissolved in *tert*-butanol (150 mL) by warming to 40°C. Ethyl bromoacetate 5 mL) was added over a period of 10 minutes. After stirring for 2 hours solvent was evaporated. The residue was dissolved in 100 mL of 2N NaOH. After 2 hours at room temperature, the pH of the reaction mixture was adjusted to 2. CM-PEG was extracted into $CHCl_3$ (two 200 ml portions). After drying over Na_2SO_4, $CHCl_3$ was evaporated to yield 12g of CM-PEG.

H-Phe-OEt·HCl (2.3g) and CM-PEG (3.4g) were dissolved in 35 mL of H_2O. The pH was adjusted to 6.0 with NEt_3· EDC(2.0g) was added, and the pH was maintained by automatic addition of 0.1N HCl. After 3 hours at room temperature, the reaction mixture was acidified to pH 2 and passed through a column of Dowex-50 (42x3.8cm, flow rate 1ml/minute, monitored by reading absorbance at 257nm). Alternatively, PEG-Peptide was extracted into 250 ml of $CHCl_3$ and the solvent evaporated (yield, 3.3g).

The ethyl ester group was removed by immobilized chymotrypsin[7] at pH 8.0. Both base uptake and amino acid analysis indicated the presence of 100 nmoles of the phenylalanine per mg of CM-PEG.

H-Met-OEt was coupled similarly and CM-PEG-Phe-Met-OEt was subjected to treatment with I-CPY at pH 8.5. The details of synthesis of H-Leu-Phe-Leu-OEt and H-Ala-Ala-Cys(Bzl)-Lys(Z)-OEt on this handle are presented in Table II. Thus side chain protection could be conveniently used in our procedure of peptide synthesis.

Table II. Peptides Synthesized

Peptides Synthesized	Yield[a]	Rf[b]	Amino acid analysis
H-Leu-Phe-Leu-OEt	61%	0.8	a. Acid hydrolysis 2.10 Leu; 1.00 Phe b. I-APM digestion 2.00 Leu; 1.00 Phe
H-Ala-Ala-Cys(Bzl)-Lys(Z)-OEt	45%	0.52	Ala 2.03, Lys 1.00, Cys(Bzl) 1.08
H-Ala-Ala-Cys(Bzl)-Lys(Z)-Asn-Phe-OEt	To be characterized.		

[a] Based on amino acid analysis of the Phe content on CM-PEG-Phe
[b] Rf on TLC (silica gel) n-BuOH:AcOH:Water::4:1:5

The question of racemization arises when chain elongation is done with the C-terminus of growing peptide chain. The generally accepted mechanism for racemization is the formation of azlactone and its deprotonation.[8] We reasoned that this deprotonation would not be significant at pH 6.0 in water. Z-Leu-Phe-Leu-OEt made by our procedure had optical rotation identical with that of the product obtained by the excess mixed anhydride procedure.[9] Base uptake during deblocking steps matched the predicted amount, amino acid analyses of samples prepared by acid and enzymatic hydrolysis of H-Leu-Phe-Leu-OEt were in agreement. These data strongly suggest that our method yields peptides of high optical purity. For a more sensitive test for racemization, we adopted the method of Kitada and Fujino.[10] Leu-Leu prepared by our procedure showed less than 0.7% of D-Leu-L-Leu isomer on a column of Beckman AA-15 which can separate D-Leu-L-Leu from L-Leu-L-Leu.

Conclusions

1. Harsh conditions normally used in peptide synthesis can be avoided.

2. All operations are carried out in water near neutral pH thus eliminating many side reactions.

3. C-Terminal deblocking of peptides made by conventional approaches can be accomplished using immobilized CPY without any side reactions even when Asn and/or Asp(Bzl) are present in the sequence.

Acknowledgement

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A PHENOLIC POLYMER: A NEW TOOL FOR THE PREPARATION OF PROTECTED PEPTIDE FRAGMENTS AND FOR THEIR CONSECUTIVE CONDENSATION: AN EXAMPLE LH-RH

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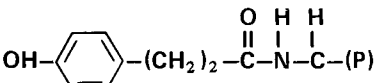
Introduction

p-hydroxyphenyl-propionic-resin was used to prepare the 1-6 protected fragment of LH-RH.



(P=polystyrene 1% divinylbenzene)

1 5 10
p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

By means of benzotriazolyl-N-oxy trisdimethyl amino phosphonium hexafluorophosphate (BOP) as coupling reagent[1], the fragment 1-6 was then condensed to the fragment 7-10 prepared on the same polymer.

The peptidyl-resin conjugate was then separated in two aliquots in order to obtain: 1 — LH-RH after aminolysis and treatment with liquid hydrogen fluoride. 2 — LH-RH COOH after saponification followed by a hydrogenation or a treatment with liquid hydrogen fluoride.

Results

Stepwise elongation of each fragment was performed by the DCC-HOBt method according to the traditional solid phase procedure. To prevent any risk of transesterification dry acetone or *t*-butanol in methylene chloride was used to shrink the resin.

The 1-6 Protected Fragment was split from the matrix according to

the following procedures. 1 — Saponification in dimethylformamide with sodium hydroxide, or 2 — Transesterification in dimethylformamide with dimethylamino-ethanol followed by hydrolysis as described by Barton.[2]

After purification on Sephadex LH 20 with dimethylformamide as an eluent, the 1-6 fragment was coupled to 7-10-peptidyl resin with BOP. This coupling reagent is much more handy to use than the mixture DCC-HOBt due to: — avoidance of dicyclohexylurea formation, — solubility of each by products, and — possibility to reuse the reaction mixture until the end of the coupling.

1 — LH-RH (Luliberin)

Aminolysis in DMF split the peptide from the resin. Reaction was completed within 6 hours. Side chain function protecting group removal was performed by liquid HF and gave crude LH-RH which was analyzed by HPLC and compared to crude LH-RH prepared stepwise on benzhydrylamine resin. Comparison of elution profiles clearly shows the improvement brought by this new strategy (Figure 1). LH-RH was purified by two successive gel sievings. Each chromatography was controlled with HPLC with regard to standard LH-RH in order to select fractions containing this releasing factor (Figure 2).

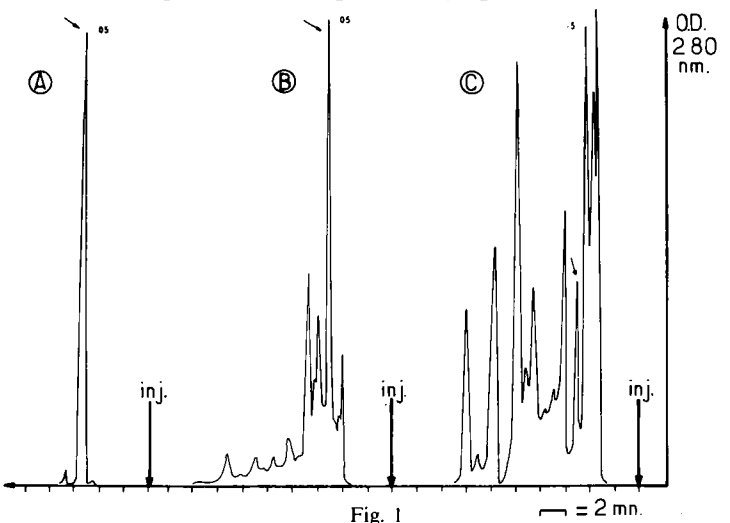


Fig. 1

A — Standard LH-RH.
B — Crude LH-RH prepared with phenolic resin by fragment condensation.
C — Crude LH-RH prepared stepwise on benzhydrylamine resin.

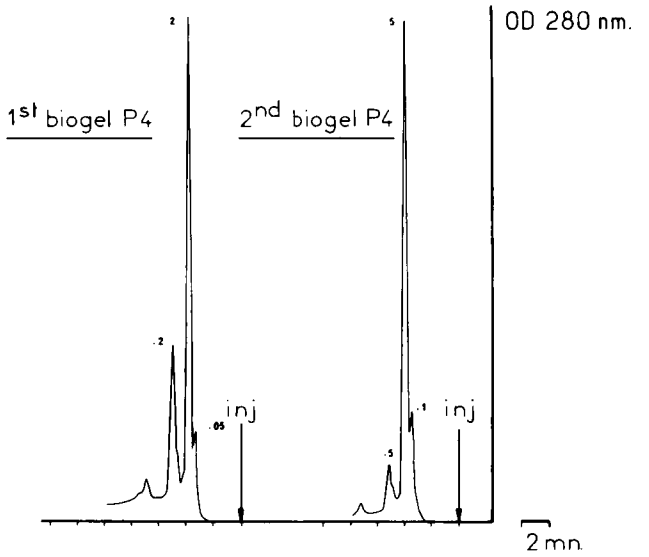


Fig. 2. Purity control of LH-RH prepared on phenolic resin after lyophilization of selected fractions obtained by two successive chromatographies on biogel P_4.

μ Bondapak C_{18}(L = 60 cm ϕ 4 m) 1.5 ml/mn 2500 psi solvent HCOOH 0,25 N + Et_3N pH 3, CH_3 CN 7- : 30 V/V.[3]

2 — LH-RH-COOH

After saponification protecting groups were removed by hydrogenolysis or treatment with hydrogen fluoride. Both products obtained were purified by two successive gel sievings and were chromatographically and analytically identical.

Biological assays

In radioimmunoassay system (Figure 3A) the synthetic decapaptide (x—x) and a hypothalamic extract (•—•) showed parallel curves to the standard LH—RH (•—•).[4,5]

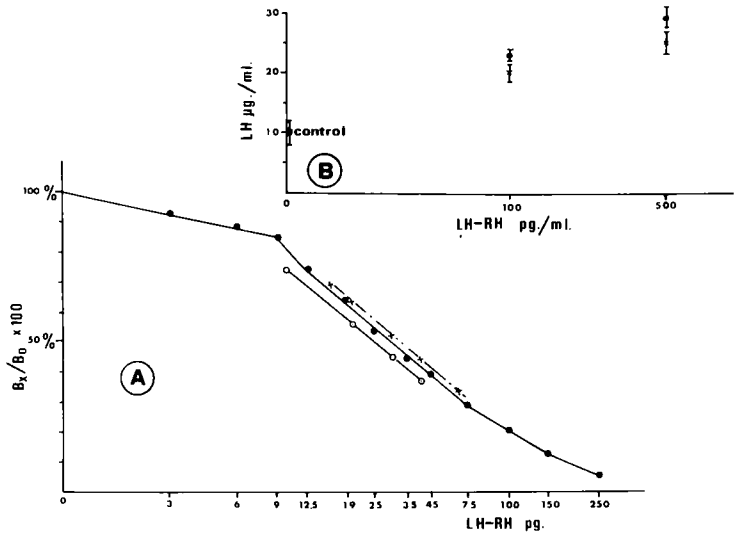


Fig. 3

In bio-assay system (Figure 3B), the standard LH-RH • and the synthetic decapeptide × released similar amounts of LH.

Conclusion

The decapeptide synthetized on phenolic resin is identical to endogenous and synthetic LH-RH. This resin is therefore a convenient and rapid tool to prepare protected fragments as building units for peptide synthesis, and offers the following advantages: 1 — According to the method of cleavage the peptide can be obtained in its amide (aminolysis) or carboxylic form (saponification). 2 — The disadvantage of liquid hydrogen fluoride treatment can be avoided in many cases. 3 — The resin may be reused indefinitely since elimination of the peptide chain is complete by saponification.

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IMPROVED PREPARATION OF ACID STABLE SUPPORTS FOR SOLID-PHASE PEPTIDE SYNTHESIS

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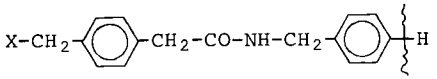
We have been engaged in synthesizing and studying the immunological usefulness of amino terminal peptide sequences of Type C RNA tumor virus p30 antigens.[1] The following is a report on the synthesis of the 1-28 sequence (1) of the p30s of Gibbon ape leukemia virus (GALV) and Simian sarcoma associated virus (SSAV), isolated from Woolly monkey.[2]

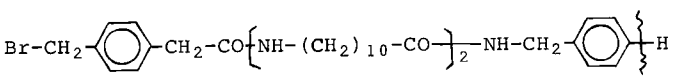
Pro-Leu-Arg-Ala-Ile-Gly-Pro-Pro-Ala-Glu-Pro-Asn-Gly-Leu-Val-Pro-Leu-Gln-Tyr-Trp-Pro-Phe-Ser-Ser-Ala-Asp-Leu-Tyr

1

For the synthesis of this octacosapaptide (1) the acid stable Pam-(2a)[3] and Sparrow-resins (3)[4] promised to prevent any significant acidolytic peptide loss from the solid support.



2a: X = Br; 2b: X = OH

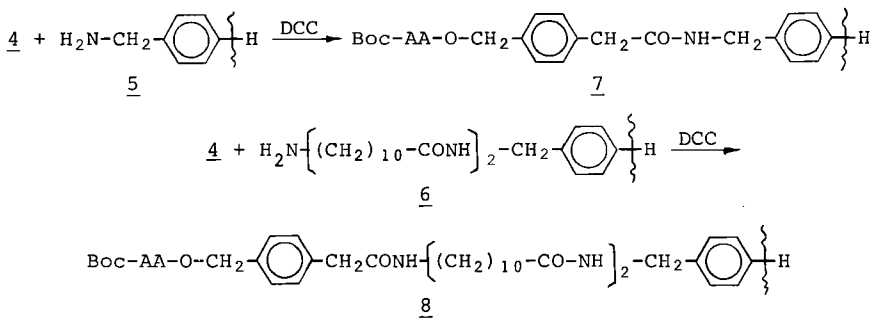


3

In order to avoid the undesired N-benzylation which is inherent in the synthesis of these resins,[3] the 4-(hydroxymethyl)-phenylacetamido-methyl-polystyrene support (2b) was developed by the Merrifield school.[3]

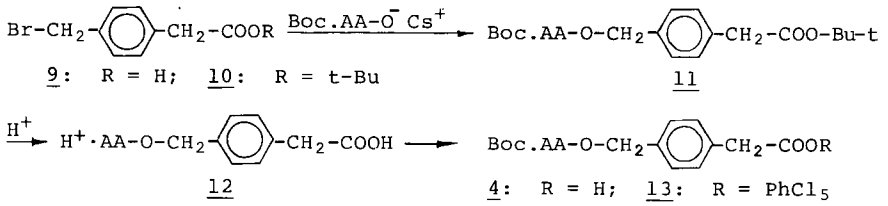
As an alternate route to a by-product free Boc-aminoacyl-4-(oxymethyl)-Pam resin (7)[3] and Boc-aminoacyl-4-(oxymethyl)-phenyl-acetamido-bis-(undecanoylamido)-methyl resin (8)[4] an unambiguous synthesis of key-intermediate Boc-aminoacyl-4-(oxymethyl)-phenyl-acetic acid (4)[3] was developed (Scheme I).

A DCC activated coupling of 4 to the amino-methyl-polystyrene resin (5)[3,5] or amino-bis-(undecanoylamido)-methyl-polystyrene support (6)[4] led to the acid stable Boc-aminoacyl-4-(oxymethyl)-Pam-(7) and Boc-aminoacyloxy-Sparrow-resins (8), respectively.



Derivative 4 and its active ester (13), prepared from Tyr-(OBzl-2, 6-Cl_2) were coupled to 5 and 6 and depending upon the reaction conditions 0.16-0.55 mmole/g resin incorporations were attained.

The synthesis of 4 derivatives is outlined in Scheme I. Sulfuric acid catalyzed esterification of 4-(bromomethyl)-phenylacetic acid (9) with isobutylene in dichloromethane produced its t-butyl ester (10) in 71% yield (m.p. 42-43°; λ_{max}^{neat} 1720 cm^{-1}). Cesium-salt of Boc-amino acids[6] with 10 provided diesters (11) in > 90% yields. Trifluoroacetic acid (40% in CH_2Cl_2 30 minutes) hydrolyzed both *t*-butyl groups leading to the amino acid salts (12) in good yields. Then, *t*-butyloxycarbonylation of 12 produced the desired 4 derivatives in excellent yields. Some of these products were transformed into their active esters (13).



Scheme I

The following derivatives of 4 were prepared (AA., R, m.p., yield %): Ala (H, 100-101, 100); Glu-(γ-OBzl) (H, 114-116, 91); Gly (H, 113-114, 76); Leu (H, liquid, 75); Leu (PCP[a], 86-87.5, 67); Pro (H, 72-73, 93); Pro (DCHA salt, 110-112, 90); Thr-(OBzl) (H, 90-91, 60); Tyr-(OBzl-2, 6-Cl_2) (t-Bu, 56-57, 88); Tyr-(OBzl-2, 6-Cl_2) (H, 121-122, 95); Tyr-(OBzl-2, 6-Cl_2) (TCP[b], 119-121, 85); Trp (H, 48-51, 92) and Val (H, 63-64, 73); (a = pentachlorophenyl; b = 2,4,5-trichlorophenyl). All derivatives showed correct elemental analyses and the expected ir, nmr spectra.

For the synthesis of the octacosapeptide (1) 5 g Boc-Tyr-(OBzl-2, 6-Cl_2)-4-(oxymethyl)-Pam resin (7) was prepared (0.37 mmole Tyr/g.). The unreacted amino sites were blocked by acetic anhydride-pyridine mixture (1:1;3 hours). For the synthesis of 1 the following amino acid derivatives were applied: Boc-Ala, Boc-Arg-(N^g-tosyl), Boc-Asn-OTCP, Boc-Asp-(OBzl-4-Cl), Boc-Gly-OTCP, Boc-Gln-OPCP, Boc-Glu-(γ-OBzl), Boc-Ile, Boc-Leu, Boc-Phe, Boc-Pro, Boc-Ser-(OBzl), Boc-Trp-(N^{in}-formyl), Boc-Tyr-(OBzl-2, 6-Cl_2) and Boc-Val.

Deblocking reactions were performed by 50% TFA-CH_2Cl_2 mixture (30 minutes). For the coupling reactions 2 mole excess of symmetric anhydrides[7] were used, unless the application of active esters were warranted (Gly, Gln and Asn).

In order to select the most available Tyr sites for further peptide growth, half amount of Boc-Leu anhydride was coupled to the Tyr-charged resin during the second cycle of the synthesis. Thus, a total of 0.85 mmole Leu-Tyr dipeptide resin load was assured. The uncoupled Tyr sites were terminated with 0.1 M 3-nitrophthalic anhydride-pyridine (30 minutes).[8] The same reagent was used for all termination steps after the completion of every coupling step which was monitored by the fluorescamine test.[9]

After the asssembly of the twenty-eight amino acid residues, (3.6 g weight increase; 94%), the peptide was cleaved from the resin in liquid HF-anisole mixture at 0°C/1 hour. Water-acetic acid mixture (85:15; 350 ml) extracted 2.14 g peptide from the resin. Then, the Trp residue of the product was N-deformylated (liq·NH_3/$H_3N^+OHCl^-$). The crude peptide mixture was stirred in 60 ml distilled water and the insoluble peptide fraction was purified on a low-pressure AG-1-X2 (acetate) column, using a water-acetic acid (9:1) gradient which eluated 1.06 g (0.34 mmole) octacosapeptide (1) (yield 40%, calculated on the original 0.85 mmole peptide load). The pure peptide (1) showed the following correct analysis: Ala 3.03, Arg 0.99, Asx 2.18, Gly 2.05, Glx 2.22, Ile 0.94, Leu 3.94, Phe 0.98, Pro 6.09, Ser 1.88, Trp 0.99, Tyr 2.00 and Val 1.00.

The immunological study of the octacosapeptide (1) is underway.

Acknowledgement

This work was supported by Contract N01-CP-53530 from the Special Virus Cancer Program, National Institutes of Health-National Cancer Institute, Bethesda, Md. One of us (S.I.S) was also generously supported by the Allen and Huntington County Cancer Societies, Indiana. The amino acid analyses were performed by Prof. Marc Loudon's group at Purdue University. The technical assistance of Ms. Gail Kerchner is greatly appreciated.

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CARRIER MEDIATED SYNTHESIS OF N^G-ALKYL ARGININES AND THEIR PEPTIDES

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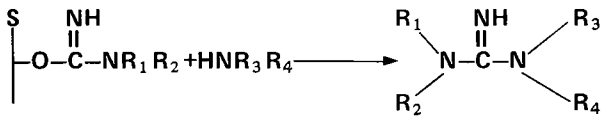
N^G-MeArg, N^G,N^G-Me_2Arg and N^G,N^G-Me_2Arg occur in several proteins among which are myelin basic protein, histones, myosin, actin and ribosomal proteins.[1] They are also present in the free state of blood and urine.[2] The wide distribution of these derivatives indicate that they have an important biological role.

Recently we have shown that the product of the reaction between polysaccharides and cyanogen bromide results in cyanoesters (R-O-C ≡ N).[3] Reaction of these esters with amines gives polymeric N-substituted isoureas (pseudourea RO-C(=NH)—NR_1NR_2)[4] (Figure 1). Since methylpseudoureas were used to convert ornithine to arginine and to their guanidomethyl derivatives[1,2], we assumed that the polymeric pseudoureas should also be useful for the same purpose. Moreover it can be used for synthesis of any nonsymmetrical or symmetrical guanidines which by other methods can only be done with great difficulty if at all, since any amine can be coupled to the polysaccharide and any amine can be used to remove it from the polymer.



Where R_1 R_2 R_3 and R_4 = H or $(CH_2)_n$–CH_2X; X=H or other functional groups.

Up to date no methods have been published for the synthesis of N^G-alkyl arginines except for methyl derivatives, the synthesis of which is quite complicated and requires more than a week for reaction and subsequent purification.[1,2]

In this paper we describe the synthesis of different N^G-alkyl arginines and N^G-alkyl homoarginines from ornithine, Table I, and lysine coupled to polysaccharides, respectively. The reaction is fast and the yields are high. The method is not suitable for the preparation of N^G,N^G-alkyl derivatives.

Table I. Synthesis and properties of N^{G}-alkyl arginines

Derivative[a]	Ornithine bound[b] µmole/g	Release with amine: Derivative µmole/g	Release with amine: Ornithine[c] µmole/g	Yield of derivative %	R_f[d] on electrophoresis pH 3.5	Retention time on A.A.A.[e] min
Arg	74.3	49.5	6.9	66.4	1.00	50
N^{G}-Me-Arg	72.8	48.2	7.5	66.2	0.95	48
N^{G},-N^{G}-Me_2-Arg	66.3	46.2	7.3	69.7	0.90	48
N^{G}-Et-Arg	68.4	43.6	2.9	63.6	0.90	60
N^{G}-Bu-Arg	58.6	38.2	5.4	66.2	0.83	97
N^{G}-He-Arg	78.6	51.3	7.4	65.2	0.76	132

[a] Abbreviation used: Et = Ethyl, Bu = Butyl, He = Hexyl, A.A.A. = Amino acid analysis.
[b] Each experiment was performed on newly coupled ornithine to cellulose.
[c] Product of hydrolysis.
[d] The electrophoresis was performed in pyridine-acetate buffer pH 3.5 for 35 minutes at 70V.
[e] On the short column in sodium citrate buffer pH 5.28.

The polysaccharides, agarose, Sephadex and cellulose were activated with cyanogen bromide under conditions which we found to give the highest activation yield.[3] Excess copper complex of ornithine and lysine were coupled to the activated polymers. The use of copper complex enabled us to isolate pure arginine derivatives without the use of protecting groups. The excess of the copper complex was used in order to receive maximum coupling yield. The excess complex was not lost since after the reaction it was filtered off, the amount of complex left was determined and used again. The use of the copper complex also enabled the fast determination of the amount of ornithine coupled by copper analysis which was verified also by amino acid analysis. The copper was removed by washing of the polymers with acetic acid or hydrochloric acid. Incubation of the ornithine-containing polymers with different amines for 24 hours at 55°C gave the required arginines in high yield. No racemization was detected in the arginines synthesized. The polysaccharides can be used again for the complete cycle of reactions. The cellulose was used at least three times. The overall reaction is summarized in Figure 1 which describes the particular case of synthesis of N^G-Me-L-arginine and homoarginine. This procedure was also used for the synthesis of agmatine, creatin, α-guanido amino acids and other guanidines.

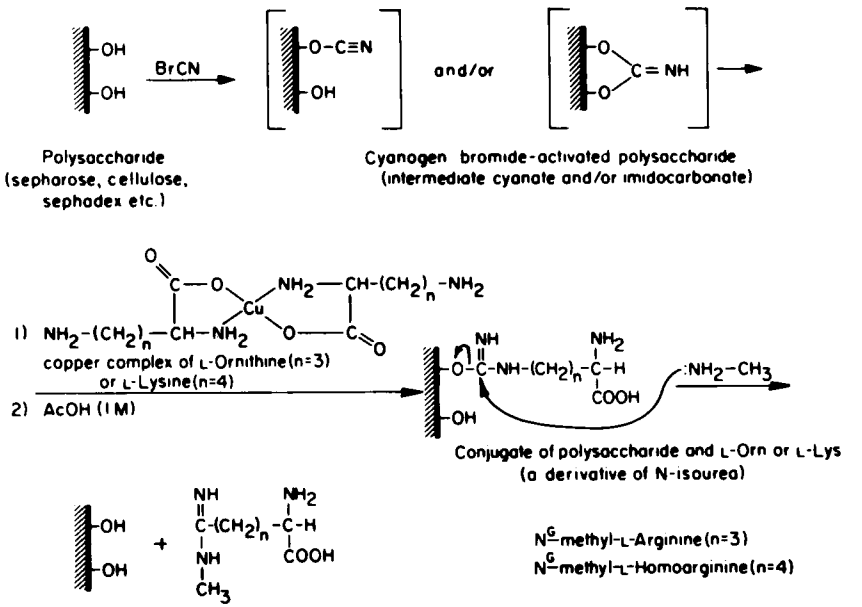


Fig. 1. Mechanism of activation of polysaccharides and the synthesis of arginine derivatives.

Coupling of peptides through a free δ-amino group of ornithine or ε-amino group of lysine in which the α-amino group is protected, followed by reaction with different amines, enabled the preparation of up to now unknown N^G-alkyl arginine peptides in high yield. The advantage of these peptides is that the charge of the guanido group is not drastically altered even though alkyl groups with different functions were introduced. These derivatives will enable the study of the importance of guanido groups in biologically active peptides. Coupling of proteins to polysaccharide, followed by reaction with amines, enabled the introduction of N^G-alkyl homoarginine into proteins. Their effect on activity and structure is now being studied.

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A CHLORANIL COLOR TEST FOR MONITORING COUPLING COMPLETENESS IN SOLID PHASE PEPTIDE SYNTHESIS

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Introduction

Chloranil, 2,3,5,6-tetrachloro-1,4-benzoquinone, has been used for qualitative[1] as well as quantitative determination of amines.[2] Several qualitative tests for checking the coupling completeness in solid phase peptide synthesis have been reported.[3–5] However, to carry out these tests, the reaction mixture has to be completely removed from the resin. This paper describes a qualitative test for coupling completeness, which can be carried out in the presence of the reaction mixture as well, based upon the reaction of chloranil with free amino groups. If free amino groups are present, a green to blue color develops on the resin in less than five minutes. The test can also be used, when the N-terminal amino acid residue is proline, where other qualitative tests fail[4] or are unsatisfactory. The sensitivity is in the order of 5 μmole/g resin, which is comparable to the sensitivity of the ninhydrin test. By applying the chloranil test during the synthesis of a hexapeptide different coupling rates at different stages of the synthesis are demonstrated. The test is also used with success to follow DCC couplings with trifunctional amino acids.

Experimental and Results

Procedure for the chloranil test. — 1. The chloranil reagent is made by preparing a saturated solution of chloranil in toluene. 2. ca. 1 mg of peptide resin is transferred to a test tube (50 x 6 mm). 3. a) For the detection of secondary amino groups 200 μl acetone is added. b) For the detection of primary amino groups 200 μl acetaldehyde is added. 4. 50 μl chloranil reagent is added and the test tube is swirled 5 minutes at room temperature. If free amino groups are present, a green or blue color is formed on the resin beads.

Syntheses were performed on an automated peptide synthesizer with feed back control based upon perchloric acid titration.[6] Boc-amino acids and DCC were dissolved in methylene chloride. The Boc-group was removed by 1 hour treatment with 1 N hydrogen chloride in acetic acid/methylene chloride, 9 : 1 (v/v). Boc-amino acid resins were prepared using the cesium salt method.[7] In order to evaluate the sensitivity of the method, different peptide resins were synthesized by coupling Boc-Pro-OH to H-Ala-Gly-O-S-X2 and Boc-Ala-OH to Pro-Gly-O-S-X2, using DCC in various degrees of deficiency. The results for the coupling of Boc-Ala-OH to Pro-Gly-O-S-X2 are shown in Table I.

In a second experiment H-Gly-O-S-X2 and H-Ala-Gly-O-S-X2 were coupled with 0.01 equivalents Boc-Ala-OH and Boc-Pro-OH, respectively, using DCC in excess. After 15 minutes the free amino groups were acetylated quantitatively, which rendered the chloranil test negative. After removal of the Boc-groups, neutralization with triethylamine, and 6 times washing with methylene chloride, the chloranil test was positive for both peptide resin samples.

Table I. Comparison of the chloranil test with the ninhydrin test for the coupling of Boc-Ala-OH to Pro-Gly-O-S-X2, and quantitative determination of the coupling yields by amino acid analysis and perchloric acid titration.

			mole Ala/mole Pro	
chloranil test	ninhydrin test		amino acid analysis	perchloric acid titration
strongly positive	resin: dark brown	liquid: blue	0.69	0.71
strongly positive	resin: light brown	liquid: yellow	0.87	0.89
positive	resin: light brown	Liquid: yellow	0.97	0.95
slightly positive	resin: light brown	liquid: yellow	0.97	1.00
negative	resin: yellow to brown	liquid: yellow	1.01	1.00

A quantitative evaluation of the results showed, that the sensitivity of the chloranil test is ca. 2-3 μmole/g resin for N-terminal proline and ca. 5-8 μmole/g resin for N-terminal glycine and alanine, when 1 mg peptide resin is used. The applicability of the chloranil test for solid phase peptide synthesis was further demonstrated during the synthesis of the hexapeptide resin, Ala-Val-Pro-Pro-Phe-Gly-O-S-X2. Couplings were performed in methylene chloride with Boc-amino acids in fourfold excess and DCC in twofold excess. The chloranil test was employed at different time intervals during the couplings. When a coupling was considered complete based upon a negative chloranil test, the coupling was discontinued and after thoroughly washing of the resin the ninhydrin test was performed. In all cases the ninhydrin test turned out to be negative as well. The results of this experiment are shown in Table II.

Table II. Application of the chloranil test during the synthesis of Ala-Val-Pro-Pro-Phe-Gly-O-S-X2. The test result at different time intervals is symbolized by: + positive, (+) slightly positive, (-) questionable, - negative.

Resin -	Gly -	Phe -	Pro -	Pro -	Val -	Ala
1 min		(-)	+	+	+	+
6 min		-	(+)	+	+	-
12 min			(-)	+	+	
18 min			-	(+)	(+)	
24 min				-	-	
total coupling time		12 min	23 min	30 min	30 min	12 min

Several peptides containing trifunctional amino acids were synthesized, using DCC as coupling reagent, and monitored by the chloranil test. His(Ztf) containing peptide resins develop a faint violet to rose color with chloranil, which may render the test difficult to use for histidine peptide resins.

Advantages of the chloranil test are that it is easily and quickly performed and that it can be applied to a resin, which is in contact with the reaction mixture. Thus the chloranil test allows the determination of optimal reaction times for each coupling step of the synthesis, which may reduce coupling times and in consequence of this possible side reactions.

Acknowledgement

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KINETIC STUDY OF COUPLING AND RACEMIZATION: INVESTIGATION OF PEPTIDE RACEMIZATION MECHANISM AND AN APPROACH TO REDUCE RACEMIZATION

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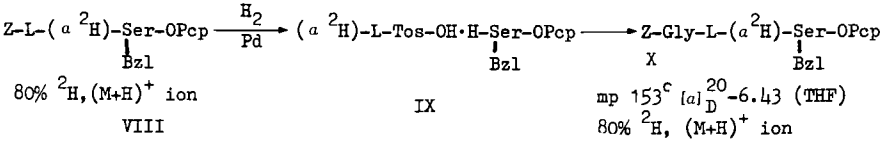
Our systematic study of the racemization rates of N-protected L-amino acid and dipeptide active esters indicates that the dipeptide pentachlorophenyl esters (OPcp) usually racemize 94 to 280 times faster[1] than the corresponding monomers with the exception of cysteine and serine (in THF solution in the presence of NEt_3). This ratio for cysteine is 3, and for the corresponding p-nitrophenyl esters it is only 1.8, which strongly suggests that the mechanism of racemization of the dipeptide active esters is not the usual 5(4H)-oxazolone formation but proceeds through enolization.[1] Similarly the Z-Gly-Ser(Bzl)-OPcp racemizes only 10 times faster than the Z-Ser(Bzl)-OPcp, which again seems to indicate that the serine dipeptide also racemizes, at least partially, by the α-hydrogen abstraction. At the Fifth American Peptide Symposium[2a] (1977) and at the XV European Peptide Symposium,[2b] we reported evidence that Z-Gly-Cys(Bzl)-ONp racemizes mostly through the enolization mechanism. Furthermore this deviation from the 5(4H)-oxazolone mechanism is due to the presence of the sulfur in β-position. It was concluded that the mechanism of racemization is side chain dependent.[2b]

The study of the mechanism of racemization of Z-Gly-Ser(Bzl)-OPcp is reported here using the deuterium isotope effect to distinguish between the enolization and oxazolone mechanism.[3] It was necessary to prepare deuterium labelled Z-Gly-L-(α^2H)-Ser(Bzl)-OPcp(X) and racemize it under the same condition as the unlabelled X. Deuteration was carried out by treating the 5(4H)-oxazolone II with AcOD: II was obtained from Ac-Ser(Bzl)-OH(I) using DCC. The deuterated 5(4H)-oxazolone III was hydrolyzed with D_2O to Ac-DL-(α^2H)-Ser(Bzl)-OH(IV). The acetyl group was removed by 1 N HCl to obtain DL-(α^2H)-Ser(Bzl)-OH(V), which was converted to Z-DL-(α^2H)-Ser(Bzl)-OH(VI).

The deuterium content was 92% as determined by chemical ionization mass spectrometry. Racemic VI was resolved using optically active α-phenylethylamine to obtain Z-L-(α^2H)-Ser(Bzl)-OH(VII) which was converted to the pentachlorophenyl ester VIII. After catalytic hydrogenation VIII gave IX, which was then coupled with Z-Gly-OH to obtain X.



Racemization of labelled dipeptide X in THF solution in the presence of 7 equivalents of NEt_3 gave $k_r^D = 574 \times 10^{-6} M^{-1}sec^{-1}$ and that for the unlabelled dipeptide was $k_r^H = 690$ giving an isotope effect of $k_r^H/k_r^D \simeq 1.2$. This value is much smaller than the k_r^H/k_r^D value for the Z-Gly-L-Cys(Bzl)-ONp, which is 2.5, and therefore it seems to indicate, together with the k_{r2}/k_{r1} value, that the racemization probably proceeds mainly through 5(4H)-oxazolone and partially through the enolization mechanism. This conclusion is valid provided the oxazolone formation is the rate determining step and the racemization of the oxazolone is much faster than the coupling of the oxazolone ($k_r \gg k_c$).

Optically active 5(4H)-oxazolone XII was obtained under controlled conditions from Z-Gly-L-Ser(Bzl)-OH(XI) and DCC. The 5(4H)-oxazolone XII racemizes fast by autoracemization in THF solution $k_{r\,auto} = 420 \times 10^{-6} sec^{-1}$ or $7500 \times 10^{-6} M^{-1}sec^{-1}$ if XII is considered a base. Racemization of XII by NEt_3 was measured on a Cary 60 Spectropolarimeter: $k_r = 35{,}000 \times 10^{-6} M^{-1}sec^{-1}$, but it racemizes slower than the corresponding 5(4H)-oxazolone of Z-Gly-Cys(Bzl)-OH (within 8 seconds). Coupling of XII with H-Val-OMe was followed by i.r. and $k_c = 4.7 \times 10^{-2} M^{-1}sec^{-1}$ was obtained. These data indicate that XII couples approximately at the same rate as it racemizes by NEt_3, that is $k_r \simeq k_c$!

It must be concluded that Z-Gly-Ser(Bzl)-OPcp(X) racemizes by NEt_3 mainly through the 5(4H)-oxazolone and to a lesser degree through the enolization mechanism and the evidence suggests that during coupling both mechanisms are involved.

It is interesting to note that the Z-Ser(Bzl)-OPcp coupling rate with H-Val-OMe is similar to the other amino acids ($k_c = 0.33$) but the dipeptide X, (most dipeptides couple 10 times faster than the corresponding monomers) couples only slowly ($k_c = 0.42$) giving k_c/k_r values of 51 and 6 respectively. This indicates the extent of racemization expected during coupling: serine, especially serine dipeptide

pentachlorophenyl or other active esters, will couple with substantial racemization when compared with other amino acids and their dipeptides, with the exception of Z-His(Bzl)-OPcp (k_c/k_r = 1.6) and Z-Gly-Asp(OMe) (k_c/k_r = 6). Another exception is Z-Ser-OPfp (pentafluorophenyl ester) which gives k_c/k_r value of 650; therefore this active ester should be preferred in the synthesis of serine peptides.

Rapid racemization of histidine was observed during actual peptide synthesis by several authors. We carried out additional racemization and coupling rate studies on τ-protected[4] Z-His-OPcp esters with NEt_3 and H-Val-OMe respectively.

The autoracemization rate constant of Z-His(Bzl)-OPcp is approximately independent (at a value of about 27 x $10^{-6}sec^{-1}$) of ester concentration in the range from 0.05 to 0.15 M, indicating that the autoracemization is probably intramolecular. At an ester concentration of 0.05 M in the presence of NEt_3, it was found that the rate of racemization increases rapidly (though apparently not linearly) with increasing base concentration up to about 0.05 M (1 equiv.); then increases less rapidly, and finally is nearly constant at 280 x $10^{-6}sec^{-1}$ for NEt_3 conc. between 0.1 (2 equiv.) and 0.7 M (14 equiv.). Further study is in progress to explain this unusual behavior.

When the imidazole ring is protected in the τ position by Bzl, Tos, Z, or DNp groups, the second order racemization rate constant, k_r, decreases in the following order: Bzl(850)$\gg$Tos(4.2)>Z(2.2)>DNp(1.1). These values clearly indicate that the racemization rate decreases with increasing electron withdrawing ability of the imidazole protecting group: this will decrease the acidity of the α-hydrogen as well as the basicity of the imidazole ring and in turn prevent the intramolecular autoracemization which is very fast (28, for the benzyl protecting group). However, the coupling rate constants, k_c, are less affected by the imidazole protecting group and the decreasing order is: Bzl(0.86)>Tos (0.31)>Z(0.27)>DNp(0.26). The k_c/k_r values are 10<740<1200<2500 for Bzl, Tos, Z and Dnp groups. These numbers indicate the relative extent of racemization which can be expected during coupling. It is concluded that the best imidazole protecting group is Dnp, followed by Z and Tos; the Bzl group should not be used. For example the Z-Gly-L-His(Bzl)-OPcp cannot be prepared from Z-Gly-OH and HBr·H-His(Bzl)-OPcp since complete racemization takes place during coupling through the mixed anhydride procedure.

We have previously noted[2b] that in a second order coupling reaction, the time required for a given extent of coupling (e.g. 99%) is dramatically

reduced by using an excess of one reagent which is usually the active ester. We now wish to calculate the effect that this shortening of reaction time might have on the optical purity of the product. For this purpose we assume the following reaction scheme:

$$\text{L-peptide} \xleftarrow{k_c} \text{L-ester} + \text{L-amine} \xleftrightarrow{k_r} \text{D-ester} + \text{L-amine} \xrightarrow{k_c} \text{DL-peptide}$$

and calculate F, the fraction of the product in the DL form. Analysis of this scheme yields the following result:

$$F = \frac{\frac{m}{2} - \frac{(n-x)}{2x}\left[1 - \left(\frac{n-x}{n}\right)^m\right]}{(m+1)}$$

in which the fraction of the undesired diastereomeric peptide depends on three variables: m, the ratio of rate constants k_r/k_c; n, the ratio of initial concentrations of ester to amine, and x, the fractional extent of coupling. In general we reach the following conclusions: The fraction of racemized product increases with the extent of reaction, because as the reaction is prolonged, the fraction of the product formed last will be from the most racemized ester. But usually we require x to be 0.99 or more in a synthesis. In general a significant improvement in optical purity can be obtained by using an excess of ester as well as by choosing a small m value ($m \ll 1$). For example: for m=1, if the ester to amine ratio is 1.5, 2 or 10 the fraction of the undesired diasteriomeric peptide will be reduced by a factor of 1.5, 2 and 10 respectively and so is inversely proportional to n. If m is small (i.e. $K_c/K_r = 100$), the percent DL form will be reduced by a factor of nearly 2n for x near 1.

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NEW ADDITIVES SUPPRESSING RACEMIZATION IN THE SYNTHESIS OF PEPTIDES BY THE DICYCLOHEXYLCARBODIIMIDE AND MIXED ANHYDRIDES METHODS AS DETERMINED BY THE ANDERSON TEST

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Racemization of amino acid residues is known to proceed via oxazolone derivatives which are formed during peptide synthesis. In the dicyclohexylcarbodiimide method this process can be suppressed by addition of different additives to the reaction mixture.[1]

Our previous study[2] on the additives suppressing racemization in peptide synthesis by the DCC method showed that a reagent used as an additive should display the so-called alpha effect, i.e. its molecule should possess two nucleophilic centers at the α position. One of the centers, that is not involved in the nucleophilic attack, should be attached to a substituent that meets two requirements. First, it should ensure appropriate acidity for the bound proton with the attacking center (mostly a hydroxyl group) and, secondly, the substituent should not deprive the center of the nucleophilicity by carrying too strong electron-attracting groups.

We also found previously that the most effective pK_a range of the additives extends from 4.60 to 8.40.

All additives used to date in the DCC method proved not to be very effective in the mixed anhydride method, mainly on account of side reactions occurring between the acyl chloride and the additive. These reactions eliminated a major part of the reagents from the desired reaction pathway, thereby reducing the yield of the peptide. The advantage of the mixed anhydride method of synthesis of the peptide bond, is its outstanding simplicity. However, it has a shortcoming; it yields mostly racemized products.

The objective of this work was to find new additives which would be equally effective in both the DCC and the mixed anhydride methods. By employing the Anderson test we investigated suppression of racemization by additives which were characterized by the lack of both the alpha effect and appropriate acidity. These were: azobenzene, azoxybenzene, 4,4′-dimethoxy-azoxybenzene, hydrazobenzene, biphenyl, benzophenone, benzylideneacetophenone (chalcone) and stilbene.

Table I. The Degree of Racemization of the Ethyl Ester of Benzyloxycarbonylglycyl-L-phenylalanylglycine Obtained by the DCC Method Plus Additive

	Additive	Overall yield,%	Yield of DL,%	Yield of L,%
1	No additive	71.0	8.8	62.2
2	$C_6H_5-N=N-C_6H_5$ Azobenzene	79.3	-	79.3
3	$C_6H_5-\overset{O}{\overset{\downarrow}{N}}=N-C_6H_5$ Azoxybenzene	79.0	-	79.0
4	$(p)CH_3O-C_6H_4-\overset{O}{\overset{\downarrow}{N}}=N-C_6H_4-OCH_3(p)$ 4,4′-dimethoxyazoxybenzene	83.5	-	83.5
5	$C_6H_5-NH-NH-C_6H_5$ Hydrazobenzene	69.0	7.1	61.9
6	$C_6H_5-C_6H_5$ Biphenyl	66.6	2.3	64.3
7	$C_6H_5-CO-C_6H_5$ Benzophenone	66.8	0.8	66.0
8	$C_6H_5-CH=CH-CO-C_6H_5$ Benzylideneacetophenone	78.5	3.4	75.1
9	$C_6H_5-CH=CH-C_6H_5$ Stilbene	72.6	2.4	70.2

With dicyclohexylcarbodiimide (DCC), the reactions were carried out in tetrahydrofuran in the presence of 0.1 mol of the additive for 2 hours at –5°C and for 20 hours at room temperature (Table I). In the mixed anhydride method, ethyl chloroformate, triethylamine and 0.1 mol of the additive were used as reagents in the same solvent. The reactions were carried out for 2 hours at –5° C and for 2 hours at room temperature (Table II).

Table II. The Degree of Racemization of the Ethyl Ester of Benzyloxycarbonylglycyl-L-phenylalanylglycine Obtained by Mixed Anhydrides Plus Additive

	Additive	Overall yield,%	Yield of DL,%	Yield of L,%
1	No additive	89.0	28.8	60.2
2	$C_6H_5-N=N-C_6H_5$ Azobenzene	68.7	-	68.7
3	$C_6H_5-\overset{O}{\overset{\downarrow}{N}}=N-C_6H_5$ Azoxybenzene	69.3	-	69.3
4	(p)$CH_3O-C_6H_4-\overset{O}{\overset{\downarrow}{N}}=N-C_6H_4-OCH_3$(p) 4,4′-dimethoxyazoxybenzene	78.5	-	78.5
5	$C_6H_5-NH-NH-C_6H_5$ Hydrazobenzene	68.6	-	68.6
6	$C_6H_5-C_6H_5$ Biphenyl	63.2	3.4	59.8
7	$C_6H_5-CO-C_6H_5$ Benzophenone	69.3	2.4	66.9
8	$C_6H_5-CH=CH-CO-C_6H_5$ Benzylideneacetophenone	70.0	-	70.0
9	$C_6H_5-CH=CH-C_6H_5$ Stilbene	81.6	-	81.6

In both methods, azobenzene, azoxybenzene and 4,4′-dimethoxyazoxybenzene proved to be fully effective. To check their suitability for the synthesis of biologically active peptides, the synthesis of Leu-enkephalin was carried out with fragment condensation (1+3+1) by the mixed anhydrides method (ethyl chloroformate plus triethylamine), using 4,4′-dimethoxyazoxybenzene as the additive. The physical constants of the product were identical with those reported in the literature[3]: m.p. 207 - 209°C, lit. 206°C; $[\alpha]_D^{20} = -26.3°$ (c = 1, DMF), lit. $[\alpha]_D^{22} = -23.4°$ (c = 1, DMF).

As far as the mechanism of the action of these additives is concerned, the following suggestion can be offered: both azobenzene and azoxybenzene, and particularly 4,4′-dimethoxyazoxybenzene, are characterized by lyotropic liquid-crystalline behaviour owing to a high degree of ordering of their molecules in solution. In our opinion, such an ordering of the reaction medium may determine the stereospecific course of all reactions including peptide bond synthesis.

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ALTERNATIVE ROUTES IN THE SYNTHESIS OF THYMOSIN α_1 AND SOME OF ITS BIOLOGICAL ACTIVITIES

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The regulation of the immune defense mechanism of the body by polypeptides of the thymus gland is being studied with increasing interest and great success particularly in the United States. This is due to the fact that cell-free protein extracts from calf thymus — such as the standardized preparation named thymosin fraction no. 5 — were found to suppress with varying success immune deficiency effects, e.g. diminished rejection of skin grafts, increasing susceptibility to infections, accelerated aging and enhanced probability of tumor formation.[1] These kinds of studies were mainly performed with genetically athymic and thymectomized mice. Nowadays, several clinical applications of thymosin fraction no. 5 in patients with leukemia and other types of cancer are already reported to show curative effects.[2] The potentials of polypeptides from the thymus for becoming helpful pharmaceuticals against immune deficiency diseases in man are best indicated by the great financial investments of industry internationally and by the substantial research grant support by, e.g., the American cancer research plan and by other scientific organizations.

In 1977, A.L. Goldstein and the others[3] separated from the thymosin polypeptide mixture an acidic component in pure form and published the sequence of a peptide molecule named thymosin α_1. The total synthesis, simultaneously announced, was undertaken in the peptide research group headed by J. Meienhofer at Hoffmann — La Roche. Early this year this work was published by S. S. Wang and others.[4]

Having a long-standing fascination in the effects of thymus polypeptides on the differentiation and maturation of pre-T-lymphocytes into immune-competent T-lymphocytes, the publication of the sequence of thymosin α_1 kindled our eager interest also to develop a synthesis of this molecule.

In the recent past we had experienced[5] the particular usefulness of excess 2-(3,5-dimethyloxyphenyl)propyl(2)oxycarbonyl (Ddz) amino acids in the famous mixed anhydride method of Th. Wieland and others. This modernized efficient method of synthesis needs no intermediate work-up and results in analytically pure peptides ready for further elongation within 24 hours. As indicated in Figure 1, one can calculate from all mixed anhydride syntheses, described below, of the Ddz-fragments of thymosin α_1 an average yield per stepwise N-terminal peptide elongation of 80%.

Because of the lability of the temporary Ddz-protector to 1-5% trifluoroacetic acid, the glutamic and aspartic acid as well as serine and threonine residues in the fragments were advantageously protected on side chain functions by the *tert*-butyl group. This enhanced the lipophilicity of the thymosin α_1 fragments shown in Figure 1 to such an extent that all building blocks were soluble in dichloromethane. This is worth mentioning since the 28 amino acids of thymosin α_1 include 6 glutamic acid and 3 each of aspartic acid, serine and threonine residues. Additionally, it should be mentioned that the N-terminus of thymosin α_1 was found to be blocked by an acetyl group.[3]

	DDZ-PEPTIDE ESTERS OF THYMOSIN α_1 FRAGMENTS	AVERAGE YIELD/STEP[a] [%]	
I	DDZ-SER(BU^t)-ASP(OBU^t)-ALA-ALA-VAL-ASP(OBU^t)-OME	66	83[b]
II	DDZ-THR(BU^t)-SER(BU^t)-SER(BU^t)-GLU(OBU^t)-ILE-THR(BU^t)-OME	73	
II	DDZ-THR(BU^t)-SER(BU^t)-SER(BU^t)-GLU(OBU^t)-ILE-THR(BU^t)-OBZL	86	
III	DDZ-THR(BU^t)-LYS(Z)-ASP(OBU^t)-LEU-LYS(Z)-GLU(OBU^t)-LYS(Z)-OME	84	83[b]
IV	DDZ-LYS(Z)-GLU(OBU^t)-VAL-VAL-GLU(OBU^t)-OME	88	
V	DDZ-GLU(OBU^t)-ALA-GLU(OBU^t)-ASN(MBH)-OBZL	76	
		80[c]	

a) stepwise N-terminal elongation of the peptides
b) repeated synthesis
c) overall

Fig. 1. Yields of Ddz-peptide esters of thymosin α_1 fragments prepared from excess Ddz-amino acid mixed anhydrides.

Next we have to explain our decision not to perform the thymosin α_1 synthesis on a polymer support, inspite of having recently demonstrated some success with the Merrifield method both in the total synthesis of the mast cell degranulating (MCD) peptide[6] and in the preparation of the insulin A chain.[7]

First, we wanted to demonstrate the ease and efficiency of the application of excess Ddz-amino acid mixed anhydrides in solution to the preparation of fragments of a biologically relevant polypeptide mainly consisting of trifunctional amino acids.

Second, in the quick search for an optimal route for the synthesis aiming at an undescribed polypeptide from fragments of unknown physicochemical characteristics, we found it less time consuming first to gather the necessary knowledge on the preparative details from an efficient method of synthesis in solution and then to transduce this knowledge into a repeated polymer-bound synthesis utilizing the same set of protective groups.

As shown in Figure 2, for the preparation of useful fragments we subdivided the sequence of thymosin α_1 into five portions. The divisions were made between identical or similar, polar amino acid residues whose side chain functions were protected by the lipophilic *tert* butyl group. The guiding idea was to introduce a lipophilic interplay of those "fatty coated" polar side chain functions. In a polar solvent such as dimethylformamide this might hold the fragments close together to favour the condensation reaction.

Though we changed the order of the fragment condensations during our search for an optimal route, all cutting positions remained constant indicating the versatility of the above mentioned concept. It was also our intention to use isoleucine 11 and leucine 16 as key amino acids in the analytical control of the fragment condensations. In our first trial (Figure 2), we used the N-terminal acetyl-hexapeptide Ac(1-6)OH, which was only slightly soluble in dichloromethane, for the combination of the fragments.

On combination with fragment II benzyl ester in dimethylformamide we obtained the sequence Ac(1-12)OBzl in 61% yield. However, the successful hydrogenolytic cleavage of the C-terminal protecting group (90% yield) resulted in an acetyl-dodecapeptide which was hardly useful in the final fragment condensation because of its very poor solubility. In the first trial we obtained the fully protected end-product only in yields of 12-17%. Also the final deprotection of the end-product with HBr/trifluoroacetic acid proceeded unsatisfactorily and resulted in a very impure crude product of thymosin α_1. Though we were not able to purify this material sufficiently to obtain an accurate amino acid analysis, it showed significant biological activity in the stimulation of human lymphocytes in the E-rosettes formation assay.

Since the fragment condensations in the C-terminal region of the thymosin α_1 molecule proceeded quite satisfactorily, our second and third trials for the total synthesis were concentrated on alterations in the (1-12) fragments.

To overcome the insolubility problem caused by the acetyl-fragment (1-6), we prepared Ddz(1-6) as shown on the right half of Figure 3.

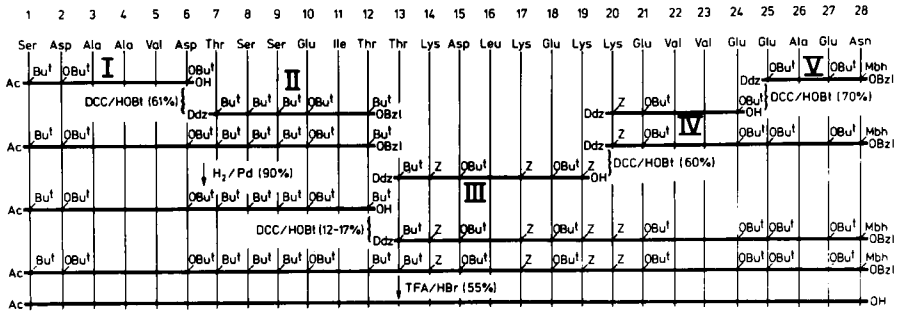

Fig. 2. Scheme of the first trial for the total synthesis of thymosin α_1 from five fragments.

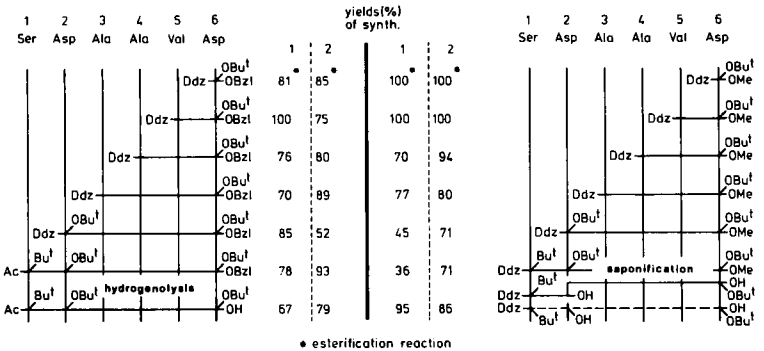

Fig. 3. Syntheses of fragment I (1-6) of thymosin α_1 by stepwise N-terminal peptide elongation with excess Ddz-amino acid mixed anhydrides.

Yet, on saponification of the Ddz-hexapeptide methyl ester we were confronted with a strange side reaction, whose elucidation is described in Figure 4. After treatment of Ddz(1-6)OMe in dioxane/methanol with aqueous sodium hydroxide we isolated a product mixture of two components. By routine ^{1}H-n.m.r. analysis (60 MHz) we detected the complete loss of one of the two *tert*-butyl ester moieties. To our surprise the mixed product showed two negative charges in the electropherogram in basic buffer, as the starting material does after treatment with non-aqueous trifluoroacetic acid. While the latter material can be degraded completely by aminopeptidase-M, only 15% of the former was totally digested by the same enzyme. On saponification with methoxylate, we obtained again a product mixture which by electrophoresis was analyzed to contain only one instead of two anionic charges. In the ^{1}H-n.m.r.

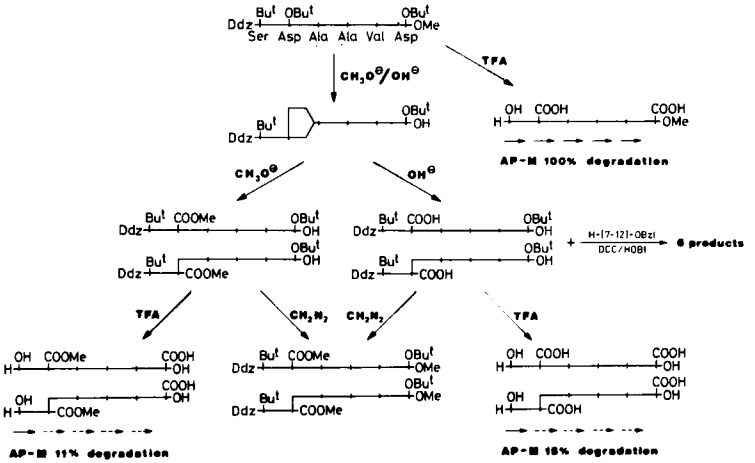

Fig. 4. Elucidation of the aspartyl-alanyl peptide bond β-rearrangement in position 2-3 of thymosin α_1

spectrum we observed one methyl ester. After treatment of this material with trifluoroacetic acid, also only 11% of the mixture containing one methyl ester group could be completely digested by aminopeptidase-M. Upon methylation with diazomethane the materials from both of the saponification pathways yielded identical products. In conclusion, these observations clearly indicate a β-rearrangement of the aspartyl-alanyl peptide bond in position 2-3 as the main reaction. To our knowledge there is no example described in the literature, which indicates the attack of an aspartyl-alanyl peptide bond amide nitrogen on the β-*tert*-butyl aspartate group. M. Bodanszky reported similar rearrangements with β-benzyl aspartate,[8] and R. Schwyzer observed already in 1963 the attack of a seryl nitrogen on β-*tert*-butyl aspartate, yet catalyzed by the free alcoholic side function of serine under alkaline conditions.[9]

As also shown in Figure 4, the above described mixed saponification product of Ddz(1-6)OMe was useless in the condensation with fragment II benzyl ester. On thin layer chromatograms we identified at least 6 products with similar amino acid compositions. Therefore, in the third trial we decided to synthesize fragment I with a C-terminal benzyl ester group. On the last stage of the hexapeptide synthesis we introduced N-acetyl serine *tert*-butyl ether, as presented on the left side of Figure 3. On hydrogenation in 2,2,2-trifluoroethanol or 2-propanol/glacial acetic acid in the presence of palladium catalyst the benzyl ester was readily cleaved from the N-acetyl hexapeptide.

The whole scheme of the final, third trial for the total synthesis[10] of thymosin α_1 is presented in Figure 5. The insolubility problem of the former, first synthesis was solved by condensation of fragment II (7-12) not with Ac(1-6) but with the bigger C-terminal unit (13-28) benzyl ester, which was built-up by stepwise N-terminal condensations starting from fragment V (25-28) benzyl ester. In these syntheses the fragment head components always were used in a twofold excess.

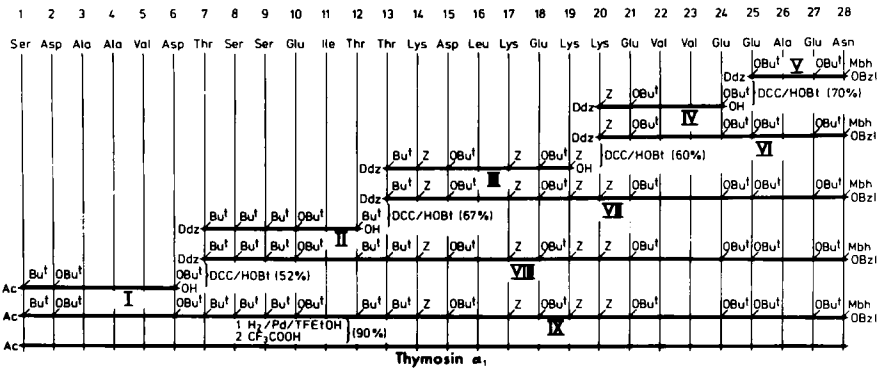

Fig. 5. Final scheme for the total synthesis of thymosin α_1.[10]

Because of our bad experiences with HBr/trifluoroacetic acid and with HF/pyridine in the first trial for the final deprotection of thymosin α_1, we performed some model studies directed toward the C-terminal asparagine. The results are summarized in Figure 6. On two hours treatment with HBr/trifluoroacetic acid, β,(4,4^1-dimethoxybenzhydryl)asparagine benzyl ester [H-Asn(Mbh)OBzl] reacts incompletely yielding partly α-amino-succinimide. About 10% of the material is still protected on the imide nitrogen by the Mbh group. On mild saponification with bicarbonate we obtained both the α- and β-amides of aspartate as well as their Mbh-protected derivatives. In contrary, the hydrogenolysis of H-Asn(Mbh)OBzl in 2,2,2-trifluoroethanol in the presence of palladium catalyst followed by treatment with trifluoroacetic acid in the presence of anisole yielded pure asparagine.

Therefore, by this last mentioned procedure we obtained thymosin α_1 from the final deprotection in a yield of 90% after chromatographic purification on Bio gel P-6 in 1% acetic acid (containing 10% of trifluoroethanol).

Amino acid analysis:	Asp	Thr	Ser	Glu	Ala	Val	Ile	Leu	Lys
110°C, 24-72 h, 6n HCl	4	3	3	6	3	3	1	1	4
fully protected	3,65	2,76	2,45	6,05	2,23	2,51	1,00	1,09	4,08
free Thymosin α_1	3,54	2,70	2,58	6,16	3,13	2,46	0,96	1,00	4,08
electrofocusing	4,00	2,80	2,58	5,31	2,96	2,71	0,95	1,00	4,54

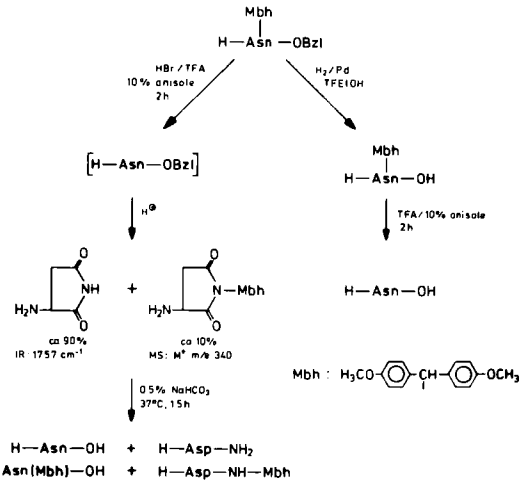

Fig. 6. Model studies for the deprotection of the C-terminal thymosin α_1 H-Asn(MbH)OBzl.

On electrofocusing in comparison with thymosin fraction no. 5 our synthetic thymosin α_1 showed one acidic band at pI of about 3.9, as presented in Figure 7. In a discontinuous ORD measurement thymosin α_1 shows unusually large negative [α] values. Figure 8 presents these data together with the CD spectrum and the secondary structure of thymosin α_1 as estimated by the method of Chou and Fasman.[11] The immune stimulatory potential of the synthetic thymosin α_1 was compared to a highly purified natural thymus polypeptide, prepared by one of us (H.G.M.) from calf thymus. This latter preparation consists only of one immune stimulatory active and another inert polypeptide. In the E-rosettes assay thymosin α_1 was three times more potent than the natural product, as presented in Table I.

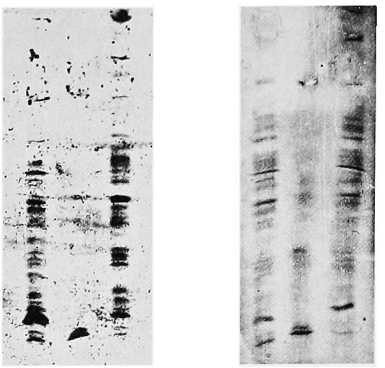

Fig. 7. Isoelectric focusing at pH 3.5 — 5.5 of synthetic thymosin α_1 in comparison to natural thymus polypeptides.

The E-rosette assay was performed on human peripheral lymphocytes, whose RNA-polymerase was inhibited by α-amanitine.[12] In 100μL of a suspension containing 5 x 10^5 cells only 1.6 nMoles (0.5 μgrams) of synthetic thymosin α_1 completely restores the capacity to form E-rosettes of lymphocytes, which previously were blocked by α-amanitine. It is worth mentioning that also the impure preparation of

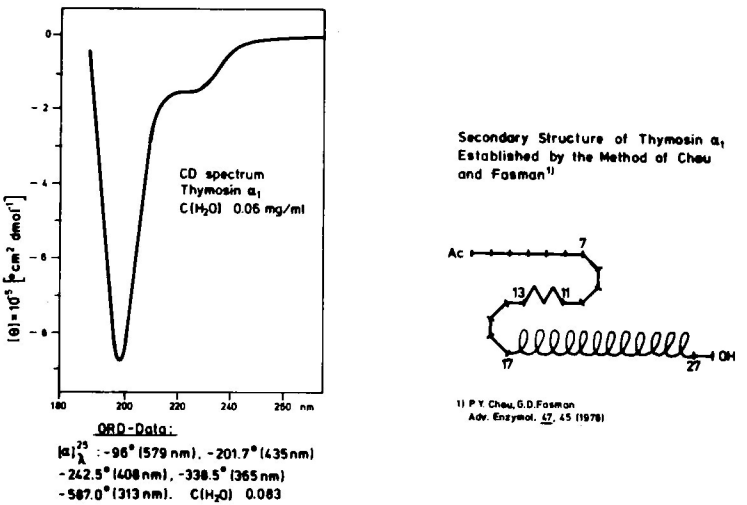

Fig. 8. CD spectrum, ORD data and an estimation of the secondary structure of thymosin α_1.

Table I. Comparative data of synthetic thymosin α_1 and of a natural thymus polypeptide in the E-rosettes assay with α-amanitine inhibition.

normal cell population: 64%[a] E-rosettes
inhibited cell popul. : 25%[a] E-rosettes

thymus polypeptide[b] µg/culture	[%][e]	synth. thymosin α_1[c] µg/culture	[%][e]	synth. thymosin α_1[d] µg/culture	[%][e]
6.25	10	5.00	33	1.00	55
3.12	67	2.50	57	0.50	39
1.25	100	1.00	64	0.25	48
0.62	62	0.50	100	0.12	48
0.31	6	0.25	39	0.06	3

[a] absolute value
[b] natural
[c] analyt.pure
[d] impure, first trial of total synthesis
[e] relative amounts of E-rosettes restored on stimulation by thymus peptides of α-amanitine inhibited peripheral human lymphocytes

thymosin α_1 from our first trial of the total synthesis showed about 50% restoration of the formation of E-rosettes, however, at ten times higher concentrations.

In the mitogen response assay, performed on human peripheral lymphocytes inhibited by α-amanitine,[13] the synthetic thymosin α_1 shows both stimulatory and inhibitory activities in the presence of varied concentrations of the mitogens phytohaemagglutinin (PHA) and concanavalin A (ConA), as presented in Table II.

Table II. Activity of synthetic thymosin α_1 in the mitogen response assay on human peripheral lymphocytes inhibited by α-amanitine.

	PHA[a]		[%]	PHA[b]		[%]
thymosin α_1 1µg/ml	-	+		-	+	
α-amanitine 10 µg/ml	452	640	41[c]	50	35	-30[d]
1 µg/ml	46912	46090	- 2	495	366	-26
0.1µg/ml	63647	65804	3	1053	1392	32
0 µg/ml	81290	88880	9	1213	1061	-12

	Con A[a]		[%]	Con A[e]		[%]
thymosin α_1 1µg/ml	-	+		-	+	
α-amanitine 10 µg/ml	107	57	-47	172	81	-50
1 µg/ml	8736	9730	11	2947	2109	-28
0.1µg/ml	25841	30647	18	2719	6857	150
0 µg/ml	38146	36674	- 4	8670	8583	- 1

[a] optimal concentration
[b] 1/100 optimal concentration
[c] stimulation
[d] inhibition
[e] 1/10 optimal concentration

Table III. Allogenic one-way mixed lymphocytes response stimulated by thymosin α_1.

Peripheral human lymphocytes, names of donors: S., We., Wa.
α-amanitine and thymosin α_1 1µg/ml cell suspension each
120 h incubation
r: responder cell, s: stimulator cell (mitomycin blocked)

		thymosin α_1 -	thymosin α_1 +	stimulation [%]
S_r /Wa_s	a)	100521	120887	20
	b)	52439	97649	86
Wa_r/S_s	a)	66012	83800	27
	b)	53570	78552	47
S_r /We_s	a)	75994	79392	4
	b)	17314	23747	37
We_r/S_s	a)	20042	28821	44
	b)	4822	4809	0
We_r/Wa_s	a)	76610	97259	27
	b)	4228	69967	1550
Wa_r/We_s	a)	59543	86379	45
	b)	27271	41073	51

a) non inhibited culture
b) inhibited by α-amanitine

As summarized in Table III, a most surprising effect of our synthetic thymosin α_1 was observed in the allogenic one-way mixed lymphocytes response with normal human peripheral lymphocytes. In several cell cultures, thymosin α_1 stimulated the responder cells on an average of 28% over the stimulatory effect of the mitomycin blocked lymphocytes. This effect on the average was doubled if the responder cells previously were inhibited with α-amanitine. In contrast, thymosin α_1 preparations from natural sources in another type of mixed lymphocytes culture assay as performed by A.L. Goldstein and others were inactive.[13]

Detailed discussions of the biological meaning of the data obtained with our synthetic thymosin α_1 would go beyond the scope of this contribution and are therefore omitted. Investigations of the effect of synthetic thymosin α_1 on different types of animal cancers are in progress.

Acknowledgements

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CONFORMATIONAL CONSIDERATIONS IN THE DESIGN OF SOMATOSTATIN ANALOGS SHOWING INCREASED METABOLIC STABILITY

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Many approaches are available toward the design of peptide analogs of improved biological activity. An approach taken in our laboratory involves analog design based on a continuously evolving view of what we believe to represent the biologically active conformation. A specific target of our studies has been the design of somatostatin analogs for use in the treatment of diabetes.

Through synthesis of a series of conformationally restricted analogs of somatostatin and evaluation of their physical and biological properties, we have arrived at a proposed biologically active conformation for somatostatin. These studies have resulted in the design of a highly active analog which shows increased resistance to hydrolysis catalyzed by trypsin *in vitro,* increased duration of action *in vivo* and a biologic response after oral administration.

Before proceeding with a discussion of our studies, I would like to define my use of the term "biologically active conformation". I refer to the conformation of the peptide when bound to the receptor at the moment that a given biological response is elicited. This conformation may be one of the solution conformations or it might arise subsequent to receptor binding. Whatever the case, determination of this conformation requires reference to the biological activity.

Our first information regarding the biologically active conformation of somatostatin came from the observation of high activity from bis-carba analogs (eg., I and II, see Table I).[1] This result requires that the biologically active conformer(s) fall within those conformations allowed to the cyclic structure, a small but real reduction of the number of possibilities to be considered. Further limitations of the conformation possibilities are established by the observation of high potency for the analogs of II having proline in positions 5 or 13 and the low or absent activity of the analog having proline in position 10.[3] (Numbering refers to the residue position in somatostatin.)

Table I. Inhibition of Release[a] (Relative Potency of Somatostatin 1)[b]

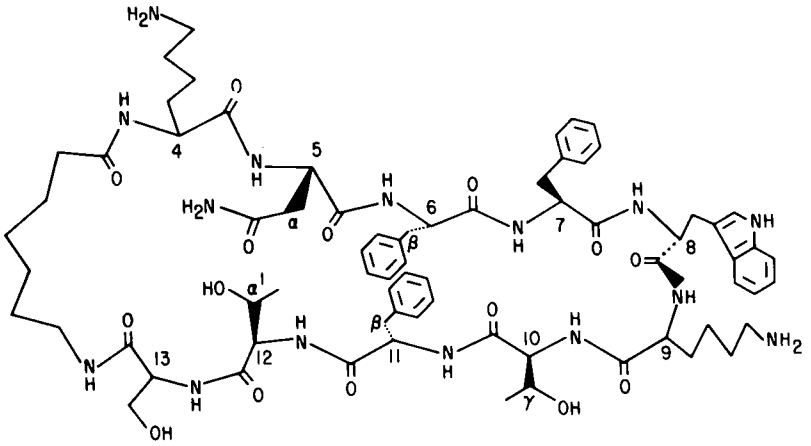
Compound	Glucagon	Insulin	Growth Hormone		Gastric Secretion
			In Vitro	In Vivo	
I C(Aha-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser)	0.62(0.10-1.41)	0.26(0.08-0.60)	0.60(0.40-0.90)	----	----
II C(Aha-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser)	1.40(0.26-12.40)	1.53(0.92-2.66)	0.37(0.29-0.48)	1.88(0.35-8.33)	0.10
III C(Aha-Lys-Cys-Phe-Phe-D-Trp-Lys-Thr-Phe-Cys-Ser)	1.40(0.3-12.4)	1.5(0.9-2.7)	0.37(0.29-0.48)	1.9(0.35-8.33)	0.1
IV C(Aha-Lys-Abu-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-Ser)	1.3(0.28-8.4)	1.1(0.64-1.94)	0.88(0.82-0.95	0.9(0.14-15.2)	0.1
V C(Aha-Phe-Phe-D-Trp-Lys-Thr-Phe)	0.86(0.44-1.53)	0.88(0.30-2.45)	0.93(0.69-1.2)	0.65(0.20-4.61)	0.03
VI C(Aha-Cys(Acm)-Phe-D-Trp-Lys-Thr-Cys(Acm))	c	0.020(0.003-0.060)	0.03(0.02-0.04)	0.14(0.05-0.29)	0.03
VII C(Aha-Cys-Phe-D-Trp-Lys-Thr-Cys)	2.66(1.32-6.10)	3.50(2.31-6.38)	1.24(0.81-1.88)	2.55(0.99-11.1)	0.05

[a] The biological effects were measured using methods described previously.[1] Inhibition of glucagon and insulin release was measured in urethan anesthetized rats. Growth hormone release inhibition *in vivo* was measured in pentobarbital treated rats. *In vitro* inhibition of growth hormone release was measured using dispersed pituitary cells. Inhibition of gastric secretion was measured in dogs stimulated by pentagastrin. [b] 95% confidence limits given in parentheses. [c] Low level of activity observed, no dose response.—Aha, ω-aminoheptanoic acid.

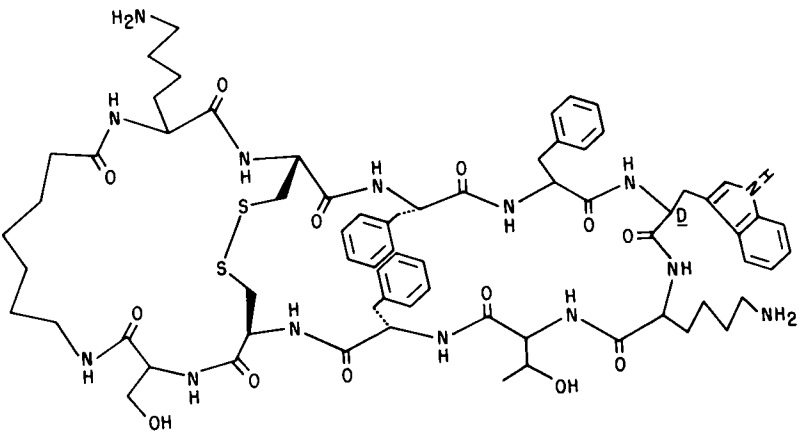
The high potency of analogs of somatostatin having D-tryptophan in position 8[4,5] can be understood in terms of a type I β-turn in somatostatin involving residues 7, 8, 9, and 10. In this conformation the side chains of residues 8 and 9 of somatostatin will bear an equatorial relationship to the peptide backbone. A change at position 8 to the D configuration could result in a change in the backbone conformation to a type II' β-turn, thereby retaining the equatorial relationship of the side chains of residues 8 and 9.[6] NMR evidence for the existence of such an equatorial relationship of the lysine and tryptophan side chains in D-tryptophan containing analogs has been presented, based on an upfield shift of the lysine γ-methylene of about 1 ppm and a correlation of the shift with the conformation at biological receptors.[3] Since the upfield shift is not observed in somatostatin, it has also been proposed that the conformation(s) which interact with receptors represent only a minor contribution to the overall population of conformations in the natural hormone.

Using this limited information derived from conformational constraint of the peptide, a conformation for II capable of receptor interaction was proposed[2] which is different from conformations proposed based on physical studies in solution.[7] This model led to the design of analogs of compound II having Asn-5 and Thr-12 and Phe-6 and Phe-11 replaced by cystine (III and IV respectively).[2] Each of these analogs shows activity at least as great as that observed for somatostatin. The monocyclic precursor to III is also highly active, confirming the unimportance of the side chains at positions 5 and 12 as first observed by replacement of each of these by alanine.[8] In contrast, replacement of either Phe-6 or Phe-11 by Ala in somatostatin results in a substantial loss of activity.[8] That high activity is seen when a disulfide bond replaces the two aromatic rings, while simple removal of these rings lowers activity, was interpreted by us as a hydrophobic bond stabilization of the active conformation of somatostatin.[2]

On the basis of the high biologic activity of III we concluded that the β-carbons of residues 5 and 12 in somatostatin are in close proximity when somatostatin is bound to the receptors involved in the inhibition of insulin, glucagon, and growth hormone release. Evidence has been presented by us that *in vivo* reduction of the disulfide bridge of III to give the monocyclic, dihydro derivative is not the explanation for the observed high biologic activity of III *in vitro* and *in vivo*.[2] These studies also focused attention on amino acids 7-10 of somatostatin as important



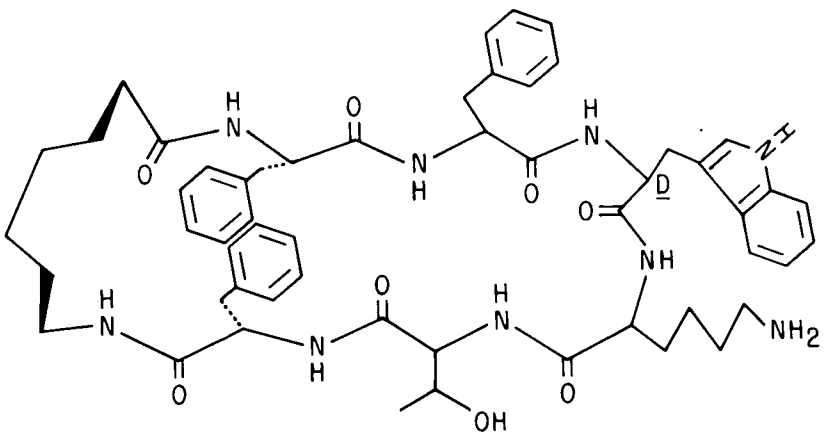
I(L-Trp^8) II(D-Trp^8)



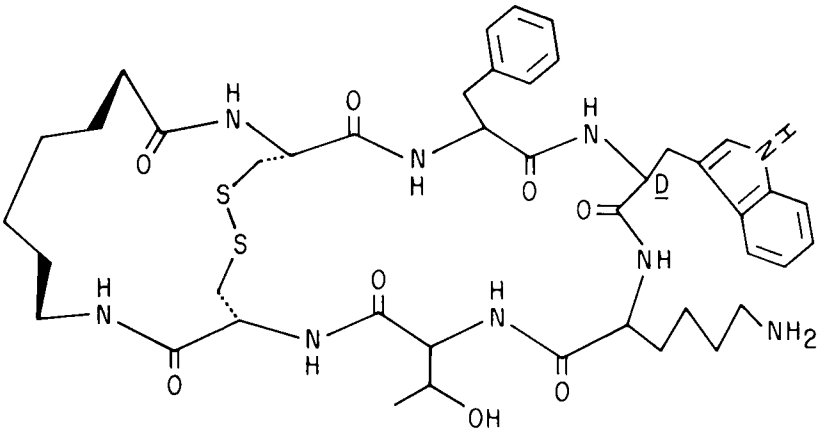
III

in interaction with receptors while amino acids, 5, 6, 11 and 12 are not. It had also been shown that the amino acids 4 and 13 could be individually deleted with the retention of biologic activity.[9] These observations taken together suggested that the amino acids to the left of the disulfide bridge of III might be deleted and the disulfide bridge replaced by methylene groups with retention of biologic activity. Thus, we undertook the synthesis of analog V, cyclo (Aha-Phe-Phe-D-Trp-Lys-Thr-Phe), which was also designed as an analog of III incapable of *in vivo* reduction.[10,11] A disulfide-containing analog of ring size similar to V has also been reported which shows significant biologic activities.[12] A further test of the minimum requirements for biologic activity could be obtained through replacement of phenylalanines 6 and 11 of V by cystine as we have previously reported for the analog IV having the full ring size of somatostatin.[2] We therefore also synthesized the bicyclic analog VII, cyclo-(Aha-Cys-Phe-D-Trp-Lys-Thr-Cys), which retains only the amino acids 7-10 of somatostatin.

The biologic potencies of the novel analogs in a variety of biological tests are summarized in Table I. It is seen that the biologic properties of V, VII and somatostatin are nearly the same except for the reduced gastric secretion inhibition potency of V and VII relative to somatostatin. Indeed these and our previous studies indicate different structural requirements for gastric secretion inhibition than for the other biological properties.[2] The high potency for inhibition of insulin, glucagon, and growth hormone release by V is additional evidence against *in vivo* disulfide reduction as a requisite of biologic activity of I.



V



VII

The roles of Phe-6 and Phe-11 have already been implicated, at least in part, to stabilization of peptide conformation through formation of a hydrophobic bond. This conclusion is further strengthened by the high biologic activity of VII relative to VI. The disulfide bridge of VII appears to be a requisite of high biologic potency. The lack of possible bonding between amino acids 6 and 11, either covalent (disulfide) or hydrophobic (non-bonded, as when the aromatic rings are present), results in lowered potency as is seen in VI. The high biologic potency of V and VII indicates that all of the information required for interaction with receptors for inhibition of insulin, glucagon and growth hormone release appears to be present in the four amino acids of the proposed β-turn, provided that the biologically active conformation is attained.

The bicyclic analog VII was found to be more resistant to enzymatic cleavage as demonstrated by its relative resistance to hydrolysis between lysine and threonine by trypsin *in vitro*. The monocyclic analog V is cleaved by trypsin at about 100 times the rate of hydrolysis of VII under identical conditions. The rates of hydrolysis were determined concurrently under identical conditions (1.5×10^{-3}M peptide). The course of hydrolysis was followed by high pressure liquid chromatographic analysis assessing the ratio of substrate to product at various time intervals. Noteworthy is the observation that VII in the presence of 1.5×10^{-3}M mercaptoethanol is hydrolyzed at about the same rate as V. Thus the disulfide bond adds enzymatic stability apparently through conformational or steric constraint. Furthermore, this observed decrease in the rate of tryptic hydrolysis was shown to result from reduced binding

to trypsin since III does not inhibit the tryptic hydrolysis of IIa even when present at 10-fold higher concentration. The introduction of conformational constraint appears to have predominantly increased K_M rather than prevented some needed conformational change along the pathway of hydrolysis.

An increased duration of action after subcutaneous administration of VII was seen relative to V and somatostatin (Figure 1). The data in Figure 1 at 75 minutes after injection show that both V and VII have a longer duration of action than somatostatin. The data at 135 minutes show VII to have a longer duration than V. Indeed, VII has been found to demonstrate significant inhibition of growth hormone release in this system at time intervals up to 16 hours after subcutaneous administration.

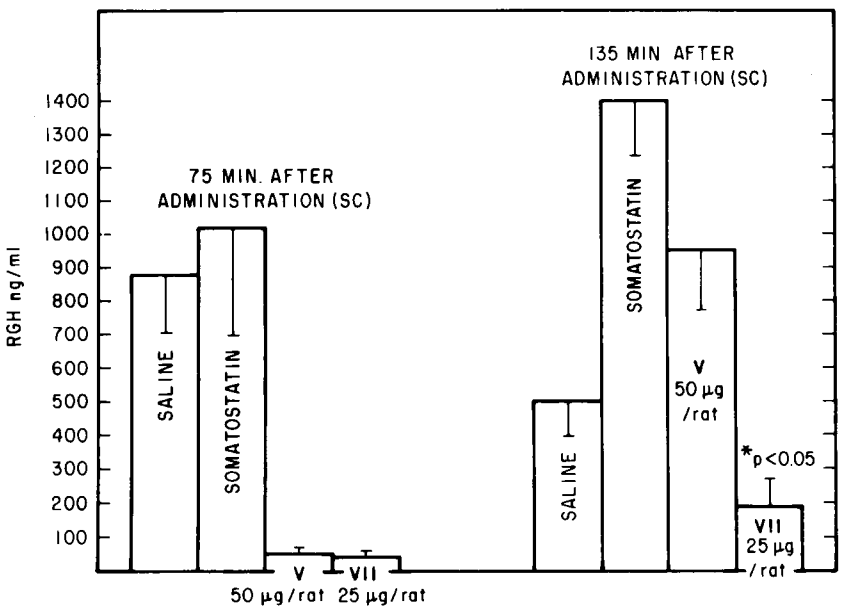


Fig. 1. Duration of action of somatostatin and analogs on the inhibition of growth hormone release. Male Sprague-Dawley rats (190-200 g) were injected with saline or compound (S.C.). One hour or two hours later sodium pentobarbital (17 mg/kg, i.v.) was injected. Fifteen minutes later, the rats were bled via the orbital sinus. The plasma was collected and assayed for GH content by radioimmunoassay. The data are presented as the mean ± S.E.M. (6 rats/group). Doses of somatostatin (50 µg), V (50 µg) and VII (25 µg) were chosen to attain equipotence.

Although it seems reasonable that the increased duration of action is a consequence of the enhanced enzymatic stability of VII, we cannot exclude the possibility that it relates also in part to solubility changes which lead, in effect, to depot forms of the analog.[5]

In addition to the increased duration of action observed after subcutaneous administration, VII also shows inhibition of growth hormone release after oral administration. When given to rats at doses of 2.5-25 mg/kg, significant suppression of pentobarbital stimulated growth hormone release is observed 1 hour later. Suppression of growth hormone levels is not seen at two hours after the oral doses. Thus, the oral activity seen is of relatively short duration and requires high doses. Improvement is still needed for development of an orally administered agent for use in therapy.

The bicyclic analog clearly has fewer degrees of conformational freedom in solution than are available to somatostatin. We have concluded therefore that solution measurements on VII will yield more information about the bioactive conformation of the active part of somatostatin than will be obtained from somatostatin or other less constrained analogs.

We have studied the NMR spectra at 300 MHz of VII in methanol, DMSO, and water (D_2O). The fact that the upfield shift of the γ-CH_2 of lysine in methanol at sufficiently low temperature (-16° C) resembles that in water at higher temperature (22° C) (Table II) suggests that in methanol the lowest energy conformation(s) in this part of the molecule are similar to those in water. Because we feel that hydrophobic interactions (e.g., Phe^6-Phe^{11} and Trp^8-Lys^9) in somatostatin tend to create the bioactive conformation, it is our bias that the data in water is more pertinent to describing that conformation. Organic solvents such as methanol tend to overcome the internal hydrophobic interaction through lipophylic interactions with the solvent.

We have been able to obtain a nearly complete assignment of the ^1H NMR spectrum by detailed ^1H and ^{13}C studies of VII compared with a variety of closely related analogs. The large differences in the two CαH-CβH coupling constants in water for one of the cystine α-protons (10 and 4 Hz), the lysine α-proton (11 and 4 Hz) and the tryptophan (10 and 6 Hz) indicates a high population of a single rotamer and is consistent with a great deal of structural rigidity both within and outside the ring structure. We have also been able to obtain the NH-CαH coupling constants in water. These in turn allow estimation of the dihedral angles.[13] These

Table II. Temperature Dependence of NMR Features of VII

	δ-CH_2-Lys		NH	$\Delta\delta$/deg (CH_3OH, 20^o-62^o)
Temp.	$\delta(CH_3OH)$	$\delta(H_2O)$	Thr	0.0031
-47^o	0.34,0.15	----	Aha	0.0029
-16^o	0.42,0.31	----	Lys	0.0064
0^o	0.43		Cys	0.0074
3^o	----	0.44,0.23	Cys	0.0059-0.0116[a]
22^o	0.49	0.48,0.32	Trp	0.0062-0.0101[a]
51^o	0.64	----	Phe	0.0050
61^o	----	0.54,0.41		

[a]Non-linear—Aha, ω-amino-heptanoic acid.

angles are consistent with our working model of the bicyclic analog VII and that model has in turn been modified to a fit with the angles derived from these coupling constants. A stereoview of the model so obtained is shown in Figure 2. The temperature dependence of the N-H resonances is

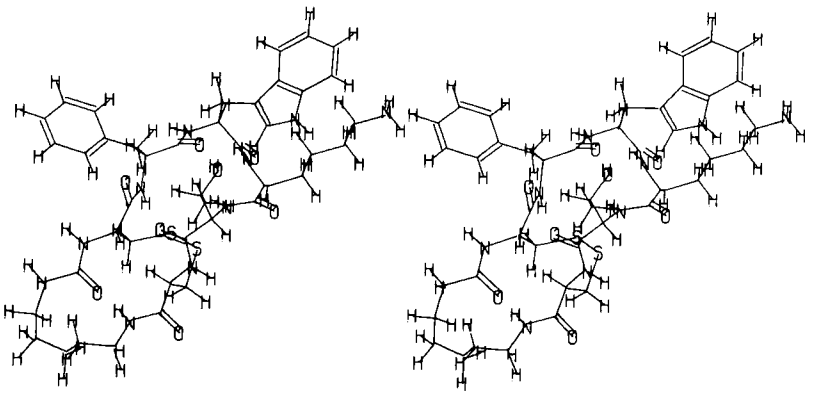


Fig. 2. Stereo view of proposed bioactive conformation of II having NHCH dihedral angles defined by NMR studies in D_2O.

also consistent with this structure. The small change in chemical shift of the threonine and ω-aminoheptanoic NH's is consistent with internal hydrogen bonding (Table II).[14] These hydrogen bonds are a part of our proposed bioactive conformation. This model is presently our best picture of both the solution and biologically active conformation of VII. We believe it to represent the bioactive conformation of the active portion of somatostatin.

It is worth noting that the disulfide ring of IV and VII is of the same size as that found in oxytocin, vasopressin and insulin (A chain 6-11). Hodgkin has proposed a similar structure for the ring of oxytocin and the ring she had observed in insulin.[15] Both of these include what is in essence a β-turn, although β-turns had not been defined at that time. A more precise definition of this type of system and the recognition of the turn as being of type II in oxytocin and vasopressin has been more recently described.[16] This more precise definition then gives a description of side chain stereochemical relationships to the backbone. In our model the same ring is proposed to have a type II' β-turn (when D-Trp is present). Thus the original implication of Hodgkin, that the conformation of the ring found in insulin (a β-turn) may have more general biological importance[13] is again indicated in our studies with somatostatin. The biological significance of β-turns as proposed by Walter[17] is also further supported by this work.

Thus through a series of studies involving analog synthesis, biological evaluation of analogs and physical measurements, we have proposed and refined a postulated bioactive conformation. This approach has led to the design of a conformationally restricted analog having only 4 of the original amino acids of somatostatin. In addition to limiting potential sites for metabolism by proteases, the designed analog binds less well to one known metabolizing protease, trypsin. The approach of analog design guided by conformational consideration can lead to analogs showing increased duration of action. This is one important aspect of the development of therapeutically useful peptides.

Acknowledgement

The work that I have reported clearly involves the efforts of a large number of individuals in a variety of scientific disciplines whose efforts I wish to acknowledge. Synthetic effort was supplied by Miss Susan J. Bergstrand, Dr. Stephen F. Brady, Dr. Frederick W. Holly, Mrs. Ruth F. Nutt, Mr. William J. Paleveda, Jr., and Mr. Robert G. Strachan.

Biological evaluation of the analogs was carried out by Dr. Marylou Torchiana (gastric) and Dr. Monroe S. Glitzer and Richard Saperstein (hormone). NMR studies were carried out by Dr. Byron Arison and conformational evaluation using the Merck computer and graphics systems by Drs. Roger Freidinger, Peter Gund and Graham Smith. Thanks also to Dr. Kenneth Kopple for helpful discussion. Finally I wish to acknowledge the important support given to this entire effort by Dr. Ralph Hirschmann.

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SOME ASPECTS OF SYNTHESIS OF EDEINE ANTIBIOTICS

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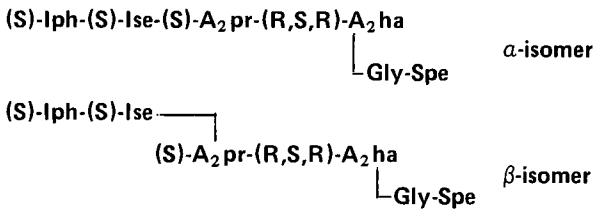
Edeines are polypeptide antibiotics produced by the Bacillus brevis Vm_4 strain. At minimum growth inhibitory concentration edeines inhibit DNA synthesis, in prokaryota (bacteria) while the inhibition of growth of eukaryotic organisms is a result of inhibition of protein synthesis and not of DNA.[1,2]

A mixture of antibiotics formed during biosynthesis contains mainly four closely related compounds named edeines A,B,D and F. These antibiotics are pentapeptide amides containing four nonprotein amino acids, glycine and polyamine.[3,4,5] Edeine D as well as other edeines are mixtures of two isomeric compounds, but only one each of these exhibits the biological activity. Its antibacterial activity probably depends on the mode of linkage of the isoserine moiety with α- or β-amino groups of 2,3-diaminopropionic acid residue[6] (Figure 1).



Abbreviations: **Iph, β-phenyl-β-alanine; Ise, isoserine; A_2pr, 2,3-diaminopropionic acid; A_2ha, 2,6-diamino-7-hydroxyazelaic acid; Spe, spermidine.**

Fig. 1. The structure of edeine D isomers

The structure of both edeine D isomers has been ultimately established by total synthesis of these compounds. This allowed to demonstrate which of two the isomers is the active one.

The development of total synthesis of edeines opened the possibility of chemical modification for the establishment of structure-activity relationships.

The general concept of the synthesis was based on the coupling of N-terminal tripeptides with dipeptide amide using the Z, Boc and Trt groups as amino and methyl or *t*-butyl esters as carboxyl protection (Figure 2).

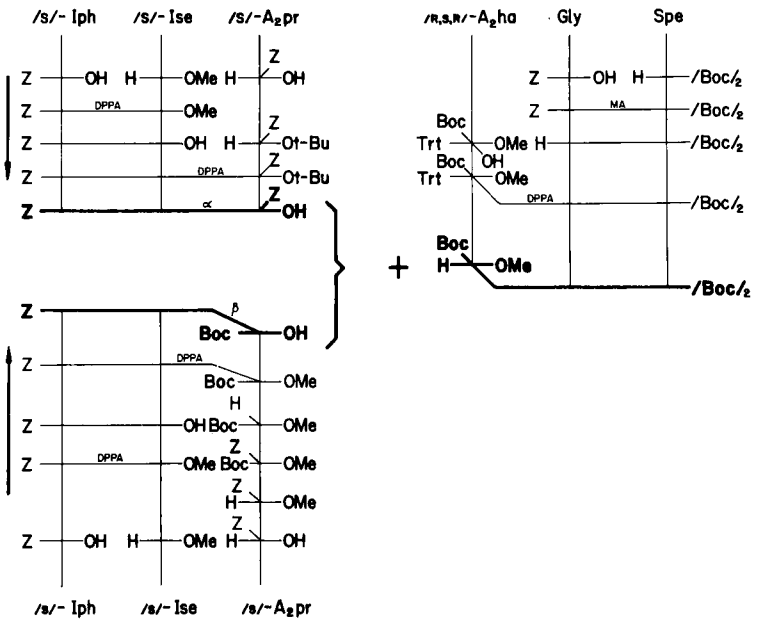
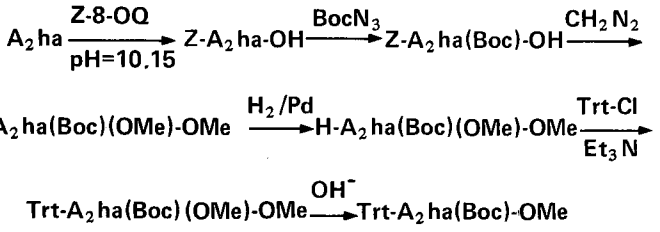


Fig. 2. The synthetic scheme for protected edeine D isomers. See Fig. 1 for amino acid abbreviations; Boc, *t*-butyloxycarbonyl; DPPA, diphenylphosphorazidate; MA, mixed anhydride; OMe, methyl ester; OtBu, *t*-butyl ester; Trt, trityl; Z, benzyloxycarbonyl.

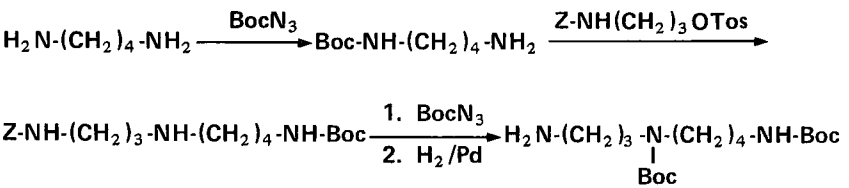
N-Terminal tripeptides were synthesized applying elongation of the peptide chain from the N-terminal amino acid (Iph) without racemization in high yield. Synthesis starting from the C-terminal 2,3-diaminopropionic acid gave the same products but in poor yield.[7]

2,6-Diamino-7-hydroxyazelaic acid has been isolated from the acid hydrolysate of edeine and transformed into the derivative suitable for synthesis as follows:[8]



Abbreviation Z-8-OQ, benzyl-8-hydroxyquinolyl carbonate

Spermidine with protected secondary and primary amino groups at the tetramethylene fragment has been obtained by the following sequence of reactions:[9]



The protected spermidine was coupled with *Z*-Gly-OH using the mixed anhydride method (MA). From the product obtained, the benzyloxycarbonyl group was removed and the protected glycine (spermidin) amide was coupled with protected A_2ha by the azide method with diphenylphosphorazidate (DPPA). After cleavage of the trityl group the protected dipeptide amide was coupled with both isomeric tripeptides using DPPA. The obtained protected pentapeptide amides were submitted to alkaline hydrolysis and catalytic hydrogenolysis in anhydrous formic acid yielding the edeine D α- and β-isomers. The antimicrobial activity was exhibited only by the edeine D α-isomer.

The synthesis of other edeines and their analogs can be performed in similar manner.

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EFFECT OF COUPLING OF ACYL MOIETY TO IMMUNOADJUVANT MURAMYLPEPTIDE

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Introduction

Recent developments in the chemistry of immunoadjuvant activity of the bacterial cell wall enable us to discuss and elucidate many biological phenomena related to immunoreactions at the molecular level. Findings that the muramyl peptide corresponding to the partial structure of the cell wall is responsible for adjuvant activity have been reported independently by our group[1] and French researchers[2]. It is now well established that *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) involved in all bacterial cell walls as a common structural moiety is the least unit required to exhibit adjuvant activity for humoral as well as cell-mediated immunities. Concerning a relationship between structure and adjuvant activity, our previous synthetic studies have revealed that the presence of the D-isoglutamine residue is a strict requirement, whereas the L-alanine residue and the sugar moiety can be changed to analogous structures without severe loss in activity[1,3,4,5].

For the Freund's test, either the cell wall itself or the synthetic muramyl peptide must be injected into the foot pad of animals in the form of a water in oil emulsion using mineral oils, such as Drakeol, otherwise no response for humoral or cellular immunity is observed. In order to clarify the role of such lipophilic medium, investigations on the effect of acyl groups introduced to the molecule of the muramyl peptide seemed to us very important.

Results and Conclusion

For the acylation of the muramyl dipeptide, the hydroxyl group in position 6 of the muramic acid moiety was chosen. The acylation was carried out via coupling reactions with acid chloride or an exchange reaction of tosylate with various kinds of fatty acids (Figure 1).

Among a series of 6-O-acyl-N-acetylmuramyl-L-alanyl-D-isoglutamine derivatives with various straight chain fatty acids (L type), the stearoyl (L18) derivative gave a minimum value of critical micelle con-

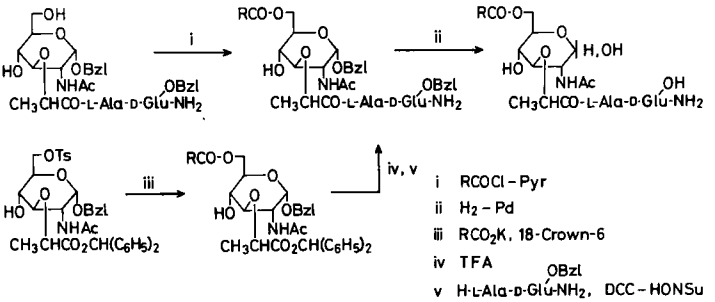

Fig. 1. Synthesis of 6-O-Acyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine.

centration as shown in Table I. This indicates that the stearoyl derivative (L18-MDP) has a special physical property to facilitate the micelle formation. Enhanced effects in adjuvant activity, i.e., corneal response, antibody production, and swelling of lymph nodes, were observed, particularly in the case of the stearoyl muramyl dipeptide (Figure 2). Furthermore, nonspecific protective activities of 6-O-acylmuramyl dipep-

Table I. Physical Properties of 6-O-Acyl-*N*-Acetylmuramyl-L-alanyl-D-isoglutamine.

Compd	Acyl group	Mp (°C, dec)	$[\alpha]_D$	Rf on TLC [a]	CMC [b]
L2-MDP	CH_3CO	132-134	+38.6°	0.14	
L4-MDP	C_3H_7CO	105-107	+35.6°	0.18	
L8-MDP	$C_7H_{15}CO$	66- 72	+32.6°	0.22	3.2
L10-MDP	$C_9H_{19}CO$	178-180	+50.3°		
L12-MDP	$C_{11}H_{23}CO$	181-182	+35.6°	0.29	0.54
L14-MDP	$C_{13}H_{27}CO$	127-129	+36.0°		
L16-MDP	$C_{15}H_{31}CO$	127-129	+37.3°		0.027
L17-MDP	$C_{16}H_{33}CO$	152-155	+37.7°		
L18-MDP	$C_{17}H_{35}CO$	171-172	+29.3°	0.35	0.013
L19-MDP	$C_{18}H_{37}CO$	128-138	+36.2°		
L20-MDP	$C_{19}H_{39}CO$	135-136	+35.1°		0.042
L22-MDP	$C_{21}H_{43}CO$	153-155	+37.0°	0.49	
L24-MDP	$C_{23}H_{47}CO$	158-160	+33.8°		0.047

[a] Silica gel, $CHCl_3$-MeOH-AcOH (5:1:0.05).
[b] Critical micelle concentration (μmol/ml) in phosphate buffer (pH 7.0) at 35° C.

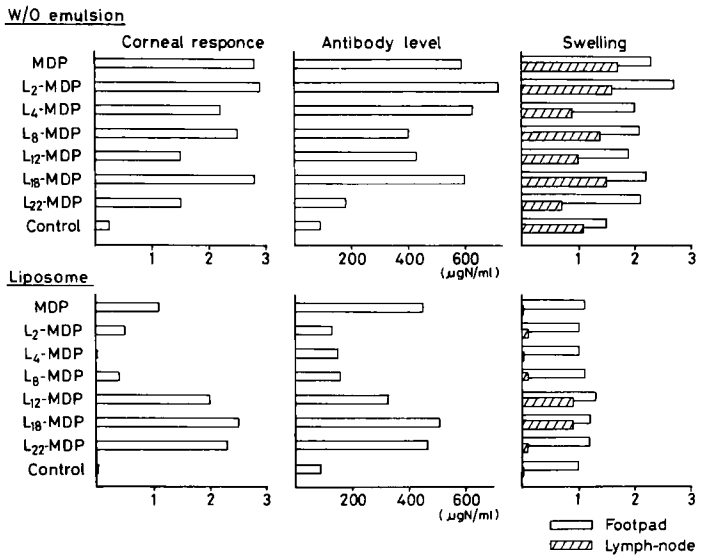

Fig. 2. Adjuvant activity of 6-O-Acyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine derivatives.

tides against bacterial infections were recognized with higher levels of the palmitoyl, stearoyl, and eicosanoyl derivatives. It should be emphasized that synergistic effects were observed for the stearoyl muramyl dipeptide p(L18-MDP) with the cephalosporin-type antibiotic cefazolin for the protection against *Escherichia coli* or with the aminoglycoside gentamycin against *Pseudomonas aeruginosa.* Another remarkable activity of 6-O-stearoyl-*N*-acetylmuramyl dipeptide (L18-MDP) was observed by Dr. Siddiqui of the University of Hawaii. The derivative can replace Freund's complete adjuvant in the immunization of owl monkeys against infections with human malaria parasites *(Plasmodium falciparum).*[6] One can now possibly avoid unsafe features in the use of Freund's adjuvant by employing the synthetic compound in the form of liposomes. Here we see an encouraging step toward the development of an effective and safe vaccine for human malaria.

Considering the structure of mycolic acid and its presence in the outermost layer of the cell wall of mycobacteria, we prepared various kinds of 6-O-acyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine with either α-branched (B type) or α-branched-β-hydroxy (BH type) fatty acid as acyl moiety (Figure 3).[7]

Most of these acylmuramyl peptides exhibited adjuvant activities to the extent shown in Figure 4. It is noteworthy that 6-O-(2-tetradecylhexadecanoyl)-N-acetylmuramyl-L-alanyl-D-isogluta-mine (B30-

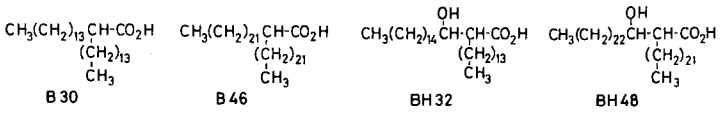

Fig. 3. Structures of High Molecular Weight Fatty Acids Used In This Study.

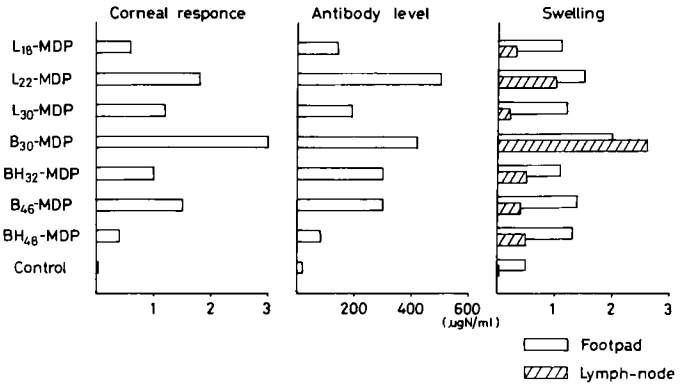

Fig. 4. Adjuvant Activity of 6-O-Acyl-N-acetylmuramyl-L-alanyl-D-isoglutamines (PBS Suspension).

MDP) showed particularly high activity for the corneal response and antibody production in guinea pigs even in the form of phosphate buffered saline suspension. A most striking effect of B30-MDP was observed in granuloma formation which is believed to be closely related to the activation of macrophages (Figure 5). There is reason for the assumption of a relationship between the biological activities stated above and the physical properties of the acylmuramyl dipeptides. In experiments for the measurement of critical micelle concentration (CMC), changes in the solubility of the dye, 4-(dimethylamino)azobenzene, in phosphate buffer upon the addition of acylmuramyl dipeptides were plotted against concentrations of acylmuramyl dipeptides (Figure 6). While the straight line for L18-MDP showed an inflection point at 0.013 μmol/ml corresponding to CMC value, such point was not observed in the line of B30-MDP as far as 0.003 μmol/ml. In the latter case, the CMC point may conceivably be located at a lower concentration, indicating that B30-MDP is more likely to form micelles than L18-MDP. This special physical characteristic of B30-MDP may be reflected in the unique adjuvant activities as well as the specific activation of macrophages. On the other hand, L30-MDP and BH32-MDP with less adjuvant activities did not give straight lines in the range of the concentrations tested, indicating that these analogs are not favorable to micelle formation.

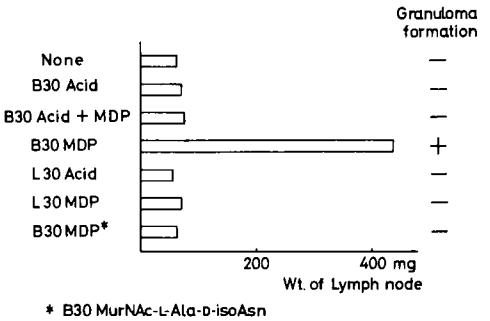

Fig. 5. Granuloma Formation by 6-O-Acyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine.

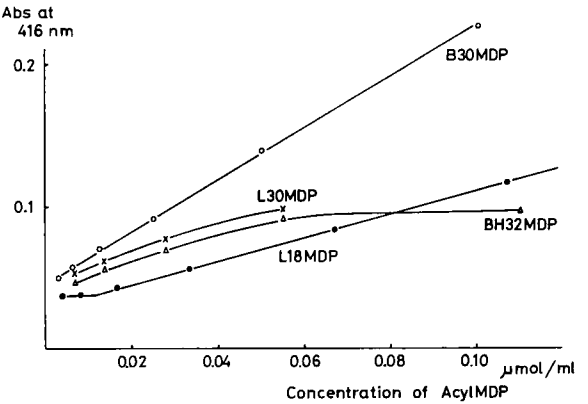

Fig. 6. Solubilization of 4-(N,N-Dimethylamino)azobenzene.

Table II. Adjuvant and Antitumor Activities of 6-O-Acyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine.

Compound	Corneal response [a]	Cell-mediated cytotoxicity [b]	Tumor-suppression [c] (Tumor-free mice / Mice tested)
L30 - MDP	2.1	60 %	0 / 10
B30 - MDP	1.2	18	1 / 10
BH32-MDP	1.6	38	2 / 10
B46 - MDP	2.6	45	4 / 10
BH48-MDP	2.4	59	7 / 10
Nocardia CWS			10 / 10
control	0.2	15	0 / 10

[a] w/o emulsion, in guinea pigs.
[b] Adjuvant activity on cell-mediated cytotoxic cells of allogeneic mice; specific lysis of target cells.
[c] 3-Methylcholanthrene-induced ascites tumor cells (5×10^4) in BALB/c mice.

Finally, the antitumor activity of 6-O-acyl-*N*-acetylmuramyl dipeptide was tested in 3-methylcholanthrene-induced ascites tumor cells of mice.[8] As shown in Table II, BH48-MDP inhibited significantly tumor growth although to a lesser degree than the *Nocardia* cell wall skeleton. It may be concluded that α-branched structures and also the β-hydroxyl function are required in the acyl moiety to enhance the antitumor activity of 6-O-acylmuramyl dipeptides. The development of synthetic analogs of 6-O-acyl-*N*-acetyl-muramyl peptides may provide us with promising and entirely new approaches to the immunotherapy of cancer.

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SYNTHETIC STUDIES DIRECTED TOWARDS THE SYNTHESIS OF A LYSOZYME ANALOG

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The total synthesis of a protein is a daunting task and is the ultimate objective for many peptide chemists. In order to be a target for total synthesis the protein must have an unequivocal primary structure and preferably a known three dimensional structure provided by X-ray analysis, also it is highly advantageous if the biological activity of the protein is well understood.

The initial objective of this work was to synthesise a protein consisting of 129 residues analogous to hen egg white Lysozyme, this particular molecule being chosen for study as it is one of the most fully charaterised proteins.[1] The ultimate purpose of the program was to shed light on the mechanism of action of the enzyme and to examine the effects of variations in structure on enzymic activity. Many Lysozymes are known with varying degrees of sequence homology but throughout a very similar tertiary structure is maintained. The target analogue which has 28 changes in sequence is shown in Figure 1. Residue changes were made in order to alleviate synthetic difficulties which are often encountered in the presence of arginine, histidine and methionine and to introduce the diagnostic residues norleucine and norvaline. All the changes were at selected surface residues leaving the active-site region unaltered. Admittedly there is some possibility that the sequence changes would alter the folding characteristics but one of the most important questions which may be posed is whether or not a polypeptide chain of this size can be synthesised by solution methods to the normally accepted standards of organic chemistry. The project was also seen as a testing ground for new protecting groups, coupling and purification methods which might be generally useful in the synthesis of smaller molecules.

The synthetic strategy employs maximal protection of side-chain functions in order to minimize side-reactions. This tactic makes for protected intermediates which are soluble in organic solvents but

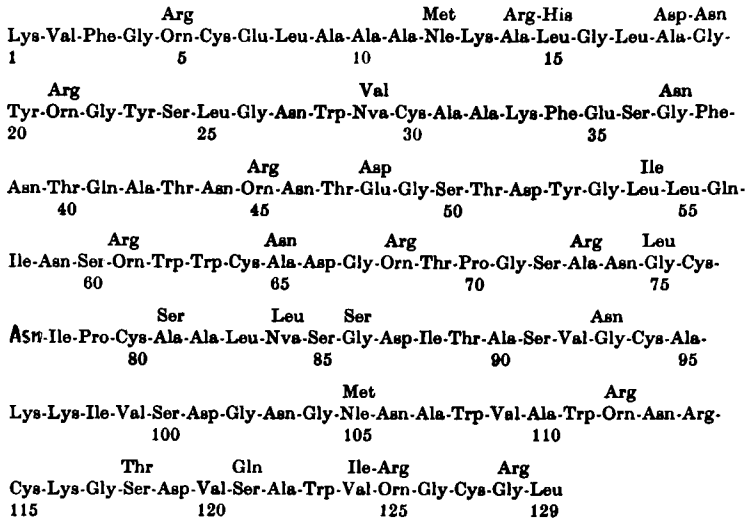


Fig. 1. Sequence of the Lysozyme analogue. Superimposed residues are those of hen egg white Lysozyme.

occasionally increased hydrophobicity led to the protected peptides becoming very insoluble, this being particularly evident in the 1-37 region of the analogue. The protecting groups (as shown in Figure 2) have generally been well tested although the phenyl ester group [2,3] has only really become proven through its use in this synthesis.

PROTECTING GROUPS

$-NH_2$

-Z ; -Bpoc ; (-BOC) ; - Adoc

$-CO_2H, OH$

$-OBu^t$; -OPh

Cysteine -Acm

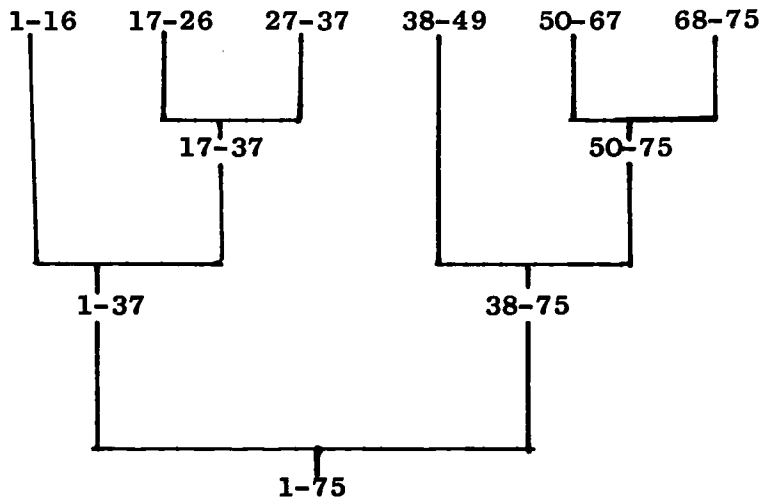
Fig. 2. Protecting groups used in the synthesis.

Phenyl esters were chosen for carboxy protection in contrast to the more widely used methyl esters as we believe that the use of the latter is only marginally acceptable in terms of racemisation. The phenyl esters may readily be prepared by condensation of N-protected amino-acids with phenol using *N,N'*-dicyclohexylcarbodi-imide (DCC) in pyridine. Removal is usually achieved in under 30 minutes in a wide range of solvents and racemisation has been shown to be zero when one equivalent of hydrogen peroxide is used at pH 10.5. The medium should ideally be 20% aqueous and should contain 50 equivalents of dimethylsulphide in order to prevent deleterious side-reactions with tryptophan or methionine.

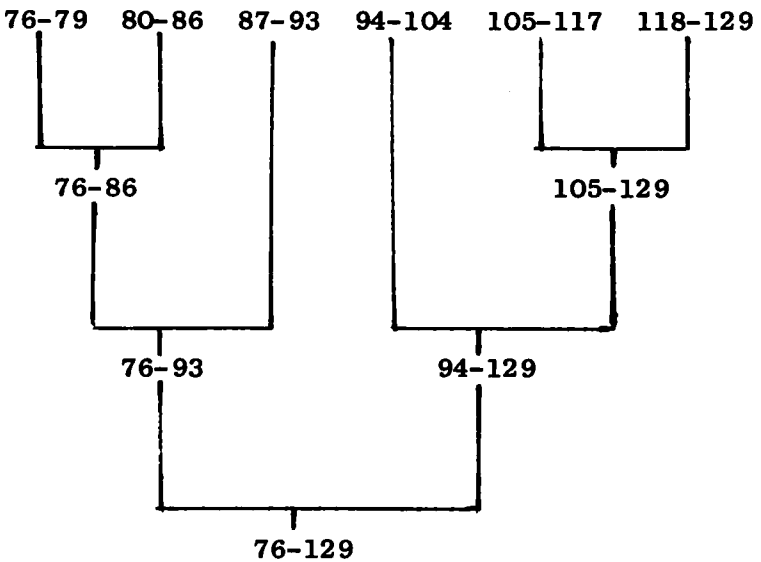
The coupling methods employed have in the main been conventional, i.e. a variety of active esters, isobutyroyl or pivaloyl mixed anhydrides and DCC in the presence of *N*-hydroxybenzotriazole[4] or *N*-hydroxysuccinimide.[5] Two new methods have however evolved during this program. The first, "Bates reagent"[6] is formed by condensing two moles of hexamethylphosphoramide in the presence of *p*-toluenesulphonylanhydride. It gives some racemisation when used in fragment coupling and thus is best restricted to use at glycine residues. In the 76-93 portion of the analogue several fragment couplings were effected with the reagent in the presence of *N*-hydroxysuccinimide which increased the coupling yield.

A second new coupling method which has been developed during the project was the diphenylphosphinic mixed anhydrides.[7] These mixed anhydrides are formed by reaction of the N-protected amino-acid with diphenylphosphinyl chloride in the presence of *N*-methylmorpholine. Couplings with this reagent are particularly efficient with no loss of regiospecificity in the opening of the activated intermediate; the levels of racemisation being similar to those encountered with other mixed anhydride methods. This means of coupling was found to be particularly useful when hindered residues such as side-chain *N*-adamantyloxycarbonyl lysine or ornithine were being coupled.

The chain was divided into twelve subfragments each terminating in glycine. These subfragments were assembled using the protecting groups and coupling methods outlined above, to yield fragments spanning the two halves of the protein 1-75[8] and 76-129.[9] The fragment couplings were carried out according to Schemes 1 and 2.



Scheme 1. Fragment couplings used to prepare Adoc(1-75)OPh.



Scheme 2. Fragment couplings used to prepare Bpoc(76-129)OBu^t.

The purification of intermediate fragments was achieved using Sephadex LH20 eluting with DMF.[10] Unfortunately this matrix has a molecular weight limit of approximately 3000 thus the larger fragments could not be purified in this way. During this work the polyacrylmorpholide, Enzacryl K_2 became available and using *N*-methylpyrrolidone (NMP) as solvent an improvement in purification was achieved in the higher molecular weight area.[11] A more significant advance occurred with the commercial availability of Sephadex LH60. Using this matrix and eluting with NMP considerable improvements were achieved.[12] Two points became apparent; firstly the fully protected 1-37 fragment which seemed to be homogeneous on Enzacryl K_2 was in fact heterogeneous although several criteria had indicated to the contrary. Secondly that considerable aggregation of the protected peptides was occurring; this being evidenced by material which eluted from the column at the void volume. Deprotection of this large molecular weight fraction gave a material which was identical by electrophoresis and isoelectric focusing to the monomeric product which eluted rather later. The Bpoc (94-129) OBu^t was particularly prone to aggregation but the aggregate could be disrupted considerably by the addition of urea to the eluant; unfortunately this complicated the isolation procedure as the urea had to be removed after chromatography before the peptide could be isolated.

Using one or more of the above gel filtration methods the protected Adoc (1-75)OPh and Bpoc (76-129)OBu^t fragments were isolated. One of the major problems encountered in syntheses of this magnitude is the difficulty in characterising intermediates.

Generally we have checked homogeneity by tlc of the protected material in several solvent systems and having established the degree of purity in this way the fragments were deprotected and inspected by electrophoresis at two pHs and isoelectric focusing. The deprotected fragments were also subjected to ion-exchange chromatography on Sephadex CM25 on a gradient running from 0.1M ammonium acetate (pH 6.5) to 0.4M "ammonium carbonate" (pH8.5). The amino-acid analyses of the protected and deprotected 1-75 and 76-129 portions are shown in Table 1. On assessing the available data we were satisfied that the two halves of the molecule were sufficiently pure for us to consider assembly of the total linear sequence of the enzyme analogue.

The phenyl ester was removed from the Adoc(1-75)OPh by treatment with hydrogen peroxide at pH 10.5 in the presence of dimethylsulphide, using trifluoroethanol as solvent. The cleavage could be followed by tlc and a qualitative check on the extent of cleavage made

Table I. Amino-acid analyses for 1-75 and 76-129 fragments.

	1-75			76-129		
Residue	Protected	Deprotected		Protected	Deprotected	
Lys/Orn	7.78	8.53	(8)	4.46	5.05	(5)
Arg	-	-	-	1.01	0.98	(1)
Asp	7.79	7.73	(8)	7.12	7.00	(7)
Thr	4.35	3.77	(5)	1.12	0.90	(1)
Ser	4.63	4.40	(5)	4.57	4.67	(5)
Glu	5.29	5.37	(5)	-	-	-
Pro	0.80	0.95	(1)	1.01	0.98	(1)
Gly	11.00	10.40	(11)	7.12	7.15	(7)
Ala	10.10	9.94	(10)	7.31	6.93	(7)
Val	0.89	1.14	(1)	4.71	4.97	(5)
Nva	1.20	0.84	(1)	1.14	1.05	(1)
Ile	1.12	1.18	(1)	3.01	2.64	(3)
Leu	6.37	5.98	(6)	2.10	2.18	(2)
Nle	0.96	1.10	(1)	1.01	1.05	(1)
Tyr	3.04	2.78	(3)	-	-	-
Phe	2.92	3.05	(3)	-	-	-

Theoretical value show in parenthesis, no correction has been made for the partial destruction of serine and threonine.

by UV estimation of the liberated phenol. Cleavage of the Bpoc function from Bpoc(76-129)OBu^t was rapidly achieved using 0.05M HCl in 90% aqueous trifluoroethanol, the cleavage again being monitored by tlc and UV. Coupling was carried out over 3 days using DCC/*N*-hydroxysuccinimide (1.2 equivalents of DCC and 2 equivalents of *N*-hydroxysuccimide being added initially and a further 0.5 and 1 equivalent respectively after 24 hours) employing a mixture of hexamethylphosphoramide and DMF as solvent. The crude product was precipitated with water and washed with a variety of solvents, to give material having the amino acid analysis: Lys/Orn 12.08 (13), Arg 1.00 (1), Asp 14.81 (15),

Thr 5.50 (6), Ser 8.27 (10), Glu 5.27 (5), Pro 2.08 (2), Gly 17.93 (18), Ala 16.98 (17), Cys 8.06 (8), Val 5.93 (6), Nva 2.51^{+} (2), Ile 4.29 (4), Leu 8.27 (8), Nle 2.04 (2), Tyr 2.94 (3), Phe 2.82 (3). (Carried out in the presence of 2-nitrophenylsulphenylchloride with theoretical values in parenthesis; + occurs at the buffer change).

This material was then deprotected under nitrogen by treatment with 90% trifluoroacetic acid for 3 hours using anisole and mercaptoethanol as scavengers. The deprotected product was precipitated with ether, then chromatographed on Sephadex G50 eluting with 50% acetic acid. The major product (Ve/Vt 0.4) which represented a rather low overall recovery was rechromatographed on Sephadex CM25 in the system described earlier. Two peaks were observed the second of which gave the best amino-acid analysis, again however the recovery was low. This product which was homogeneous by isoelectric focusing with an isoelectric point between 10.5 and 11 was then dissolved in 50% acetic acid and treated with mercuric acetate for 70 minutes, the free thiol being obtained by treatment with mercaptoethanol and subsequent chromatography on Sephadex G15 eluting with 0.1M acetic acid. An initial figure of approximately four, (4.01, 4.16) out of a possible eight, thiol functions was obtained using the Ellman reagent. The removal of the *S*-acetamidomethyl function was then repeated in the presence of 8M urea and the liberated thiol figure rose to approximately six (6.15, 6.00) but still unacceptably short of the anticipated value. Although this figure was considered to be too low for the oxidation to stand a reasonable chance of success, the oxidative final step was attempted. Using this material no Lysozyme activity was observed by the standard assay.

From this work it is clear that the *S*-acetamidomethyl function is far from adequate as a cysteine protecting group. Using mercuric acetate the recovery of thiol was only about 75% of the expected figure, in addition the integrity of tyrosine residues must be checked as mercuration can be a serious side-reaction. Probably direct formation of disulphide using iodine is the best method for *S*-acetamidomethyl group removal although in many peptides including Lysozyme this is prevented by the presence of tryptophan. Thus from our work it is clear that development of new S-protecting groups is vital if large peptide and proteins are to be synthesised. The improvement of purification and characterisation methods must also be a priority, this is particularly true for large fully protected fragments as there are a much wider range of methods available for free peptides.

Acknowledgements

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SYNTHESIS OF CYCLOTETRAPEPTIDES, AM-TOXINS AND ANALOGS OF CYL-2

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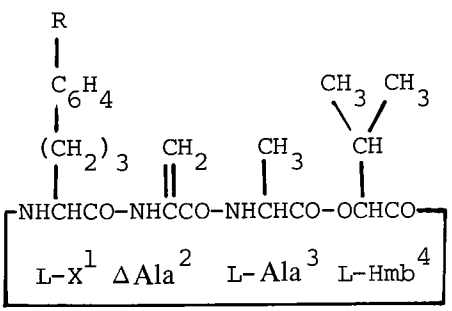
AM-toxins and Cyl-2 are phytotoxins with cyclic tetrapeptide structures. AM-toxin I *(1),* II *(2),* and III *(3)* are host-specific toxins causing necrosis on apple leaves, their structures being *cyclo*(-L-X-ΔAla-L-Ala-L-Hmb-) : X in *1,* Amp, 2-amino-5-*p*-methoxyphenylpentanoic acid; X in *2,* App, 2-amino-5-phenylpentanoic acid; X in *3,* Ahp, 2-amino-5-*p*-hydroxyphenylpentanoic acid; ΔAla, dehydroalanine; and Hmb, 2-hydroxy-3-methylbutanoic acid. We attempted to establish the synthetic confirmation of the structures of AM-toxins I,[1,2] II,[1] and III[1] and clarify the structure-activity relationships of AM-toxins through the syntheses of analogs.



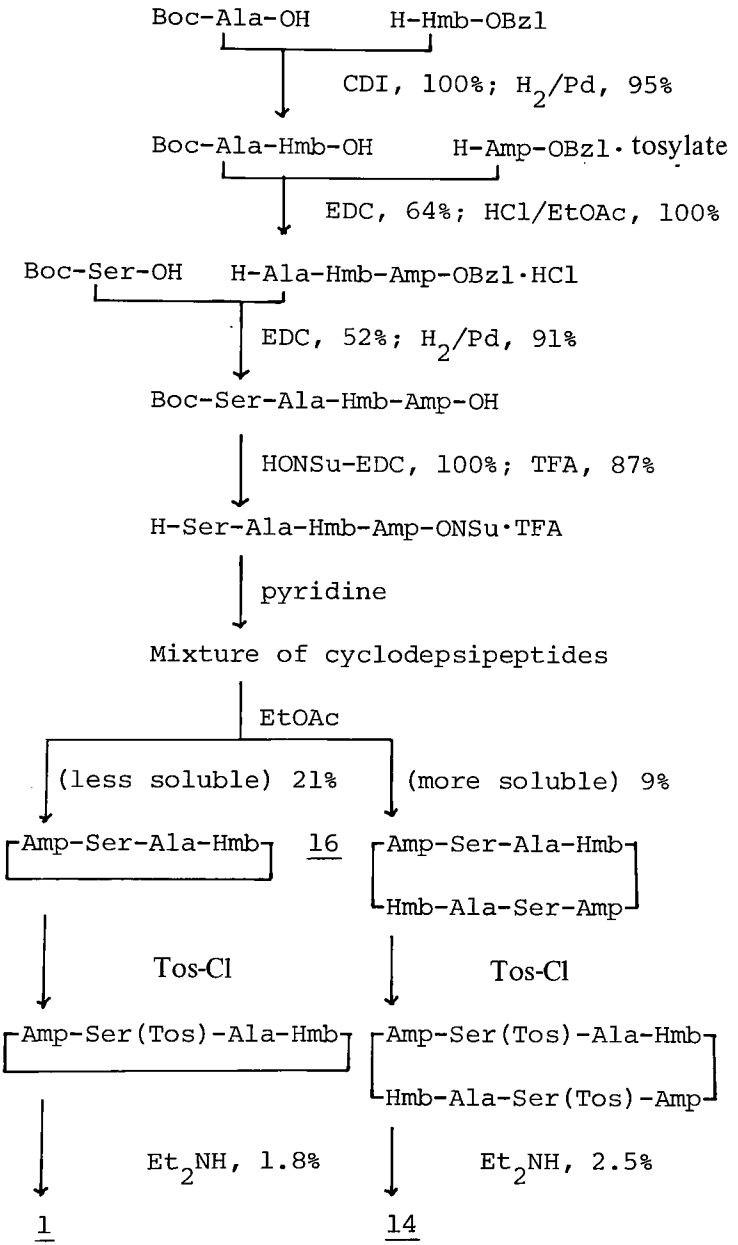
		R	X
AM-toxin I	(1)	OCH_3	Amp
AM-toxin II	(2)	H	App
AM-toxin III	(3)	OH	Ahp

Fig. 1. Structure of AM-toxins.

As a preliminary study, we prepared a simple analog, [L-Tyr-$(Me)^1$]-AM-toxin *(15),* Table I, as a model peptide of *1,* and studied the mode of cyclization and dehydration reactions. The synthesis of *15* was designed in a way that six different routes were used. The six tetrapeptide

intermediates, H-Ser-Ala-Hmb-Tyr(Me)-ONSu *(4)*, H-Tyr(Me)-Ser-Ala-Hmb-ONSu *(5)*, H-Ala-Hmb-Tyr(Me)-Ser-ONSu *(6)*, H-Tyr(Me)-ΔAla-Ala-Hmb-ONSu *(7)*, H-Ala-Hmb-Tyr(Me)-ΔAla-N_3 *(8)*, and H-Ala-Hmb-Tyr(Me)-Ser(Tos)-N_3 *(9)*, were subjected to the cyclization reaction. Only compound *4* afforded cyclic monomer in good yield (32%), the ratio of monomer to concomitant cyclic dimer being 3:2. Compounds containing ΔAla *(7* and *8)* or azides *(8* and *9)* did not afford *15*. Compounds *5* and *6* gave mainly cyclic dimer (the ratio of monomer to dimer, 5:95). These cyclization reactions were carried out in high dilution (3 mM) in pyridine. In case of the cyclization of compound *4*, crude cyclic peptides were purified by fractional precipitation. The L-Ser residue in the purified cyclic peptides was quantitatively converted to Ser(Tos) residue with *p*-toluenesulfonyl chloride (Tos-Cl), and then converted again to ΔAla residue with Et_2NH. Aromatic amino acids contained in AM-toxins I and II were prepared from diethyl acetamidomalonate and arylpropyl bromides and then resolved with Taka-acylase.[4] AM-toxin I *(1)* was synthesized in a similar manner as shown in Scheme I.[5,6] The yield of the conversion reaction to ΔAla residue was lower because of steric hindrance of the bulky Amp side chain.

In order to study the biological role of ΔAla, saturated analogs containing D-Ala and L-Ala in place of ΔAla were synthesized.[7] In this case, cyclization of the D-Ala-containing precursor yielded exclusively monomer (50%). Therefore, the synthesis of AM-toxin II *(2)* was carried out using D-Ser, and the cyclic monomer, *cyclo*(-L-App-D-Ser-L-Ala-L-Hmb-) *(10)*, was obtained in high yield (50%) as expected. However, the tosylation reaction of *10* was difficult to achieve and, furthermore, tosylated product *(11)* was quite stable in the treatment of bases. Vigorous conditions made *11* to decompose. The use of methanesulfonyl chloride (MsCl) containing sulfur dioxide finally provided esterification of *10* to [D-Ser(Ms)^2]-AM-toxin, which was converted into cyclotetradepsipeptide *2* by treatment with Et_2NH. These results suggest that the difference in behavior to form sulfonic esters between cyclotetradepsipeptides containing D-Ser and those containing L-Ser may be attributed to differences in the conformations, and that the difficulty in the elimination of tosyl and phenylmethanesulfonyl groups may be due to steric hindrance of their bulky phenyl groups preventing the access of the base. Synthetic AM-toxins I *(1)* and II *(2)* showed same R_f in TLC, mass and UV spectra, and toxic activities (Table I) as those of natural ones.



Scheme 1. Synthesis of AM-toxin I *(1)* and its dimer *(14)*.

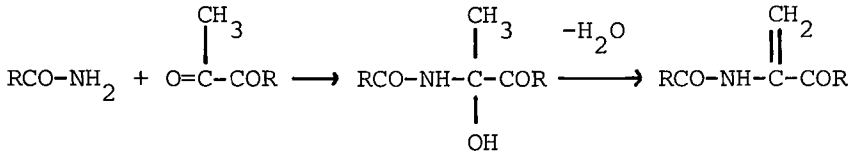
Table I. Necrotic Activities of Synthetic AM-Toxins Against Apple Leaves

Leaves

AM-toxins		Side chain position 1	Side chain position 2	Necrotic activity, μg/ml
AM-toxin I	(1)	$-(CH_2)_3-C_6H_4-OCH_3$	$=CH_2$	0.002
AM-toxin II	(2)	$-(CH_2)_3-C_6H_5$	$=CH_2$	0.02
[D-Ala^2]-AM-toxin I	(12)	$-(CH_2)_3-C_6H_4-OCH_3$	$-CH_3$(D)	5-10
[L-Phe^1]-AM-toxin	(13)	$-CH_2-C_6H_5$	$=CH_2$	20
AM-toxin I dimer	(14)	$(-(CH_2)_3-C_6H_4-OCH_3)$	$(=CH_2)$	20-40
[L-Tyr(Me)^1]-AM-toxin	(15)	$-CH_2-C_6H_4-OCH_3$	$=CH_2$	25-50
[L-Ser^2]-AM-toxin I	(16)	$-(CH_2)_3-C_6H_4-OCH_3$	$-CH_2OH$(L)	30
[L-Ala^2]-AM-toxin I	(17)	$-(CH_2)_3-C_6H_4-OCH_3$	$-CH_3$(L)	>100

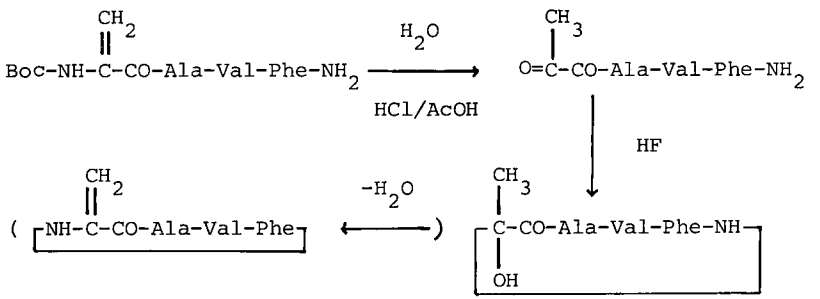
Table I shows the structures of synthesized analogs and their activities. Activity of △Ala-containing dimers, which were prepared from L-Ser-containing dimers, was very low, suggesting the importance of ring sizes. Distinct difference in activities between *12* and *17* clearly shows the importance of spatial structure. Conformational analysis of *12* and *17* suggests the occurrence of all-trans-3-1 type backbone for *12* and all-trans-4-0 type for *17*. All four CO groups in *17* face to one side of the ring, whereas, the CO group of the aromatic residue in *12* faces to the other side.[9] The backbone conformation of AM-toxins is expected to be all-trans-3-1 type as expected from biological consideration. We can summarize that the presence of the △Ala or Ser residue, the presence of the arylpentanoic residue, and the specific conformation are important for showing activity.

The synthetic methods used for AM-toxins I and II can hardly be utilized for AM-toxin III *(3)* because *3* contains the free hydroxyl group. Therefore, we tried to apply the condensation of amide with keto acid (Scheme 2). We attempted to synthesize simple AM-toxin analogs by the intramolecular condensation of pyruvyl tripeptide amides, which were expected to give cyclized peptides with a △Ala residue. Boc-△Ala-L-Ala-L-Val-L-Phe-NH_2, which was prepared from Boc-△Ala-OH and the



Scheme 2. Condensation of acylamide with keto acid.

tripeptide amide by the conventional method, was converted to pyruvyl-Ala-Val-Phe-NH_2 by the treatment with 1 M HCl in AcOH in the presence of an equivalent amount of water. The pyruvyl tripeptide amide was treated with anhydrous HF for 1 hour at 0°C, and after purification by column chromatography using silicic acid, a pure product was obtained in high yield (60-80%). By elemental analysis, mass spectrum and NMR study, the compound was identified to be *cyclo*(-Hyala-Ala-Val-Phe-) : Hyala, α-hydroxyalanine. No dimer was detected. The treatment of Boc-ΔAla-Ala-Val-Phe-NH_2 with anhydrous HF afforded the same product. *Cyclo*(-Hyala-Ala-Hmb-Phe-) was also obtained in the same manner from Boc-ΔAla-Ala-Hmb-Phe-NH_2 (74%). Conversion of Hyala residue to ΔAla residue is under investigation (Scheme 3).



Scheme 3. Synthesis of AM-toxin analog containing an α-hydroxyalanine residue.

Cyl-2 inhibits the root growth of lettuce seedlings, and its structure is shown to be *cyclo*(-D-Tyr(Me)^1-L-Ile^2-L-Pip^3-Aoe^4) : Pip, pipecolic acid; Aoe, 2-amino-8-oxo-9,10-epoxydecanoic acid. The configuration of Aoe is unknown. We synthesized simple analogs, [L-Pro^3, L-Leu^4]-, [L-Tyr(Me)^1, L-Pro^3, L-Leu^4]-, [L-Leu^4]-, and [D-Leu^4] Cyl-2 *(18, 19, 20,* and *21,* respectively). In case of *18,* Boc-Ile-Pro-OH was synthesized at

first and peptide bonds were extended to both N and C directions. H-D-Tyr(Me)-L-Ile-L-Pro-L-Leu-ONSu was subjected to cyclization reaction and yielded monomer *18* (50%). Other analogs were prepared similarly, silica gel chromatography being used for purification. ^{1}H-NMR spectra of *18, 20* and natural Cyl-2 showed a similar pattern. On the other hand, *19* and *21* showed different patterns. These results suggest the configuration of Aoe in natural Cyl-2 to be L and the conformation of Cyl-2 to be similar to those of *18* and *20*. A unique *trans-trans-cis-trans* backbone conformation with a *cis* Ile-Pro bond is proposed for *18* on the basis of ^{1}H- and ^{13}C-NMR results.

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APPROACHES TO THE SOLID PHASE SYNTHESIS OF PORCINE RELAXIN

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Relaxin is a 51-amino acid ovarian peptide hormone whose main biological function is to soften the ligaments of the pubic symphysis and other pelvic joints during pregnancy, thus facilitating parturition. Another important biological effect of relaxin is to inhibit contractions of the uterus, an effect which may contribute to prevention of the premature onset of labour. Relaxin also causes dilatation and softening of the cervix and may stimulate mammary gland development (See Schwabe *et al.*,[1] for review).

The isolation and purification of relaxin from the pregnant pig ovary[2] and from the rat[3] have been described. To date only the amino acid sequence of the pig hormone is known although discrepancies exist in the structures reported from two different laboratories.[4,5]

Figure 1 describes the sequence of the major form of the pig relaxin isolated from pregnant ovaries as determined in our laboratory.[6] The sequence shown differs slightly from that reported by Schwabe & McDonald[4] and from our own previously published sequence.[5]

A striking feature of the structure is the similarity with insulin. Relaxin is a two-chain molecule comprising an A-chain of 22 residues and a B-chain of 29 residues. The A-peptide has an intra-chain disulfide loop and is joined to the B-peptide via inter-chain disulfide bonds. The disposition of the disulfide bridges has been completely conserved in insulin and relaxin.[4] However, apart from these general structural similarities there is little sequence homology. Relaxin, unlike insulin, contains methionine and tryptophan, and has a pyroglutamyl residue at the amino terminus of the B-chain. There is no histidine or tyrosine in

relaxin. During sequence analysis the isolated B-chain was found to be poorly soluble in aqueous media.

As with insulin, there is now evidence that relaxin is biosynthesized via a prohormone structure involving a C-peptide linking the carboxyl terminal of the B-chain with the amino terminal of the A-chain.[5,7] These close structural similarities between relaxin, insulin and other peptide growth factors suggests that they may have evolved from a common ancestral gene.[5,8] On the basis of the amino acid sequence and the analogy with insulin, models of the tertiary structure of relaxin have been proposed.[9,10] A key feature of the models is the close proximity of the carboxyl end of the B-chain to the amino terminus of the A-chain. Again, this is similar to the situation found with insulin.[11,12]

A CHAIN

```
 1                   5                  10
H-ARG-MET-THR-LEU-SER-GLU-LYS-CYS-CYS-GLN-VAL-GLY-CYS

      15                  20      22
ILE-ARG-LYS-ASP-ILE-ALA-ARG-LEU-CYS-OH
```

B CHAIN

```
 1               6                  10
PCA-SER-THR-ASN-ASP-PHE-ILE-LYS-ALA-CYS-GLY-ARG-GLU

    15                  20                  25
LEU-VAL-ARG-LEU-TRP-VAL-GLU-ILE-CYS-GLY-SER-VAL-SER

        29
TRP-GLY-ARG-OH
```

Fig. 1. Amino acid sequence of porcine ovarian relaxin.

The close structural similarities of relaxin and insulin suggested to us that the procedures developed for the synthesis of insulin should be directly applicable to relaxin. The two main approaches we have explored include (1) synthesis of the separate A and B peptides with appropriate selective protection of the functional groups followed by chain combination, and (2) the assembly of an artificial pro-hormone with the B-chain joined to the A-peptide via linkages that can be removed following chain combination.

Separate-Chain Approach — *Solid phase synthesis of relaxin A-chain.* The 22-peptide chain representing the sequence of relaxin A-chain

was assembled by standard solid phase procedures on a phenylacetamidomethyl-1% cross-linked polystyrene (PAM) resin.[13] In three separate syntheses the following A-chain peptides were prepared: (Acm-Cys-9,22) Relaxin A (1-22); (SO_3^--Cys-8,9,13,22) Relaxin A (1-22) and (Nle-2,SO_3^--Cys-8,9,13,22) Relaxin A (1-22). Purification of the crude peptide following HF-anisole cleavage was by gel-filtration on BioGel P10 in 0.1*M* acetic acid followed by ion-exchange chromatography. In the case of the Acm-Cys peptide purification was performed on a CM-cellulose column with elution by an ammonium acetate gradient at pH 6.7. The purified peptide eluted at a conductivity of 7.0 mS. The S-sulfonated peptides, prepared according to the procedure at Katsoyannis *et al.*[14,15] were purified on DEAE cellulose equilibrated in 0.1 *M*tris-HCl, pH 8.5 and eluted with a NaCl gradient (to 0.5*M* NaCl). The S-sulfonated peptide eluted with a conductivity of 3.0 mS. The purified peptides were found to be homogeneous as assessed by amino acid analysis, TLC and Edman sequence analysis. The overall yield of pure product was in the range of 12-20% based on the crude peptide obtained after HF cleavage. The major side products isolated and identified were termination peptides arising from cyclization of Gln-10, (8 to 21%); Lys blocked peptides from failure to completely remove trifluoroacetyl protecting groups (47%) and peptide polymers arising from incomplete S-sulfonation (28 to 36%). Approximately 18% of the Acm groups on Cys were lost during HF cleavage. The poor efficiency observed for the removal of the TFA groups from Lys using standard conditions was surprising and may be sequence related. Use of the Cl-Z protecting group eliminated the problem. The formation of Glu-anisole adducts and trifluoroacetylated termination peptides was negligible.

Solid phase synthesis of Relaxin B-chain. Relaxin B-chain was synthesized according to the general procedures described above commencing with a Kel F-g-polystyrene PAM resin. Protection of Cys was via the *p*-methoxybenzyl group. Immediately after cleavage with HF-anisole the crude peptide was converted to the S-sulfonate derivative. Relaxin B-chain whether in the free sulfhydryl form or as the S-sulfonate derivative was found to be completely insoluble in most aqueous and organic solvents, thus severely hampering purification and characterization procedures. The only solvent in which the B-chain would completely dissolve was 6 *M* urea. Initial purification was by gel-filtration on BioGel P4 in 6 *M* urea; however, attempts to purify the peptide further by ion-exchange chromatography in urea resulted in the peptide precipitating

out on the column as the salt concentration increased. The synthetic B-chain could only be partially purified by separating the soluble impurities. In this way it has been possible to identify the major side products as arising from chain termination at Glu-20, Cys-23 and Cys-10. However, because of solubility difficulties it has not been possible to arrive at accurate yield figures.

Attempted combination of synthetic Relaxin A and B chains. The method of chain combination found to be satisfactory for synthetic insulin preparations was attempted with the synthetic relaxin A and B peptides. This procedure developed by Schwartz and Katsoyannis[15] involves reduction of $A(S\text{-}SO_3^-)_4$ completely reduced form with mercaptoethanol at pH 8.3 followed by reaction with $B(S\text{-}SO_3^-)_2$ at pH 10.6. A 2-fold molar excess of A-chain to B-chain was used. The chain combination experiments were monitored by radioimmunoassay using an antibody raised to native porcine ovarian relaxin, referred to as CMa′ according to the nomenclature of Sherwood & O'Byrne.[2] Figure 2 shows that the antibody system does not recognize synthetic A-chain but has a very slight recognition of the B-chain at high concentrations. Neither synthetic $A(S\text{-}SO_3^-)_4$ nor native $A(S\text{-}SO_3^-)_4$ was detected by the assay system. Equimolar mixtures of A and B S-sulfonated peptides likewise were not recognized by the antibody.

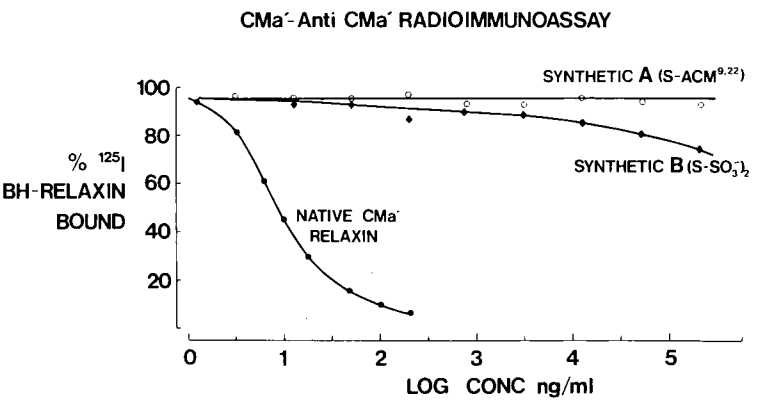

Fig. 2. Radioimmunoassay of native porcine relaxin and synthetic A and B chains. Labelled tracer was prepared by the Bolton-Hunter (BH) procedure.

Other chain combination experiments were attempted in which the Acm-Cys-A-chain was deprotected with iodine in 60% acetic acid in the presence of fully reduced B-chain.

Figure 3 illustrates a representative sample of numerous chain combination experiments that have been carried out. Using the standard Katsoyannis procedure or the Acm-Cys/iodine method described above, only very weak relaxin immunoreactivity could be detected. Analysis of the reaction mixture indicated that the major product was the A-chain dimer which arises because of the poor solubility charateristics of the B-chain peptide. A similarly poor chain combination yield was observed with S-sulfonated chains isolated from native relaxin, again because of the insolubility of the B-chain in the aqueous solvents used. A significant improvement in yield was observed when the combination experiment was performed in 6 *M* urea — the only solvent found so far in which the B S-sulfonated chain is completely soluble. Following addition of reduced A-chain to the B S-sulfonate the reaction mixture was transferred to a dialysis bag and dialyzed against a 0.1 *M* sodium glycinate buffer, pH 10.6, overnight at room temperature and finally against distilled water at 4°C for 24 hours. The peptide was recovered by lyophilization. The average yield when the combination reaction was performed in the presence of urea was approximately 1%. Whereas this yield is very low it should be compared with the usual yields reported for the combination of synthetic insulin chains which are of the order of 6 to 16%.[16]

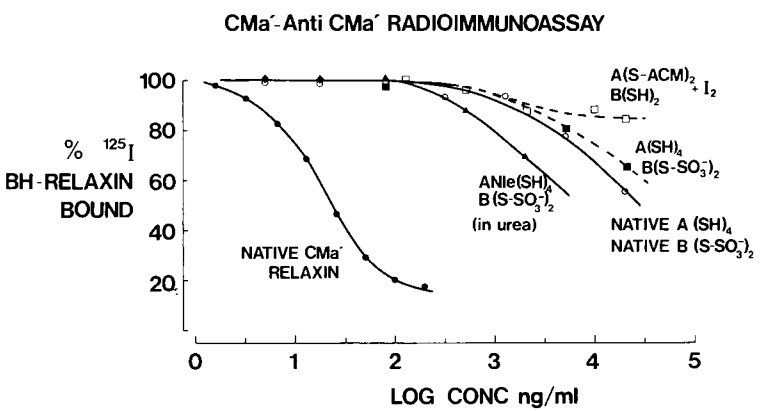

Fig. 3. Attempted chain combination experiments monitored by radioimmunoassay.

Single-Chain Artificial Pro-Hormone Approach — The poor solubility characteristics of the B-chain and the low combination yields experienced even with native relaxin suggested that an approach via an artificial pro-hormone would be worthy of investigation. The use of a reversible cross-linking agent to improve combination yields of insulin chains has been reported by Carpenter *et al.*[17] and Geiger & Obermeier.[18] Yields of 25 to 38% have been reported via this procedure. Rather than attempt to combine the separate chains with a cross-linking agent (relaxin does not have a Lys close to the amino terminus of the A-chain) we chose to synthesize an artificial pro-hormone in one step by the solid phase procedure. Initially the Nle-2 analogue of the relaxin A-chain was assembled on a PAM resin using the conditions described above. This was followed by the spacer arm MET-GLY-MET (MGM) and then the B-chain sequence. All the side-chain protecting groups were chosen to be simultaneously removed upon cleavage of the peptide from the resin (see Figure 4). After purification the experimental plan was to attempt a controlled oxidation step to fold the chain in the correct orientation for disulfide formation, followed by cyanogen bromide cleavage to remove the spacer arm and a Cpase A digestion to remove the carboxyl terminal homoserine generated during this cleavage.

The synthesis proceeded smoothly and preliminary chemical characterization of the synthetic pro-hormone product has been most

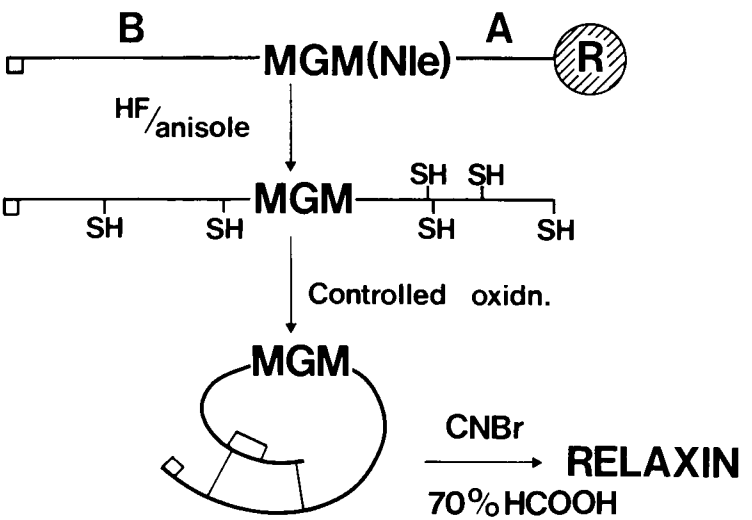

Fig. 4. Strategy for the solid phase synthesis of relaxin by the artificial pro-hormone approach.

satisfactory. Figure 5 illustrates the elution profile of the crude peptide after gel-filtration on BioGel P10 in 30% acetic acid. A majority of the product elutes as polymers or dimers. Peak III represents single-chain MGM-pro-hormone. Peak I and II could be converted back to the monomer form with reducing agent; however, the reduced form of the pro-hormone was found to be very insoluble, requiring the presence of urea or guanidine for solubilisation during the re-oxidation step.

Figure 5 describes the immunoreactivity of the various forms of the pro-hormone preparations. The reduced and re-oxidised MGM-pro-hormone peptide had 1.5% of the activity of native relaxin. In preliminary experiments this immunoreactivity was not further improved by removing the MGM spacer peptide.

In summary, the solid phase synthesis of the separate chains of relaxin has been achieved. The poor solubility characteristics of the B-chain severely hampered purification procedures and prevented effective chain combination with A-chain using conditions which have been reported to achieve satisfactory yields with the structurally related insulin molecule. The use of 6 *M* urea to solubilize the separate chains during the

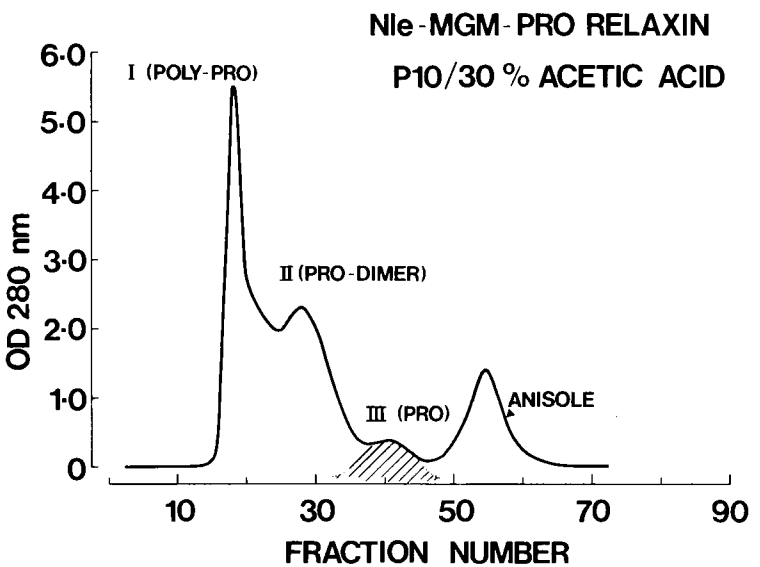

Fig. 5. Gel filtration profile of crude Nle-2, MGM-pro-relaxin peptide.

combination experiment results in a significant improvement but the yields are very low. The use of an artificial pro-hormone synthesized in one step by the solid phase procedure appears to be a more satisfactory approach. Appropriate conditions for chain folding and optimal length and composition of the artificial linking arm are currently under investigation.

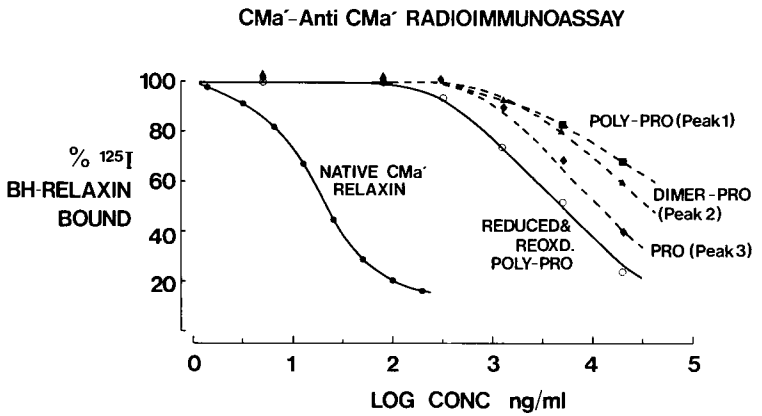

Fig. 6. Immunoactivity of synthetic Nle-2, MGM-pro-relaxin peptides.

Acknowledgements

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NOVEL PARTIALLY MODIFIED RETRO-INVERSO ANALOGS OF BIOLOGICALLY ACTIVE PEPTIDES

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Partial retro-inverso modification of biologically active peptides was suggested by us as the most generally applicable approach to topochemically related analogs[1]. The ultimate requirement is to reproduce the side chain topochemistry of the extended conformation of the parent peptide as closely as possible in analogs in which part of the sequence is reversed. This topochemical transformation results in the incorporation of two residues which may not be amino acid residues at the sites of initiation and termination of the retro modified section. The amino acid residue at the N-terminus of the modified section is transformed *via* Curtius rearrangement to a corresponding *gem*-diaminoalkyl residue. The C-terminal amino acid residue is replaced by a malonic acid residue substituted on the C^{α} position by the side chain of the original amino acid (see Structure *A* in Figure 1). In those cases where the modification involves more than a single peptide bond, the reversed sequence between the two non-amino acid residues is comprised of the enantiomeric amino acids (see Structure *B* in Figure 1).

The synthetic methodology involved in the preparation of the modified retro-inverso peptides was described in our previous publications[1,2].

In these proceedings we report our recent applications of our approach to investigate partially modified retro-inverso analogs of hypothalamic hormones such as LH-RH and TRH and of the morphinomimetic peptide such as enkephelin.

Studies on the enzymatic mechanism for biodegradation of biologically active peptides suggested which of the peptide bonds are susceptible to proteolytic cleavage[3]. One reason for choosing these bonds for modification was to enhance the stability of the analogs by introducing retro-inverso modifications.

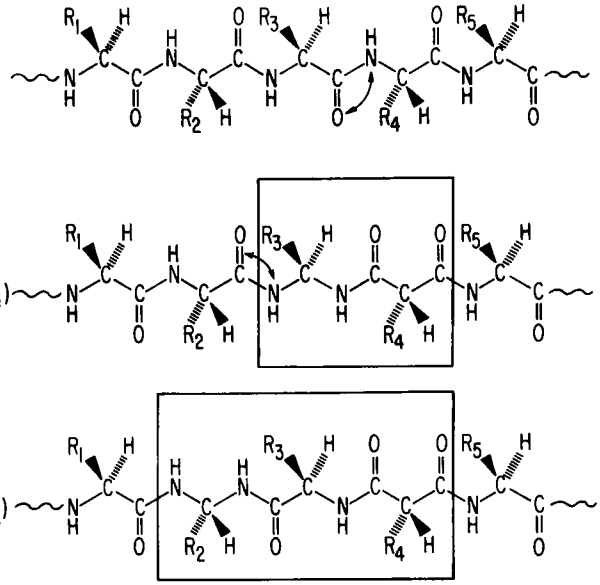

Fig. 1. Schematic representation of partially modified retro-inverso analogs (*A* & *B*) of the parent peptide. The arrow indicates the peptide bond subjected to modification. The structure enclosed indicates the modified sections.

Figure 2 outlines the various partially modified retro-inverso analogs of LH-RH and TRH prepared by us. In Compounds I and II the modification was directed to the peptide bonds susceptible to degradation by endopeptidases[4], and included a reversal of a single peptide bond (Tyr[5]-Gly[6]) or two consecutive bonds (Tyr[5]-Gly[6] and Gly[6]-Leu[7]). The stimulation of release of LH and FSH by analogs I and II was determined *in vitro*[5] and was found to be less than 0.01% and 2%, respectively, as compared to the LH-RH, and no antagonist activity could be demonstrated. Analogs III-VIII were prepared, characterized, and the tests for *in vitro* biological activity are under way. Figure 3 outlines the synthesis of Compound I which is a typical synthetic scheme for the various partially modified retro-inverso analogs.

Analog III is a C-terminally modified analog of des-Gly[10]-[Pro[9]-NEt]-LH-RH found to be a powerful agonist[6]. Analogs IV and V include modifications at the N-terminal and are comprised of a single and two consecutive peptide bonds, respectively. In analogs VI-VIII of TRH the modification is in the N-terminal peptide bond (pGlu[1]-His[2]) or in a carboxamide function yielding an N-formylated modified C-terminus or a combination of both modifications as in VIII. The biodegradation studies of analogs VI-VIII are in progress.

The most encouraging results to date have been obtained when this topochemical approach was applied to the C-terminal modification of [D-Ala[2]]-enkephalinamides[7]. Table I outlines the various analogs and

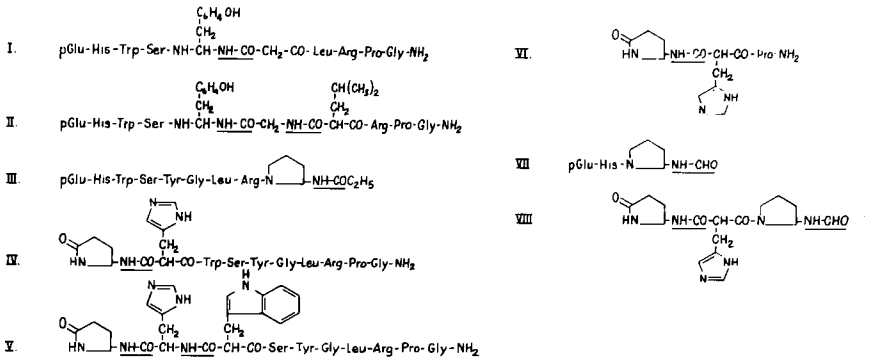

Fig. 2. Partially modified retro-inverso analogs of LH-RH (I-V) and TRH (VI-VIII). The reversed peptide bonds are underlined.

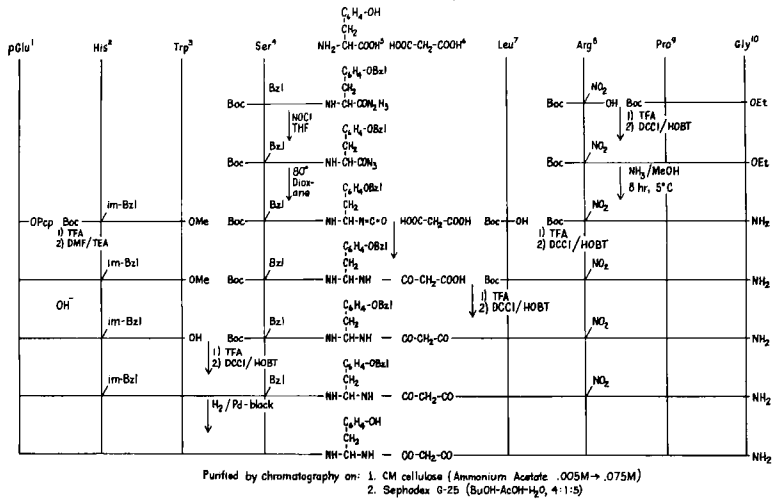

Fig. 3. Synthetic scheme of a partially modified retro-inverso LH-RH analog (Analog I).

their *in vitro* biological activity[8]. Single peptide bond modifications between residues 4 and 5 resulted in analogs IX and X and additional modifications on the carboxamide C-terminus resulted in the replacement of an L-amino acid amide by an N-formyl-D-amino acid residue. The *in vivo* assay[9] of analogs XI and XII demonstrated analgesic activity similar in duration and potency as that observed for β-endorphin on the same weight basis.

Table I. *In Vitro* Activity of Partially Modified Retro-inverso Analogs of [D-Ala^2]-Enkephalinamides. The Modified Peptide Bonds are Underlined.

COMPOUND NO.	COMPOUND	Rel. Potency	95% Confidence Limits
	Tyr-Gly-Gly-Phe-Met-OH (Met^5-Enkephalin)	100	--
IX.	Tyr-D-Ala-Gly-NH-CH(CH_2-C_6H_5)-<u>NH-CO</u>-CH(CH_2-$CH(CH_3)_2$)-CO-NH_2	200	(174-240)
X.	Tyr-D-Ala-Gly-NH-CH(CH_2-C_6H_5)-<u>NH-CO</u>-CH($(CH_2)_3$-S-CH_3)-CO-NH_2	175	(150-205)
XI.	Tyr-D-Ala-Gly-NH-CH(CH_2-C_6H_5)-<u>NH-D-Leu-For</u>	1464	(1255-1668)
XII.	Tyr-D-Ala-Gly-NH-CH(CH_2-C_6H_5)-<u>NH-D-Met-For</u>	1499	(1314-1710)

In summary, we have developed a methodology for the synthesis of partially modified retro-inverso peptides. Thus, applications of this transformation to a variety of peptides can be foreseen. The partial retro-inverso modifications of biologically active peptides, where topology of the side chains in the extended conformation is maintained, may in fact result in prolonged and enhanced biological activity.

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ROLE OF LEUCINE RESIDUES IN THE ACTIVE SITE OF C3a ANAPHYLATOXIN

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During activation of the serum complement (C) system, C3 is cleaved into protein C3b, an opsonin and mediator of cellular lysis, and C3a anaphylatoxin, a primary mediator of acute inflammation.[1] In man, C3a is a 77-residue polypeptide that produces degranulation of mast cells and basophils, increases vascular permeability, and contracts a variety of smooth muscle tissues.[2]

Our initial study of biological effects of synthetic peptides from human C3a showed that the active site of C3a lies within the COOH-terminal octapeptide.[3] This peptide exhibited the specificity and all the biological activities of natural C3a, but was only about 1% as potent as C3a on a molar basis. Recently, the active site has been more precisely localized to the COOH-terminal pentapeptide (Leu-Gly-Leu-Ala-Arg)[4] which is about 10% as active as human C3a in releasing histamine from human mononuclear leukocytes.[5]

This paper deals with the functional role of leucine-73 and leucine-75 in the active-site C3a-(73-77)-pentapeptide, which is conserved in man, pig, and rat. Several analogues of this peptide were synthesized by the solid-phase method[6] and were purified by reverse-phase high-pressure liquid chromatography (HPLC). The synthetic peptides gave correct amino acid ratios after hydrolysis and were homogeneous by analytical HLPC and thin-layer chromatography.

Each synthetic active-site analogue was assayed[3] for the ability to contract isolated strips of smooth muscle from guinea pig ileum (Table I). Contraction is mainly due to the release of histamine from mast cells within the muscle. Preincubation of the muscle with the antihistamine chlorpheniramine abolishes the ability of C3a or its synthetic analogues to induce contraction. Although the active-site pentapeptide *1* produced full contraction at doses as low as 15-20 nmol (about 10 μg), it was 150 times less active on a molar basis than the very potent natural human C3a.

Peptide *1* desensitized the muscle strip to subsequent stimulation of contraction by itself or by human C3a but did not block stimulation by human C5a.

Replacement of leucine-73 in pentapeptide *1* by tyrosine *(2)* or phenylalanine *(3)* produced no significant loss in activity, whereas replacement by methionine *(4)* decreased the activity 2-fold. Thus leucine-73 can be replaced by an amino acid with an aromatic or long aliphatic side chain with little or no loss in activity. But replacement of leucine-73 with alanine *(5)* decreased smooth muscle contracting activity 14-fold. These position-73 analogues were substantially more active than tetrapeptide *6* lacking leucine-73, which was 70 times less active than pentapeptide *1*. The presence of a large hydrophobic residue at position 73 is functionally important, since removal of leucine-73 from

Table I. Smooth Muscle Contracting Activity of Synthetic C3a Peptides

Code	Peptide structure 73 74 75 76 77	Minimum effective dose[a] (nmol)	Relative molar activity	Desensitization[b] self	C3a	C5a
C3a	[human C3a-(1-77)]	0.12	15,000		+	-
1[c]	Leu-Gly-Leu-Ala-Arg	15-20	[100]	+	+	-
2	Tyr-Gly-Leu-Ala-Arg	18	100	+	+	-
3	Phe-Gly-Leu-Ala-Arg	20-25	80	+	+	
4	Met-Gly-Leu-Ala-Arg	40	45	+	+	-
5[c]	Ala-Gly-Leu-Ala-Arg	250	7	d	+	-
6[c]	Gly-Leu-Ala-Arg	1200-1400	1.4	d	±	-
7[c]	Leu-Gly-Ala-Ala-Arg	>1750	<1	d	-	-
8	Leu-Gly-Phe-Ala-Arg	>950	<2	d	-	-

[a] Treatment of isolated strips of smooth muscle from guinea pig ileum with peptide dissolved in Tyrode's solution at 37°C. The minimum effective dose is the minimum amount that produces full contraction of the ileal strip in a 10-ml bath. ">X" indicates that no contraction was seen at X, the highest dose tested.

[b] Treatment with peptide desensitized the muscle to contraction when subsequently tested with the agent shown.

[c] References 4 and 7.

[d] Not determined.

Table II. Relative Activity of Isomeric C3a Active-site Analogues

Sidechain:	$-CH_3$	$-CH_2-CH(CH_3)_2$	$-CH_2-C_6H_5$
X:	Ala	Leu	Phe
X -Gly-Leu-Ala-Arg	7	[100]	80
Leu-Gly- X -Ala-Arg	<1	[100]	<2

pentapeptide *1* produces nearly as large a decrease in activity as does removal of the NH_2-terminal 72 residues of human C3a. Shortening the isobutyl side chain of leucine-73 in pentapeptide *1* to the smaller methyl group of alanine to give *5* decreases the activity 14-fold and removal of alanine-73 to give *6* decreases the activity yet another 5-fold.

Replacement of leucine-75 in pentapeptide *1* by alanine or phenylalanine gave pentapeptides *7* and *8*, respectively. These peptides failed to show even slight contractile activity at the highest doses tested and failed to desensitize muscle strips to later stimulation by intact C3a. These peptides are thus at least 50-fold less active than the active-site pentapeptide *1*. The relatively inactive position-75 analogues are contrasted with the active position-73 analogues in Table II. The methyl side chain of alanine is considerably smaller than the isobutyl side chain of leucine, which in turn is less bulky than the benzyl side chain of phenylalanine. Although the larger side chains of Leu, Tyr, or Phe have nearly equivalent activity at position 73, only the isobutyl side chain of leucine provided significant activity as position 75. The larger benzyl group of phenylalanine-75 evidently prevents the peptide from properly binding to the mast cell sites that trigger histamine release. The methyl group of alanine-75 is apparently too small to produce significant activity. Thus leucine-73 is relatively insensitive while leucine-75 is quite sensitive to replacement by large hydrophobic residues for the maintenance of smooth muscle contracting activity.

Acknowledgements

We thank Mrs. Maria Pospischil, Mrs. Laura Benedetto, and Mrs. Marlene Kawahara for their excellent technical assistance. This research

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MERCAPTOALKANOYL DERIVATIVES OF CYCLIC IMINO ACIDS AS INHIBITORS OF ANGIOTENSIN-CONVERTING ENZYME

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Introduction

Markedly potent competitive inhibitors of angiotensin-converting enzyme (ACE) have been developed utilizing a hypothetical model of the active site (Figure 1)[1]. The most potent of these inhibitors, *(S)*-3-mercapto-2-methylpropanoyl-L-proline (SQ 14,225, captopril) is undergoing clinical trials as an antihypertensive agent of wide applicability. The purpose of the present study is to further clarify the nature of the interactions between the amino acid residue of such mercaptoalkanoyl proline inhibitors and the enzyme active-site.

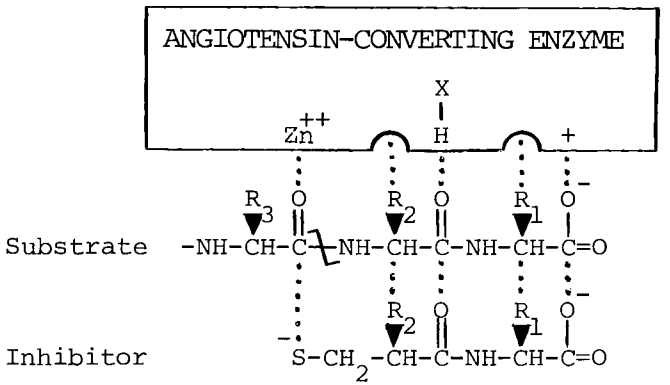
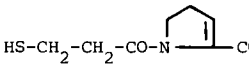
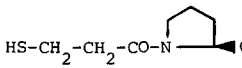
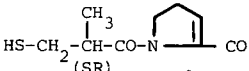
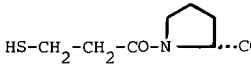
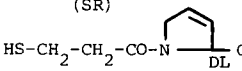
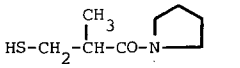
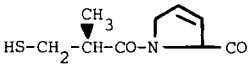
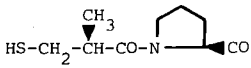


Fig. 1.

Synthesis

2,3-Dehydroproline Analogs: Addition of acetylthiopropionyl chlorides across the double bond of 1,2-dehydroproline *tert*-butyl ester, followed by dehydrohalogenation, yielded the *tert*-butyl esters of 3-acetylthiopropanoyl-2,3-dehydroproline, from which the final products *1* and *2* (Table I) were obtained by a two-step deprotection procedure.

Table I. Inhibitory Activity of 3-Mercaptopropanoylpyrrolidine Derivatives.

No.	Structure	I_{50} μM	No.	Structure	I_{50} μM
1.		0.65	5.		0.24
2.	 (SR)	0.27	6.		1800
3.	 DL	0.33	7.		240
4.		0.009	8.	 (captopril)	0.02

3,4-Dehydroproline Analogs: DL-3,4-Dehydroproline was acylated with acetylthiopropionyl chlorides under Schotten-Baumann conditions. In the case of the 3-mercapto-2-methylpropanoyl analog *4*, the optical active *(S)*-3-acetylthio-2-methylpropionyl chloride[2] was used. The diastereoisomers obtained in the latter case were separated by fractional crystallization of their dicyclohexylammonium salts. The optically active acid chloride was prepared from S-3-mercaptoisobutyric acid obtained by acid hydrolysis of captopril *(8)*. Since captopril was synthesized from L-proline and racemic 3-acetylthio-2-methylpropionyl chloride, the synthesis of analog *4* represents a resolution of 3,4-dehydroproline utilizing L-proline as a resolving agent.

Enzyme Assays

The inhibitory activity against rabbit lung ACE of the analogs listed in Table I was determined by the procedure described previously[3].

Discussion

In the active-site model of ACE, the carboxyl group of the amino acid moiety of the inhibitor is proposed to bind to a positively charged group on the enzyme, probably the guanidinium group of arginine. Earlier structure activity studies had shown that the L-configuration of the amino acid moiety was required for optimal activity, since the D analog was three orders of magnitude less active (*5* and *6*). The most effective interaction between a carboxylate anion and a guanidinium cation requires a precise alignment of atoms to achieve a double hydrogen bonded structure such as in Figure 2a, but a single hydrogen bonded

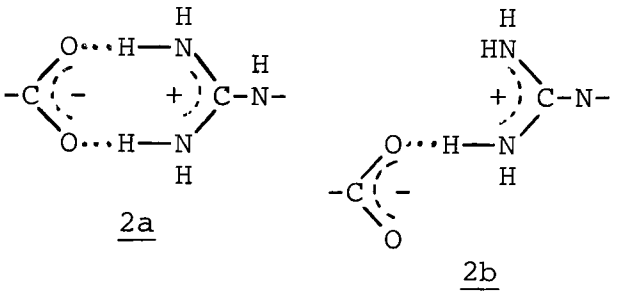


Fig. 2.

interaction is also possible (Figure 2b), although it is not as energetically favorable as that of Figure 2a[4]. In the D-proline analog *6*, neither of the two alternatives is probably possible, since its inhibitory activity is close to that of *7*, the analog without the carboxyl group. To investigate the possibility of partially restoring some of this ionic binding by locating the carboxyl group in a position closer to that obtained in the L-analog, we synthesized the 2,3-dehydroproline analogs in which the carboxyl group is located in the plane of the pyrrolidine ring. The significant increase in inhibition observed in analog *1*, as compared with the D-proline derivative *6*, indicates that we have partially retored the interaction between the carboxylate anion of the inhibitor and the guanidine cationic group on the enzyme, probably as depicted in Figure 2b.

In order to achieve this intermediate positioning of the carboxyl group we had to introduce a double bond into the pyrrolidine ring, which raises the question of how this modification would influence activity. However, it is not likely that it might have influenced activity unfavorably, since the 1-(3-mercapto-2-methylpropanoyl)-L-3,4-dehydroproline *(4)* showed inhibitory activity slightly higher than that of the saturated ring analog *8*. A number of analogs of proline containing peptides have been reported in which the replacement of proline by the 3,4-dehydroproline residue leads to full retention or enhancement of biological activity. In most of these cases an additional π-π bond interaction between the peptide and the corresponding receptor has been postulated to explain the increase in activity[5,6,7]. A similar interaction may be operative in the case of the 1-*(S)*-(3-mercapto-2-methylpropanoyl)-3,4-dehydro-L-proline analog and the terminal amino acid binding site of the angiotensin-converting enzyme.

All of the analogs synthesized in this study inhibited the contractile activity of angiotensin I in the guinea-pig ileum strip, and potentiated the contractile activity of bradykinin on the same tissue, without affecting

the response to angiotensin II and acetylcholine. These results indicate that these analogs are functioning as specific ACE inhibitors in the guinea-pig ileum[8].

Analog *4* was also shown to produce a marked inhibition of the pressor effect of exogeneous angiotensin I in the unanesthetized rat[8] when administered orally in a dose of 1 mg/kg.

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SYNTHESIS OF ANGIOTENSIN II ANALOGS WITH GREATER RESISTANCE TO ENZYMATIC DEGRADATION

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Introduction

One of the limitations of angiotensin II antagonists is that they have short *in vivo* half-lives and are not orally active. Our endeavor, therefore, has been to introduce modifications in these molecules that would increase duration of action without decreasing antagonistic potency.

Since short *in vivo* half-life is presumably due to degradation by peptidases, attempts have been made to make these peptides resistent to enzymatic degradation by replacing natural amino acid residues with N-methylamino acids,[1] O-methylthreonine (replacing isoleucine),[2] or α-methylamino acids.[3] Likewise, the chain-lengths at the N-terminus were increased with $(Pro)_n$- or $(Sar)_n$- moieties.[4] This work has now been extended to: a) replacement of tyrosine in position 4 with β-homotyrosine or α-methyltyrosine, and b) studies on comparative enzymatic degradation of the above and other analogs.

Methods

Synthesis of *tert*-butyloxycarbonyl β-homotyrosine was based on the Arndt-Eistert reaction and Wolf rearrangement. The experimental conditions adopted were particularly suitable for acid sensitive blocking groups.[5,6] The analogs were synthesized by the solid-phase procedure of Merrifield.

Determination of pressor activity of analogs was carried out on vagotomized ganglion blocked rats. The duration of action was calculated from the time of injection of the peptide to the time the blood pressure levels returned to base-line. The pressor activity and the duration of action of each analog was compared to angiotensin II.

Results

Table I describes the results of rat pressor bioassays and enzymatic degradation of angiotensin II analogs with leucine amino peptidase (Microsomal, Sigma Chemical Co.). The degradation of angiotensin III was much faster than that of angiotensin II. In contrast, degradation of [Sar^1]angiotensin II was much slower, and even after 2 hours of incubation a considerable quantity of [Sar^1]angiotensin II was present. Although degradation of [β-$HIle^5$]angiotensin II was fast, substitution of position 1 with sarcosine considerably slowed its hydrolysis.[6]

With sarcosyl-angiotensin II degradation was detected within 15 minutes. However, even after 18 hours of incubation a considerable quantity of sarcosyl-angiotensin II was present. This type of degradation was absent with $(Sar)_3$- or $(Sar)_5$ angiotensin II.

When incubated with alpha chymotrypsin (beef pancreas, Nutritional Biochemical Corp.) angiotensin II was degraded rapidly. Similar results were obtained with [Sar^1,Ile^8]-, [Sar^1,$Thr(Me)^5$,Ile^8]-, [Sar^1,$Thr(Me)^8$]- and [Sar^1,$Thr(Me)^5$,$Thr(Me)^8$]angiotensin II. The pattern of degradation of [Asn^1,Ile^5]-, [Sar^1]- and [Asn^1,Val^5]angiotensin II was also not grossly different from angiotensin II. $(Pro)_n$- and $(Sar)_n$ angiotensin II were degraded into two fragments within 1 hour of incubation. [Sar^1,β-$HTyr^4$]-, [β-$HIle^5$]- or [Sar^1,$\beta HIle^5$]angiotensin II showed very little degradation up to 3 hours,[6] while [α-$MeTyr^4$] angiotensin II showed no degradation under the same conditions.

Discussion

Analogs substituted with α-methyltyrosine in position 4 or those with Pro- or $(Sar)_3$ linkages at the N-terminus showed pressor activity equal to or greater than angiotensin II. $(Pro)_3$-, $(Pro)_5$-, $(Sar)_3$-, and $(Sar)_5$ angiotensin II showed no degradation even after 18 hours of incubation with leucine aminopeptidase M, but were easily degraded with chymotrypsin. In contrast, analogs substituted with α-methylamino acids and β-homoamino acids in position 4 or 5 were fairly stable to chymotrypsin but were easily degraded with leucine aminopeptidase M.

The results suggest that it may be possible to synthesize potent angiotensin II analogs with greater resistance to enzymatic degradation by increasing the chain-length at the N-terminus and by replacing position 4 (or 5) with α-methylamino acids. However, low *in vivo* duration of action with all the analogs (Table I) suggests that modifications at position 4 or 5 and at the N-terminus are not alone

sufficient to increase the duration of action. Multiple modifications are necessary to stabilize these peptides against enzymatic attack by both endopeptidases and exopeptidases. The modifications introduced should not disturb the side chain conformation (particularly of tyrosine) or overall conformation of angiotensin II.

Table I. Enzymatic Degradation of Angiotensin II Analogs with Leucine Aminopeptidase Microsomal

	% free amino acid after 1 hr incubation					Rat Pressor Bioassay	
Analogs of [Asp^1,Ile^5] Ang II	0 Sar (Pro)	1 Asp (Sar)	2 Arg	3 Val	4 Tyr	Pressor activity[a]	Duration of action[b]
[Asp^1,Ile^5]-		18.2	16.2	14.1	7.8	100	4.2-6.1
[Sar^1]-		11.8	5.5	4.4	0.3	150	
des-Asp^1-			89.7	39.9	14.0	30-50	
(Sar)-	6.4	5.9	-	-	-	71.5	5.7
$(Sar)_3$-			no degradation			136.7	6.3
$(Sar)_5$-			no degradation			58.1	7.1
(Pro)-	84.6	38.9	29.4	27.0	18.0	114.5	4.5
$(Pro)_3$-			no degradation			45.2	4.3
$(Pro)_5$-			no degradation			44.2	4.0
[α-MeTyr^4]-						97.6	4.9
[Sar^1, βHTyr^4]-						2.0	5.6
[βHIle^5]		35.1	33.0	32.8	-	2.82	5.8
[Sar^1,βHIle^5]		15.5	1.4	-	-	29.2	6.3

[a] Relative to [Asp^1,Ile^5]angiotensin II = 100
[b] At a dose level of 1.8 ng/ml

Acknowledgement

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STRUCTURAL REQUIREMENTS FOR ANGIOTENSIN'S EFFECT ON CALCIUM TRANSLOCATION IN SMOOTH MUSCLE CELLS

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Introduction

Angiotensin II (AII), like other myotropic substances, is thought to exert its smooth muscle contracting activity by increasing the Ca^{2+} concentration in the cytoplasm both through release of that ion from intracellular stores and through promotion of Ca^{2+} translocation from the external medium. The organ's response is usually composed of a fast transient (phasic) component and a slower sustained (tonic) component. In the case of the guinea pig ileum we have observed (unpublished) that the contraction does not depend on intracellular Ca^{2+} sources, and that both phasic and tonic components are due to Ca^{2+} translocation from the external medium.

In order to better understand the mechanism of AII's action on the guinea pig ileum, we have explored the acute specific desensitization (tachyphylaxis) which is characteristic of that hormone's myotropic activity.[1] Since it was previously observed that the desensitization caused by repeated treatments with AII is mainly due to a loss of the phasic component of the response,[2] we have now studied further the ion dependence of the two components of the contraction as well as the structural requirements for angiotensin's ability to induce desensitization.

Methods

The isolated guinea pig ileum was prepared and its isometric contractions were recorded as previously described.[2] The normal medium had the following millimolar composition: NaCl, 138; $CaCl_2$, 1.3; $MgCl_2$, 0.52; KCl, 2.7; $NaHCO_3$, 11.2; NaH_2PO_4, 0.33; glucose, 5.5. The

pH of this solution was 8.0 when equilibrated with air. To obtain a pH of 7.0, the medium was equilibrated with a gas mixture containing 95% O_2 and 5% CO_2. The low sodium medium differed from the normal one by having 68 mM NaCl and 140 mM sucrose. The degree of tachyphylaxis induced by AII and its analog was estimated from the reduction of the isotonic responses to repeated treatments, as previously described.[3]

The peptides were synthesized by the solid phase method,[4] and purified by ion exchange and gel permeation chromatography until homogeneous behavior on TLC in three solvent systems and on electrophoresis at three different pH's was attained.

Results and Discussion

Lowering of the sodium concentration in the medium from 150 to 80 mM, as well as treatment with tetrodotoxin (an inhibitor of voltage-dependent sodium channels in excitable membranes), produced changes in the response to AII (Figure 1) which were very similar to those previously observed in tachyphylactic organs,[2] namely a marked loss of the phasic component of the contraction. On the other hand, amiloride (an inhibitor of non voltage-dependent sodium channels) blocked preferentially the tonic component of the contraction (Figure 1). Apparently, the inward calcium movements responsible for the contractions are activated through a primary effect of the hormone on two different sodium channels: a tetrodotoxin-sensitive channel, triggering the phasic component, and an amiloride-sensitive channel, responsible for the tonic component of the contraction. Desensitization (tachyphylaxis) would then be the result of residual blocking, by AII, of the tetrodotoxin-sensitive channel, which we think might be the previously proposed[1,2] "tachyphylactic site" at or near that hormone's receptor. According to this hypothesis, the hormone-receptor complex, after triggering the cell's contractile response, would slowly rearrange to a refractory configuration in which the "tachyphylactic site" would be blocked.

A study of the ability of many AII analogs to induce desensitization at different pH's has indicated that the presence of a protonated amino group is essential for this phenomenon to occur.[4,5] Of all the analogs studied before, there was only one that had a protonated amino group at physiological pH and still was unable to induce tachyphylaxis,[4] namely, $|Gly^1,Gly^2|$-AII. Since $|Gly^1|$-AII was very tachyphylactic, we decided to

investigate the importance of the Arg^2 side-chain in AII for that phenomenon. Table I shows that, in normal medium ($|Na^+|$ = 150mM), $|Lys^2|$-AII and $|Orn^2|$-AII did not induce desensitization, similarly to what was observed with $|Suc^1|$-AII (desamino-AII). This contrasts with the significant tachyphylaxis produced by AII and $|Gly^1|$-AII. When the sodium ion concentration in the medium was lowered from 150 to 80 mM, a much more pronounced desensitization was induced by AII and $|Gly^1|$-AII and some desensitization to $|Suc^1|$-AII became apparent (Table I). However, even in low sodium medium, $|Lys^2|$- and $|Orn^2|$-AII did not desensitize the guinea pig ileum.

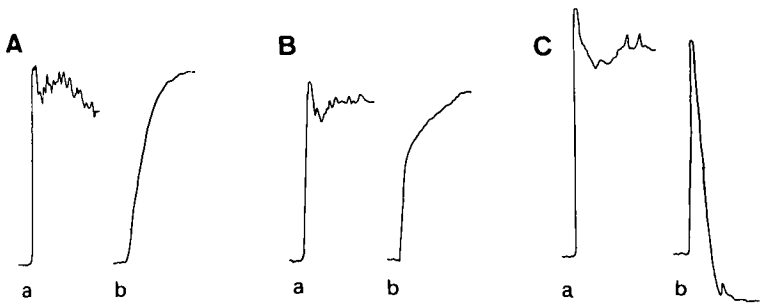

Fig. 1. Isometric recordings of the responses of three isolated guinea pig ileum preparations to 80 mM AII before (a) and after (b) the following modifications of the normal medium: A, reduction of Na^+ concentration to 80 mM; B, addition of 10^{-6} M tetrodotoxin; C, addition of 10^{-4} M amiloride.

Table I. Desensitization of the Guinea Pig Ileum to AII and to some Analog Peptides, and its Dependence on Sodium Concentration and pH.

	Desensitization[a]		
Peptide	$\|Na^+\|$ = 150mM pH = 8.0	$\|Na^+\|$ = 80mM pH = 8.0	$\|Na^+\|$ = 150mM pH = 7.0
AII	25.5 ± 4.5	71.5 ± 2.8	51.6 ± 6.3
$\|Gly^1\|$-AII	28.7 ± 6.1	76.2 ± 4.4	
$\|Suc^1\|$-AII	- 3.7 ± 7.4	26.3 ± 2.9	
$\|Lys^2\|$-AII	- 5.7 ± 5.2	8.7 ± 7.6	- 2.2 ± 7.0
$\|Orn^2\|$-AII	1.8 ± 2.7	2.5 ± 5.6	- 5.0 ± 3.9

[a] Desensitization was estimated by the % decrease in isotonic response between the first and third administration of a maximal dose of the peptide. Averages of 6-7 determinations ± s.d. are shown. Negative values indicate that the average response to the third dose was greater than that to the first one.

These results indicate that the guanido group of the Arg^2 side-chain, as well as the protonated amino group, are essential for inducing desensitization of the guinea pig ileum. The desensitization was more pronounced in low sodium medium, in which tachyphylaxis was induced even by an analog lacking the amino group ($|Suc^1|$-AII), but not by those without the guanido group. This suggests that the latter group may compete with sodium ions for the proposed "tachyphylactic site". If the "tachyphylactic site" is a tetrodotoxin-sensitive sodium channel, as suggested above, one might speculate with basis on the evidence that the guanido group is the portion of the tetrodotoxin molecule responsible for blocking the sodium channel.[6] Our previously proposed model[3] might then be modified by supposing that the "tachyphylactic site" is a tetrodotoxin-sensitive sodium channel, which may be blocked by interaction with the Arg^2 guanido group in AII. The positive charge of the protonated amino group is also important, possibly for promoting binding of AII to the "tachyphylactic site".

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ANGIOTENSIN II ANALOGS CONTAINING UNSATURATED AMINO ACIDS

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[Sar^1, ΔAla^8] angiotensin II was isolated as a major by-product of the synthesis of [Sar^1, Cys(Bzl)^8] angiotensin II. It was characterized by NMR spectroscopy which had olefinic proton signals at 6.03 ppm and 6.52 ppm, by hydrogenation of the double bond followed by amino acid analysis, and by enzymatic degradation[1]. *In vivo*, the peptide showed potent angiotensin II(AII) blocker activity. To confirm its structure, the synthesis of [Sar^1, ΔAla^8] AII was undertaken.

Many methods of preparing α, β-unsaturated amino acids have been reported[2]. The problem that arose was to find a suitable method of generating the double bond of dehydroalanine which was compatible with the other amino acid residues i.e. Arg, Tyr, His. Since dehydroalanine had been successfully used to make peptide amides in solid phase peptide synthesis[3], it would seem that dehydroalanine could withstand the rigors of solid phase synthesis. The problem would be the successful removal of the peptide from the resin.

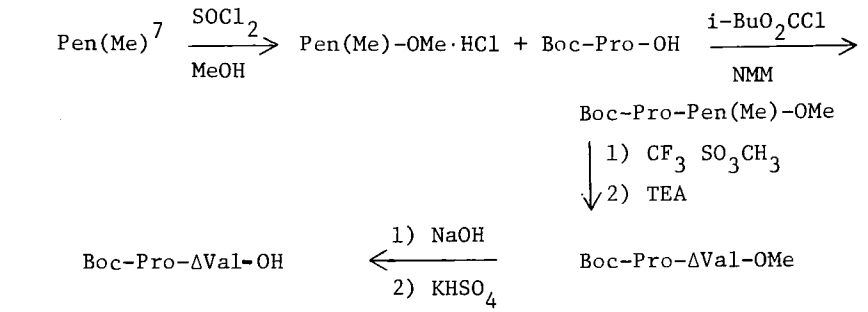
Boc-Pro-ΔAla^3 was attached to chloromethylated resin by a modified cesium salt method[4]. The yield of attachment was 75%. A conventional solid phase peptide synthesis was undertaken to produce Boc-Sar-Arg(NO_2)-Val-Tyr(2-BrZ)-Ile-His(Tos)-Pro-ΔAla-Ⓟ. The only precaution taken was to dry all solvents and liquid reagents over 4A sieves except for the methylene chloride which was distilled.

To remove the peptide from the resin, it was treated with HF for twenty minutes at 0° C. After the removal of HF, the resin was stirred for an hour in 30% N,N-diisopropylethylamine in THF. Failure to treat the resin with base resulted in complete loss of product. The product was purified on a column 15 x 1000 mm of Waters Porasil B C_{18} (37-75μ) using a stepwise gradient of CH_3CN/0.1M NH_4OAc.

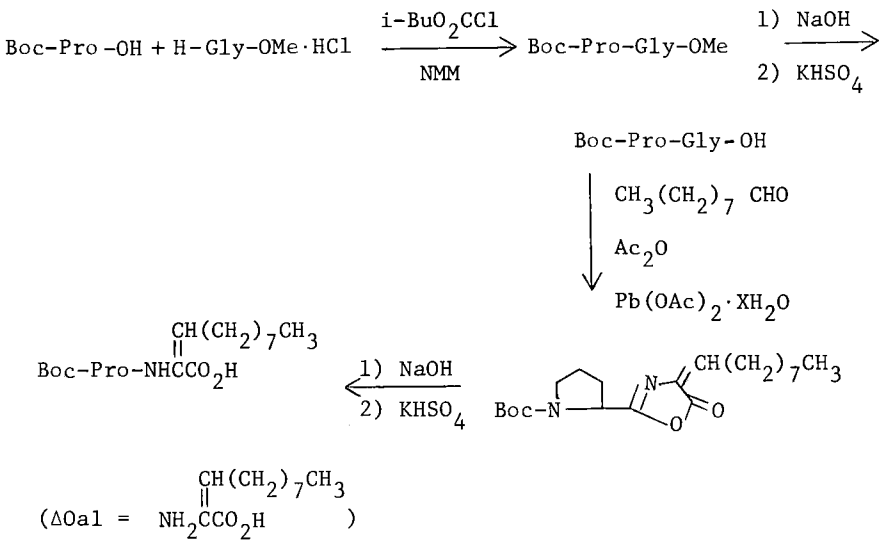
The solid phase peptide synthesis of the three analogs was essentially the same as described for [Sar^1, ΔAla^8]AII. In order to investigate the variety of methods for the synthesis of unsaturated amino acids the three dipeptides were prepared each using a different technique.

Boc-Pro-ΔPhe-OH was prepared by dehydration of Boc-Pro-Phe(β-OH)-OH by a method which was originally reported by Bergmann[5] and more recently by Stammer[6].

Boc-Pro-ΔVal was synthesized by elimination of the sulfonium ion formed from penicillamine by treatment with base.



The method with the potential for the widest utility is Robinson's[8] preparation of unsaturated azlactones.



(ΔOal = $NH_2\overset{\overset{CH(CH_2)_7CH_3}{\|}}{C}CO_2H$)

The compounds were tested in an AII receptor binding assay[9] and for inhibition of aldosterone biosynthesis in isolated rat adrenal glomerulosa cells[1,10]. [Sar[1], ΔAla[8]] AII was the most active AII blocker in the series. [Sar[1], ΔPhe[8]] AII was an agonist. [Sar[1], ΔVal[8]] AII was somewhat less active than [Sar[1], ΔAla[8]] AII and [Sar[1], ΔOal[8]] AII was a mixed agonist-antagonist. The data are shown in Table I.

Table I

Compound	Receptor Binding Assay	Aldosterone Biosynthesis Assay Agonist	Antagonist (ID_{50}, M)
AII	1.00	100%	
Saralasin	0.7		3.5×10^{-7}
[Sar^1,Ile^8]AII	1.2		1.0×10^{-8}
[Sar^1,Cys(Bzl)^8]AII	4.0	44%	8.0×10^{-8}
[Sar^1,ΔAla^8]AII	1.4		3.9×10^{-8}
[Sar^1,ΔVal^8]AII	0.4		2.7×10^{-7}
[Sar^1,ΔPhe^8]AII	0.6	100%	
[Sar^1,ΔOal^8]AII	0.4	55%	$\sim 10^{-7}$

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ORGAN SELECTIVE ANTAGONISTS OF THE RENIN/ANGIOTENSIN SYSTEM

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Introduction

It has been known for some years that the "angiotensin receptor" in fact represents a heterogeneous population of receptors.[1] This heterogeneity is obvious with both agonists and antagonists of the renin/angiotensin system with many tissues and organs showing even rank order differences in sensitivity to analogs and homologs (e.g. des-Asp^1-A-II; A-III) of the parent hormone. By exploiting this property it should be possible therefore to prepare organ selective reagents. To this end we have prepared Sarcosyl^1-Cysteinyl(S-methyl)^8-Angiotensin II [Sar^1-Cys(Me)^8-A-II] and Sar^1-Cys(Me)^8-A-I.

Methods

Peptides were prepared by standard solid-phase methods[2] as modified in Day and Freer.[3] They were removed from the resin by reaction in anhydrous HF and purified by countercurrent distribution (200 transfers) in n-BuOH:HOAc:H_2O (4:1:5). The compounds were homogenous as determined by thin layer chromatography (3 systems), high voltage electrophoresis (pH 5), amino acid analysis and in the case of Sar^1-Cys(Me)^8-A-I by HPLC.

Isolated smooth muscle assays were as described by Freer[4] and the *in vivo* blood pressure assay was as previously described.[5]

Results and Discussion

The rationale for synthesis of the Sar^1-Cys(Me)^8-A-II and Sar^1-Cys(Me)^8-A-I was as follows: Firstly, it has been consistently noted that the presence of sarcosine in position 1 of A-II enhances potency of both antagonists and agonists and, in fact, has resulted in analogs exhibiting some non-competitive antagonism.[6,7] The insertion of Cys(Me) into the 8 position was prompted by the observation that Sar^1-Thr(Me)^8-A-II was

an extremely potent antagonist — especially on vascular smooth muscle.[8] In addition, the linear Cys(Me)[8] side chain should also be compatible with its incorporation into prohormone A-II antagonists of the type described by Chiu *et al.*[9].

As predicted the Sar[1]-Cys(Me)[8]-A-II was an extremely potent A-II antagonist which had its most marked effect on vascular smooth muscle (Table I). At 25 ng/ml the dose-response curve for A-II on rabbit aortic strip (RAS) was shifted 460 fold to the right ($pA_2 \simeq 9.2$) while a 5.0 μg/kg/min infusion shifted the responsiveness of the rat blood pressure (RBP) assay by nearly 3 logs. In addition, the slope and maximum response of both the RAS and RBP were both markedly depressed. The visceral smooth muscle preparations, guinea pig ileum (GPI) and rat uterus (RU), were quite a bit less sensitive to the antagonist and showed smaller, although significant (GPI 35%; RU 12%), depression of the maximum response. The relative potency was RBP>RAS$\gg$GPI>RU. Interestingly, the well known A-II antagonist, Sar[1]-Ala[8]-A-II (Saralasin^R) shows qualitatively the same result but was considerably less potent (approximately 10 times).

The Sar[1]-Cys(Me)[8]-A-I, however, shows a different pattern — presumably due to differing availability of converting enzyme in the various assay systems. As predicted by our prohormone studies,[9] *in vivo,* where there is a large excess of converting enzyme, the A-I analog is as good and possibly a somewhat better inhibitor than its octapeptide counterpart. This might suggest that the active antagonist is being released at or near the receptor. All inhibitory effects of the analog were reversed by SQ14225 (Captopril^R). Conversely in the RAS and RU there is little or no conversion of the A-I analog to active inhibitor. The GPI was intermediate with the Sar[1]-Cys(Me)[8]-A-I having about 50% of the activity of Sar[1]-Cys(Me)[8]-A-II. Changes in slope of dose-response curves and maximum response were proportionately attenuated in the *in vitro* assays. The relative potency for this analog was RBP>GPI$\gg$RAS, RU inactive.

In summary then it is clear that the reported antagonists do show some organ selectivity. The Sar[1]-Cys(Me)[8]-A-II is extremely potent on vascular smooth muscle where, in fact, it exhibits noncompetitive dynamics. The reason for this is not known but probably is related to the presence of the sarcosine residue in position 1. A greater affinity for the receptor has been reported for sarcosine analogs,[10] but it is also possible that a specific desensitization is occurring. This suggestion is supported

Table I. Pharmacological Evaluation of Sar^1-Cys(Me)^8-A-II and Sar^1-Cys(Me)^8-A-I

Antagonist	Assay	pA_2*	Antagonist dose**	Dose ratio***	% maximum response
Sar^1-Cys(Me)^8-A-II	Rabbit aorta	9.2	25 ng/ml	460	23 ± 3
	Guinea Pig ileum	8.5	25 ng/ml	37	65 ± 5
	Rat uterus	7.9	25 ng/ml	25	88 ± 4
	Rat blood pressure	---	5.0 µg/kg/min	938	---
Sar^1-Ala^8-A-II	Rabbit aorta	8.3	50 ng/ml	58	62 ± 2.5
Sar^1-Cys(Me)^8-A-I	Rabbit aorta	8.1	25 ng/ml	2.9	85 ± 6
	Guinea pig ileum	8.2	25 ng/ml	17	60 ± 4
	Rat uterus	inactive	250 ng/ml	1	95 ± 5.5
	Rat blood pressure	---	5.0 µg/kg/min	1300	---

*pA_2 as defined by Schild *(Brit. J. Pharmacol.* **2,** 189-206, 1947).

**Antagonist pre-incubated for 10 min *in vitro* and infused *in vivo* (via femoral vein) for 10 min before generating dose response data.

***Dose ratio = A-II in presence of antagonist/A-II control. Ratios determined at ED_{50} level (except ED_{25} for Sar^1-Cys(Me)^8-A-II on rabbit aorta) *in vitro* and ED_{20} mm Hg level *in vivo*.

by the finding that Sar[1]-A-II produces tachyphylaxis in RAS while A-II itself does not.[11] The correct explanation must await further investigation.

Acknowledgements

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SYNTHESIS OF TRITIUM LABELLED HUMAN CALCITONIN

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Introduction

Catalytic tritium/halogen exchange using a suitable protected intermediate has proved to be a fruitful route to the preparation of highly radioactive peptides.[1] It has been found possible to prepare several labelled corticotrophins by this means notwithstanding the presence of methionine in the molecule which might have been expected to behave as a catalyst poison. We wished to prepare human calcitonin similarly labelled but this molecule contains a cystine disulphide bridge in addition to methionine and was therefore expected to present additional problems.

Results

We have examined various approaches to the synthesis of tritium labelled calcitonin. The most direct method, which has been used by Fromageot to prepare several labelled peptides,[2] involved iodination of the free peptide followed by catalytic deiodination in aqueous solution. We successfully obtained and characterised an iodinated product but attempts to label by exchange using a variety of catalysts were unsuccessful, although Fromageot has reported executing a similar reaction successfully in the case of oxytocin which contains a disulphide linkage.

It was therefore decided to prepare an iodine-containing fragment and follow a route analogous to that adopted by us previously for the corticotrophins.[1] The final coupling in the synthesis of calcitonin[3] involves linking 1-10 and 11-32 fragments. The C-terminal sequence contains no sulphur. Tyrosine and histidine are the only amino acids present which are susceptible to reaction on iodination.

H-Thr(Bu^t)-Tyr(Bu^t)-Thr(Bu^t)-Gln-Asp(OBu^t)-Phe-Asn-Lys(Boc)-Phe-
His-Thr(Bu^t)-Phe-Pro-Gln-Thr(Bu^t)-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH_2

I

In the selected intermediate (I) tyrosine was protected as the O-*tert*-butyl ether and was expected not to react with iodine chloride in methanol. This was confirmed in practice, iodination of (I) occurring only at histidine. Exhaustive iodination was not attempted due to the danger of scission of the imidazole ring. Using one molar equivalent of iodine chloride the product (II) was found to contain 0.73 atoms iodine per molecule peptide and was a mixture of docosapeptides containing diiodo- and monoiodohistidine in addition to histidine itself. Catalytic reduction of (II) gave pure (I) indicating that no ring breakdown had occurred.

H-Thr(Bu^t)----------His(I)----------Pro-NH_2

II

Boc-Cys-Gly-Asn-Leu-Ser(Bu^t)-Thr(Bu^t)-Cys-Met-Leu-Gly-OH

III

Compound (II) was then coupled with N-terminal decapeptide (III) to give the full calcitonin sequence containing iodinated histidine. Attempts to reduce this intermediate with the usual catalysts were unsuccessful due to poisoning.

Trt Trt
| |
Boc-Cys-------Cys-------His(I)-------Pro-NH_2

IV

We then prepared the protected full sequence with the disulphide bridge opened and the cysteine thiol groups protected as the trityl derivatives (IV). Model experiments had shown that a mixture of (II) and ditrityl (1-10) decapeptide could be dehalogenated catalytically without poisoning the catalyst. However it transpired that (IV) was extremely insoluble and could only be handled in hot dimethylformamide, trifluoroethanol, or hexamethylphosphoramide (HMPA). Compound (IV) was purified by gel filtration on Sephadex G-50 in 5% aqueous HMPA and gave satisfactory amino acid analyses. Attempts to dehalogenate catalytically were unsuccessful, it is suggested that the

handling may have resulted in some detritylation and the liberated mercapto groups were poisoning the catalyst.

The next approach attempted, which was in fact the successful route to the labelled compounds, was to tritiate (II) catalytically and subsequently couple with (III) and deprotect. This necessitated developing conditions for coupling which were rapid, would maximise utilisation of the radioactive intermediate and would avoid the use of countercurrent distribution for purification of the protected dotriacontapeptide. Model experiments showed that reaction of the ten-fold excess of the hydroxybenzotriazole ester of (III) with (I) had consumed all of (I) in 5 minutes. A repeat of this experiment using (I) derived from (II) by catalytic tritiation gave the protected dotriacontapeptide which was separated from excess reagent and byproducts by gel filtration. Protecting groups were removed by treatment with 90% trifluoroacetic acid for 1 hour and the product was purified by chromatography on carboxymethylcellulose using a linear gradient (0-0.2 M) of pH 5.0 trimethylammonium acetate. The main peak was collected, solvent and buffer were evaporated off, the residue was dissolved in water and chromatographed by reversed phase HPLC on Nucleosil using a gradient from $MeOH:H_2O:H_3PO_4$ = 400:600:1 (by volume) to 800:200:1. The main peak was again collected, phosphate exchanged for acetate, and the product stored in aqueous solution at −195°C. [^{3}H-His^{20}]-Calcitonin M had a specific activity of 18.2 Ci/mmole. Examination of a portion of an acid hydrolysate showed that the radioactivity was stably bound and was associated with histidine alone.

Acknowledgements

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SYNTHESIS AND CONFORMATIONAL ANALYSIS OF CYCLIC TETRAPEPTIDES RELATED TO CHLAMYDOCIN. STEREOSPECIFIC REDUCTION OF DEHYDROPHENYLALANINE TO L-PHENYLALANINE

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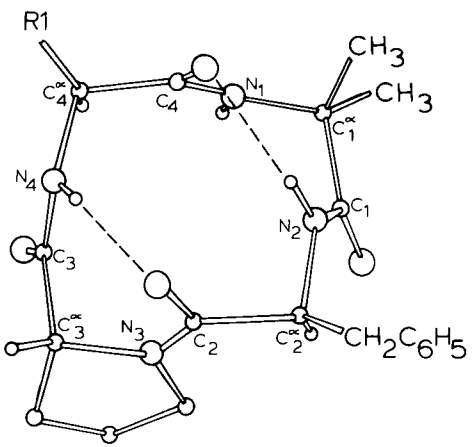
Chlamydocin (1a) is a cytostatic cyclic tetrapeptide produced by *Diheterospora chlamydosporia.*[1] It is the first cyclic tetrapeptide shown to be cytostatic,[2] the first shown to contain four *transoid* amide bonds (S_4 symmetry),[3] and one of the two peptides known to contain the epoxy-ketone moiety.[4] We report here the synthesis of cyclo(A^{t}bu-L-Phe-D-Pro-L-Ala), a model for the chlamydocin ring system in which L-Ala replaces the epoxy ketone amino acid, L-2-amino-8-oxo-9,10-epoxidecanoic acid.



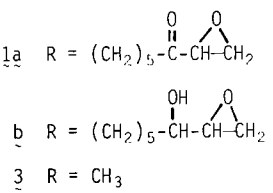


Fig. 1.

Cyclic tetrapeptide rings this highly substituted (5α-substituents) have not been synthesized previously and work with less substituted structures[5] suggested that direct cyclization of the precursor H-A^{i}bu-L-Phe-D-Pro-L-Ala-OTcp (2) might not proceed in acceptable yield. In fact direct cyclization of 2 gave little (<1%) of the desired product under standard reaction conditions.[6] Consequently we developed an alternate method for synthesizing 3 in which a dehydrophenylalanine residue (Phe[(Z)Δ])[7] is used to facilitate the cyclization reaction and then is reduced stereospecifically to L-Phe in the cyclic tetrapeptide.

The synthesis of cyclic tetrapeptide 3 is outlined in Scheme 1. Linear tetrapeptide 4 was prepared by stepwise addition of protected amino acids in solution to H-L-Ala-OMe. Conversion of 4 to the methyl sulfonium salt followed by base elimination[8] gave the *(Z)* isomer 5a which was converted to the trichlorophenyl(OTcp) ester 5b.[6] Removal of the p-methoxybenzyloxycarbonyl(pMZ) group (4 N HCl in dioxane; 30 minutes, 25°C) followed by cyclization (1.0 mM, 90°C, pyridine, 8 hours) gave cyclic tetrapeptides 6a (10% yield; MS m/e 398.1949, calc. 398.1953; A^{i}bu, 1.15; D-Pro, 1.0; L-Ala, 1.08) and 6b (5%, MS m/e 398.1958, calc. 398.1953; A^{i}bu, 1.22; D-Pro, 1.0; D-Ala, 1.08). The diastereomers were separated by chromatography over silica gel and the optical configuration of alanine in each established by hydrolysis followed by amino acid analysis before and after reaction with L-amino acid oxidase.[9] Hydrogenation of the L-Ala diastereomer 6a (24 hours, 1 atm., Pd/C 10%, methanol) gave 3 (60%; MS m/e 400.2107, calc. 400.2111; A^{i}bu, 1.13; L-Phe, 1.0; D-Pro, 1.0; L-Ala, 1.04). The configuration of L-Phe (>98% L) was established as described above using the L-amino acid oxidase procedure.

Scheme 1. R_1-X-Phe[3-SBzl]-D-Pro-L-Ala-OCH_3
4: X = A^{i}bu; R_1 = pMZ
R_1-X-Phe[(Z)Δ]-D-Pro-L-Ala-OR_2
5: X = A^{i}bu; R_1 = pMZ a: R_2=CH_3 b: R_2=Tcp
cyclo(X-Phe[(Z)Δ]-D-Pro-Y-Ala)
6: X = A^{i}bu a: Y = L b: Y =D
cyclo(X-L-Phe-D-Pro-L-Ala)
3: X = A^{i}bu

The use of a dehydrophenylalanyl residue in place of L-Phe appears to substantially increase the yield of cyclic tetrapeptide formed under

identical reaction conditions. The best yield we have obtained for 3 beginning with 2 is only about 1%. The 10% yield of 6a is sufficient to provide a useable pathway to this highly substituted cyclic tetrapeptide ring system and extension to the synthesis of chlamydocin itself appears feasible.

^{1}H and ^{13}C NMR spectroscopy of Ala^{4}-chlamydocin in $CDCl_3$ is consistent with the conformation shown in structure 3. The principle evidence supporting this conformation is summarized. The chemical shifts of Pro-C$^{\beta}$ and C$^{\gamma}$ (24.68 and 24.46 ppm) establish a *trans* X-Pro amide bond.[10] The variations in chemical shift of Ala-NH and Phe-NH with temperature (0.34 and 1.90 ppb/°C) or solute concentration are small and much less than found for the A^{i}bu-NH (3.5 ppb/°C). These data suggest the Ala and Phe amide protons are solvent shielded. This is supported by the observation that addition of DMSO-d_6 to $CDCl_3$ solutions of 3 causes the A^{i}bu-NH resonance to shift 3.0 ppm downfield while the Ala-NH and Phe-NH resonances are shifted only 0.15 and 0.24 ppm. The unusually high chemical shift for the Pro-C$^{\beta}$ is characteristic of a γ-turn.[11,12] In $CHCl_3$, the CD spectrum of 3 shows an $n \rightarrow \Pi^*$ transition at 243 nm ($\Phi_m = -16{,}000$) which is consistent with a γ-turn. Together these data indicate that in $CHCl_3$ solution. Ala^{4}-chlamydocin 3 has a conformation with 3 → 1 intramolecular hydrogen-bonds between the Ala-NH and Phe-CO and between Phe-NH and Ala-CO. The conformation of 3 in chloroform solution thus is very similar to that observed for dihydrochlamydocin 1b in the crystalline state.

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SYNTHESIS OF FRAGMENTS OF THE PROα1 CHAIN OF TYPE I PROCOLLAGEN: INHIBITORS OF THE N-PROTEASE REQUIRED FOR THE SYNTHESIS OF COLLAGEN

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Collagen is first synthesized as procollagen, a larger precursor with an extension "propeptide" of about 100 amino acids on the N-terminus and an extension propeptide of about 250 amino acids at the C-terminus of each of the three α chains of the molecule (for review, see ref. 1). We have recently purified about 300-fold a procollagen *N*-protease from chick embryo tendons which cleaves *en bloc* the *N*-propeptides from the two proα1 and the one proα2 chains found in Type I procollagen. Here we describe synthesis of a series of peptides with the same amino acid sequences as the cleavage site (Figure 1) at which procollagen *N*-protease cleaves the *N*-propeptide from the proα1 chain of Type I procollagen.

The peptides were synthesized by step-wise elongation and fragment condensation in solution. α-Amino and α-carboxyl groups were protected with *t*-butyloxycarbonyl (Boc) and benzyl ester groups. Functional groups attached to side chains of Ser, Tyr, Lys, Asp and Glu were protected with benzyl, 2,6-dichlorobenzyl, 1-chlorobenzyloxy-carbonyl, and benzyl ester groups, respectively. All coupling reactions of fragments were carried out in DMF using the DCC-HOBt method.[2] Finally, protecting groups of fully protected linear peptides were removed simultaneously by a single treatment with HF at 0°C for 30 minutes in the presence of anisole.[3] The products were purified by gel filtration on Biogel P2 or P4 after treatment with Dowex 1x4 (AcO^- form). Dinitrophenyl peptides were prepared by the following procedure: (a) The Boc-group was removed from fully protected linear peptide by trifluoroacetic acid. (b) The partially protected peptide was dissolved in DMF and the pH was adjusted to 8 with triethylamine. (c) A 5-fold excess of 2,4-dinitrofluorobenzene was added and the solution vigorously

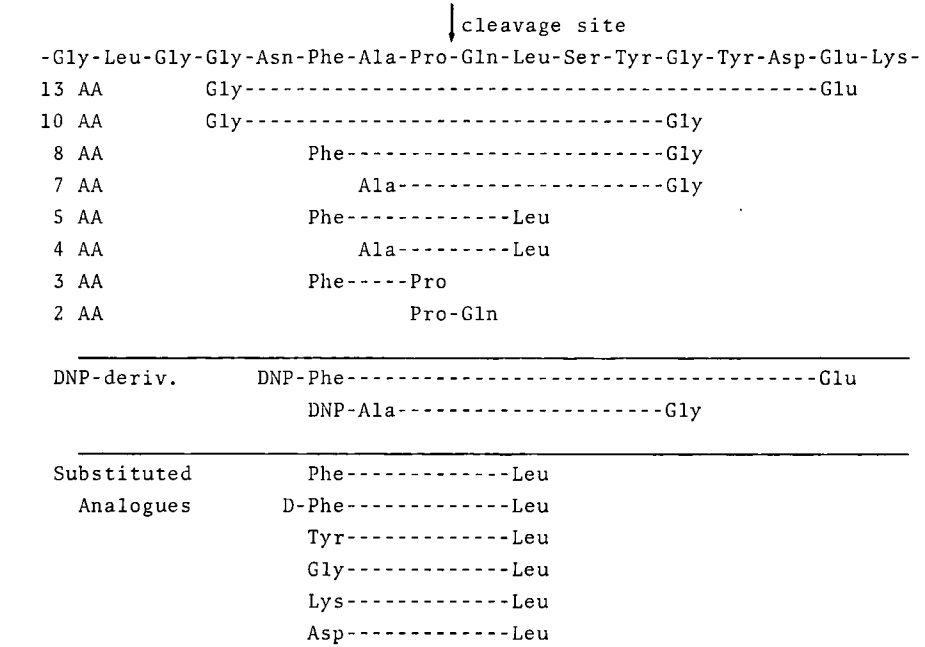

Fig. 1. Amino acid sequences of synthetic peptides. Sequence at top is from calf proα1(I) chain (5).

stirred in the dark at room temperature. (d) Protecting groups of DNP-peptide were removed with HF as described above. Substituted analogues were synthesized by adding several different amino acids to the N-terminal end of the tetrapeptide benzyl ester (Ala-Pro-Gln-Leu-OBzl) by the active ester method, to give five different substituted pentapeptides. Protecting groups were removed in the same manner as above. Homogeneity of the final products (inhibitors, DNP-derivatives and substituted analogues) was confirmed by thin-layer chromatography, paper chromatography, paper electrophoresis and amino acid analysis.

Studies with DNP-derivatives of several peptides demonstrated that the peptides prepared here are not cleaved by procollagen *N*-protease. Procollagen *N*-protease, about 0.4 units, was incubated in 100 μl with either 0.5 or 1.0 mg of DNP-undecapeptide for 2 hours or 24 hours.[4] The reaction mixture was then examined by thin-layer chromatography in butanol:acetic acid:water (4:1:1) and chloroform:methanol:acetic acid (85:10:5). The results indicated that the migration of the DNP-undecapeptide was not changed when it was incubated with enzyme. Also, there was not evidence of the expected cleavage product, DNP-Phe-Ala-Pro. The failure of the enzyme to cleave the peptides is consistent with the observation that procollagen itself must be in a native triple-helical conformation to serve as a substrate.[4]

Most of the peptides however were found to be effective inhibitors of procollagen *N*-protease. The cleavage of ^{14}C-labeled procollagen[4] was inhibited about 50% with 1-2 mM octapeptide, and 5 mM heptapeptide. The dipeptide, tripeptide and tetrapeptide did not inhibit.

The pentapeptide with the sequence Phe-Ala-Pro-Gln-Leu was used as a model inhibitor and analogues were prepared in which the Phe was systematically substituted with a variety of other amino acids. The results demonstrated that replacement of L-Phe with D-Phe reduced the inhibitor activity from 86% to 17% with a peptide concentration of 3 mg/ml. Replacement of the L-Phe with Tyr reduced the inhibitor activity to 18%. Replacement of the L-Phe with Gly reduced the inhibitor activity to 0, replacement of the L-Phe with Lys reduced the inhibitor activity to 16%, and replacement of the L-Phe with Asp reduced the inhibitor activity to 6%. The results suggested therefore that the L-Phe residue in the third position to the left of the cleavage site was important for inhibitor activity.

Several of the peptides synthesized here were used for developing an affinity column for purifying the *N*-protease. The affinity column was prepared with pNcollagen prepared from the skin of dermatosparactic sheep. Because the sheep have a genetic defect in procollagen *N*-protease, the skin contains pNcollagen which retains the *N*-propeptide of procollagen but is lacking the *C*-propeptide. The pNcollagen was linked to agarose with cyanogen bromide activation technique and a partially purified preparation of *N*-protease was passed through the affinity column. The affinity column was then eluted with the octapeptide synthesized here. A 20-fold additional purification of the enzyme was obtained. The recovery of enzymic activity was about 70%.

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SYNTHESES OF FLAVIN CONTAINING PEPTIDES: A NOVEL AMINO ACID BLOCKING AGENT

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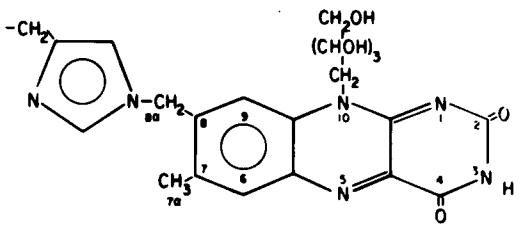
Introduction

Twelve flavoenzymes have now been isolated with covalently bound flavins.[1,2] In eleven of these, the attachment is through the 8α-C of the flavin, while in one case it is to the C6 of the isoalloxazine ring.[3] The apoenzyme is attached either through an imidazole nitrogen from histidine or the sulfur of cysteine.

The flavin containing pentapeptide obtained by tryptic/chymotryptic digestion from bovine succinate dehydrogenase (SD)[4] has been shown to contain the sequence:[5]

Ser-His-Thr-Val-Ala

with the flavin attached as:



An example of flavin attachment to a cysteinyl sulfur is found in *Chromatium* cytochrome c552 in the tryptic/chymotryptic tetrapeptide: Tyr-Thr-Cys-Tyr.[6] The flavin is again linked through the 8α-C as above. The N-terminal tyrosine has been shown to markedly affect the properties of the flavin.

Since the majority of these proteins containing covalently attached flavins can be obtained only with difficulty in low yield, we sought methods to obtain suitable quantities for peptide characterization. Besides our initial effort at solid phase synthesis of the SD flavin pentapeptide,[7] flavin containing peptides similar to monoamine oxidase have been synthesized in McCormick's laboratory.[8,9] Their approach was to synthesize the peptide first and then attach the flavin, a tedious and

only partially successful approach. We attached the flavin first, then synthesized peptides found in the enzymes.

Methods and Materials

Covalently Bound Flavin Preparation — Peracetylated riboflavin was prepared according to McCormick,[10] except that the solution was heated to boiling for one minute before cooling and extraction of product. The product was recrystallized from water and gave a 97% yield and a melting point of 250-252°C. 8-α-Bromotetraacetylriboflavin (bromoTARF) was obtained by the synthesis of Walker *et. al.*[11] A yield of 89% was obtained and the product gave similar melting points, halogen tests and NMR spectra to a sample kindly provided by Dr. P. Hemmerick of the University of Konstanz.

Solid Phase Synthesis — The 8α-[*N*-Boc-histidinyl(3)]-TARF was obtained by refluxing a 2.5 fold excess of Boc-histidine with TARF in absolute dimethyl formamide at 90°C for 25 hours. The product was recrystallized from absolute methanol and isopropanol with 52% yield. This material was used directly in the solid phase synthesis. The flavin containing SD pentapeptide was synthesized by solid phase procedures using *tert*--butyloxycarbonyl (Boc) amino acids (Beckman), Boc-histidinyltetraacetylriboflavin, dicyclohexylcarbodiimide, and resin bound alanine (Sigma) by procedures described in Stewart and Young.[12] A manual shaker was constructed according to the instructions of the authors. Cleavage by HBr/trifluoroacetic acid was sufficient to remove the acetyl groups from the ribityl side chain as well as release the peptide from the resin. The product was chromatographed on G-25 and lyophilized. Crude product yield was 0.412 g (95%) from 1.00 g of alanine-resin. It was further purified by pH 5.0 high voltage paper electrophoresis.[13]

Syntheses in Solution — Benzoyl histidine[11] and cysteine[14] were attached to 8α-bromoTARF essentially as published. The benzoyl group was removed by refluxing in 6N HCl for 15 hours. The appropriate amino acids and flavin derivatives were converted to the *N*-carboxy anhydrides under darkened conditions using the procedures of Hirschmann *et al.*[15] Serine and threonine were converted to their silver salts first and then modified to the o-trimethylsilyl derivatives before condensation. The coupling reactions were conducted according to

Hirschmann *et al.*[16] except for the substitution of a Virtis homogenizer. The pH was maintained at 10.2 by 5N KOH, until stabilized. The reactions were conducted consecutively, without isolation of intermediate products. Upon completion of the total reaction, the pH was lowered to 3.5 and the products quickly chromatographed on Sephadex G-25. The SD pentapeptide was observed by a single flavin band in 95% yield and three flavin containing bands were found for the C tetrapeptide with a total yield of 95%. The first band of the C peptide gave the best composition, so it was used exclusively. Characterization of these peptides will be presented elsewhere, but the peptidic flavin properties resemble the histidyl and cyteinyl flavins more than the holoenzyme properties.

Acknowledgements

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MILD DEPROTECTION OF N^{α}-NPS-AMINO ACIDS USING 2-THIOPYRIDONE TRIFLUOROACETATE: SOLID PHASE SYNTHESIS OF HUMAN GASTRIN I

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In conventional solid-phase peptide synthesis using Boc-amino acids, yields are limited by repeated exposure to strongly acidic deblocking reagents, e.g., 33% TFA, and by subjection to final cleavage from the resin by HF or similar reagents. Furthermore, strongly acidic deblocking prohibits the use of acid-labile side-chain protecting groups, such as Boc, *t*-butyl or trityl. The harsh peptide-resin bond cleavage can be circumvented with Wang's recently described *p*-alkoxybenzyl alcohol resin, which enables rapid scission by 50% TFA[1]. Such a resin requires that α-amino protecting groups be removable under extremely mild conditions. We have found new deblocking conditions which now make the *o*-nitrophenylsulfenyl (NPS) group well-suited for synthesis on Wang's resin and for synthesis of peptides bearing sensitive side-chain protecting groups.

The NPS deprotection by dilute HCl originally proposed by Zervas *et al.*[2] has been proven to derivatize Trp residues[3] and to be insufficiently selective for general use with Boc and *t*-butyl side chain protection[4]. Numerous alternatives to acid cleavage of the NPS moiety have been suggested, but each has a drawback for routine solid-phase synthesis, being either too non-selective, too slow, or producing insoluble by-products. Recently, Tun-Kyi reported[5] selective N^{α}-NPS deblocking with a 5 to 10-molar excess of a 1:1 mixture of 2-thiopyridone (2-TP)/acetic acid in CH_2Cl_2 with no affect on Boc, *t*-butyl, or benzyl protecting groups. Although quantitative cleavage in 30 minutes was claimed for solid-phase synthesis, in our hands, 3.5-6% of the NPS groups remained uncleaved, making even moderate peptide yields unfeasible.

We found that the use of TFA instead of acetic acid with 2-TP markedly accelerates N^{α}-NPS deprotection on solid-phase. A 10-molar

excess of 1:1 2-TP/TFA in CH_2Cl_2 (2-TPTFA) (I) leaves no N^{α}-NPS groups intact after 30 minutes. Percent uncleaved NPS after deblocking treatment was determined spectrophotometrically by removal of remaining NPS groups using 50% TFA for 20 min and measurement of the NPS absorption at 362.5 nm. A TLC study of the stabilities of sensitive protecting groups in solution showed that *t*-butyl esters and ethers, Boc and *S*-trityl groups are unchanged after at least 19 hr exposure to 2-TPTFA at room temperature. No detectable derivatization of Trp occurred when N^{α}-NPS-Trp was treated with 2-TPTFA under the same conditions.

The reactive deprotecting species is presumably protonated 2-thiopyridone (I), which is sterically well-suited for attack on the N^{α}-NPS sulfur atom and simultaneous protonation and displacement of the resulting uncharged α-amino group (Figure 1). A similar mechanism has been proposed by Stern *et al.* for N^{α}-NPS deprotection by the thione tautomer of unprotonated 2-TP in solution[6]. The freely soluble mixed disulfide (II) is the major side-product, as was also found with Tun-Kyi's conditions[5].

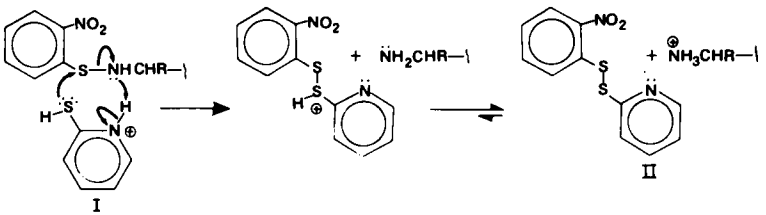

Fig. 1. Proposed mechanism for deprotection by 2-TPTFA.

2-TPTFA is quite stable at room temperature in the dark, with no detectable change in its effectiveness after 3 months. Using 2-TPTFA deprotection of N^{α}-NPS groups, we synthesized leucine-enkephalin on Wang's resin in 70% yield. Having established that these deprotection conditons will work for a simple, small peptide, we then undertook the synthesis of human gastrin I, with 17 residues including six with acidic side chains. HG I was synthesized in 16% yield in this, the first reported solid-phase synthesis.

Synthesis of Model Peptides

Leucine-enkephalin and human gastrin I were each synthesized on a Beckman 990 synthesizer on ~ 0.5 mmole scale. Each NPS-L-amino

acid, prepared by modifications of the methods of Zervas *et al.*[2] was coupled in CH_2Cl_2 using 1.5 mmole of amino acid and of DIC[7]. When necessary, coupling was completed by the symmetric anhydride method[8]. The resin-bound peptide was deblocked at r.t. for 30 min with 5 mmole 2-TPTFA (5 mmole 2-thiopyridone and 5 mmole TFA in 30 ml CH_2Cl_2), washed, neutralized with 2% Et_3N, and washed again before coupling to the next amino acid.

Leucine-enkephalin, Tyr-Gly-Gly-Phe-Leu, was synthesized on p-alkoxybenzyl alcohol resin prepared from 1% crosslinked Merrifield resin by Wang's method[1]. NPS-L-Leucine was esterified to the resin using 4-dimethylaminopyridine catalyst[9] in CH_2Cl_2 with DIC to give 0.45 mmole/g. Unreacted benzyl alcohol groups on the resin were benzoylated at room temperature. The remaining NPS-amino acids were coupled and deprotected as described above, except that Boc-L-Tyr-OH was used to complete the peptide. Leu-enkephalin was cleaved from the resin in 1½ hrs with 50% TFA. Purification by standard techniques gave 70% yield of pure peptide, identical with authentic Leu-enkephalin by TLC and amino acid analysis.

Human gastrin I, pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2, was assembled on a Boc-Phe-Merrifield resin. NPS-Glu and NPS-Asp side chains were protected by *t*-butyl ester groups; the phenol ring of NPS-Tyr was unprotected[10]. Ammonolysis of the protected peptide from the resin at r.t. for 2 days in 1:1 DMF/MeOH freed approximately 50% of the resin-bound peptide as the peptide amide. Removal of the *t*-butyl protecting groups without peptide breakdown or derivatization of Trp proved to be difficult for HG I. The most successful deprotection was done by stirring the peptide in a mixture of 97% TFA, 2% ethanedithiol and 1% water for 1 hr, removing TFA *in vacuo*, and triturating with ethyl ether. The best procedure for purification of HG I was found to be ion-exchange chromatography on Sephadex DEAE using a linear concentration gradient of 0.5-1.5 M NH_4HCO_3, followed by partition chromatography on Sephadex G-50 with 11:5:3 0.03 M AcOH/BuOH/Pyr. The final yield of HG I, 16% based on ammonolyzed material (8% from the first amino acyl-resin), could be improved at least 3-4 times by combining the most effective side chain deprotection method and purification procedures. HG I gave the correct amino acid analysis after acid and enzymatic hydrolyses. TLC was done on silica gel in the following solvents: 4:1:1 BuOH/AcOH/H_2O, R_f = 0.42; 5:5:1:3 EtOAc/Pyr/AcOH/H_2O, R_f = 0.81. In both TLC systems, the peptide was identical to authentic HG I prepared by

solution techniques. HPLC on a μ-Bondapak C_{18} column (0.4 x 25 cm) using 0.01 M NH_4OAc (A) and 2-PrOH (B) with linear gradient elution of 25-35% B in 5 min gave R_f = 6.56 min, identical to that of authentic HG I. The specific rotation of HG I in 0.03 M NH_4OH is $[a]_D^{25}$ — 60° (C, 0.10).

Acknowledgments

We thank Dr. J. S. Morley for his gift of authentic human gastrin I.

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THE CORRECTED STRUCTURES AND SYNTHESIS OF MINIGASTRIN

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A pair of peptides, termed "Minigastrins", were isolated by Gregory and Tracy[1] from hepatic metastasis. Both peptides were shown to be potent stimulators of gastric acid secretion. Amino acid analysis[2] gave the result $Leu_{1.0}$ $Glu_{5.0}$ $Ala_{1.0}$ $Tyr_{1.0}$ $Gly_{1.0}$ $Trp_{1.0}$ $Met_{1.0}$ $Asp_{1.0}$ $Phe_{1.0}$. Alkaline hydrolysis indicated that one of the peptides was sulphated on the tyrosine hydroxyl whilst the other was free tyrosine. Thus these two minigastrins were considered to be the C-terminal-tridecapeptides of Gastrins I and II.

Comparison of the relative potencies of synthetic unsulphated minigastrin and the natural material indicated that the potency of the synthetic material was twice that of the natural material. It was suggested that this discrepancy may be due to the presence of an extra tryptophan residue. This observation was due to the fact that the dispensation of gastrin peptides is based on the UV absorption at 280 nm.

Dansylation[3] of Minigastrin followed by hydrolysis and two dimensional thin layer chromatography on polyamide sheets[4] indicated the presence of dansyl-tryptophan only. Minigastrin was taken through one cycle of Edman degradation[5] followed by dansylation and hydrolysis. This time the amino terminal residue was shown to be leucine. Thus it would seem that the correct sequence of the minigastrins must be Trp-Leu-$(Glu)_5$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2 i.e. the C-terminal tetradecapeptides of Gastrins I and II.

The synthesis of this new sequence was undertaken. The general synthesis is outlined in Figure 1. Fragment couplings were mediated by either the DCC-HONSu[6,7] or DCC-HOBt[8] procedure.

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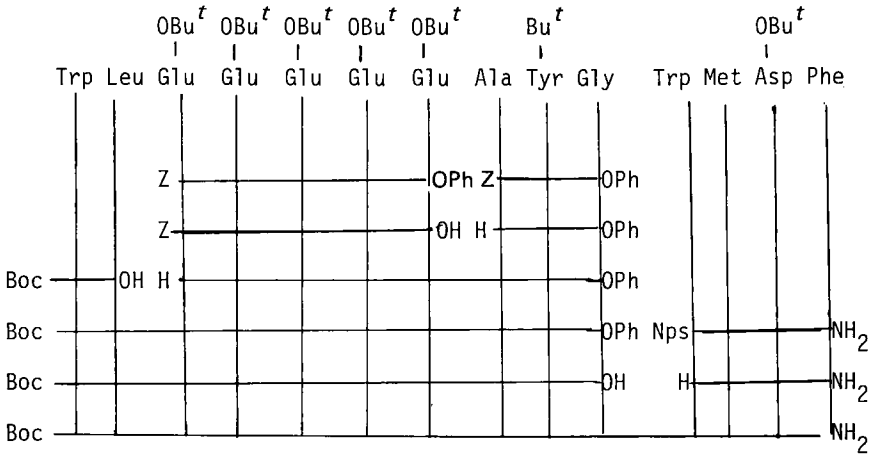

Fig. 1. General scheme of minigastrin synthesis.

Individual fragments were synthesised in a stepwise manner. Temporary carboxyl protection was afforded by the phenyl ester (OPh) group,[9] and the benzyloxycarbonyl group was utilised as temporary amine protection. In the case of the protected pentapeptide Z-[Glu(OBu^t)]$_5$-OPh the major coupling procedure utilised was the active ester method using 2,4,5-trichlorophenyl active esters (OTcp)[10] (Figure 2). Tripeptide formation required the faster pivalic mixed anhydride procedure to avoid diketopiperazine formation. The fragment Z-Ala-Tyr(Bu^t)-Gly-OPh was synthesised by the procedure outlined in Figure 3.

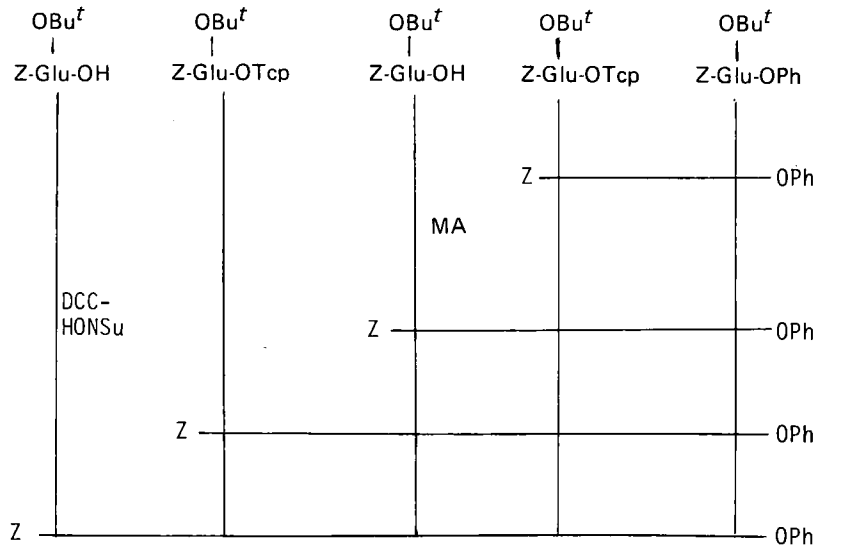

Fig. 2. Scheme of synthesis of Z-[Glu(OBu^t)]$_5$-OPh. MA = Mixed pivalic anhydride

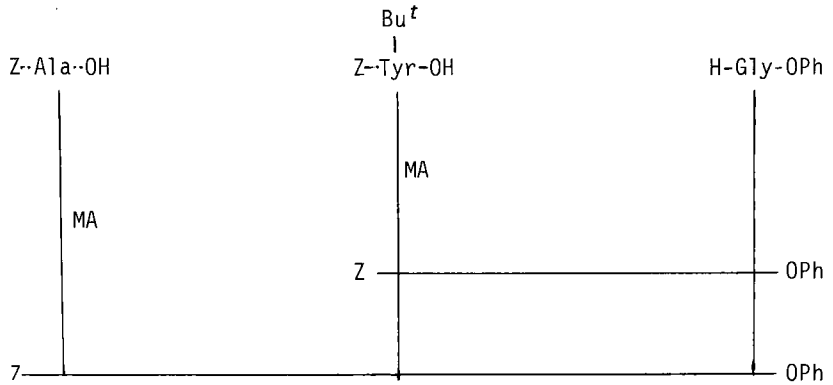

Fig. 3. Scheme of synthesis of Z-Ala-Tyr(Bu^t)-Gly-OPh. MA = Mixed pivalic anhydride

The two fragments were coupled by the DCC-HONSu procedure.[6,7] The resulting protected octapeptide was purified by Sephadex LH-20 chromatography.[11] Detection of the peptide was by UV absorption at 280 nm and the optical rotation of the solution. The dipeptide Boc-Trp-Leu-OH was synthesised by a salt coupling using Boc-Trp-ONSu and the triethylammonium salt of leucine. Again formation of the protected decapeptide was mediated by the DCC—HONSu coupling procedure and purification was by Sephadex LH-20 chromatography. Cleavage of the NPS-group from the C-terminal tetrapeptide NPS-Trp-Met-Asp-(OBu^t)-Phe-NH_2[12] was achieved by treatment with aqueous thioglycollic acid. The C-terminal phenyl ester of Boc-[1-10]-OPh was cleaved by hydrogen peroxide catalysed hydrolysis at pH 10.5. The final fragment coupling was performed using the DCC—HOBt procedure[8] and the product purified by Sephadex LH-20 chromatography. All the protecting groups were removed by a single treatment with 90% aqueous trifluoracetic acid in the presence of a large excess of 2-mercaptoethanol and anisole. Under these conditions there was no evidence for *t*-butylation of Trp and Tyr residues as shown by NMR studies of deprotected peptides at 220 MHZ.

Purification achieved by aminoethyl-cellulose chromatography with a linear gradient of triethylammonium carbonate (0.05M to 0.5M, pH 5.7 to 7.4) gave the product Trp-Leu-$[Glu]_5$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2. This material was homogenous on thin layer chromatography and paper electrophoresis (pH 6.5) and had the appropriate amino acid composition i.e. $Glu_{5.01}$ $Leu_{0.94}$ $Asp_{0.98}$ $Gly_{1.00}$ $Ala_{1.06}$ $Met_{0.98}$ $Phe_{0.94}$ $Tyr_{1.02}$. The UV spectrum was in accordance with the structure. Bioassay

of this material showed that it had the same potency and activity as the natural material.

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SOLID-PHASE SYNTHESIS OF GRAMICIDIN S, USING 2-DIMETHYLAMINOETHANOL-THALLOUS ETHOXIDE FOR THE TRANSESTERIFICATION STEP

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Introduction

Transesterification with 2-dimethylaminoethanol/thallous ethoxide, which was introduced by Savoie *et al.*,[1] was found to provide a useful method for the cleavage of the protected peptides from the Merrifield resin after solid phase synthesis. The extent of racemization at the C-terminal residue was tested mainly on dipeptides. In order to demonstrate the usefulness of this procedure in the preparation of larger biologically active peptides, the present study deals with its application in the synthesis of the well characterized gramicidin S[2-6] on the solid support.

Synthesis of Peptides and Results

The corresponding protected open-chain decapeptide was assembled by standard solid phase method,[7] beginning with Boc-Pro-resin. Nine cycles of deprotection, neutralization and coupling were carried out with the appropriate Boc-amino acids, producing the fully protected decapeptide esterified to the resin (I) with the following sequence: Boc-Val-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-resin.

In this synthesis using 2% cross-linked resin, the Boc-group was used as α-amino protection. For side chain protection of ornithine the Z-group was used. The Boc-groups were removed by a 30 minute treatment with 1M HCl in glacial acetic acid. The deprotection steps were monitored by means of chloride titrations. The couplings were carried out with DCC (4 equivalents). The couplings were repeated until no decrease of the number of the residual free amino groups could be found by means of Dorman determinations.[8]

The protected peptide was cleaved quantitatively from the resin in the form of 2-dimethylaminoethyl ester (96% yield) by double treatment with 2-dimethylaminoethanol (DMAE) in the presence of 0.2 equivalent thallous ethoxide (20 hours).

After removal of the thallium salt on Amberlite IRC-50, the obtained ester was hydrolysed by pH-stat procedure at pH 10.7, affording the Boc-di-benzyloxycarbonyl-substituted decapeptide acid (II). After purification of the crude product by precipitation from DMF with water, a white crystalline product was obtained in 80% yield. The peptide had a correct amino acid analysis; $[\alpha]_D^{25}$ — 67.2°(C 0.2, DMF) R_f 0.76 (tlc, 1-butanol: acetic acid: water, 4:1:5).

For comparison, the protected decapeptide resin (I) was transesterified by another reagent. The peptide was cleaved from the resin with a mixture of Et_3N, MeOH and DMF.[9,10] The saponification of the resultant peptide methyl ester proceeded smoothly in DMF-water mixture to afford a peptide indistinguishable from peptide (II), obtained from the DMAE-thallous ethoxide transesterification step.

After removal of the Boc-group, the linear peptide was cyclized with dicyclohexylcarbodiimide-p-nitrophenol in pyridine (concentration 0.1 mmol/litre) at room temperature. Purification of the crude cyclic peptide by ion-exchange chromatography on Dowex-50 (H^+ form), gave a chromatographically homogeneous product (III) in 68% yield.

Catalytic hydrogenation of (III) afforded synthetic gramicidin S dihydrochloride trihydrate (IV) as fine needles (EtOH-1M HCl) in 52% yield. The synthesized (IV) and natural gramicidin S dihydrochloride had the same melting point, specific rotation and showed indistinguishable chromatographic and electrophoretic patterns; m.p. 275-277°, $[\alpha]_D^{23}$ — 263 (C 0.4, EtOH), amino acid ratio: Pro, 0.82; Val, 1.00; Leu, 1.13; Phe, 1.23; Orn, 1.13.

The synthetic gramicidin S dihydrochloride exhibited the same antimicrobial activities as those of the natural antibiotic. The minimum inhibitory concentration of (IV) was 2.28 μg/ml on *S. aureus,* 6.8 μg/ml on *B. subtilis* and 61.7 μg/ml on *E. coli.*

From the above mentioned findings, it is obvious that DMAE thallous ethoxide reagent afforded a racemization-free cleavage of the peptide from the resin.

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SELECTIVE PROTEIN MODIFICATION BY APPROPRIATE CHOICE OF REACTION MEDIUM AND REAGENT. GUANIDINATION OF INSULINS IN GUANYLDIMETHYLPYRAZOLE/DIMETHYL SULFOXIDE AND APPLICATION OF DERIVATIVES.

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Introduction

The guanidination of protein amino groups has been carried out with *S*- and *O*-methylisourea and guanyldimethylpyrazole (GDMP) in aqueous solution at alkaline pH (see ref. 1, 2 for reviews).

Protected peptides can be guanidinated at the free amino sides in DMF with GDMP/triethylamine[3]. Recently it was shown by us, that the Gly^{A1}-amino group in the corresponding di-Boc-insulin could be quantitatively converted into a guanidino group[4,5] in DMSO.

Conservation of a positive charge at the insulin A-chain N-terminus is important for its bioactivity[5,6]. Of the three amino groups the Al-side is most sensitive to modification if measured by vitro bioassay (data in ref. 6, 7, 8). Of 24 Al-substituted insulins,[6] the [Al-guanidinoacetyl]-insulin[4,5] was the only one with almost unimpaired bioactivity. This derivative was synthesized using GDMP for the first time for protein modification in an aprotic solvent to give homogeneous guanidino-insulin.

Results and Discussion

Guanidination reactions under the same conditions with partially aminoprotected insulins (Al-Boc-, Al-citraconyl-, AlBl-di-citraconyl-, AlB29-di-Boc-insulin) as well as native insulin were investigated. Amino acid analysis revealed that free Al and B29 amino groups were guanidi-

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nated to ca. 80% and B1 to 20% or less. These results correlated with pH 8.6 electrophoresis patterns, which give information about the extent of reaction at α-amino groups. When the reaction was carried out at higher protein and reagent concentration a mixture could be obtained, where the A1 and B29 amino groups were guanidinated to more than 99% while B1 was reacted less than 20%. Using the same reaction conditions with B1-Boc-insulin quantitative reaction at the free amino groups was obtained (to 1 ml stirred DMSO are added 20-40 mg insulin, 100 μl triethylamine, 200 mg GDMP-nitrate; after 16-18 hr 3-5 vol. 10% acetic acid and solid urea to obtain a clear solution are added, followed by chromatography over Sephadex G-15 or G-25 in 1 M acetic acid and freeze-drying). The guanidination reaction with partially protected as well as native insulin was followed by reaction of free amino groups with methylsulfonylethoxycarbonyl-hydroxysuccinimide ester (Msc-ONSu) in DMSO (1-3 mM insulin, 10-30 mM Msc-ONSu, 10-30 mM triethylamine) followed by electrophoretic analysis. Quantitation of pH 2 patterns[9] reveals the degree of substitution. The pH 8.6 pattern shows the degree of substitution of the α-amino groups. After sulfitolysis the difference in reaction between A- and B-chain is seen. These analyses on guanidination of native insulin revealed the A1B29-di-guanidino-insulin to be the main product (⩾80%). The A1B1B29-tri-guanidino-insulin was the only detected minor product (⩽20%). Amidination at imidazole residues would have been revealed in pH 8.6 electrophoresis. End group determination by the dansyl-technique[10] showed only Dns-Phe and the Dns-O-Tyr spots with crude and purified (QAE-Sephadex, pH 8.7, salt gradient) di-guanidino-insulin (see ref. 4, 6, 9).

In conclusion, the amino group with the more hydrophobic surrounding (B1) reacts considerably slower. This is also found in other reactions in DMSO leading to the selective synthesis of A1B29-di-substituted insulins[9].

Di-guanidino-insulin obtained by this procedure can be reacted at the free B1-amino group to yield the corresponding substituted insulin. Edman degradation followed by acylation also yielded derivatives[13] with replacements in the N-terminal positions of the B-chain, as was demonstrated with A1B29-di-phthaloyl[11] or A1B29-di-Msc-insulin[12] as starting material. Thus, derivatives can be synthesized which carry radiolabels or fluorescent labels and have almost unimpaired bioactivities compared to the native hormone. Table I gives examples of syntheses, *in vitro* bioactivities and receptor binding data of guanidinated and non-guanidinated insulins.

Dimers have been prepared with succinyl-, adipoyl-, and suberoyl-bridges between B1 and B1' using A. Schuttler's approach (PhD thesis in prep.) (D. J. Saunders, to be published). Coupling of di-guanidino-insulin to solid supports should be of interest. If an active ester coupling (ONSu-ester) at pH 6 is used, the coupling reaction would be selective and no protecting groups have to be removed. B1-biotinyl-, di-guanidino-insulin could be bound to avidin-Sepharose (analogous to ref. 14). Such bound insulins are useful for antibody and receptor isolation work. Further, the reports that mature lymphocytes carry insulin receptors while immature lymphocytes do not[17], suggests the support bound insulin to be useful for cell separation purposes.

Table I. Examplary Synthesis and Bioactivities of Guanidinated and B1-substituted Insulins.

derivative	synthesis	in vitro bioactivity[a)]	receptor binding[b)]
A1-guanidino-ins.	ref. 4,5	88 ± 5 %	84 ± 3 %
A1B29-di-guanidino-insulin (crude)	as described here	75 - 100 %	100 ± 5 %
B1-Boc-,A1B29-di-guanidino-insulin	"	101 ± 6 %	n.d.
B1-carbamoyl-,A1B29-di-guanidino-ins.	c)	100 ± 10 %	n.d
B1 Boc-insulin	d)	106 ± 11 %	n.d.
B1-carbamoyl-ins.	e)	87 ± 4 %	n.d.
B1-biotinyl-ins.	ref. 14	94 ± 9.6 %	100 ± 3 %

[a] Free fat cell assay acc. to Moody *et al.*[15]

[b] Binding measured in a competitive binding assay using ^{125}I-insulin and beef liver membranes (P. Rosen, M. Simon, H. Reinauer, H.-J. Friesen, C. Diaconescu, D. Brandenburg, manuscript in prep.)

[c] Crude di-guanidino-insulin was carbamylated,[16] followed by SP-Sephadex chromatography[7].

[d] Insulin was reacted at pH 6 with di-*tert*-butyl-dicarbonate followed by DEAE-ion exchange chromatography[9]. The B1-Boc-insulin is the main product of the reaction and obtained in pure form after the purification.

[e] A1-trifluoroacetyl-insulin (ref. 5, 9) was carbamylated as for c), deblocked at A1[4] and purified by DEAE-ion exchange[9].

The preparation of the crude di-guanidino involves only an overnight reaction and a gel filtration. For affinity approaches, where the insulin would have to be coupled to a support (e.g. glass, nylon, Sepharose), crude di-guanidino-insulin can be used as well as purified product, because the contaminating tri-guanidino-insulin would not couple if an active ester approach at pH 6 is used.

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TOWARDS THE SYNTHESIS OF [A-19]3-(IODO)-, AND 3,5(DI-IODO)-TYROSINE PORCINE INSULINS

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Tyrosines in insulin have been iodinated to prepare labelled tracers for the study of insulin action, metabolism, and the immunoassay of the hormone in blood or other body fluids. Direct iodination of insulin yields heterogenous products with various distributions of iodine between the A and B chains[1]. Definite conclusions on the chemical and biological effects of the iodination on insulin cannot be drawn from such materials. We have used 3-(iodo)-tyrosine and 3,5-(di-iodo)-tyrosine in the synthesis of homogeneous iodinated porcine insulin analogues.

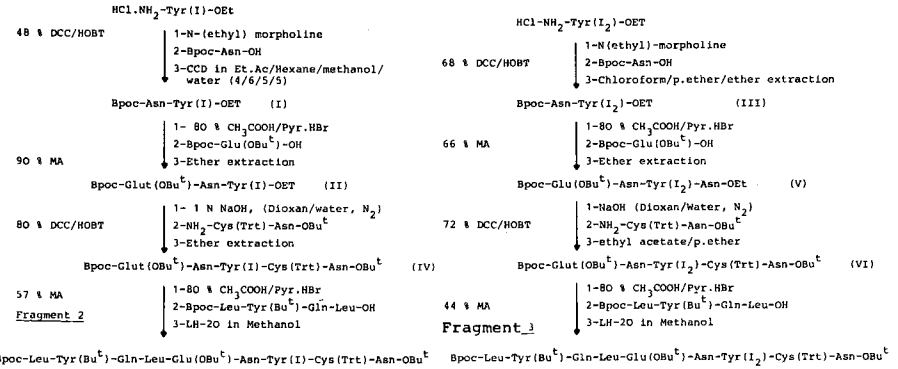


Fig. 1. Outline of the synthesis of 3-(iodo) and 3,5-(di-iodo) tyrosine containing nonapeptide derivatives. Fragments 2 and 3.

Tyrosine [A19] is on the surface of the monomer and may be involved in the interaction between the hormone and its receptor[2]. Pure [A19] 3-(iodo)- and 3,5-(di-iodo)-tyrosine insulins are required to obtain information about the role of tyrosine [A19] in the receptor-binding region.

The synthesis of [A19] 3-(iodo)- and 3,5-(di-iodo)-tyrosine S-sulphonated A chains used the strategy of fragment condensation in combination with acid labile protecting groups. Cysteine residues were protected by trityl groups. C-terminal and γ-carboxyl functions were

protected as *t*-butyl esters. Hydroxyl functions were protected as *t*-butyl ethers except for the 3-(iodo)-tyrosine and 3,5-(di-iodo)-tyrosine residues for which no protection was needed. For the temporary protection of α-amino functions, the benzyloxycarbonyl- and the 2-(4-biphenylyl)isopropyloxycarbonyl-groups were used. The fragments synthesized were:

1 Boc-Gly-Ile-Val-Glu(OBu^t)-Gln-Cys(Trt)-Cys(Trt)-Thr(Bu^t)-Ser-(Bu^t)-Ile-Cys(Trt)-Ser(OBu^t)-OH

2 Bpoc-Leu-Tyr(Bu^t)-Gln-Leu-Glu(OBu^t)-Asn-Tyr(I)-Cys-Trt-Asn-OBu^t

3 Bpoc-Leu-Tyr(Bu^t)-Gln-Leu-Glu(OBu^t)-Asn-Tyr(I_2)-Cys(Trt)-Asn-OBu^t

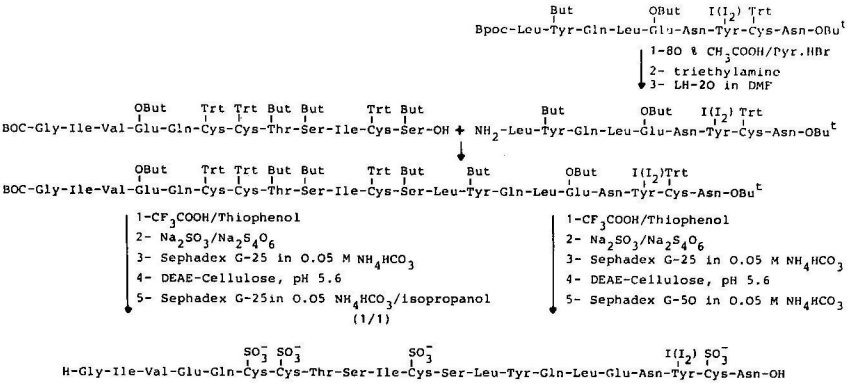
The protected peptide fragment 1 was synthesized via a procedure previously published[3]. The schemes for the synthesis of fragments 2 and 3 are outlined in Figure 1. The protected peptides were purified via crystallization, gel-filtration or countercurrent distribution, and characterized by TLC, elemental and amino acid analysis. (Table I).

Table I. Analytical Data for Iodo-peptides of Figure 1.

Compound	State (Melting Point °C)	R_f*	$(\alpha)^D_{22}$ (C in MeOH)	Iodine analysis (Theor. value)
Bpoc-Asn-Tyr(I)-OEt I	Cryst., (83-85)	0.83	-6.2 (2)	18.46 (18.76)
Bpoc-Asn-Tyr(I_2)-OEt II	Cryst., (117-118)	0.71	-8.2 (2)	31.20 (30.37)
Bpoc-Glu(OBu^t)-Asn-Tyr(I)-OEt III	Cryst., (105-106)	0.42	-3.3 (2)	-14.54 (14.89)
Bpoc-Glu(OBu^t)-Asn-Tyr(I_2)-OEt IV	Cryst., (149-150)	0.54	-7.8 (2)	25.41 25.24
Bpoc-Glu(OBu^t)-Asn-Tyr(I)-Cys(Trt)-Asn-OBu^t V	Cryst., (144-145)	0.58	-7.1 (1.5)	9.33 (9.58)
Bpoc-Glu(OBu^t)-Asn-Tyr(I_2)-Cys(Trt)-Asn-OBu^t VI	Cryst., (154)	0.69	-2.2 (1)	17.08 (17.25)
Bpoc-Leu-Tyr(Bu^t)-Gln-Leu-Glu(OBu^t)-Asn-Tyr(I)-Cys(Trt)-Asn-OBu^t. Fragment 2	Cryst., (180-182)	-	-12.5 (2)	6.56 (7.02)
Bpoc-Leu-Tyr(Bu^t)-Gln-Leu-Glu(OBu^t)-Asn-Tyr(I_2)-Cys(Trt)-Asn-OBu^t.Fragment 3	amorph., (200 d)	-	-11.9 (1.5)	12.32 (12.44)

Amino Acid Analysis: V, Glu 1.07, Asp 2.00, Tyr 0.92; VI, Glu 0.88, Asp 2.00, Tyr 0.95; Fragment 2, Asp 1.99, Glu 2.03, Leu 2.00, Tyr 1.9; Fragment 3, Asp 2.04, Glu 1.93, Leu 2.00, Tyr 2.01.

The protected chains were synthesized by coupling fragment 1 and 2 (for the 3-(iodo)-tyrosine chain) and 1 with 3 (for the 3,5-(di-iodo)-tyrosine chain) using DCCI/HOBT with N-methylpyrolidone as solvent. Purification was achieved on Sephadex LH-20 in the lower phase of n-butanol/methanol/chloroform/water (1/1/1/1).



Amino acid	Asp	Thr	Ser	Glu	Gly	Val	Ile	Leu	Tyr
Theory	2.00	1.00	2.00	4.00	1.00	1.00	2.00	2.00	2.00
[A 19] 3-(iodo)-tyrosine S-sulphonated A-chain	1.77	1.08	2.23	3.83	1.00	0.66	1.71	1.85	1.80
[A 19] 3,5-(di-iodo)-tyrosine S-sulphonated A-chain	2.00	1.12	2.06	3.96	1.00	0.82	1.70	2.00	1.60

Fig. 2. Synthesis of [A19] 3-(iodo)-, and 3,5-(di-iodo)-tyrosine S-sulphonated A-chains with amino acid analysis.

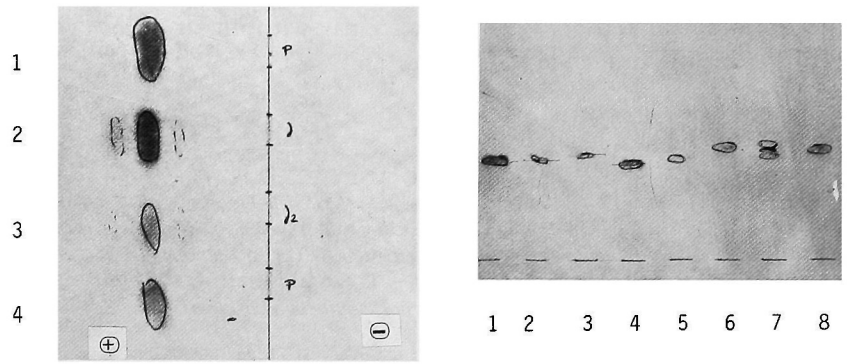


Fig. 3. Electrophoretic pattern of the A-chains at pH 2.2. 1.4 S-sulphonated porcine A chain. 2. [A19] 3-(iodo)-tyrosine S-sulphosulphonated A chain. 3. [A19] 3,5-(di-iodo)-tyrosine S-sulphonated A chain.

Fig. 4. Paper print of TLC in Butanol/Pyridine/acetic acid/water (15/10/3/12 v/v). 1.4.8 Porcine S-sulphonated A chain. 2.5 [A19] 3-(iodo)-tyrosine S-sulphonated A chain. 3.6 [A19] 3,5-(di-iodo)-tyrosine S-sulphonated 7 Mixture of the 3-chains.

The synthetic products were deprotected with trifluoroacetic acid using thiophenol as trityl-cation scavenger[4]. The thiol chains were converted to S-sulphonates without observable reduction in iodine content, and purified on DEAE-cellulose at pH 5.6. Gel-filtration on Sephadex G-25 in 0.05 M NH_4HCO_3/isopropanol (1/1) was needed for the 3,5-(di-

iodo)-tyrosine chain. The purity of the [A19]3-(iodo)- and 3,5-(di-iodo)-tyrosine S-sulphonated chains as checked by electrophoresis and TLC, Figures 3 and 4.

The [A19] 3-(iodo)-tyrosine S-sulphonated A chain and native S-sulphonated B chain were reduced together and the mixture was oxidized at pH 10.6 to yield [A19] 3-(iodo)-tyrosine porcine insulin. The crude product was fractionated on Sephadex G-50 at pH 8.2. The yield of pure (electrophoresis and TLC) analogue was 9% based on the A-chain. Amino acid analysis showed: Asp 3.27(3), Thr 2.14(2), Ser 3.28(3), Glu 6.95(7), gly 4.14(4), Ala 2.08(2), Cys 2.87(3), Val 0.60(1), Ile 1.24(2), Leu 5.66(6), Tyr 3.54(4), Phe, 2.80(3), Lys 1.02(1), His 1.87(2), Arg 1.2391). *In vivo* activity of 24 ± 2% was determined by rat equididymal adipoxytes by the method of Moody *et al*[5].

In conclusion, the low biological activity of the analogue may be caused either by the single site modification (tyrosine to iodo-tyrosine) or by concerted structural changes in the molecule. Sonne *et al.*[6] found a similar reduction in binding activity for an [A19]-iodo insulin.

Another [A19] analogue[3], [A19]-Phe porcine insulin, also had reduced biological activity (22%), apparently without structural distortion of the insulin molecule (CD data).

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SYNTHETIC APOLIPOPROTEIN MODEL PEPTIDES: STRUCTURAL DETERMINANTS OF PEPTIDE-PHOSPHOLIPID INTERACTIONS

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Plasma apolipoprotein A-I (apo A-I) is the principal structural protein of high density lipoprotein (HDL). We have been engaged in studies of the surface properties and lipid binding activity of synthetic fragments and of a peptide model of apo A-I in order to determine whether the "amphiphilic α-helix", believed to be the principal secondary structural feature of this macromolecule[1], is the major determinant of its biological activity. An important indicator of a successful model for apo A-I is its affinity for polar lipid surfaces, exemplified by the egg lecithin single bilayer vesicles (SBV) employed in our experiments.

Table I. Sequences of Peptides Synthesized

A 158-168 (A-I)

His-Val-Asp-Ala-Leu-Arg-Thr-His-Leu-Ala-Pro

B 147-168 (A-I)

Leu-Gly-Glu-Glu-Met-Arg-Asp-Arg-Ala-Arg-Ala-
His-Val-Asp-Ala-Leu-Arg-Thr-His-Leu-Ala-Pro

C 114-133 (A-I)

CH_3-C(=O)-Glu-Met-Glu-Leu-Tyr-Arg-Gln-Lys-Val-Glu-
Pro-Leu-Arg-Ala-Glu-Leu-Gln-Glu-Gly-Ala

D 22-31 (A-II)

Gly-Lys-Asp-Leu-Met-Glu-Lys-Val-Lys-Ser

E Synthetic Super A-I

Pro-Lys-Leu-Glu-Glu-Leu-Lys-Glu-Lys-Leu-Lys
Glu-Leu-Leu-Glu-Lys-Leu-Lys-Glu-Lys-Leu-Ala

F 124-167 (A-I)

Pro-Leu-Arg-Ala-Glu-Leu-Gln-Glu-Gly-Ala-Arg-
Gln-Lys-Leu-His-Glu-Leu-Gln-Glu-Lys-Leu-Ser-
Pro-Leu-Gly-Gln-Gln-Met-Arg-Asp-Arg-Ala-Arg-
Ala-His-Val-Asp-Ala-Leu-Arg-Thr-His-Leu-Ala

The primary sequences of our model peptides are shown in Table I. Peptides A, B, C and F are fragments of apo A-I, and peptide D is a fragment of plasma apolipoprotein A-II. Peptide E is a docosapeptide designed to have high amphiphilicity when in the α-helical conformation. The most apo A-I like behavior is shown by peptides B, E and F. The peptides were synthesized using the Merrifield solid phase method.[2] Purification was accomplished chromatographically. No impurities in the peptide were detected by

chromatographic and electrophoretic methods or by automated Edman degradation.

Table II summarizes the physical properties of the peptides. P_α and P_β are, respectively, the α-helix and β-sheet potential of the Chou and Fasman predictive scheme.[3] The per cent α-helix for each peptide solution was estimated from circular dichroism measurements.[4] The helix content of peptides B, C and F greatly increases as the polarity of the solvent decreases.[5] $\pi_{collapse}$ is the highest surface pressure at which monolayers of a given molecule are stable at an interface (air-water in this case). K_d is the dissociation constant of the peptide- SBV complex, and N is the number of amino acids bound to each SBV at saturation. The affinity of peptides B and C for SBV is pH dependent; the affinity of B is governed by the protonation of one group of pK = 6.2 (presumably His) having higher affinity at low pH. In peptide E the acidic and basic groups are "fixed" near neutral pH, and its binding to SBV shows no pH dependence. The π-A work, calculated from the π-A curves,is the work required to remove a molecule from an amphiphilic interface and is a good index of the intrinsic amphiphilicity of a molecule.[6]

Apo A-I and the synthetic peptides may be grouped into two families, the *homologous* series including peptides A, B, C, F and apo A-I and an *analogous* series including peptides B, C and E. Correlations are most easily made among the members of each series although some may be made among all of the peptides. For all of the peptides, $\pi_{collapse}$ is directly proportional to $-RT \ln (K_d) \equiv \Delta G_{aff}$, although peptides C and E do deviate. For the homologous series, $\pi_{collapse}$ is proportional to ln (number of helical segments) and $K_a \equiv 1/K_d$ is directly proportional to the number of helical segments. Within the analogous series $-RT \ln (K_d) \equiv \Delta G_{aff}$ is proportional to the π-A work.

High helicity and amphiphilicity are required for apo A-I like properties. In all of the peptides studied, the secondary structure having the highest amphiphilicity is the α-helix. Thus, increasing the helix content increases the surface affinity. Peptide E is indeed a "super peptide" showing the highest per-residue helicity, amphiphilicity and phospholipid surface affinity. The number of amino acyl residues bound per SBV stays constant over the entire series of peptides and probably represents the "free surface area" between the lecithin head groups.

The meaning of the correlations between the physical properties and the SBV surface affinities of the peptides is complex. It is expected that the SBV surface affinity (ΔG_{aff}) should be proportional to the intrinsic amphiphilicity (π-A work) and for the *analogous* series this is true. This

Table II. Physical Properties of the Peptides

Peptide	P_α	P_β	% α-helix	$\pi_{collapse}$ (dyne-cm^{-1})	$K_d \times 10^6$ (M)	N (a.a./SBV)$_a$	π-A Work (kcal-mole^{-1})	ΔG_{aff} (Kcal-mole^{-1})$_b$
D	1.072	0.935	8$_c$	∿ 1.5	> 3000$_d$	-	0	< 3.5$_d$
A	1.125	0.997	8$_c$	∿ 3	> 600$_d$	-	0	< 4.4$_d$
C	1.164	0.946	13$_c$	∿ 6	∿ 20	∿ 770	∿ 0.3	6.5
B	1.13	0.94	15$_c$	9.5	6.4$_c$	∿ 770$_c$	2.6	7.2
F	1.13	0.97	18	13.5	∿ 3.9	∿ 650	∿ 3	7.5
E	1.28	<1.0	50$_e$	22$_e$	1.9$_e$	∿ 1050$_e$	5.6	7.9
apo A-I	1.10$_f$	0.93$_f$	61$_g$	22$_h$	0.94$_e$	∿ 1000$_{e,i}$	∿ 18	8.3

[a] Assuming 3200 PC/SBV. [b] $\Delta G \equiv -RT \ln K_d$. [c] K_d value is that measured at optimum pH. D. J. Kroon, J. P. Kupferberg, E. T. Kaiser and F. J. Kezdy. *J. Amer. Chem. Soc.*, **100**, 5975-5977. [d] Based upon limits of detection of binding. [e] D. Fukushima, J. P. Kupferberg, S. Yokoyama, D. J. Kroon, E. T. Kaiser and F. J. Kezdy, *J. Amer. Chem. Soc.*, **101**, 3703-3704, peptide as tetramer. [f] C. Edelstein, F. J. Kezdy, A. M. Scanu and B. W. Shen, *J. Lipid Res.*, 1979, **20**, 143-153. [g] L. B. Vitello and A. M. Scanu, *J. Biol. Chem.*, 1976, **251**, 1131-1136; A. M. Scanu and R. Hirz. *Proc. Natl. Acad. Sci. (U.S.A.)* 1968, **59**, 890-894. [h] B. W. Shen, A. M. Scanu and F. J. Kezdy, *Circulation*, 1973, **48**, suppl. IV, 218, [i] Assuming only 2/3 of apo A-I residues participate in binding.

is not true with the *homologous* series where ΔG_{aff} is proportional to $\pi_{collapse}$ which is in turn roughly proportional to the square root of the π-A work. Furthermore, the number of 22 residue helical segments per peptide is not proportional to ΔG_{aff} as one might think, but rather to exp $(\frac{\Delta G_{aff}}{RT}) \equiv K_a$. While the helix intrinsic amphiphilicity (π-A work) seems to be additive in the homologous series, the SBV surface affinities (ΔG_{aff}) are not. The difference between the stability of the peptide or protein in solution and the same molecule at the SBV surface is reflected by ΔG_{aff}. The increased stability of any molecule at the SBV surface is probably due to the reduced hydration of its lipophilic moieties. The members of the analogous series must have their lipophilic side chains completely hydrated when they are monomers in aqueous solution. Their SBV surface affinities directly reflect intrinsic amphiphilicity. The higher members of the homologous series may have their lipophilic side chains only partially hydrated as monomers in aqueous solution because of intramolecular interchain association. Such an association would be second order or higher so that the number of lipophilic residues hydrated would be expected to be proportional to the *n*th root of the local chain concentration. The difference between the ΔG_{aff} of apo A-I and the ΔG_{aff} expected on the basis of its intrinsic amphiphilicity implies that 30% or fewer of the lipophilic side chains of apo A-I are hydrated when apo A-I is a monomer in solution. This is supported by the tendency of apo A-I and

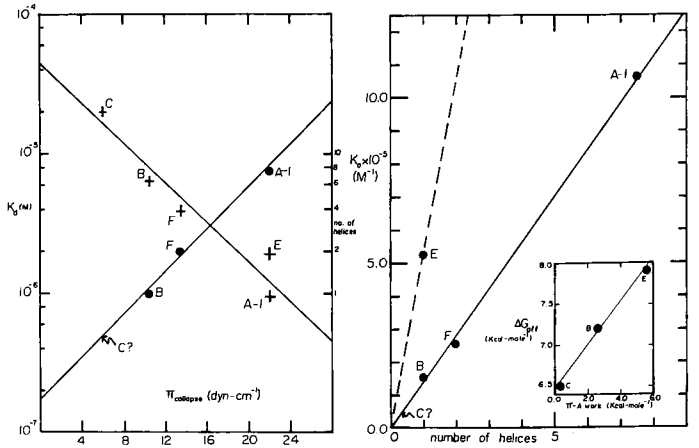

Fig. 1. Correlations among collapse pressure, number of helices and SBV affinity. *Left panel* (+) surface affinity (ln K_d) *vs* collapse pressure ($\pi_{collapse}$), (●) ln (no. of helices) *vs* $\pi_{collapse}$. *Right panel* SBV-peptide association constant (K_a) *vs* no. of helices. *Inset* binding affinity (ΔG_{aff}) *vs* π-A work.

peptide E towards self-aggregation in solution and by the fact that tetramers and octomers of apo A-I do not bind to lipid surfaces reversibly.[7]

The question may now be posed why apo A-I is such a large polypeptide containing many imperfect amphiphilic helices. It may be physiologically important for apo A-I to have no higher an affinity than it does so that it is still free to exchange. While the amphiphilic helix is necessary for all and sufficient for many of apo A-I's functions, there may be structural or sequence elements of apo A-I which are necessary for other functions but which detract from the stability of the amphiphilic helical conformation. A new series of peptides is being designed and synthesized to test this last possibility.

Acknowledgment

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LIPID BINDING BY APOLIPOPROTEIN A-I: SYNTHETIC STUDIES

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Introduction

The sequence of apolipoprotein A-I (apoA-I) from human high density lipoproteins has been determined.[1,2] The apoprotein has been shown to recombine with phospholipid and phospholipid-cholesterol dispersions.[3] The apoprotein has been shown to activate the enzyme lecithin-cholesterol acyltransferase (LCAT),[4] the enzyme responsible for cholesterol ester synthesis in plasma. Since the carboxyl-terminal cyanogen bromide fragment binds phospholipid and also activates LCAT,[4] we have synthesized a portion of this peptide (Figure 1) and report here the results of the lipid binding studies.

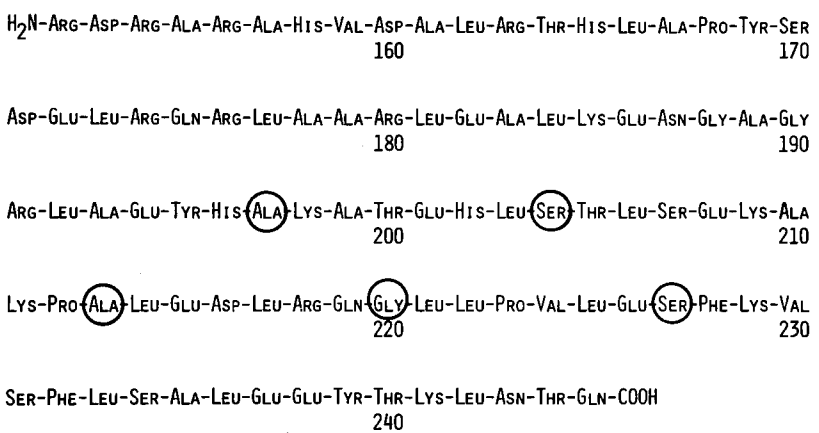

Fig. 1. Amino acid sequence[1] of cyanogen bromide fragment I of apolipoprotein A-I. The circled residues are the amino termini of the five peptides synthesized and studied for lipid binding.

Results

The five fragments (Figure 1) were synthesized on a modified polystyrene resin.[5] The peptides were cleaved from the resin with HF at 0°C in the presence of ethanedithiol and anisole. The extracted peptides were purified by ion exchange chromatography on DEAE-Sephadex. The purified peptides had the expected amino acid analysis (Table I) and showed a single band on electrophoresis in urea-polyacrylamide gels at pH 4.3 and 8.2.

Table I. Amino Acid Analyses of Synthetic Peptides of Apolipoprotein A-I.

AMINO ACID	227-245	220-245	213-245	204-245	197-245
ASPARTIC ACID	0.9 (1)	1.0 (1)	1.7 (2)	2.1 (2)	2.0 (2)
THREONINE	1.5 (2)	1.8 (2)	1.6 (2)	2.6 (3)	3.5 (4)
SERINE	2.1 (3)	2.4 (3)	2.2 (3)	4.6 (5)	4.7 (5)
GLUTAMIC ACID	2.9 (3)	4.5 (4)	5.7 (6)	7.1 (7)	8.1 (8)
PROLINE	0	1.0 (1)	0.9 (1)	2.1 (2)	2.5 (2)
GLYCINE	0	1.0 (1)	0.9 (1)	1.1 (1)	1.1 (1)
ALANINE	1.1 (1)	1.2 (1)	2.0 (2)	3.1 (3)	5.0 (5)
VALINE	1.0 (1)	2.0 (2)	1.9 (2)	1.9 (2)	2.2 (2)
LEUCINE	2.7 (3)	6.2 (6)	7.6 (8)	9.0 (9)	9.9 (10)
TYROSINE	1.0 (1)	1.2 (1)	0.9 (1)	0.9 (1)	0.8 (1)
PHENYLALANINE	2.2 (2)	2.1 (2)	2.5 (2)	2.2 (2)	2.0 (2)
LYSINE	1.6 (2)	1.9 (2)	1.9 (2)	3.9 (4)	4.7 (5)
HISTIDINE	0	0	0	0	0.8 (1)
ARGININE	0	0	0.9 (1)	1.0 (1)	1.1 (1)

The phospholipid binding by the peptides was studied by observing the C.D. spectrum upon the addition of dimyristoylphosphatidylcholine (DMPC) and by isolation of stable peptide-lipid complexes by density gradient ultracentrifugation in cesium chloride. The smallest peptide (227-245) showed no changes in the C.D. spectrum (Figure 2A) in the presence of DMPC. The remaining peptides displayed C.D. spectral changes which have been associated with the interaction of the apolipoproteins with phospholipid (Figure 2B-E). By density gradient ultracentrifugation, peptide 227-245 did not form a stable complex with lipid (Figure 3A). The other peptides formed complexes (Figure 3B-E)

with molar ratios of lipid to protein of 40:1 to 100:1. When analyzed for LCAT activation using DMPC-cholesterol dispersions, none of the peptides were active.

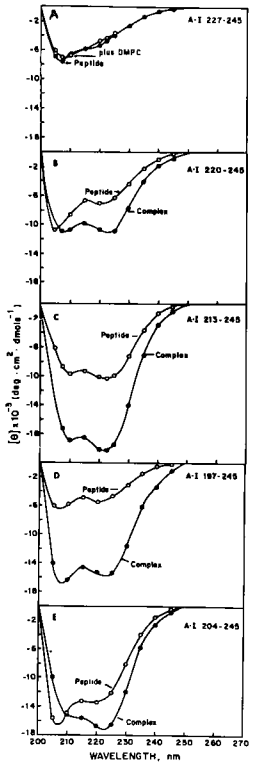

Fig. 2

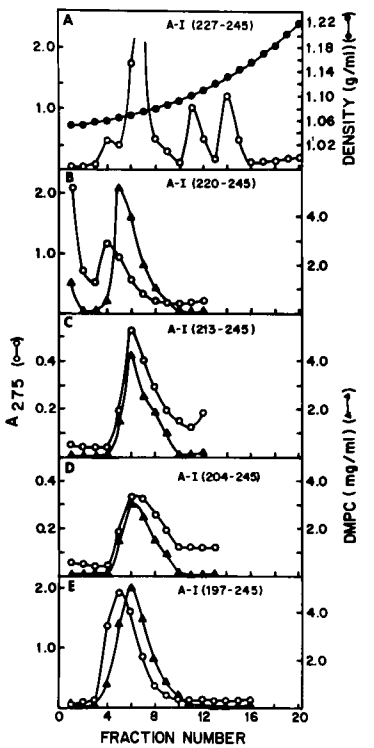

Fig. 3

Fig. 2. Circular dichroic spectra of the synthetic peptides and the isolated complexes from the density gradients.

Fig. 3. Density gradient ultracentrifugation of peptide/dimyristoylphosphatidylcholine mixtures.

Discussion

We have demonstrated[6] that the requirements for phospholipid binding by the apolipoproteins are: 1) the ability to form amphipathic helical segments, 2) the hydrophobicity of these segments and 3) a critical length of the amphipathic helix. From the C.D. spectrum and the

distribution of phospholipid in the density gradient, we conclude that the highly hydrophobic peptide 227-245 interacts with the lipid but it does not have sufficient length to form a stable complex, whereas, peptide 220-245 and the longer peptides do have sufficient length and hydrophobicity to permit the isolation of a complex. Since none of the peptides were found to activate LCAT, we conclude that the requirements for activation by the cyanogen bromide peptide are contained between residues 152 and 197.

Acknowledgement

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MASTOPARAN, A POTENT MAST CELL DEGRANULATING PEPTIDE: SYNTHESIS AND RADIOIMMUNOASSAY

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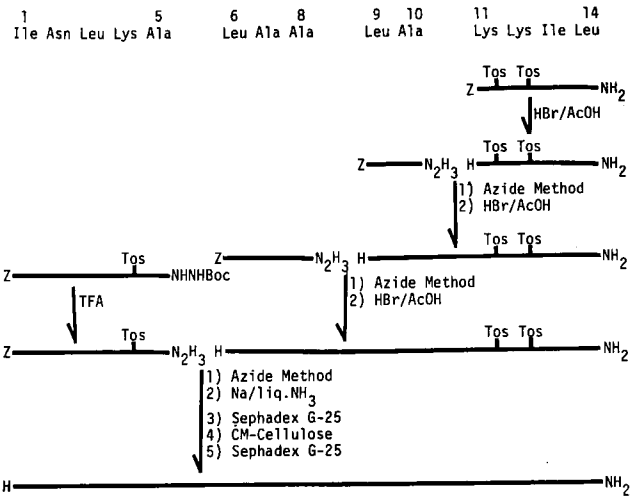
Mastoparan possessing a potent mast cell degranulating activity was isolated from venum of *Vespula lewisi,* and its structure has been proposed to be a tetradecapeptide amide: H-Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Ala-NH_2.[1] In the present study, the synthesis of mastoparan was performed in order to confirm the proposed structure and to develop a radioimmunoassay specific for mastoparan. As a substrate for preparation of ^{125}I-labelled antigen, N^{α}-tyrosylmastoparan was also synthesized.

Synthesis of Mastoparan and N^{α}-Tyrosylmastoparan

Our approach for the synthesis of mastoparan involved the preparation of four fragments: H-Lys(Tos)-Lys(Tos)-Ile-Leu-NH_2, Z-Leu-Ala-N_2H_3, Z-Leu-Ala-Ala-N_2H_3 and Z-Ile-Asn-Leu-Lys(Tos)-Ala-N_2H_3, by stepwise elongation using mainly the mixed anhydride[2] or active ester method.[3] Azide fragment condensation was selected for the chain elongation (Scheme I). The yield in each coupling stage was over 70%. The protected tetradecapeptide thus obtained was deblocked by means of sodium in liquid ammonia.[4,5] The reaction was terminated by addition of dry ammonium chloride. After evaporation of ammonia, the residue was gel-filtered on Sephadex G-25 using 50% acetic acid as eluent. The product was further purified by ion exchange chromatography on CM-cellulose with a linear gradient of H_2O — 0.2M ammonium acetate buffer (pH 6.7). Finally, the product was desalted by gel filtration on Sephadex G-25. The synthetic tetradecapeptide amide behaved as a homogeneous component on tlc in different solvent systems. Acid hydrolysate and



Scheme I. Synthesis of mastoparan.

AP-M digest of the peptide contained the constituent amino acids in theoretical ratios: $[\alpha]_D^{24} = -77.8°$ (C 1.0, 1M acetic acid); amino acid ratios in acid hydrolysate (6N HCl, 48 hours) Lys(3)3.03, Asp(1)0.94, Ala(4)4.09, Ile(2)1.78, Leu(4)4.14; amino acid ratios in AP-M digest Lys 2.82, Asp 0.87, Ala 4.04, Ile 2.09, Leu 4.17. The chromatographic patterns of dansylated derivatives of the synthetic tetradecapeptide amide and its tryptic digest were identical with those of the corresponding derivatives of the natural mastoparan.

N^{α}-tyrosylmastoparan was prepared in a manner similar to that used for synthesis of mastoparan. Purity of this analog was assessed by tlc in different solvent systems and amino acid analysis: $[\alpha]_D^{24} = -85.0°$ (C 1.0, 1M acetic acid); amino acid ratios in acid hydrolysate (6N HCl, 48 hours) Lys(3)2.99, Asp(1)1.07, Ala(4)3.87, Ile(2)2.06, Leu(4)3.99, Tyr(1)1.02.

Biological Activity

Biological property of the synthetic mastoparan and N^{α}-tyrosylmastoparan was examined in terms of effects on degranulation and histamine release from the rat peritoneal mast cell. The activities were compared with those of the natural mastoparan. Assay for the degranulating activity was carried out under phase contrast microscopic

observation. The results are summarized in Table I. The synthetic mastoparan was found to have the biological properties equivalent to those of the natural one. The activities of N^{α}-tyrosylmastoparan were also almost identical with those of mastoparan in both degranulating and histamine releasing effects.

Development of Radioimmunoassay for Mastoparan

Anti-mastoparan serum (R-3801, final dilution 1:14,000) was generated in a rabbit using synthetic mastoparan-BSA conjugate as immunogen, which had been prepared by the action of glutaraldehyde.

Table I. Relative Potency of Natural and Synthetic Mastoparan.

Compound	Dose	Mast cell degranulation	Histamine release
Triton	0.1%	Accepted as 100	
Compound 48/80	1 ug/ml	+	18.8
Natural mastoparan	0.5 nmol/ml	-	ND
	5.0	+	17.2
	50	+++	72.5
Synthetic mastoparan	0.5 nmol/ml	-	ND
	5.0	+	15.9
	50	+++	72.5

Labelled antigen was prepared by radioiodination of N^{α}-tyrosylmastoparan according to the method by Hunter and Greenwood.[6] The antigen-antibody reaction mixture in 0.01M phosphate buffer (pH 7.4) was incubated at 4° C for 48 hours, when the bound and free antigens were separated by the dextran-coated charcoal method. The radioimmunoas-

say system was able to detect standard synthetic mastoparan added in doses ranging from 80 pg to 1280 pg per tube.

Conclusion

In the present study, a synthetic tetradecapeptide amide having the proposed structure of mastoparan was found to be identical with the natural material in regard to chemical and biological properties examined. Biological activities of N^{α}-tyrosylmastoparan was shown to be almost equipotent to mastoparan itself. A radioimmunoassay system for mastoparan was developed using synthetic mastoparan and N^{α}-tyrosylmastoparan. The system will be useful for search for mastoparan-like immunoreactivity in tissues of various animals.

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STRUCTURE-ACTIVITY STUDIES OF NEUROTENSIN IN CARDIAC AND SMOOTH MUSCLE TISSUES

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Few structure-activity studies oriented toward the identification of the chemical groups responsible for the biological activity of neurotensin (NT) have been published[1,2,3]. These studies provided evidence that the major determinants of biological activity (e.g. hypotension, hyperglycemia, hypothermia, gut contraction) of NT reside primarily in its COOH-terminal sequence. Whether the receptors mediating the various biological effects of NT are all of the same type, is an unanswered question. More information is needed on the structural requirements for the biological activity of NT in various tissues before attempts can be made to differentiate NT receptors.

Recently, we described the powerful cardiostimulant effect of NT in isolated, spontaneously beating auricles of guinea pigs[4]. Further studies were undertaken to identify the chemical groups responsible for the inotropic action of NT. Several NT fragments lacking 1, 2, 3 or more amino acids at their N- or C-terminal end, and analogs in which the Tyr^{11} residue was replaced with Ala, Leu, D-Tyr or D-Phe, were synthesized by the solid-phase method[5] and tested in isolated, spontaneously beating auricles of guinea pigs. The contractile effect of NT fragments and NT derivatives was also assessed in rat isolated stomach strips[4,6]. The apparent affinities of these peptides were calculated from concentration-response curves and are expressed as pD_2 (- log of the molar concentration of agonist producing 50% of the maximum response)[7]. The intrinsic activity (α^E), a measure of the ability of an agonist to activate its receptors[7], was evaluated by dividing the maximum response obtainable with a NT fragment or analog in a given preparation, by that obtained with NT in the same preparation.

Gradual shortening of the NT molecule from the N-terminus produced relatively minor decreases in the affinity (pD_2) of NT for its

receptors and no change in its ability to produce the maximum response (α^E = 1.0, see compounds 2, 3, 4, 5, 6, and 7, Table I). NT (9-13) (compound no. 8) exhibited a more important loss of affinity, specially in the guinea pig auricles, but it was still capable of producing the same maximum response as NT. NT (10-13) (compound no. 9) was completely inactive as agonist or antagonist in the two preparations.

The removal of Leu^{13} (compound no. 10) markedly reduced the affinity (pD_2) of NT for its receptors without modifying its intrinsic activity thus suggesting that this amino acid contributes more to the binding than to the stimulation of NT receptors. The deletion of Ile^{12} and Leu^{13} (compound no. 11) produced much larger decrease in affinity than removing Leu^{13} alone, but again, without producing major reduction in the ability of NT to give the maximum response (α^E = 0.8-1.0). The absence of the 3 C-terminal amino acids, Tyr^{11}, Ile^{12}, and Leu^{13} (compound no. 12), caused complete loss of biological activity. Compound no. 12 was also unable to act as antagonist of NT, suggesting that this NT fragment binds no longer to NT receptors. The absence of effect of NT (1-10) and NT (10-13) was taken as an indication that the sequence 9-11 (Arg^9-Pro^{10}-Tyr^{11}) contains the chemical groups responsible for the ability of NT to stimulate its cardiac and smooth muscle receptors.

Substitution of Tyr^{11} with Ala (compound no. 13), Leu (compound no. 14), D-Tyr (compound no. 15) or D-Phe (compound no. 16) gave products with very low affinity but relatively high intrinsic activity. These results suggest that Tyr^{11} contributes more to the affinity of NT for its receptors than to their stimulation and that it is important to have an aromatic side-chain with proper spatial configuration in position 11 for optimum affinity of NT for its cardiac and smooth muscle receptors. Compounds no. 13, 14, 15, and 16 did not behave as antagonists of NT in the two preparations used.

There are a few differences and similarities between the results of our structure-activity studies and those of others. [D-Tyr^{11}]-NT and [D-Phe^{11}]-NT were reported to be 10 times more potent than NT in the test of hypothermia[2]. However, the same compounds exhibited an affinity 10-100 times lower than NT for specific mast cell binding sites[2]. In our preparations, the affinities of these two compounds were reduced by a factor of 5,000-10,000 compared to NT (Table I). The discrepancies may be due to differences in rate and/or extent of NT degradation and/or due to the existence of different NT receptors in various tissues. The relative affinities (e.g. potencies) of NT fragments presented in Table I correlated relatively well with those described in various assays (e.g. hypotension,

hyperglycemia, vascular permeability, and ileal contractility[1], hypothermia, and mast cell binding[2]). However, Uhl *et al.*[8] reported that NT (4-13), NT (6-13), and NT (8-13) exhibit greater affinity than NT for binding to specific NT receptors in rat brain membranes. We could not confirm this result using rat isolated stomach strips and guinea pig auricles.

Table I. Intrinsic Activities (α^E), Affinities (pD_2) and Relative Affinities (R.A) of Neurotensin (NT), NT Fragments and Analogs as Determined *In Vitro* on Rat Stomach Strips and Guinea Pig Auricles[a].

		1 2 3 4 5 6 7 8 9 10 11 12 13					
		< Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH					
		Rat Stomach Strips			Guinea Pig Auricles		
No	Peptide	α^E	pD_2	R.A (%)	α^E	pD_2	R.A (%)
1	NT	1.0	7.96	100	1.0	8.04	100
2	NT (2-13)	1.0	7.92	90	1.0	8.0	94
3	NT (3-13)	1.0	7.92	90	1.0	7.96	84
4	NT (4-13)	1.0	7.88	85	1.0	8.02	99
5	NT (6-13)	1.0	7.85	78	1.0	7.88	73
6	NT (7-13)	1.0	7.77	65	1.0	7.72	49
7	NT (8-13)	1.0	7.79	69	1.0	7.74	52
8	NT (9-13)	1.0	7.0	11	1.0	6.16	1.3
9	NT (10-13)	0	0	—	0	0	—
10	NT (1-12)	1.0	5.8	0.65	1.0	5.0	0.09
11	NT (1-11)	0.8-1.0	3.4-3.6	<0.01	0.8-1.0	3.3-3.4	<0.01
12	NT (1-10)	0	0	—	0	0	—
13	[Ala^{11}]-NT	0.97	4.07	0.01	>0.4	—	<0.01
14	[Leu^{11}]-NT	0.7	4.65	0.05	0.8-1.0	3.9-4.2	≤0.01
15	[D-Tyr^{11}]-NT	0.76	4.75	0.06	0.7-1.0	3.8-4.2	≤0.01
16	[D-Phe^{11}]-NT	0.63	4.78	0.07	0.7-1.0	3.9-4.3	≤0.01

Our results indicate that the sequence < Glu[1]-Leu[2]-Tyr[3]-Glu[4]-Asn[5]-Lys[6]-Pro[7]-Arg[8] contributes very little to the affinity of NT for its cardiac and smooth muscle receptors in comparison with the sequence H-Arg[9]-Pro[10]-Tyr[11]-Ile[12]-Leu[13]-OH. the chemical groups responsible for the itrinsic activity of NT in these tissues appear to be located in the sequence -Arg[9]-Pro-[10]-Tyr[11]-. Preliminary studies with NT analogs substituted in position 11 reveal the extensive contribution of Tyr[11] to affinity and possibly, to the intrinsic activity of NT.

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THE PRECURSOR-SPECIFIC REGION OF PRE-PROPARATHYROID HORMONE: CHEMICAL SYNTHESIS AND PRELIMINARY STUDIES OF ITS EFFECT ON POST-TRANSLATIONAL MODIFICATION OF HORMONE

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Introduction

Parathyroid hormone (PTH) is biosynthesized as a 115-amino acid precursor termed Pre-ProPTH.[1] The NH_2-terminal 25-amino acids are representative of sequences found in precursor forms of other proteins. These leader or signal regions[2] are similar in length and hydrophobicity, and are thought to promote nascent protein association with rough endoplasmic reticulum and entry into the cisternal space. Transmembrane transport and post- or co-translational modification of Pre-ProPTH may involve microsomal receptors or enzymes. To investigate hormone processing in a cell-free system that contains intracellular membranes, we chemically synthesized the hormone precursor-specific region.

Methods

Synthesis of the Precursor-Specific Peptide — Synthesis was performed by Merrifield solid-phase method.[3] Specialized blocking groups (Figure 1) were used to avoid anticipated side-reactions.[4] Purification was facilitated by use of denaturing conditions and synthetically-incorporated radioactive amino acids near each terminus of the molecule (Figure 1). Homogeneity was assessed by sequence analysis, isoelectric focusing and analysis of tryptic fragments.[4]

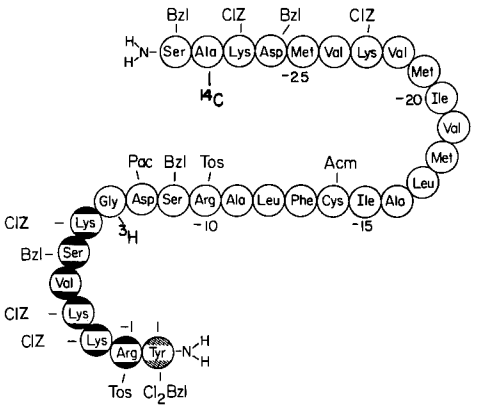

Fig. 1. Sequence of [D-Tyr^{+1}]Pre-ProPTH-(-29—+1)amide showing protecting groups and radioactive amino acids.

Inhibition of Conversion of Pre-ProPTH to Pro-PTH — Purified PTH mRNA[5] was translated in the reticulocyte lysate system[6,7] in the presence of canine pancreatic membranes[8] and [^{35}S]methionine. Pre-Pro peptide was added in concentrations of 50 to 1000 nM. Products were analyzed by SDS discontinuous gel electrophoresis,[9] followed by fluorography[10] and densitometric scanning. Densitometrograms were also computer-analyzed according to best-fit Gaussian distributions.

Results

Peptide addition in concentrations up to 100 nM caused apparent dose-dependent inhibition of Pre-ProPTH conversion to ProPTH (Figure 2). Computer analysis revealed comparable inhibition. Higher concentrations of peptide caused generalized decreases in protein synthesis, precluding analysis of inhibition of conversion.

Discussion

Chemical synthesis and purification of the precursor region of Pre-ProPTH has been achieved, permitting preliminary studies of the nature and structural requirements of the membrane apparatus that processes Pre-ProPTH. Although studies are still in progress, initial findings indicate that addition of low concentrations of precursor peptide to a translation system inhibits conversion of Pre-ProPTH to ProPTH in a

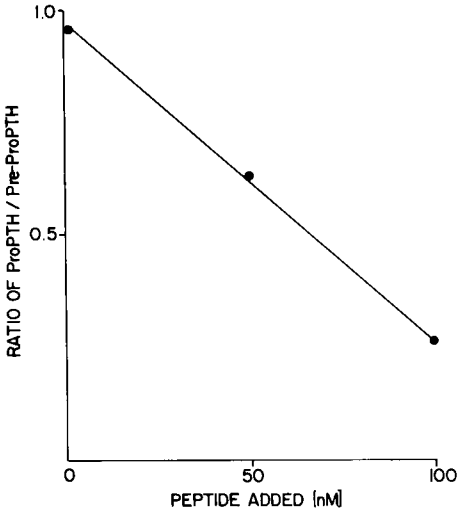

Fig. 2. Ratio of ProPTH to Pre-ProPTH produced in the cell-free translation system as a function of added peptide.

dose-dependent fashion. Higher doses of peptide cause generally diminished protein synthesis. These early studies may indicate that processing involves interaction of precursor forms with microsomal receptors, or that microsomal enzymes can be competitively inhibited by synthetic precursor structures. If such microsomal receptors or enzymes exist, they may serve a universal role in processing. More detailed studies are needed to elucidate the mechanism of Pre-ProPTH attachment to, transport across, and proteolytic cleavage by rough endoplasmic reticulum. The synthetic precursor peptide may facilitate these studies.

Acknowledgements

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SECRETIN ANALOGS MODIFIED IN POSITION 1, 4 OR 6

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It has been suggested that the His residue in the secretin molecule is probably important for pancreatic secretion in dogs, since both secretin (2-27)[1] and [1-Tyr]-secretin[2] showed extremely low activity. [6-Tyr]-secretin was also demonstrated to possess significantly lower activity in dogs[2,3] and in rats[3], when compared with natural or synthetic secretin. On the other hand, [4-D-Ala]-secretin[4,5] retained considerable activity. In order to derive more information with regard to the role of the His, Gly and Phe residues in the N-terminal portion for pancreatic secretory activity of secretin, we prepared [1-Pyr(3)ala]-secretin, [4-D-Trp]-secretin and [6-D-Phe]-secretin. [4-D-Ala]-secretin was also synthesized. The present paper describes the syntheses and biological activities of these analogs.

The syntheses of the secretin analogs were achieved by following our method which had been established for the preparation of secretin and its phenolic group-containing analogs.[2] As acylating agents, *Z*-Pyr(3)ala-Ser-Asp-Gly-Thr-Phe-NHNH-Boc, *Z*-His-Ser-Asp-D-Trp-Thr-Phe-NHNH-Boc and *Z*-His-Ser-Asp-Gly-Thr-D-Phe-NHNH-Boc were synthesized in a stepwise manner. After acylation of secretin (7-27)[2] with azide derived from the respective protected hexapeptide, the crude protected heptacosapeptide amide was hydrogenated and the product was purified by droplet countercurrent distribution followed by gel filtration on Sephadex G-25 or/and LH-20. For the synthesis of [4-D-Ala]-secretin, *Z*-D-Ala-Thr-Phe-Thr-Ser-Glu-Leu-$NHNH_2$ (positions 4-10) and *Z*-His-Ser-Asp-$NHNH_2$ (positions 1-3) were prepared in a stepwise manner. Using azides derived from these protected peptide hydrazides, the peptide chain was successively elongated starting from secretin (11-27)[2]. Deblocking and purification of the final product were performed in the same manner as described above.

Each of the synthetic analogs behaved as a homogeneous component on TLC and in electrofocusing. Acid hydrolysates of the synthetic peptides contained the constituent amino acids in theoretical ratios. The CD spectra of [1-Pyr(3)ala]-secretin, [4-D-Ala]-secretin and [6-D-Phe]-secretin in water were found to be essentially superimposable on that of secretin itself (Figure 1). Although a marked change was not observed, the CD curve of [4-D-Trp]-secretin seems to reflect some influence of the side chain of the Trp in position 4. No significant dependence of the spectra on pH could be observed in the range between pH 5.3 and 8.0.

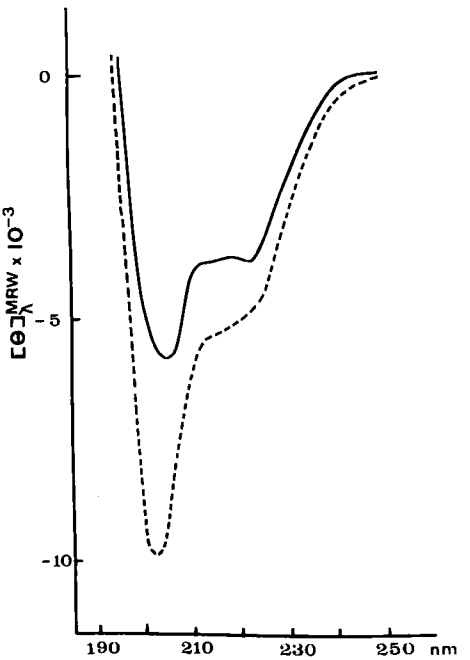

Fig. 1. CD Spectra of secretin and its analogs. (---) Secretin, [1-Pyr(3)ala]-secretin, [4-D-Ala]-secretin and [6-D-Phe]-secretin (—) [4-D-Trp]-secretin. The spectra were taken in water at 26°C with a JASCO Model J-40A automatic recording spectropolarimeter.

Amounts of pancreatic juice secreted in anesthetized dogs after intravenous injection of the synthetic analogs were compared with that observed by administration of pure synthetic secretin (n=5). Relative potencies of the analogs were: [1-Pyr(3)ala]-secretin 88.3 ± 16.8%, [4-D-Ala]-secretin 36.8 ± 1.6%, [4-D-Trp]-secretin 5.3 ± 0.3% and [6-D-Phe]-secretin 19.2 ± 3.5%. The potency of [6-D-Phe]-secretin was significantly

lower in dogs. However, the secretory response produced by this analog in anesthetized Wistar male rats was nearly identical with that of synthetic secretin. The effect on amylase output of this analog as well as [4-D-Trp]-secretin was considerably lower than that of synthetic secretin. The results are shown in Figure 2.

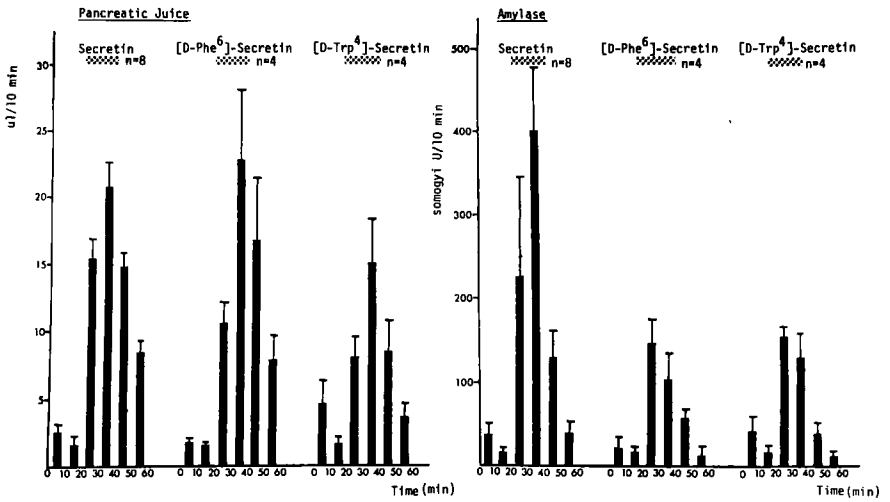

Fig. 2. Effects of secretin and its analogs on pancreatic juice and amylase secretion in Wistar male rats (250-280 g body weight).

Although the N-terminal His residue in secretin was suggested to be important for pancreatic secretory activity, [1-Pyr(3)ala]-secretin showed 88.3% activity. This finding suggests that the ionization behavior of the His residue may not be essential for the function of secretin. In contrast with the relatively high activity of [4-D-Ala]-secretin, replacement of the Gly residue in position 4 with aromatic D-Trp residue resulted in significant decrease of the activity. Possible conformational changes, reflected in the CD spectrum, may be responsible for the reduced biological activity of [4-D-Trp]-secretin.

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF GLYCOSYLATED ANALOGS OF SOMATOSTATIN

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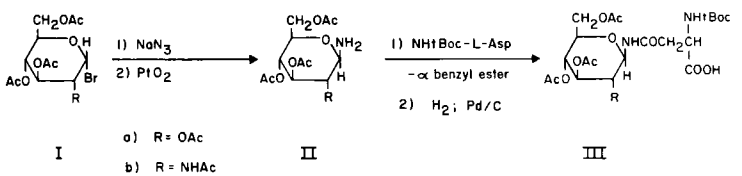
Introduction

The carbohydrate substituents of the glycoprotein hormones have specific functions, one of which is to impart an extended biological half-life to the parent protein. With an overall objective of finding longer-acting analogs of somatostatin (SS) Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH, we have consequently synthesized two glycosylated analogs of somatostatin; [Glc-Asn^5]SS and [NAcGlc-Asn^5]SS, since the Asn^5 residue of SS can be substituted, even deleted with conservation of some biological activity.[1,2]

Synthesis

The synthesis of these glycopeptides involves first the preparation of 2-acetamido-3,4,6-tri-*O*-acetyl and 2,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-*N*(*t*-butyloxycarbonyl)-L-aspartic acid III.[3]



I a) R = OAc b) R = NHAc II III

Using solid phase methodology,[4] these two molecules, IIIa and IIIb, were incorporated in the following somatostatin peptides whose properties are reported in Table I.

Table I. Physical Constants, Amino Acid Analysis and Yields of Glycopeptides

PEPTIDES	YIELD[a]	$[\alpha]_D^{23°}$[b]	HPLC[c]	TLC[d]	
				BAW	BPyA
[Glc-Asn^1]-Phe-Phe--Trp-Lys-OH	36%	-0.5	iso 21% CH_3CN 7.2 min(≥99%)	0.45	0.50
[NAcGlc-Asn^1]Phe--Phe-Trp-Lys-OH	40%	-0.2	iso 21% CH_3CN 6.85 min(≥99%)	0.46	0.52
Asn-Phe-Phe-Trp--Lys-OH	40%	-0.5	iso 21% CN_3CN 9.5 min (≥99%)	0.53	0.55
Ala-Gly-Cys-Lys--[Glc-Asn^5]-Phe-Phe--Trp-Lys-Thr-Phe--Thr-Ser-Cys-OH	16%	-29.3	iso 23.2% CH_3CN 13.9 min (96%) 15.8 min. (4%)	0.31	0.39
Ala-Gly-Cys-Lys--[NAc-Glc-Asn^5]-Phe--Phe-Trp-Lys-Thr--Phe-Thr-Ser-Cys-OH	14%	-22.4	iso 23.2% CH_3CN 11.8 min (≥99%)	0.32	0.38
Somatostatin	17%	-31.9	iso 23.2% CH_3CN 25.7 min(>99%)	0.36	0.39

[a] Yields are calculated on the basis of moles of peptides obtained after final purification relative to the total moles of starting Boc-amino acid on the resin. [b] c = 1 in 1% AcOH. [c] Details on the equipment used have been reported by Rivier and Burgus[5,6]; solvent A : 0.25N TEAP p_H:3.0; solvent B : 50% CH_3CN in solvent A. [d] R_f values were determined on Eastman No. 6061 silica gel sheets: BAW : 1 - butanol - acetic acid - water (4:1:5, upper phase); BPyA : 1 - butanol - pyridine - 0.1% acetic acid (20:12:45, upper phase). Loads varied from 20 to 40 pg per spot.

Biological Activity

In early experiments these compounds show biological activity inhibiting the release of growth hormone, in vitro[7] and in vivo, as well as that of insulin and glucagon by peripheral injection.

Acknowledgements

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PROLONGED SUPPRESSION OF INSULIN AND GH RELEASE BY A SOMATOSTATIN ANALOG

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Somatostatin, the growth hormone-release inhibiting hormone, inhibits the secretion of not only growth hormone (GH) but also insulin[1] and glucagon[2] as well as other hormones[3] in humans. Since both insulin and glucagon are lowered during somatostatin infusion into some insulinoma patients[4,5] the control of hyperinsulinemia and hypoglycemia associated with islet cell tumors has been suggested as a possible use for somatostatin.[6] Moreover, since glucagon suppression is undesirable in patients with hypoglycemia, a somatostatin analog that lowers insulin but not glucagon would be clinically useful.

Previous publications have reported analogs of somatostatin which selectively inhibit growth hormone,[7] growth hormone and glucagon,[8] and growth hormone and insulin for up to 2 hours.[9] The present paper reports on a somatostatin analog, [D-Trp^{5,8}]somatostatin (Wy-41,512), that specifically inhibits GH and insulin secretion in both unstimulated and arginine-stimulated rats, and is active for 6 hours.

Materials and Methods

Somatostatin and [D-Trp^{5,8}]somatostatin were synthesized by the Merrifield solid phase method.[10,11]

Dose response studies were conducted in male rats after stimulation of GH with Nembutal, and insulin and glucagon secretion with arginine. The animals received Nembutal 15 minutes before (sc) administration of Wy-41,512 or saline. Ten minutes after drug administration, arginine was given by intracardiac injection followed five minutes later with collection of blood in Trasylol-EDTA.[9]

The time course studies were performed by the stimulation of GH in rats with Nembutal (50 mg/kg) 20 minutes before blood collection by cardiac puncture.[12] Glucose levels were determined by the glucose-oxidase method using the Technicon autoanalyzer.

Results and Discussion

Modification of somatostatin at positions 5 and 8 with D-Trp to give Wy-41,512 was based on our observation that position 5 substitution can alter the biological specificity of somatostatin.[8,9] The D-Trp[8] modification was selected because it reportedly increases the potency of the corresponding mono-substituted somatostatin in inhibiting GH, insulin and glucagon secretion.[13] By applying these modifications we anticipated the formation of a potent analog of somatostatin having dissociated biological activity.

In assaying the biological specificity of Wy-41,512 we tested its effect on GH, insulin and glucagon secretion. As can be seen in Table I, a dose of 10 μg/kg produced a dramatic lowering of GH and insulin while not effecting glucagon levels at doses as high as 100 μg/kg. A comparison between Wy-41,512 and somatostatin (Table II) shows that Wy-41,512 is a more potent suppressor of insulin activity while having little effect on glucagon activity. Its short term action on GH is similar to that of somatostatin.

Prolonged action is essential for a somatostatin analog to be of clinical utility in treating hypoglycemia. To determine the duration of activity of Wy-41,512, animals were injected (sc) with the analog (1.0 mg/kg) and sacrificed at various time intervals. Plasma GH levels were suppressed for up to 6 hours (Table III); this prolonged GH suppression by Wy-41,512 is in marked contrast to somatostatin, which has GH lowering activity of less than 30 minutes.

Table I. Effects of Wy-41,512 on plasma hormone levels (M $\pm$ SEM) at 15 min in rats

Drug	Dose (µg/kg)	N*	GH (ng/ml)	Insulin (µU/ml)	Glucagon (pg/ml)
Wy-41,512	10	10	40 $\pm$ 6[a]	183 $\pm$ 12[a]	36 $\pm$ 5
Wy-41,512	100	10	25 $\pm$ 5[a]	105 $\pm$ 9[a]	31 $\pm$ 5
Saline	-	10	133 $\pm$ 32	348 $\pm$ 36	44 $\pm$ 8

[a] $p<0.01$; *Number of animals in test group.

Table II. Comparison of minimal effective doses (μg/kg) between somatostatin and Wy-41,512

	Somatostatin	Wy-41,512
Glucagon	50-100	1000
Insulin	100-200	1-10
GH	5-10	1-10

The time course of glucose elevation induced by Wy-41,512 was also studied. Administration of Wy-41,512 (1 mg/kg) elevated plasma glucose significantly at two and four hours (Table IV). Gerich *et al.*[14] reported a similar situation: prolonged hyperglycemia in insulin-withdrawn diabetics infused with glucagon. The unopposed action of glucagon resulted in elevated plasma glucose for the entire time that the insulin to glucagon ratio was lowered.

Table III. Duration of plasma GH suppression with Wy-41,512

Drug	Dose (mg/kg)	N*	Time (hr)	Plasma GH (ng/ml) M $\pm$ SEM
Wy-41,512	1	10	1	5 $\pm$ 2[a]
Saline	-	10	1	61 $\pm$ 14
Wy-41,512	1	10	2	16 $\pm$ 2[a]
Saline	-	9	2	227 $\pm$ 44
Wy-41,512	1	8	5	16 $\pm$ 3[a]
Saline	-	9	5	73 $\pm$ 11
Wy-41,512	1	10	6	22 $\pm$ 2[b]
Saline	-	10	6	156 $\pm$ 42
Wy-41,512	1	9	8	31 $\pm$ 11[ns]
Saline	-	8	8	55 $\pm$ 22

[a] $p<_{0.001+}$ [b] $p<0.01$, ns=not significant
*number of animals in test group

Table IV. Effect of Wy-41,512 on plasma glucose levels

Drug	Dose (mg/kg)	N*	Time (hr).	Glucose (mg/dl) M ± SEM
Wy-41,512	1	8	2	195 ± 21[a]
Saline	-	8	2	105 ± 3
Wy-41,512	1	8	4	143 ± 8[a]
Saline	-	8	4	105 ± 4

[a] $p<0.01$; *number of animals in test group

The increase in potency and the prolonged duration of action of Wy-41,512 over somatostatin may be due to the decreased degradation of the analog *in vivo,* or to the combined effects of increased affinity of the analog for insulin receptors and slow release of the compound after sc injection. This prolonged action and specificity of Wy-41,512 may be useful in controlling the hyperinsulinemia resulting from insulinomas.

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SYNTHETIC ANALOGS OF SERUM THYMIC FACTOR AND THEIR BIOLOGICAL ACTIVITIES

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Introduction

Bach and associates[1,2,3] have proposed the amino acid sequence:

$$\left.\begin{matrix}<\text{Glu} \\ \text{or} \\ \text{H. Gln}\end{matrix}\right\}\text{-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn·OH}$$

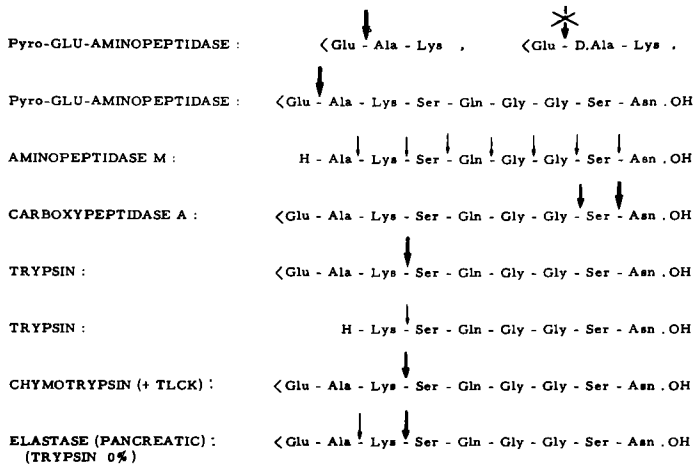
1 2 3 4 5 6 7 8 9

for a circulating serum thymic factor, called "facteur thymique sérique" FTS, isolated from pig serum. At the last American Peptide Symposium, we reported a total solution synthesis of these nonapeptides.[4] Since this communication, two other syntheses of the pyroglutamyl-nonapeptide by the solid phase method have been published.[5,6] Recently we have described[7] the synthesis of a first series of FTS analogs. We now present the biological activities of 23 structural analogs obtained by replacing one amino acid residue of these nonapeptides by enantiomer, homolog or analog compounds.

The study of the *in vitro* action of several proteases and peptidases on the synthetic pyroglutamyl-nonapeptide or shorter peptide fragments revealed the particular sensitivity of some peptide bonds of FTS to proteolysis. The results obtained are summarized in Scheme 1.

Bioassay of FTS and Its Analogs

FTS has been characterized by a bioassay based on its ability to render Θ-positive and azathioprine-sensitive the Θ-negative rosette-forming cells obtained from the spleen of adult thymectomized (ATx) mice. Azathioprine is a purine analog which, like anti-Θ serum, has been



Scheme 1. *In vitro* action of peptidases and proteases on the FTS-PyroGlu-nonapeptide and on shorter fragments.

shown to inhibit the thymus-dependent rosette-forming cells. In *normal* mice, rosette formation is inhibited by 1 μg/ml of azathioprine, whereas in *adult thymectomized* (ATx) animals, 70 μg/ml of this compound are necessary to inhibit rosette formation. FTS, as well as extracts of thymus, confer to the rosette-forming cells of ATx mice, after 90 minutes incubation at 37°, a sensitivity to azathioprine identical with that of normal mouse rosette-forming cells, thus providing the basis of a reproducible and quantitative *in vitro* and *in vivo* bioassay.[8]

In vitro rosette assay: the activity is defined as the lowest concentration of the test sample which induces inhibition of more than 50% of rosette-forming cells from spleen of ATx mice in the presence of 10 μg/ml of azathioprine.

In vivo: a) the activity in *serum* is expressed as the thymic factor level (estimated by rosette assay) in the serum of ATx mice, collected 2 or 4 hours after injection of 0.1 or 1 ng of the test sample *adsorbed to CM-cellulose;*

b) the activity in *spleen cells* is expressed as the sensitivity to azathioprine concentration of these cells from ATx mice, isolated 24 hours after injection of 0.1 or 1 ng of the test sample *adsorbed to CM-cellulose.*

Metabolic studies (retarding activity : R): Kinetics of the level of activity (defined on the basis of the rosette assay) in the serum of ATx mice were determined for analogs after injection of 1 ng of peptide sample

without CM-cellulose or, for comparison, FTS sample adsorbed to CM-cellulose.

The results of these bioassays are presented on Table I and in Figure 1.

Table I. Biological Activities of FTS Analogs: Rosette Formation.

Compounds	in vitro(pg/ml)	in vivo : serum[a]	spleen cells[b]	R[c]
FTS	0.01	1/128 000(0.1ng ; 2hrs)	0.3	
[Z-Gln^1]-FTS	0.03	1/256 000(0.1ng ;2 hrs)	0.3	
[D-Gln^1]-FTS	0.03	1/10^6 (1ng ; 2 hrs)	12	+
[D-<Glu^1]-FTS	0.03	1/512 000(1ng ;4 hrs)	6	+
[Pro^1]-FTS	0.03	1/512 000(1ng ;2 hrs)	12	
[D-Ala^2]-FTS	8	1/2 000 (1ng ; 2 hrs)	12	
[D-Lys^3]-FTS	0.03	1/512 000(0.1ng ; 4hrs)	0.7	+
[Orn^3]-FTS	>500	1/1000 (0.1ng ; 2 hrs)	50	
[Arg^3]-FTS	125	1/16 000(1ng ; 4 hrs)	12	+
[Har^3]-FTS	0.03	1/2.10^6(1ng ; 4 hrs)	0.7	+
[N$^\epsilon$-Ac-Lys^3]-FTS	>1000	1/256 000(0.1ng ; 4 hrs)	1.5	+
[D-Ser^4]-FTS	>500	<1/250 (0.1ng ; 2 hrs)	50	
[Ala^4]-FTS	>500	<1/500 (1 ng ; 2 hrs)	25	
[D-Gln^5]-FTS	>500	<1/500 (1 ng ; 2 hrs)	12	
[Asn^5]-FTS	8	1/500 (1 ng ; 2 hrs)	25	
[Glu^5]-FTS	0.01	1/512 000(1ng ; 2hrs)	3	
[Nva^5]-FTS	>500	1/250 (1ng ; 2 hrs)	12	
[D-Ala^6]-FTS	>500	1/128 000(0.1ng ; 4 hrs)	6	+
[Ala^8]-FTS	500	1/8 000 (1ng ; 2 hrs)	25	
[D-Asn^9]-FTS	>500	1/128 000(0.1ng ; 4 hrs)	6	+
[Asn-NH_2^9]-FTS	>500	1/64 000(1ng ; 4 hrs)	3	+
[Gln^9]-FTS	>500	<1/1 000(1ng ; 2 hrs)	50	
[Asp^9]-FTS	>500	1/2 000 (1ng ; 2 hrs)	25	
[β-Ala-NH_2^9]-FTS	500	1/500 (0.1ng ; 2 hrs)	25	

[a] max. active dilution (dose : time); [b] μg/ml of azathioprine; [c] retarding activity

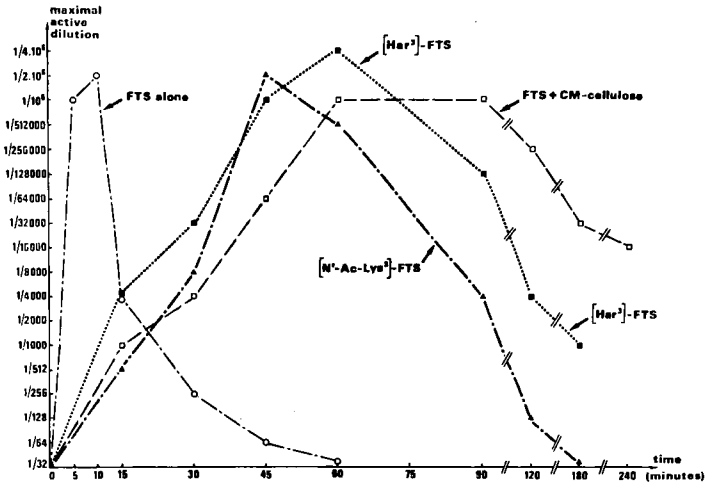


Fig. 1. Kinetics of thymic factor level in the serum of ATx mice after injection of FTS (with and without CM-cellulose) and two peptide analogs (without CM-cell).

Conclusion

The study of the biological activities of synthetic analogs, as well as those of shorter-chain fragments,[9] reveals that defined structural features are necessary for the expression of the activity. Moreover, several analogs, more resistant than FTS to proteolysis, present a retarded effect.

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REACTIVITY OF THE IMIDAZOLE SIDE CHAIN OF TRH AND RELATED COMPOUNDS

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Introduction

Conformational studies on thyrotropin releasing hormone (TRH) have been done using electrometric titration,[1] nuclear magnetic resonance spectroscopy (NMR)[2-4] and theoretical calculations.[5-7] The conformation of the histidyl residue has received special interest because methylation of the imidazole ring leads to an analog with an activity significantly greater than that of the parent compound.[8]

Grant et al.[1] explain the low pK of the imidazole ring of TRH on the basis of an intramolecular hydrogen bond between the imidazole π-nitrogen and the pyroglutamyl-histidyl peptide bond amide hydrogen. This hydrogen bond, however, it not supported by NMR[2-4] studies of TRH dissolved in water, dimethylsulfoxide or methanol. The empirical conformation calculations of Burgess et al.[5] are consistent with the NMR results. More recent studies by Flurry et al.,[7] using the CNDO/2 molecular orbital method, give evidence favorable to the existence of the proposed hydrogen bond when the TRH molecule is considered in a hydrophobic environment.

In order to obtain more information about the conformational state of the imidazole ring in TRH we have studied the interaction of protons (by electrometric titration) and of *p*-nitrophenyl acetate (NPA) with TRH, [2-N^{π}-methylhistidine]-TRH (πMeHis-TRH) and [2-N^{τ}-methylhistidine]-TRH (τMeHis-TRH). For comparison we have also studied the amino acids: Histidine, N^{π}-methylhistidine (π-MeHis) and N^{τ}-methylhistidine (τMeHis).

Methods

Electrometric titrations were done in 0.15M KCl solution as previously described,[9] in the pH interval of 2.5 to 11, and at five

temperatures in the range 10°-40°C. ΔH and ΔS values were obtained from the best fit of the data to van't Hoff's equation by the method of least squares.

The second order rate constant (k_2) of the reaction of *p*-nitrophenyl acetate (NPA) with TRH, histidine or their methylated derivatives were obtained as previously described,[10] at 25°C in the pH range 6-8 with 0.1M phosphate buffer and ionic strength adjusted to 0.2 with KCl.

TRH was synthesized without amino group protection, using only the azide coupling method. The azide of pyroglutamic acid was made to react with histidine methyl ester and the azide of the dipeptide thus obtained reacted with Pro-NH_2 giving TRH. The yields obtained in each coupling were between 40% and 60%. The methylated imidazole analogs of TRH were synthesized by the solid phase method.[11]

Results and Discussion

The imidazole pK values reported in Table I are in good agreement with previously reported data.[1,12] These results show that πMeHis-TRH and πMeHis are more basic than TRH and histidine, respectively, as expected from the inductive effect of the methyl group. The same inductive effect is not seen with τ-methylated derivatives of TRH and histidine and in these cases solvent effects must be considered. Hepler's theory[13] gives a semi-quantitative idea of solvent effects, expressed in the following equation:

$$\Delta H = \Delta H_{int} + \Delta H_{ext} = \Delta H_{int} + \beta \Delta S$$

According to this theory, ΔH_{int} arises from the enthalpy differences between the protonated and the unprotonated forms of the molecules, including the inductive effect of the methyl group, while ΔH_{ext} is associated with differences in solute-solvent interaction. Using the value of 250 for β, as previously discussed,[11] we obtained ΔH_{int} values of -308 cal/mol for πMeHis-TRH and -642 cal/mol for πMeHis, which agrees with the inductive effect of the methyl group. On the other hand, methylation at the τ position, both in TRH and in histidine, lowered the basicity, which was due mostly to ΔH_{ext} (2,152 cal/mol for τMeHis-TRH and 615 for πMeHis), since ΔH_{int} values for these two τ-methylated derivatives are positive (684 cal/mol for τMeHis-TRH and 325 cal/mol for τMeHis). These results suggest a hindrance to the interaction of the π-

nitrogen with the solvent in the τ methylated derivatives, which is more evident in τMeHis-TRH.

Table I. Ionization Parameters for TRH, Histidine and their N^{π}- and N^{τ}- Methylated Derivatives in 0.15M KCl at 25°C

	Imidazole			Amino		
Compound	pK	ΔH(kcal/mol)	ΔS(cal/^{o}mol)	pK	ΔH(kcal/mol)	ΔS(cal/^{o}mol)
TRH	6.40	7.6±0.2	- 4.0±0.5			
πMeHis-TRH	6.69	7.4±0.2	- 5.8±0.7			
τMeHis-TRH	6.23	4.8±0.2	-12.6±0.6			
His	6.14	6.8±0.2	- 5.4±0.7	9.20	10.3±0.1	-7.7±0.2
πMeHis	6.61	7.4±0.2	- 5.3±0.5	8.73	9.8±0.1	-7.2±0.4
τMeHis	5.99	5.8±0.1	- 7.8±0.3	9.27	11.1±0.2	-5.2±0.2

Table II. Second Order Rate Constants for the Reaction of NPA with the Imidazole Group of TRH, Histidine and their N^{π}- and N^{τ}- Methylated Derivatives

	$k_2(M^{-1}min^{-1})$	
Compound	Observed	Calculated[a]
TRH	7.1±0.3	3.6-10.0
πMeHis-TRH	16.0±0.4	5.5-15.4
τMeHis-TRH	0.1±0.1	2.8- 7.7
His	2.3±0.3	2.4- 6.8
πMeHis	6.9±0.2	4.9-13.6
τMeHis	0.1±0.1	2.0- 5.4

[a] The ranges represent the 95% fiducial limits calculated from the Brönsted relationship: $\log k_2 = 0.646\ pK - 3.358$, and its standard error, obtained with simple imidazole compounds.

The second order rate constants for the reaction of the imidazole groups in TRH, histidine and their π-methylated derivatives with NPA (Table II) were within the range predicted by a Brönsted relationship obtained with simple imidazole derivatives. However, the imidazole groups in τMeHis-TRH and τMeHis were unreactive towards NPA.

These results confirm those obtained by electrometric titration, suggesting that the τ-nitrogen of the imidazole rings is freely exposed to the solvent, while the π-nitrogen is hindered and with difficult access to solvent. The parallelism between the results with TRH and histidine and their respective methylated derivatives indicates that the imidazole rings of TRH and of histidine are in similar environments, agreeing with the observations of Flurry *et al,*[7] who obtained similar rotational angles in TRH and in its fragments.

Our results do not reject the $N^{\alpha} \rightarrow N^{\pi}$ hydrogen bond proposed by Grant et al.,[1] particularly in τMeHis-TRH and in τMeHis, where hindrance to solvation of the π-nitrogen would stabilize this hydrogen bond. The lower pK value of the amino group of πMeHis in comparison with that of histidine and of τMeHis and the higher ΔS value in τMeHis are also consistent with the presence of the $N^{\alpha} \rightarrow N^{\pi}$ hydrogen bond, at least in τMeHis, although the methylation of the ring's π-nitrogen may produce some hindrance to solvation of the α-amino group.

Acknowledgement

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SYNTHETIC ANALOGS OF THE TRIOSTIN DEPSIPEPTIDE ANTIBIOTICS

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There is considerable interest[1] in the mode of binding of the triostin and quinomycin depsipeptide antibiotics to DNA. The antibiotics have been shown to bind by a mechanism involving bifunctional intercalation[1b,c] of the two quinoxaline rings attached to the depsipeptide moiety. Solution nmr conformational studies have been reported[2] for triostin A *(1)* and, in more detail, for the quinomycin antibiotic, echinomycin.[3]

Preparation of analogs of the triostin antibiotics is of interest in order to further probe the structural requirements for binding of the antibiotics to DNA. We have previously reported the synthesis of triostin A *(1)*[4] and of an analog des-*N*-tetramethyltriostin A *(2)*[5]. We report in this paper the preparation of two additional analogs, namely, [L-Ser^1]-des-*N*-tetramethyltriostin A *(3)* and [Ala^3,Ala^7]-des-*N*-tetramethyltriostin A *(4)*. Analog *3* has one of the D-serine units replaced with L-serine. The other analog *4* lacks a disulfide bond due to replacement of cysteine with alanine. Both analogs lack *N*-methylamino residues common to the natural triostins.

The synthesis of [L-Ser^1]-des-*N*-tetramethyltriostin A *(3)* was accomplished as shown in Figure 2. Coupling of the known[5] tetradepsipeptides *5* and *6* via the mixed anhydride gave octadepsipeptide *7* in 80% yield. Sequential deprotection of *7* by treatment with zinc in acetic acid and trifluoroacetic acid gave a deprotected octadepsipeptide, which was cyclized with 1-hydroxybenztriazole and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in dimethylformamide-tetrahydrofuran to furnish cyclic octadepsipeptide *8* in 30% yield from *7*. Treatment of *8* with iodine in methanol effected conversion (92%) to disulfide *9*. Removal of the benzyloxycarbonyl group with HBr in acetic acid, followed by acylation with 2-quinoxalinecarbonyl chloride in dimethylformamide gave (43%) analog *3*.

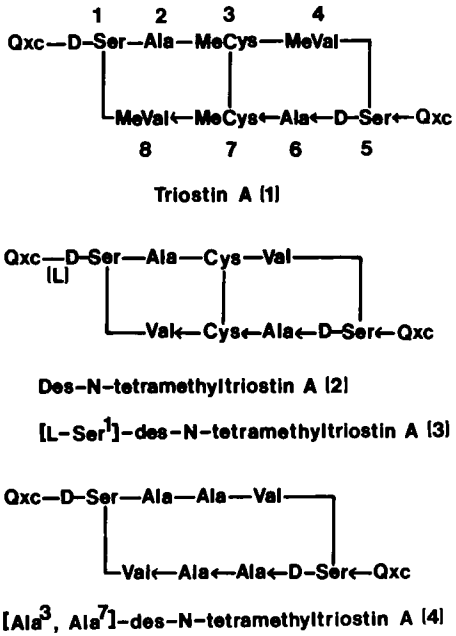

Fig. 1. Structural formulae of triostin A and analogs.

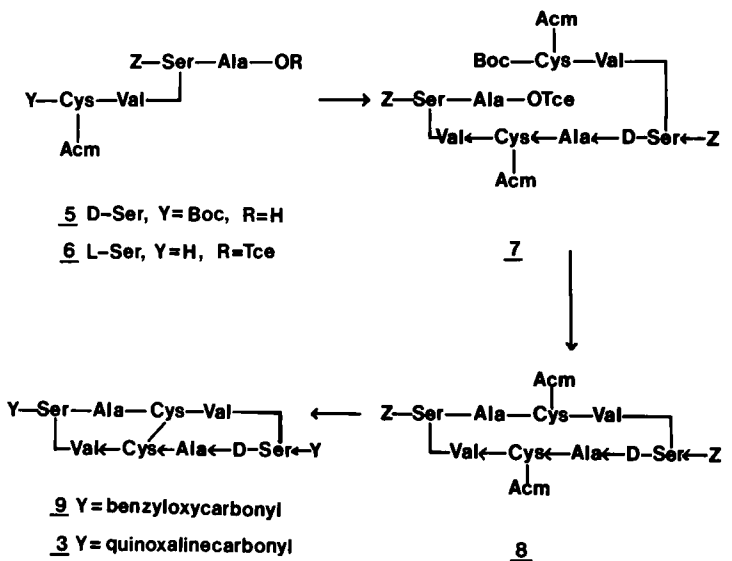

Fig. 2. Scheme for synthesis of [L-Ser[1]]-des-N-tetramethyltriostin A (3)

The preparation of analog *4* followed an analogous procedure as used for *3* (Figure 3). Thus, fragment coupling of depsitetrapeptides *11* and *12,* each prepared by appropriate deblocking of *10,* provided the linear octadepsipeptide *13* in 89% yield. Sequential deprotection of *13* (zinc in acetic acid, then trifluoroacetic acid) followed by cyclization *(N*-hydroxysuccinimide: (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) gave *14* in a yield of 34 percent. Removal of the Z group (HBr in acetic acid) in *14* and acylation with 2-quinoxalinecarbonyl chloride completed the synthesis of analog *4.*

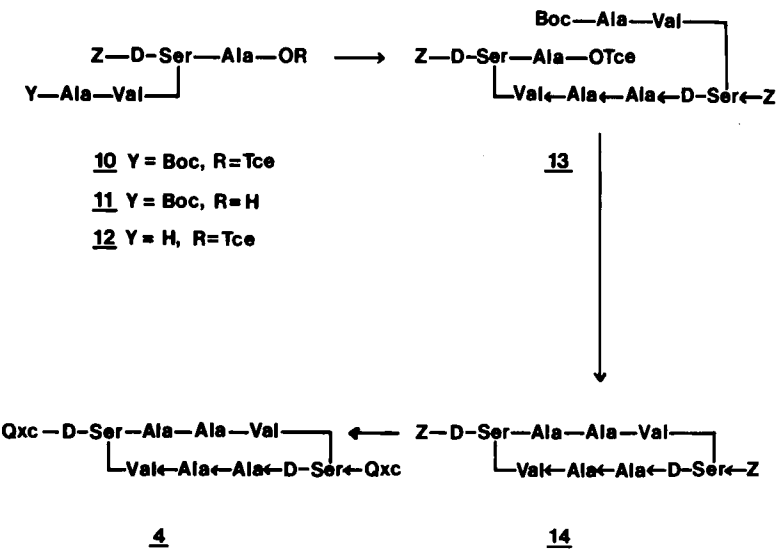

Fig. 3. Scheme for synthesis of [Ala^3,Ala^7]-des-N-tetramethyltriostin A (4)

Analogs *3* and *4* were submitted to Professor M. J. Waring, University of Cambridge, for evaluation of their binding to DNA. [L-Ser^1]-des-N-tetramethyltriostin A *(3)* showed very weak or negligible binding to DNA. In contrast, [Ala^3,Ala^7]-des-*N*-tetramethyltriostin A showed weak but measurable binding to DNA, with maximum binding observed with poly(dA-dT). Similar to the natural triostins, analog *4* was shown to bind as a bifunctional intercalating agent. These studies establish that an intact disulfide bridge is not necessary for binding to DNA, though a weaker interaction does result than that observed for the natural triostins.

Acknowledgements

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EPOXYPEPTIDES — A NEW GROUP OF INHIBITORS OF CELL WALL BIOSYNTHESIS IN SOME FUNGI

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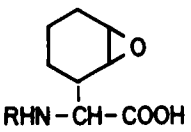
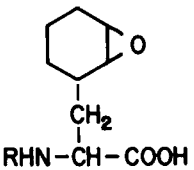
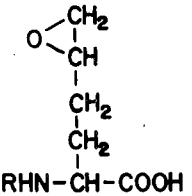
In our studies on the synthesis of analogs of the antibiotic tetaine, *S*-alanyl-(2R,3R-epoxycyclohexanone-4)-*S*-alanine[1,2], for the examination of structure-activity relationships in the group of epoxypeptides, we have synthesized the following compounds:



I R = H-Ala



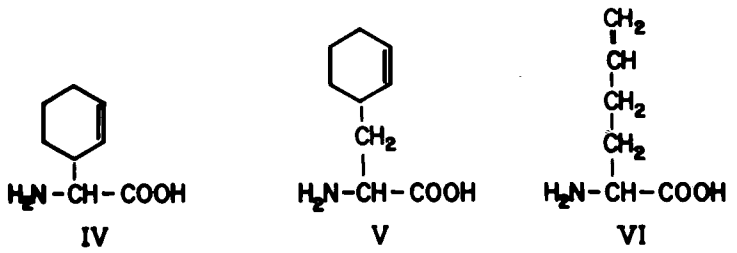
IIa R = H
IIb R = H-Ala
IIc R = Ac
IId R = Ac-Ala



III R = H-Ala

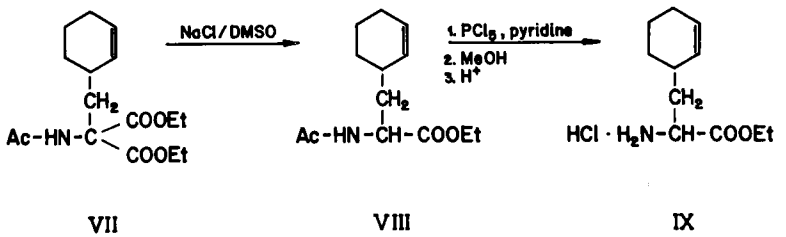
S-alanyl-2,3-epoxycyclohexylglycine (I), *S*-alanyl-β-(2,3-epoxycyclohexyl)-alanine (IIb) and *S*-alanyl-2-amino-5,6-epoxyhexenoic acid (III), the analogs of tetaine containing in the molecule modified epoxy amino acid residue, and also *N*-acetyl-β-(2,3-epoxycyclohexyl)-alanine (IIc), *N*-acetyl-S-alanyl-β-(2,3-epoxycyclohexyl)-alanine (IId) and *N*-acetyl-β-(2,3-epoxycyclohexyl)-alanyl-S-alanine, modified compounds with β-(2,3-epoxycyclohexyl)-alanine residue (IIa).

The substrates in the syntheses were unsaturated amino acids 2-cyclohexenyl-glycine (IV)[3], β-(2-cyclohexenyl)-alanine (V) and 2-amino-5-hexenoic acid (VI).[4] Compound V was obtained as described in the literature for compounds IV and VI, by malonate synthesis. The ester of the N-acetyl derivative of this amino acid (VIII) was obtained directly

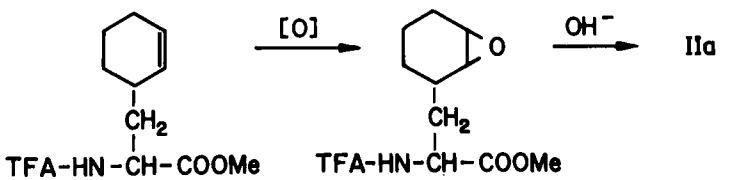


IV V VI

from the condensation product VII by decarboethoxylation with NaCl in DMSO at elevated temperature.[5] The removal of amino protection group in compound VIII, by transforming this compound to iminoether upon the action of PCl_5 and MeOH, followed by its hydrolysis,[6] gave the ester of amino acid (IX).



VII VIII IX



X XI

In the synthesis of alanyl-β-(2,3-epoxycyclohexyl)-alanine the ethyl ester of trifluoroacetylalanyl-β-(2,3-epoxycyclohexyl)-alanine (X), obtained by mixed anhydrides method,[7] was submitted to the epoxydation reaction with hydrogen peroxide in benzonitrile,[8] followed by the removal of protecting groups by alkaline hydrolysis. In a similar way compounds I, IIa, and III were obtained, with the exception that the epoxidation in the synthesis of compound III was carried out with *m*-chloroperbenzoic acid,[9] and in the synthesis of compounds IIc and IId, the corresponding *N*-acetyl-esters were epoxidized, followed by the removal of the carboxyl protection group by alkaline hydrolysis.

N-Acetyl-β-(2,3-epoxycyclohexyl)-alanyl-*S*-alanine was obtained in another procedure. S-alanine methyl ester was acylated with *N*-acetyl-β-(2,3-epoxycyclohexyl)-alanine by DCCI method, followed by removal of the carboxyl group protection by alkaline hydrolysis. Thus it has been demonstrated that it is possible to use epoxyamino acids in the elongation of peptide chains from the C-terminus.

Table I. Inhibitory Activity of Synthesized Compounds Against *Candida albicans*

Compound	Inhibition of Candida alb. growth after 180 min. at given concentration (% inhib.)	Concentration causing 50% inhibition of aminotransphe-rase reaction in cell-free extract of Candida alb.
tetaine	66 (0.02 mM)	0.002-0.005 mM
H-Ala-Ecg-OH	no effect	no effect
H-Ala-Eca-OH	72 (1 mM)	0.1 mM
H-Ala-Aeh-OH	80 (1 mM)	0.5 mM
H-Eca-OH	no effect	0.3-0.6 mM
Ac-Eca-ONa	no effect	0.45 mM
Ac-Ala-Eca-ONa	95 (0.3 mM)	0.08-0.1 mM
Ac-Eca-Ala-ONa	no effect	no effect

Ecg: 2,3-epoxycyclohexyl-glycine residue; Eca: β-(2,3-epoxycyclohexyl)-alanine residue; Aeh: 2-amino-5,6-epoxyhexenoic acid residue.

In all syntheses S-alanine and the mixture of stereoisomers of unsaturated amino acids were used. The separation of stereoisomers by column chromatography on silica gel was done only for the methyl ester of *N*-trifluoroacetylalanyl-2-amino-5-hexenoic acid. In the latter case the S-S isomer was submitted to the epoxidation.

The structures of the epoxy compounds were confirmed by ^{1}H NMR and argentometric titration.[10]

Biological properties of synthetic compounds are presented in Table I.

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CYCLIC ANALOGS OF LINEAR PEPTIDES

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Application of system analysis in the study of low-molecular weight peptides and results obtained by total semi-empirical conformational analysis have led us to the discovery of new universal principles of structural and functional organization of a number of peptide bioregulators. These approaches make possible the synthesis of analogs possessing high selectivity and more prolonged action than the natural compounds. These studies are based on the fundamental concept that biological functions of peptides and proteins are determined not so much by their chemical structure as such, but rather by the presence of a certain set of properties of the "active" sites in these structures. We use the term "signature" for such a set, and, in accordance with the principle of equivocation in general situations, it can be common to compounds of differing chemical structure[1]. In particular, our work has resulted in the discovery of so-called "common fragments" in peptides different in origin as well as in biological functions. These "common fragments" carry out similar functions in certain situations, in good agreement with the signature and equivocation principles. Common fragments contain a basic amino acid (B) situated between proline or valine residues on one side, and an amino acid with a free carboxyl group (A) or glycine on the other: ↔ Gly/A ↔ B↔ Pro/Val ↔ (↔ means "right" and "retro" direction of peptide chain). It may be noted that in certain peptides, particularly in toxins, fragments of a different type can be found, containing a doubled nucleus of common fragment: ↔B↔Pro/Val↔B↔Pro/Val↔[1].

It was shown experimentally that: (1) there exists a structural organization for certain peptides with "common fragments" adjacent in the molecule to the "active center" with minimum specificity. (2) common fragments are of important functional significance; their elimination or addition changes the specific biological activity of peptides by 3 to 5 orders of magnitude; (3) common fragments possess a wide range of

biological activities not specific, however, with respect to the parent peptide; (4) they potentiate specific effects of parent peptides and sensitize their cell receptors. One can thus distinguish in the structure of a considerable number of peptides two adjacent functionally active sites — a specific "distinguishing" one providing for specificity of ligand-receptor interaction, and a non-specific "common" one enhancing by several orders of magnitude the activity of the specific site[1].

The total semi-empirical conformational analysis, performed in vacuo for peptides, such as bradykinin, angiotensin, bradykinin potentiating factor, tuftsin, and others[2,3] showed that quasi-cyclic space structures are the most stable ones, possessing minimum energy. Such structures are formed as a result of intramolecular interaction of most amino acid residues in the molecule and in many cases are stabilized by means of ionic-type bonds between basic amino acids of the "common fragment" and an acidic (usually the C-terminal) functional group of the peptide chain. The fact that the structures of a number of peptides and proteins include cysteine containing analogs of common fragments, in which the functions otherwise attributed to basic amino acids are carried out by cysteine residues — also speaks in favor of the assumption that amino acids contained in common fragments take part in the formation of spatial structure[1]. Indeed, in some serine proteases salt and disulfide bridges are equifunctional (cf., for instance, the bonds in trypsin, Cys^{22}-Cys^{157}, and α-chymotrypsin, $Glu^{21}...Arg^{154}$.

ESR and fluorescence measurement of bradykinin analogs provided first indications for the existence of quasi-cyclic structures of linear peptides in aqueous solution[3]. On the other hand, an analysis of NMR data on bradykinin showed no evidence for the existence of a tight ionic bond between the Arg (position 1) guanido group and the Arg (position 9) carboxylate anion[4]. Our own ^{13}C-NMR studies on tuftsin — determination of pH-dependences and of spin-lattice relaxation times — do also not reveal the existence of ionic-type bonds betwen lysine and C-terminal carboxyl in aqueous medium. For such structures, limited mobility of the side-chain of lysine ought to be expected, and the NT_1 value for C^{ϵ} of lysine ought to be comparable to that of arginine C^{α}. This is however, not the case at pH = 5,7 (NT_1 = 1.8 sec. for lysine C^{ϵ} and 0.34 sec. for arginine C^{α}). It appears that formation of salt type bridges is prevented by the hydration shell of ionogenic groups. PMR spectra of tuftsin in less polar solvents (e.g. dimethylsulfoxide) are in favor of a folded structure and shielding of an arginine amide proton from the solvent. This structure is destroyed by dilution of dimethylsulfoxide with water or by

acylating the lysine ϵ-amino group. It is, thus, not unlikely that peptide quasi-cyclic structures are formed directly in the non-polar receptor biophase after dehydration of ionogenic groups. It stands to reason that the formation of compact quasi-cyclic structures (stabilized by ionogenic group interaction) in the course of binding with lipophilic receptors is one of the universal principles governing the functioning of natural peptide bioregulators[1]. This assumption explains, in particular, one of the possibilities for the dramatic decrease in specific peptide activity upon removal of dipeptide Arg/Lys-Pro/Val-containing fragments[1]: the molecule loses the elements, which stabilize the characteristic space structure of the "active center" resulting in a large drop in affinity.

A further logical step in the development of our proposed concept is the following assertion. We hold that limitation of conformational mobility of the peptide ligand molecule, accompanied by further stabilization of the so-called "biologically active" conformation of the molecule must entail an increase in ligand-receptor interaction efficiency. For experimental testing we synthesized cyclic analogs of linear peptides applying classical methods of peptide chemistry. We took care to introduce into the chemical structure of the molecules covalent bonds in such a way, as to fix the quasi-cyclic conformations predicted from conformational calculations[2]. Biological tests with the compounds obtained were carried out at the Institute of Organic Synthesis under the direction of Dr. V. Klusha.

Cyclobradykinin and Cyclokallidins. Cyclobradykinin (compound IIa), and cyclokallidin (IV) were obtained from their biologically inactive linear precursors (I, III), N-arginyl-cyclobradykinin (an isomer of cyclokallidin, IIb) — by acylation of cyclobradykinin. The CD-spectra of the cyclokinins IIa, b in aqueous solution do not differ from the bradykinin spectrum. Both cyclobradykinins (IIa, b) exhibit high and considerably prolonged depressor activity (rat blood pressure), complete absence of myotropic activity (rat uterus and colon ascendens *in vitro*), and weak effect of vascular permeability. Cyclokinins IIa, b produced depressor reaction equal to that of bradykinin in 25-50 μg/kg doses, the bradykinin effect lasting for 50 seconds, while that of cyclokinins persists for several hours with partial (up to 50%) restitution of blood pressure during this period. Contrary to cyclobradykinin II, cyclokallidin IV exerts the full myotropic activity ($\alpha = 1$) of bradykinin on the rat uterus ($pD_2 = 7.08 \pm 0.35$). Cyclobradykinin with significantly enlarged size of the cyclic structure (V) shows 20% of the bradykinin depressor activity when tested on rat blood pressure *in vivo*.

Table I. Cyclic Analogs of Kinins and Their Precursors

I Phe-Gly-Pro-Phe-Arg-[R'-Lys-Pro-Pro-Gly a) R=NO_2, R'=Z b) R=R'=H

II R-NH-CH-CO-Pro-Pro-Gly-Phe-Gly-Pro-Phe-Arg-NH-$(CH_2)_4$ a) R=H b) R=Arg

III Phe-Ser-Pro-Phe-Arg-[R'-Lys-Arg-Pro-Pro-Gly a) R' =Bzl, R* =NO_2, R''=Z
b) R' =R* =R''=H

IV NH_2-CH-CO-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-NH-$(CH_2)_4$

V [$(CH_2)_{11}$-CO-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-NH

VI Thr-NH-CH-CO-Pro-Arg-NH-$(CH_2)_4$ VII Thr-NH-CH-CO-Pro-Arg-Gly-NH-$(CH_2)_3$

VIII Lys-Val-Tyr-Val-His-Pro-Phe IX NH_2-CH-CO-Val-Tyr-Val-His-Pro-Phe-NH-$(CH_2)_4$

X NH_2-CH-CO-Val-Tyr-Val-His-Pro-Phe-Gly-NH-$(CH_2)_3$

Cyclotuftsin exhibits the phagocytosis-stimulating activity of the parent peptide (experiments with polymorphnonuclear leucocytes and with Staphylococcus aureus). The CD-spectrum of cyclotuftsin in water coincides with that of the parent compound. An increase in the size of the cyclic structure (VII) lowers phagocytic stimulation drastically.

Cycloangiotensins. The cyclopeptide IX obtained from its linear precursor — (1-des-aspartic acid, 2-lysine, 5-valine)-angiotensin (VIII) does not exhibit the pressor effect characteristic of the natural hormone on the urethan-anesthetized rat uterus *in vitro*. It shows, however, a myotropic effect as compared to angiotensin (α = 1, $pD_2 = 9.7 \pm 0.32$), in concentrations of 10^{-10}-10^{-5}M ($\alpha = 0.6 \pm 0.14$, $pD_2 = 6.87 \pm 0.83$). It is noteworthy that the cyclopeptide IX binds specifically and completely to antibodies obtained against (1-asparagine, 5-valine)-angiotensin (the binding curves of angiotensin and IX are parallel). Compound X shows pressor effects in dog (40 μg/kg; blood pressure to 20 mm), but is inactive in rats. We may thus conclude that our contention about quasi-cyclization of linear peptides in the course of their interaction with receptors has been confirmed experimentally.

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MIXED ANHYDRIDES AS REAGENTS IN SEMISYNTHESIS

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Introduction

Mixed anhydrides of carbonic and carboxylic acids readily acylate amino acids and peptides both in organic and aqueous-organic solutions.[1-3] Acylations in organic solution have been extensively studied for peptide synthesis, primarily because of high speed, yield, purity and lack of racemization.[4] The usefulness of the repetitive excess mixed anhydride method (REMA-method) has been shown by the synthesis of several pure peptides without the purification of intermediates.[5-7] Mixed anhydride synthesis in aqueous-organic solution also proceeds in high yield, but this technique has not been applied to peptide synthesis. Acylation procedures which can be used in aqueous-organic solutions will find application in semisynthesis because native polypeptides and proteins are usually soluble in such solvents. As part of our search for efficient methods applicable to our semisynthetic projects, we have investigated the potential of the mixed anhydrides as reagents in semisynthesis.

We have extended the REMA-method[5-7] to semisynthesis. Excess mixed anhydrides cause quantitative N-acylation. A minor product, a permanently blocked urethane derivative, is formed some times but this remains inert to the further acylation and deblocking steps. Thus coupling can be carried out without the necessity of purifying the intermediates. The final difference in molecular weight or net charge of the desired product from the urethane blocked sequence facilitates its purification.

Since little was known about mixed anhydride acylations in aqueous-organic solutions, a systematic study was undertaken to develop such procedures for insulin-related semisynthesis. Optimum conditions were investigated using the reaction of the mixed anhydride of Boc-Met

with insulin A-chain. Methionine was chosen as it does not occur in the native sequence and readily allowed estimation of the extent of acylation by amino acid analysis. After the conditions were standardized, bigger protected peptides were attached to the A-chain. We also attempted mixed anhydride acylations with insulin and proinsulin which differ from A-chain not only in size and charge but also in conformational and aggregation properties and in the different pKs of their groups. This would be expected to cause differences in the acylation pattern. We have observed these influences on the acylation patterns which are briefly described.

Methods

Mixed anhydrides were prepared in dimethylformamide in the usual manner at -10°C. *N*-Methylmorpholine and isobutyl chloroformate, which give high yield and no racemization, were selected to generate mixed anhydrides. Activation times were between 30 to 60 seconds. Acylations were performed in solvents such as dioxane, DMF and DMSO (the most versatile) mixed with water. Based on the results of our investigation, standard mixed anhydride syntheses were carried out for 1 minute at room temperature (18 - 25°C) as follows: 5 equivalents of the reagent was reacted with A-chain at pH 4-8 in 80% DMSO-water and 80% DMF-water concentrations. With insulin and proinsulin, 2.5 to 10 equivalents of the reagent were added in the pH range 5-8.5. The reaction was stopped by adding 1 M hydroxylamine solution pH 7.5. The reaction mixture was in general passed over Sephadex G-25 or dialyzed and then lyophylized. In the cases of insulin and proinsulin, the resolution of the mixtures was accomplished by ion-exchange chromatography before and after removal of the protecting groups. End group determination, electrophoresis of both blocked and deblocked products, and amino acid analysis were used to check the purity of the products.

Results and Discussion

Mixed anhydride reaction was initially performed in 80% DMF-water. Only subsequently 80% DMSO-water was used as it was found to be a versatile solvent. Therefore, some of the standardization experiments were done in the DMF-system. The pH-optimum for N-acylation in 80% DMF-water was 6.5. Below pH 5.5 very little N-acylation was observed although considerable urethane formation took

place, as judged from electrophoresis of the blocked and deblocked products and amino acid analysis. Little or no urethane formation was observed between pH 6.5 - 7.5, although it varies from amino acid to amino acid and peptide to peptide. For this reason we adopted pH 7 - 7.5 as the standard pH for reaction. These are the experimentally determined relative pH values because of the high organic solvent concentration. Temperature dependence studies indicated that there was little difference between the reaction done at 0°C and that at room temperature. We selected room temperature for several reasons. Mixed anhydride reactions proceeded very quickly. With 2 equivalents of reagent reaction was quantitative at pH 7.5. The time dependence studies in 80% DMSO-water using 5 equivalents of the reagent indicated the reaction to be over in less than 30 seconds. Significant O-acylation occurs only when high reagent excesses were used (20-fold). Longer reaction times (20 minutes) and lower water content also favoured this reaction. Based on these results the standard procedure involved acylation for 1 minute only. After this time hydroxylamine, a strong nucleophile, was added to inactivate the excess reagent.

For the semisynthesis of human proinsulin sequences, we used procine A-chain S-sulfonate (66-86 corresponding to human proinsulin), which is identical to human A-chain. Protected fragments of the C-peptide Boc-Val-Glu(OBu^t)-Leu-Gly-OH (42-45), Trt-Gly-Gly-Pro-Gly-Ala-Gly-Ser(Bu^t)-Leu-Gln-Pro-Leu-Ala-Leu-Glu(OBu^t)-OH (46-59), Trt-Gly-Ser(Bu^t)-Leu-Gln-OH (60-63) and Boc-Lys(Msc)-OH (64) were attached successively by the mixed anhydride method to Arg-A-chain S-tetra sulfonate (Figure 1). The protecting groups were removed by trifluoroacetic acid treatment. The fragment 42-86, obtained as mentioned above, was then extended with Boc-Val-Gly-Gln-OH (39-41). In this case end group determination showed traces of valine and the coupling was therefore repeated to yield (39-86), a 48 residue sequence.

During the course of this work it was noticed that the S-sulfonate groups of the A-chain were not stable. We are now repeating the semisynthesis with Arg-A-chain cyclic-disulfide. This was prepared by the trypsin-catalyzed condensation of Boc-Arg-OH with A-chain in 42% yield using the conditions of Inouye et al.[9]

B1,B29 di-(Boc)-B-chain cyclic disulfide, a minimally protected native sequence, contains three carboxyl groups: the two glutamate side chains B13 and B21, and the B30 C-terminal alanine (Figure 2). Activation by the mixed anhydride method followed by the reaction with

A-chain yielded a B-A product in 5% yield. It was shown that 70% of the coupling occurred at the B30 position.

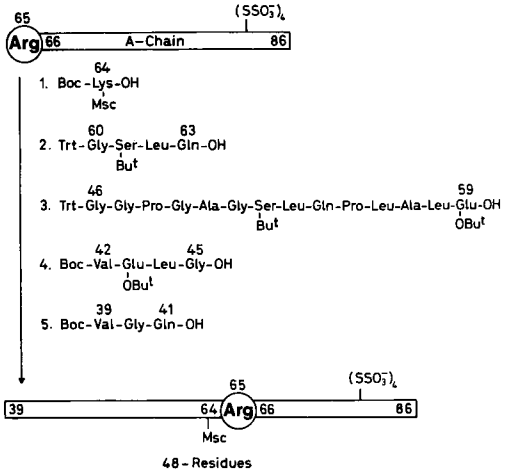

Fig. 1. Semisynthesis of human proinsulin sequence 39-86.

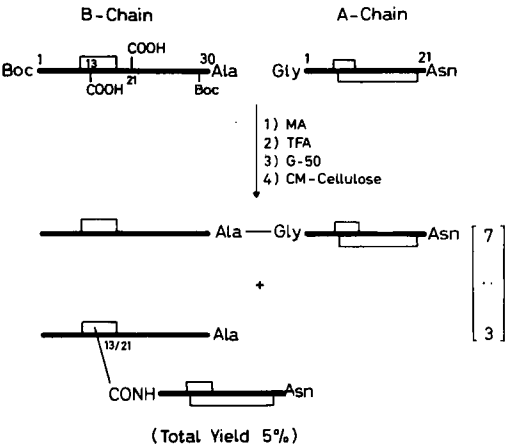

Fig. 2. Semisynthesis of B-A product.

Two insulin derivatives, A1,B1 di-(Msc)- and A1,B29 di-(Msc)-insulin were reacted with 10 equivalents of Boc-Ala mixed anhydride (Figure 3). The corresponding Ala-substituted derivatives were isolated after removal of the protecting groups. The acylation at B1 was quantitative while at B29 it was only 55%. The acylation of insulin at pH 6

and 7 with Boc-Met mixed anhydride yielded mainly B1-monosubstituted and some A1,B1 disubstituted derivatives. The extent of diacylation increased with increasing reagent concentration.

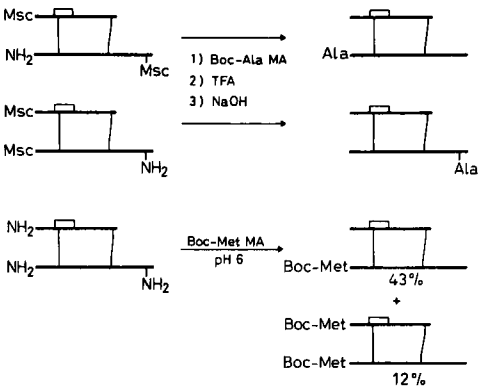

Fig. 3. Semisynthetic modification of insulin derivatives.

As we had bovine proinsulin available, and knowledge of rat prepeptide sequence,[10] we undertook the semisynthesis of a hybridpreproinsulin. A preferential acylation of the single α-amino group (Phe-1) was achieved due to the significant difference in pKs between this and the two ϵ-amino groups (Lys 29 and 59). The condensation of Boc-Ala-Gln-Ala-OH and Boc-Leu-Val-Leu-Trp-Glu(OBu^t)-Pro-Lys(Boc)-Pro-Ala-Gln-Ala-OH, corresponding to the C-terminal sequence 22-24 and 14-24 of rat prepeptide, respectively, with proinsulin at pH 6 yielded mainly a monosubstituted product reacted at Phe-1 (Figure 4). (Ala-Gln-Ala)-proinsulin and (Leu-Val-Leu-Trp-Glu-Pro-Lys-Pro-Ala-Gln-Ala)-proinsulin, represent partial preproinsulin sequences, des-(1-21)-preproinsulin and des-(1-13)-preproinsulin, respectively. These have been purified and characterized.

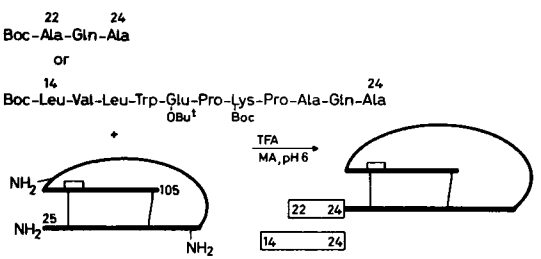

Fig. 4. Semisynthesis of des (1-21) and des (1-13) preproinsulin.

Conclusion

These studies have shown mixed anhydrides to be very powerful and efficient reagents for semisynthesis in aqueous solvent systems. The reaction is very rapid and under optimum conditions is specific for N-acylation. Side reactions, like urethane formation, can be kept minimal, if occurring at all, by selecting the correct pH; O-acylation is a slow reaction and is avoided by performing the reaction for 1 minute only. Protected amino acids and bigger fragments can be coupled quantitatively. The activation of minimally protected B-chain indicated that the side chain carboxyl groups also react. With proteins containing more than one amino group the reaction can also be specific; this is, however, dependent on a combination of several factors. This rapid method of peptide synthesis may also find application in conventional syntheses with minimum protection.

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SELECTIVE CHAIN CLEAVAGE AND COMBINATION IN PROTEIN PARTIAL SYNTHESIS

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While providing an attractive approach to the synthesis of proteins, partially synthetic methods* present many problems. Not the least of these is the difficulty of selective protection of the various side chain groupings present in polyfunctional protein fragments. The achievement of efficient bimolecular combination reactions between large and often intractable protected peptides — a problem still unsolved in the totally synthetic approach — also presents formidable difficulties. For these reasons we have considered the desirability of selective activation and combination of natural and synthetic fragments as an alternative to widespread use of protecting groups. Reformation of peptide bonds might be achieved more efficiently under unimolecular rather than bimolecular conditions. If procedures based upon these considerations could be devised they would take advantage of the properties of the protein starting material rather than attempt to mask them. Enzymic methods offer obvious potential in this respect,[2] and progress in this area has accelerated with the recognition that proteolytic enzymes may be induced to function in reverse in the presence of substantial proportions of organic solvents. This paper is concerned only with chemical methods.

It is evident that the main problems in the chemical approach are likely to be (i) efficient differentiation of the terminal carboxy-group in the carboxy-component from side chain groups of aspartic and glutamic acids elsewhere in the same molecule; and (ii) selective reaction of this uniquely activated carboxy-group with the terminal amino-group of the amino-component in the presence of multiple side chain amino and other nucleophilic functional groups in both components. We have explored solutions to these problems based on (i) differentiation of terminal carboxy-groups through chemical modification at the protein cleavage step, and (ii) selectivity induced by steric proximity and alignment.

*The term 'protein semisynthesis' is often used in this context, but has acquired a wide definition[1] including non-covalent (complementation) association of fragments and covalent linkage through non-peptide (e.g. disulphide) bonds. In this paper 'protein partial synthesis' implies *combination of natural and synthetic fragments through peptide bond formation.*

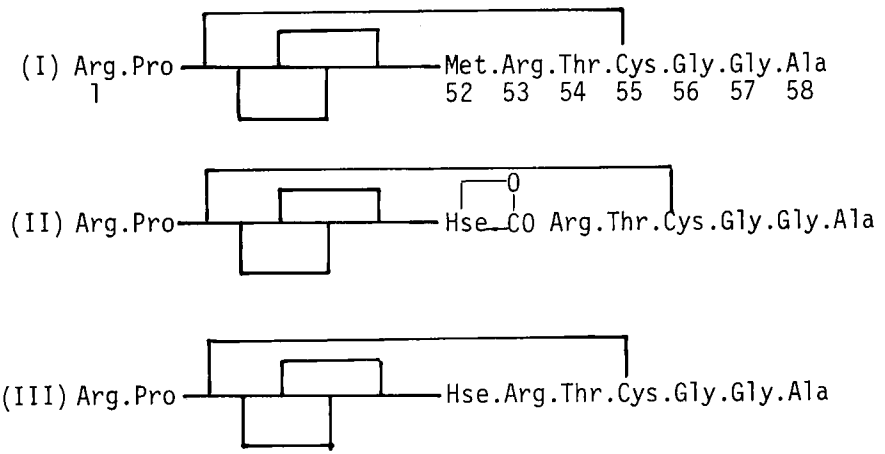
(i) Chemical procedures exist for the selective cleavage of peptide chains at methionine,[3] tryptophan,[4] and other amino-acid residues,[5] in all of which the carboxy-group derived from the cleaved peptide bond appears in the form of a five ring lactone. This provides an appropriate differentiation from other carboxy groups elsewhere in the molecule,** and at the same time a source of mild activation which proved to be of direct value in the coupling reaction. An inevitable corollary of use of cleavage methods of this type is that the side chain of the carboxy-terminal residue is transformed, for example in the case of methionine into that of the hydroxyamino-acid, homoserine. Schemes can be written for the reconversion of homoserine residues into methionine, but none that we considered was thought likely to be practical in a protein environment. Thus partially synthetic procedures based on these cleavage methods may be of value only in those cases where the particular amino-acid substitution is desired or can be tolerated.

(ii) The achievement of selective coupling reactions through steric proximity of the activated carboxy and specific amino-groups implies prior association of the fragments. This essentially converts the bimolecular system into a unimolecular one. Association could conceivably be induced by any of the factors responsible for the maintenance of tertiary structure in intact proteins, e.g. disulphide bonds, ligand or metal ion coordination, or even non-covalent complementation interactions of the hydrophobic or hydrogen bonding type. It is to be anticipated, however, that very considerable stereospecificity of association will be necessary if the subsequent intramolecular peptide bond forming step is to take place efficiently under mild conditions and with high specificity.

Our initial experiments were carried out using readily accessible basic pancreatic trypsin inhibitor (BPTI). This small (58 residue) globular protein (I) consists of a single tightly folded polypeptide chain cross linked through six cysteine residues. The single methionine at position 52 lies near the surface and within a short helical segment spanning residues 47 to 56.[7] Two disulphide cross links from cysteine residues 51 and 55 tie this helix firmly to the rest of the molecule. Treatment of the protein with excess cyanogen bromide cleaved the polypeptide chain smoothly at the methionine residue with formation of the expected seco-compound (II).[8,9] In the lactone form, (II) differs at

**This was also recognized by Offord who envisaged hydrazinolysis of the lactone ring and conversion to the acid azide.[6]

neutral or acidic pH by unit positive charge from the starting protein due to the newly liberated terminal amino-group of arginine-53. It is thus readily separated by ion-exchange chromatography and was isolated in 70% yield. Only a single product is formed from cleavage of the polypeptide chain because the carboxy terminal fragment remains attached to the rest of the molecule through the cysteine-5-cysteine-55 disulphide bond. In fact it seems that the overall tertiary structure of the



native protein (I) is largely retained in (II) since the latter possesses some 50% of the trypsin inhibitory properties of native BPTI. The structure of (II) was firmly established by tryptic digestion of the reduced and carboxymethylated derivative.[8,9]

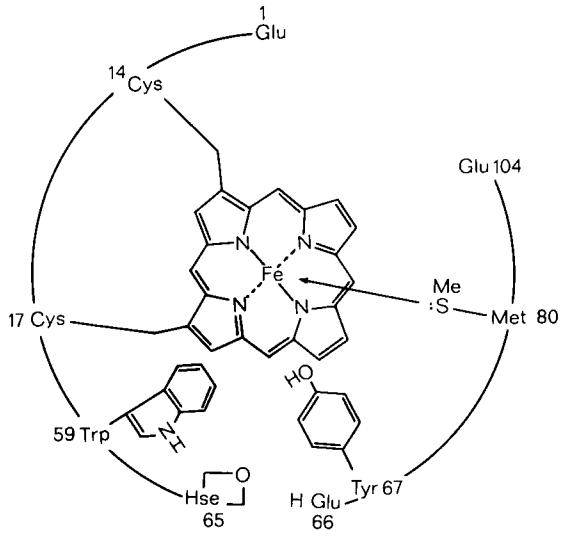
At this stage a key observation was made. On standing in neutral aqueous solution, the lactone derivative (II) was transformed into a new compound with net charge identical to that of the starting uncleaved protein (I). This is not the corresponding free acid derived by simple hydrolysis of the lactone ring. Its properties are very closely similar to those of BPTI itself. It behaves identically on ion-exchange chromatography and on electrophoresis, is biologically fully active, and folds and unfolds reversibly in the presence of thiols in a qualitatively similar manner to BPTI.[10] Tryptic digestion of the reduced and carboxymethylated product gave a pattern of peptides identical to that from BPTI and established structure (III).[8,9] Thus in the particular steric environment provided in (II), the homoserine lactone ring is sufficiently activated to undergo smooth intramolecular aminolysis (II→III) with

seemingly complete selectivity for the single α-amino-group of the terminal arginine residue 53 in the presence of five other free amino-groups in the molecule.

The t½ for this remarkably smooth reaction (the conversion is approximately 95% under optimum conditions) is ca. 29 hours at pH 6.3 and 170 minutes at pH 7.7. Clearly this rather rapid reaction of a relatively unactivated carbonyl group is a consequence of a very favourable steric relationship with the amino-group. For analogous bimolecular reactions with added amino-components, large excessess and high concentrations of the latter are required in order to achieve comparable rates.[11,12] Thus for BPTI at least, cyanogen bromide cleavage and intramolecular resynthesis fulfils many of the requirements for a selective partial synthesis system.

It would have been surprising if BPTI were completely unique in this respect, and within months a second example of the spontaneous chain resynthesis reaction was discovered. Cytochrome C contains two methionine residues at positions 65 and 80 but selective cyanogen bromide cleavage at just the former is possible. Corradin and Harbury[13] recognised that earlier observations on singly cleaved cytochrome (IV) were consistent with a chain resynthesis reaction, just as in the case of BPTI. In this instance the topology of the cleaved molecule is retained not by disulphide bonds but probably largely by non-covalent interactions centered around the haem group. Two residues (Trp-59 and Tyr-67) flanking the cleavage point are involved in hydrophobic interaction with the haem aromatic system, and the side chain of Met-80 also functions as a ligand to the haem iron atom. The haem prosthetic group is covalently attached to the N-terminal part of the molecule, and resynthesis only occurs when it is in the reduced state. As in BPTI, the residues around the reacting methionine in intact cytochrome are part of a helical system, probably aiding alignment of the reacting groups in the seco-derivative (IV).

These two examples show that the probably rather precise stereochemistry necessary for specific chain resynthesis can be maintained either largely by disulphide bonds (as in BPTI) or by weaker hydrophobic interactions (as in cytochrome). The latter provide much more favourable conditions for partial synthesis because the non-covalent nature of the interactions permits ready dissociation of the fragments and reassociation with synthetic or other peptides (see below). This is not the case with BPTI because the single disulphide bond holding



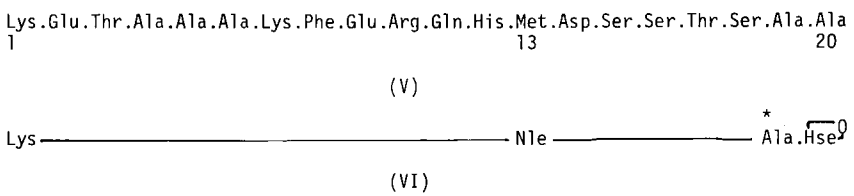
(IV)

the fragments together cannot be cleaved selectively. The bond linking cysteine residues 14 and 38 is much more reactive in the intact inhibitor (I),[14] and probably also in the seco-derivative (II). My colleague, Dr. D.A. East, has therefore investigated[15] chain synthesis in another system where two fragments are held together by weak, non-covalent forces, that of ribonuclease S. This is perhaps more typical since it lacks the highly hydrophobic haem prosthetic group of cytochrome, and the S-peptide and S-protein components in ribonuclease S appear to be held together by hydrogen bonds and other weak interactions characteristic of globular protein structures in general.

The ribonuclease A — ribonuclease S conversion constitutes perhaps the best known example of generation of a non-covalently bound complementing system by limited cleavage of a protein chain.[16] The cleaved protein dissociates readily at low pH into S-peptide and S-protein components, and the latter complements with a range of S-peptide analogues. The 20-residue S-peptide is derived from the N-terminal part of ribonuclease A through the action of an enzyme of the subtilisin type. In this case therefore, cyanogen bromide was used only to generate a homoserine lactone residue in a synthetic S-peptide analogue rather than effect the initial chain cleavage.

The sequence of natural S-peptide (V) includes a residue of methionine at position 13 which would interfere with formation of

terminal homoserine. It was therefore replaced by norleucine in the synthetic analogue, a substitution known not to reduce binding of S-peptide to S-protein seriously.[17] A new methionine residue could then be placed at position 20 for eventual conversion by cyanogen bromide to homoserine lactone, giving the S-peptide analogue (VI). Two additional residues (glycine and glutamic acid) were added at the C-terminus as aids in synthesis and isolation. Alanine-19 was radiolabelled with ^{14}C. The solid phase assembly of this 22 residue sequence was carried out by Dr. Atherton using the original version of our polydimethylacrylamide-based method.[18]



Direct detachment of the peptide from the resin by cyanogen bromide cleavage was relatively inefficient.[19] However, detachment in a more conventional manner using hydrogen fluoride, reduction of sulphoxide, cyanogen bromide cleavage, and extensive chromatographic purification furnished a low overall yield of (VI) with good analytical data.[15]

Evidence for efficient binding of this S-peptide analogue to S-protein was obtained through measurement of the quenching of the fluorescence of the latter as a function of peptide concentration. Nearly complete binding was indicated at a 1:1 stoichiometry in the presence or absence of cytidine-2'-monophosphate. This nucleotide binds to ribonuclease S through hydrogen bonds to residues 11, 12, 44, and 45;[20] it might therefore have strengthened the association between the two fragments. Resynthesis experiments were carried out[15] over a range of pH, in the presence of the above nucleotide and also in the presence of hydroxybenzotriazole. In each case the product was chromatographed on Sephadex G50 under acidic conditions which dissociate ribonuclease S, and the high molecular weight radioactive peak collected. The incorporation of radiolabel into the protein fraction reached a maximum

of only 7.5% at pH 7.0 in the presence of hydroxybenzotriazole. That the radioactivity was covalently associated with S-protein was confirmed by retention of 75% of the ribonuclease activity after digestion with trypsin under conditions in which ribonuclease S is quantitatively destroyed.

This result probably emphasises the high degree of steric alignment which is necessary for efficient intramolecular aminolysis of homoserine lactone peptides in competition with lactone hydrolysis. The X-ray crystallographic structure[20] of ribonuclease S shows that the tertiary structure is not rigidly defined around the cleavage point. The electron density map is particularly diffuse in this region and indicates mobility of the chain ends for 3 or more amino-acid residues on either side of the cleavage point. In contrast, in BPTI the terminal lactone is tied directly to the globular protein structure through the adjacent cysteine residue, and in cytochrome the terminal amino-residue is likewise immobilised by hydrophobic interaction of the adjacent tyrosine. Evidently in the ribonuclease S analogue generated by association of S-protein with (VI), mobility of both reacting groups is considerable, their effective concentrations are reduced, and hydrolysis of the lactone intervenes.

In a practical sense, the principles of selective chain cleavage and specific, non-enzymic chain resynthesis have thus far found their most important application in two partial syntheses of 65-homoserine cytochrome C[21,22] and preparation of a number of hybrid cytochromes.[23] The two partial syntheses make an interesting comparison. In the first,[21] a solid phase synthesised 66-104 peptide was combined directly with natural 1-65 homoserine lactone derivative. In the second,[22] cyanogen bromide cleavage of natural cytochrome again provided the 1-65 fragment, but additionally, cleavage of lysine side chain-protected cytochrome yielded the partly protected 81-104 fragment through cleavage at the second methionine. This shorter peptide was extended by readdition of a single methionine residue at its N-terminus, and then by a classically synthesised tetradecapeptide to yield the partially synthetic 66-104 fragment. After cleavage of the side chain protecting groups, recombination with 1-65 lactone was achieved as before. The yields for the formally identical recombination reaction in the two syntheses were 6% and 65% respectively. When the two natural fragments were similarly combined, yields of 60-75%[23] or greater than 90%[22] were obtained in the two laboratories.

These results serve to remind us that the success of partially synthetic endeavours in the protein field will depend just as much upon the quality

of the synthetic as of natural fragments. Clearly the conventional solid phase technique employed for the preparation of the 39 residue 66-104 in the first partial synthesis[21] was inadequate, and the isolation of partially synthetic protein depended very much on selectivity in the recombination step. Thus the requirement that covalent bond formation is preceded by specific association to form a defined tertiary structure was met by only a fraction of the molecules in the solid phase preparation. This selectivity is of course a very valuable feature of the method, but the formation of hybrid cytochromes[23] shows that it is not absolute.

Acknowledgements

I thank most warmly the several colleagues named in the references who have carried out much of the work described in this survey, notably D.F. Dyckes (pancreatic trypsin inhibitor), D.A. East (ribonuclease), and E. Atherton (solid phase synthesis). I thank also Dr. E. Schnabel and Bayer AG for generous gifts of pancreatic trypsin inhibitor.

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PROTEIN SEMISYNTHESIS AND THE CHEMICAL BASIS OF FOLDING AND FUNCTION

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Introduction

Semisynthesis has become an integral tool of peptide and protein chemistry for studying how the information of amino acid sequence directs the higher order properties of conformation and resultant biological function. This method generally involves chemical synthesis of limited peptide fragments which may be of native or non-native sequence, recombination of these synthetic peptides or analogues with the rest of the polypeptide chain, and characterization of the properties of the reconstituted protein species. Clearly, such sequence engineering manipulations require the existence of favorable peptide-protein systems, only a few of which exist naturally. However, controlled fragmentation of intact native proteins is an increasingly recurrent theme, due to use of enzymes with extremely limited specificity, chemical modifications of protein substrates which restrict enzymatic fragmentation, and chemical reagents which can hydrolyze peptide bonds adjacent to specific residues. The resultant increased flexibility in attaining extensive and discriminating fragmentation should make more parts of proteins accessible to sequence manipulations as parts of semisynthetic complexes. Furthermore, it now seems to be generally possible to reconvert several of these fragmented peptide-protein complexes into intact forms, so that semisynthesis offers a route to both noncovalent and covalent protein analogues.

For us, the advances in methodology for fragmentation, synthetic substitution, and reconstitution make the use of these procedures ever more appealing for answering questions about folding and function. Design of analogue sequences can answer direct questions about the roles of particular amino acid residues in producing the critical macromolecu-

lar properties of a particular globular protein. Moreover, this information then can help to define general ideas about how chemical information is used for peptides and proteins as a class. The ability to engineer protein structure and, therefore, function ultimately could lead to design of proteins with new desired specificities. In addition, the efficiency of present protein catalysts could be improved; or peptide and protein analogues could be made which retain function but have increased conformational stability or decreased antigenicity.

Several experiments which we have carried out illustrate these objectives and the types of results obtainable at this time. Our work has centered primarily around the highly characterized and convenient noncovalent protein complex, ribonuclease S', an *in vitro* proteolysis product of the intact native enzyme, bovine pancreatic ribonuclease A, which contains the full sequence in two fragments, S-peptide (residues 1-20) and S-protein (21-124).[1] As part of our work, we have made an effort to confirm and characterize the catalytic contribution of S-peptide, through His 12, to ribonuclease S' function. Interdependently, the question has been asked of how the S-peptide conformation is responsible for maintaining His 12 in the correct orientation for catalysis. Given these results, we have explored whether this interdependence of sequence, conformation, and activity could be understood well enough to try to design new sequences to mimic or improve upon existing properties of the protein.

The emphasis here is to outline some of this ongoing work, and also to describe a few new methodological applications which promise to enhance greatly the level of information attainable.

Active Site Function

Semisynthesis offers a unique opportunity to examine the role of particular amino acid residues in biological function. Residues which are suspected to have roles in catalysis based on prior evidence, from such studies as chemical modification and X-ray crystallography, can be tested for presumed roles. Where the residues of interest are accessible through replacement of a fragment containing them, modifications effected synthetically can be chosen with exquisite specificity — in contrast to what is possible with such alternative methods as chemical modification and biological mutation. The procedures allow minimal modification to be made with relative ease and can lead directly to information as to what structural or functional aspects of residues are essential for activity.

An exemplary case for us is that of the [4-fluoro-L-histidine 12] semisynthetic ribonuclease S' analogues, which were prepared in order to clarify the previously recognized participation of His 12 in enzymatic catalysis. These derivatives of normal RNase S contain a solid phase-derived synthetic S-peptide with 4-fluorohistidine(4-F-His) in place of the normal histidine at position 12. Studies on the des(16-20) analogue [4-F-His^{12}, des(16-20)]SRNase S', as well as the full sequence [4-F-His^{12}]SRNase S', have shown these species to be essentially inactive, exhibiting only about 0.2%-0.4% of native RNase S activity against uridyl-3',5'-adenosine and cyclic cytidine-2',3'-monophosphate at pH 5 and 7.[2,3] At least a large percentage of this observed low activity appears to be due to contamination with native RNase A, based on measurements of K_m and effects of dioxane. In contrast to the loss of activity, the analogues were found to have a highly conserved over-all conformation in solution.[2] Further, it was shown by direct quantitative affinity chromatography that both [4-F-His^{12}]- and [4-F-His^{12}, des(16-20)]SRNase S' have high affinity for various active site nucleotide ligands and substrates.[3] Clearly, the fluorination of the imidazole, which lowers the imidazolium pK from about 6 to 2, does not obviate substrate binding. Moreover, in recent kinetic studies, we have observed that as the pH is lowered from 5 to 3.5, there is in fact some increase in the relative enzymatic activity of the analogues. The preliminary results suggest that there indeed may be some intrinsic activity in the analogue complex which can be "switched on" by lowering the pH as the 4-F-His 12 is increasingly protonated. Taken together, the data for these derivatives provide compelling evidence consistent with the idea that His 12 acts as an obligatory catalytic component, probably dependent on its protonation at least transitorily, in the mechanism of action of native RNase.

Interpretation of the results mentioned above depend heavily on the demonstration that there is little perturbation of protein conformation, especially in the active site region, due to the incorporation of 4-F-His at position 12. We have attempted to confirm the intactness of conformation by X-ray diffraction analysis. Semisynthetic RNase S' can be crystallized under conditions similar to those used for native RNase S'. The large crystals that are obtained are of the "Y"-type, as designated by Wyckoff, Richards, and their colleagues.[4] This crystal type can be obtained not only for the normal sequence species[5] but also for des(16-20) analogues.[6] Recently, a collaborative study with H.C. Taylor, J.S. Richardson and D.C. Richardson at Duke University has shown that the

conformations of the fully active [des(16-20)]SRNase S' and the enzymatically deficient [4-F-His[12], des(16-20)]SRNase S' are essentially indistinguishable at 2.6 Å resolution. This absolute structural result confirms the assertion, inferred from spectroscopic characterization and comparison of affinities of nucleotide substrates and inhibitors, that the fluorination of histidine 12 effects little steric perturbation of the active site region.

Although the above conclusions have been made based on data for the noncovalent RNase S' complex, the implication is obviously to extrapolate to the intact parent, RNase A. Given the homology of conformational and functional properties of the A and S' species,[7] such correlation normally is reasonable. Nonetheless, the case of the 4-F-His 12 species does present difficulties in testing for possible intrinsic activity across a significant pH range, due to the innate instability of the RNase S' species at low pH. However, an opportunity to study the [4-F-His[12]]SRNase A derivative presented itself with the discovery at Purdue University, by Homandberg and Laskowski, Jr., that peptide bond formation by proteases can be favored in organic cosolvents[8] and that, as a demonstration of this concept, RNase S' could be enzymatically converted back to RNase A by equilibration of the subtilisin reaction mixture in 90% glycerol.[9] This new general approach allows covalent conversion with high yields in the absence of any side chain protection. We now have been able to resynthesize the [4-F-His[12]] analogue complex into the intact covalent form, as indicated elsewhere in this symposium.[10] At present, we are using this species to characterize the functional effect of His 12 fluorination over a wide range of pH. This study should allow a clearer definition of the extent of possible intrinsic activity. Such information could be quite helpful in understanding the role of His 12 in RNase A and the extent to which the active site might adopt mechanisms independent of this residue.

Based on the knowledge[7] that His 119 and His 12 are juxtaposed sterically at the active site of RNase and that both histidine residues have been implicated in enzyme function, a logical extension of the above work was the synthetic incorporation of 4-F-His at position 119. Use was made of the noncovalent, enzymatically active ribonuclease complex formed by the fragment (1-115), derived from RNase A by sequential digestion by pepsin, carboxypeptidase A, and carboxypeptidase Y[11-13], and the synthetic peptide corresponding to residues (116-124). Normal sequence (116-124) as well as the 4-F-His 119 analogue were prepared by Merrifield solid phase synthesis under conditions described before.[2,14] As in the case

of the [4-F-His^{12}] analogues, the complex of [4-F-His^{119}](116-124) peptide + (1-115) peptide had about 0.2% of native activity, toward cyclic cytidine-2', 3'-monophosphate at pH 7, by comparison with normal sequence complex. The overall importance of His 119 clearly is substantiated, as is the implied interdependence of His residues 12 and 119 in catalysis. The nature of this interdependence is still to be defined mechanistically.

Folding and Attainment of Active Conformation

Beyond direct study of function, semisynthesis offers an opportunity to modify amino acid residues which direct and stabilize the native secondary and tertiary structures, and thereby help to form an active conformation with an intact active site. In classifying specific amino acid residues as to their conformational roles, it is increasingly recognized that the short and long range structure-directing and stabilizing influences provided by a given residue are not equal. Among numerous structure-stabilizing interactions present in a globular protein — including apolar interactions, hydrogen-bonding and salt bridges — some of these play an essential role in directing and stabilizing tertiary structure. A key objective is to identify and test residues that provide such essential interactions.

In the RNase S' system, there is a wealth of data on conformation obtained from the pioneering semisynthetic studies on Hofmann, Scoffone, and their colleagues[15,16] as well as from more recent work.[17-19] These studies have given us extensive insight into the functions of several non-active site residues in native S-peptide sequence for the formation of a productive RNase S' complex. For example, our own study[20] on glutamic acid at position 9 has indicated that the tendency of this residue to support the integrity of α-helical conformation in this region is more important than the polarity of its side chain. This was suggested by the productive complex that results from an S-peptide with a single replacement of Glu 9 by leucine, a residue with strong side chain hydrophobicity (in contrast to Glu) but main chain α-helical propensity equally as high as that of Glu. The effect of residue replacement as well as data on sequence homology in the N-terminal region of RNase have indicated[21-23] the importance of the hydrophobic residues, methionine 13 and phenylalanine 8, for stabilizing the RNase S' complex. The one order of magnitude changes in the S-peptide:S-protein binding constant that were observed with changes at the sulfur atom of Met 13[24] or at the side

chain of Phe 8[25] tend to support the apparent importance of these apolar interactions. Some recent empirical estimations of local interaction free energy changes associated with changes in side chain to side chain interactions for the complexation reactions[26] revealed an insight into some interesting features of conformation that needed clarification. It was found that the major favorable free energy contribution came from the interactions involving Met 13 and Phe 8. Further, the analysis suggested strongly that the role of the N-terminal α-helix is mainly to provide a structural frame on which the side chains of the above two residue can be properly oriented to optimize favorable interactions with nonpolar groups on S-protein. Taken with the proven importance of His 12, as well as the above reviewed data, it may be proposed that the key minimal features for S-peptide to produce a productive binding to S-protein are a stable α-helix which can orient Met 13 and Phe 8 for complex stabilization and His 12 for active site function.

We chose to test the above postulate by designing a model peptide[27] that retains the above essential structural (α-helix), complex stabilizing (Phe 8, Met 13) and active site (His 12) features. The model peptide, of 15 amino acids, contained an α-helical matrix provided by poly-L-alanine. Besides incorporation of the above-mentioned Phe 8, His 12, and Met 13, the residues Glu 2 and Arg 10 were included to provide an ionic interaction which appears important for stabilizing the α-helix,[15] while Lys 7 was included for its contribution to the cationic environment of the active site.[28] This model sequence S-peptide, as prepared by solid phase synthesis, was found to be highly productive in generating an enzymatically active model SRNase S'.[27] From the enzymatic activation of S-protein by model S-peptide at various mole ratios of the two components the dissociation constant, K_d, was determined to be 10^{-6} M. This value is only an order of magnitude higher than that of native S-peptide. When this increased K_d value for the model peptide is compared with the same order of magnitude increase observed with peptide complexes with only a single residue replacement of native sequence (such as Glu 9 by Gly or Met 13 by methionine sulfone), the remarkable success of the model peptide is apparent. Further, the model SRNase S' exhibited about 35% of the specific enzymatic activity at saturating peptide concentrations. The substantial activity shows that the model complex can generate the RNase active site with a high degree of fidelity.

The absolute structural verification of the presumed RNase S-like conformation of the complex must rely on crystallographic analysis. As in the active site residue modification study, this is now possible. We have

obtained crystals of the model complex, once again in the native RNase S' space group. Preliminary results indicate that the crystals are suitable for structural analysis by crystallography. In addition to confirming our interpretation that the model SRNase S' retains the overall conformation of the normal sequence complex, this analysis perhaps may provide a structural clue as to a reason for the observed diminished catalytic activity of the model SRNase S'.

Remarks

Fragmentation and Reconstitution — Whereas it seems apparent that protein semisynthesis offers a clearcut advantage in studying folding and function without the need to achieve difficult total synthesis, the depth of questions asked often depend on availability of methods for controlled fragmentation and reconstitution. Chemical and enzymatic methods that are currently available should allow controlled fragmentation of almost any protein. More uncertain, it would seem, is the ability to put fragments back together to give intact, functional proteins. Chemical recoupling of fragments has been achieved in certain cases[29] but requires substantial effort and good fortune. The inherent noncovalent association properties of some protein fragments, such as in RNase S' and staphylococcal nuclease T, allows studies without re-establishing total covalency. This should be feasible even for three-fragment systems such as those of cytochrome c[30]. Nonetheless, slight differences in properties, imparted by the complex itself, may obscure differences due to sequence variation.

In this light, the enzymatic method of restitching protein fragments to give covalent forms[9] should have a strong impact on the semisynthesis approach. Moreover, this method also could be used for joining totally synthetic fragments. However, not all fragment systems can be dealt with using enzymatic reversal. For example, we were unable to couple the ribonuclease fragments (1-115) and (116-124) using α-chymotrypsin in 90% glycerol for the bond at Tyr 115. Moreover, attempts to enlarge the region of discontinuity by introducing N-terminal extensions on the (116-124) peptide also have not allowed restitching with (1-115). Although we see the approach of loop enlargement as a potentially reasonable adjunct to the use of enzymes for fragment stitching, this tactic has failed here. We interpret our preliminary failures to couple the above fragment complex to indicate that enzymatic restitching will be applicable most notably when the bond to be resynthesized is itself a site of limited proteolysis.

The advent of enzymatic reversal,[8,9] along with other avenues for fragment coupling through steric enhancement,[31] should have a major impact on the use of fragments in synthesis studies of proteins.

Modeling and Macromolecular Interactions — The model S-peptide seems prototypic of a basic approach of using generalized synthetic modification as a means of learning about the structural requirements for interactions between peptides and proteins. As shown above, with sufficient information available from a variety of sources on what might be needed for stable conformation, it was possible to design a substantially altered S-peptide and thereby to test the overall requirements for association with S-protein. This logic could be carried to the use of modeling to identify the types of interactions which occur in such associating processes as proteins with antibodies, and hormones with receptors. Fortuitously, we have found that the model SRNase-S' has a markedly reduced antigenicity toward ribonuclease antibodies that are specific for the S-peptide region in its native conformation. This preliminary result indicates that the modeling of S-peptide offers a way to identify and manipulate the constellation of residues, probably on the surface, that act as an antigenic determinant for association with antibody. In the case of peptide and protein hormones, receptors are not yet characterized at a detailed chemical and structural level. Nonetheless, as with the above cases, use of conformational propensity concepts for subsequent modeling would appear to be a reasonable approach for developing agonists and antagonists.

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ENZYMATIC CONVERSION OF SELECTED NONCOVALENT COMPLEXES OF NATIVE OR SYNTHETIC FRAGMENTS TO COVALENT FORMS

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Introduction

Limited proteolysis systems, such as ribonuclease S[1] or staphylococcal nuclease-T[2], have been the basis of detailed studies of the effect of sequence on enzymatic activity or other properties. Noncovalently bonded native fragments can be individually replaced with chemically synthesized fragments containing different folding instructions or different capabilities for interaction with the complementary partner. By appropriate substitution, the importance of individual amino acid residues can be separately determined, as has been done for the two systems mentioned above. However, the hybrid complexes often may have properties altered not only by the substitution but also by the increased chain flexibility in the regions of discontinuity. For instance, nuclease-T has only about 8% of the native nuclease activity[2]. Even in cases where the native sequence complex may have similar catalytic properties, other properties such as lower conformational stability can hinder characterization[3].

Recently it has been shown that synthesis of peptide bonds can be favored by equilibration of an enzyme-catalyzed limited proteolysis system in partially nonaqueous solvents[4]. Such solvents depress the ionization of the carboxyl group liberated during hydrolysis and thereby decrease the free energy associated with hydrolysis[4]. This concept was used to show that ribonuclease S can be enzymatically converted to ribonuclease A (50% yield) in 90% glycerol[5].

In the present study, we have applied the method of enzymatic reversal to prepare covalent [4-fluoro-His 12] semisynthetic ribonuclease A from the analogue S complex, staphylococcal nuclease derivatives from nuclease-T and covalent cytochrome c from the fragments of the

native protein. The results are indicative of the types of systems that can be dealt with in this way and of the limitations of usage.

Results and Discussion

Ribonuclease activity is dependent on the general base catalysis characteristics of two juxtaposed histidyl residues, 12 and 119, each with a pK of about 6.0 to 6.5. Earlier work has shown that the S-peptide with 4-fluoro-histidine 12 forms a stable but enzymatically deficient complex with S-protein[6]. However, since the complex dissociated at lower pH, it was felt that full characterization of enzymatic properties required a covalent form[3]. The sodium dodecyl sulfate polyacrylamide gel analysis in Figure 1 (insert) verifies the conversion of highly purified [4-F-His 12]SRNase-S′ into the covalent form (50% yield). The graph illustrates some practical points for enzymatic coupling of peptide fragments. Curve A shows that native S-peptide in up to a 4-fold excess affects a yield not greater than 50%, as expected if the equilibrium involves the complex, not free S-peptide or S-protein. Curve B shows that even with crude solid phase-derived synthetic S-peptide (normal sequence peptide of about 30-50% purity) a 30% yield of active covalent RNase-A can be obtained. The effect of added salt on synthesis is shown by Curves C and D. As predicted from the known stabilizing effect of salt on the carboxylate ion, the amount of synthesis decreases[4]. Further, added salt appears to stabilize the protein from extensive proteolysis as shown in Curves E and F, which represent the variations of content of total ribonuclease A plus S. These curves also show that the mole ratio of ribonuclease A to S remains at 1.0 at equilibrium even though the total amount of active material is decreasing due to extensive proteolysis.

Staphylococcal nuclease, in the presence of ligands, Ca^{+2} and thymidine-3′,5′-diphosphate, can be cleaved by trypsin at the Lys^5-Lys^6 peptide bond to form fully active (6-149) nuclease. The latter is further cleaved at the peptide bonds carbonyl to Lys 48 or Lys 49 to form nuclease-T_a ((6-48)+(49-149)) or nuclease-T_b (T_a with Lys 49 excised), respectively[7]. These latter two complexes are about 8% active, a point which leads to speculation on the importance of the integrity (size or sequence) of this loop region (residues 43 to 52)[8] for the necessary geometry of the nearby active site region.

This lower activity, taken with the potential for nuclease-T as a vehicle for semisynthesis, led us to attempt conversion of the complex back to (6-149)nuclease. Intriguingly, we found (Figure 2) that, although

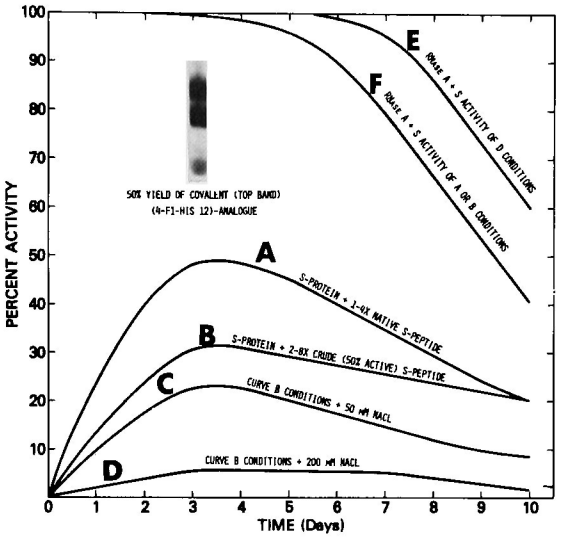

Fig. 1. Conversion of RNase complexes (1 mg/ml) to covalent forms in 90% gylcerol, 10 mM 4-Morpholine ethanesulfonic acid, MES (app pH 6.2) containing 0.05 mg/ml subtilisin BPN'. For lower curves covalent form distinguished from complex by assays against cCMP in 40% dioxane (to dissociate complex). Data for 2 upper curves are assays with dioxane omitted so total RNase A and S could be determined.

resynthesis can occur, the irreversible step of excision of Lys 49 (involved in the conversion of (6-149)nuclease to nuclease-T_b) traps the starting material (nuclease or either complex) in an equilibrium involving only nuclease-T_b and the covalent form, [des Lys 49]nuclease(6-149). However, although the Figure 2 insert of SDS gels shows the formation of the covalent material (band N, about 30%) after 2 weeks in 90% glycerol, measurement of enzymatic activity shows no increase but rather a decrease in the nuclease activity.

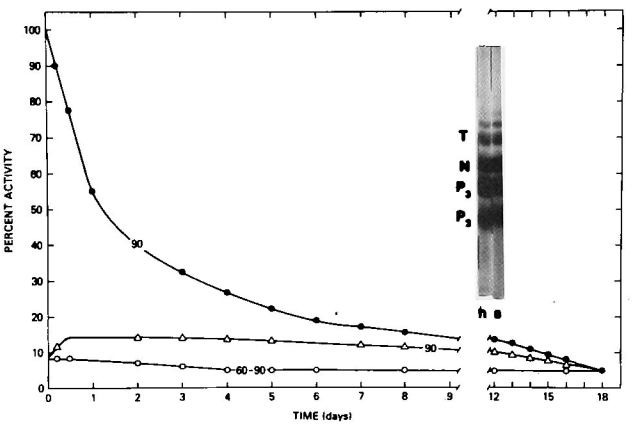

Fig. 2. Nuclease activity during resynthesis from nuclease (●), Nase-T_a (△) or Nase-T_b, (○) at 1 mg/ml in 90% glycerol, 10 mM MES (app. pH 6.2), 0.1 mg/ml trypsin, 10 mM $CaCl_2$, and 10 μM in deoxythymidine-3',5'-diphosphate. Numbers are % glycerol. SDS gels show trypsin (T), product (N) and complex components (P_2 (6-48)) and (P_{3b} (50-149)) in incubations of nuclease (h) and Nase-T_b (s).

The slight increase in activity seen in the curve of synthesis from nuclease-T_a suggests that fully active (6-149) nuclease exists only briefly before being converted to nuclease-T_b. The inactivity of the covalent derivative, [des Lys 49]nuclease (6-149), probably is due to deletion of Lys 49 and subsequent closure of the loop, which could distort the geometry of the nearby active site region.

Cytochrome c resynthesis also has been attempted, from a complex of heme-containing fragment (1-38) with (39-104) apofragment[9]. Preliminary results (Figure 3) show that incubation of the ferrous complex and clostripain in 90% glycerol, apparent pH 6.2, leads to formation of a protein with a molecular weight equal to that of cytochrome c, in a yield of at least 25%.

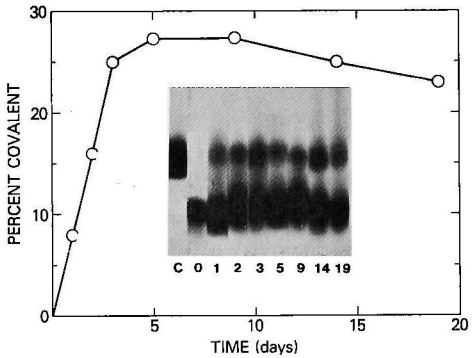

Fig. 3. Conversion of heme fragment (1-38) + apofragment (39-104) cytochrome c complex (2 mg/ml) to covalent form catalyzed by clostripain (0.1 mg/ml) in 90% glycerol, 10 mM MES (app. pH 6.2) also containing 5 mM $CaCl_2$ and 2 mg/ml ascorbate. Solution flushed with nitrogen. SDS gels, from which data on graph obtained, are for cytochrome c (C) and incubations for days as numbered.

Summary

The potential for the enzymatic restitching method lies in the yields, stereospecificity of coupling, dispensibility of protection steps, and enzymatic specificity. The success thus far indicates that, for a protein complex where the bond to be synthesized is itself the site of a limited proteolysis, the probability for conversion to a covalent form is high.

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ENZYMATIC PEPTIDE SYNTHESIS. PAPAIN CATALYZED OLIGOMERIZATION OF AMINO ACID ESTERS

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Introduction

Various proteases had been shown to synthesize water-insoluble polymers of α-amino acids. *Brenner et al.*[1,2] showed that chymotrypsin polymerized the isopropyl esters of Thr and Met. The former polymer had an estimated chain length of 10 residues whereas in the latter product dimethionine and trimethionine were isolated from the soluble fraction and a higher polymer from the insoluble one. *Dannenberg and Smith*[3] described the synthesis of an oligomer from Phe (average chain length 10 residues) by a protease from bovine lung. *Sluyterman and Wijdenes*[4] described the polymerization of the methyl ester of Leu by papain and obtained a product with 8-9 residues av. chain length. More recently, *Anderson and Luisi*[5] polymerized tyrosine methyl ester to a product of 6-7 residues av. chain length.

Due to the difficulties encountered in the analysis of water-insoluble peptides, which are equally insoluble in many organic solvents, little is known so far about the molecular heterogeneity of such polymers. With the objective of synthesizing enzymatically oligomers of some nutritionally important amino acids, we studied the incorporation of ^{14}C-labelled methionine ethyl ester into water-insoluble oligomers by papain under various reaction conditions. We chose methionine for this study because the isolated polymers could be conveniently converted into fully water-soluble sulfoxide- or sulfone derivatives which were accessible to gel-filtration and electrophoresis in aqueous systems.

Results

1. Reaction conditions, product yield, and product dispersity — When highly concentrated aqueous solutions of HCl·H-Met-OEt

including 3 μCi of L-(1-^{14}C)Met-OEt were reacted with 2% of crystalline papain of the presence of 0.05 M L-cysteine at pH 5, white precipitates formed after several hours of incubation. After the water-insoluble polymer was washed free of soluble material in an ultrafiltration cell equipped with an Amicon UM 10 membrane, radioactive oligomethionine was obtained in a yield of 3-6%. This product was resolved to give 4 components on Sephadex G-25 fine with dimethylsulfoxide as solvent. Various buffers with high buffer capacity in the pH range of 5.0-5.5 were examined as solvents for oligomer synthesis. Most remarkably, the use of carboxylic acid buffers such as sodium acetate, succinate or citrate resulted in a drastic improvement of the yield of water-insoluble product. High concentrations of citrate suppressed the formation of the lower species in favor of the larger ones. Oligomethionine synthesized in 2 M sodium citrate buffer eluted essentially as a single peak from Sephadex G-25 in dimethylsulfoxide although the peak had a shoulder which indicated the possible presence of lower peptides. Under these conditions product yields ranged regularly between 40-50%, estimated on a molar basis using amino acid analysis data. Oligomethionine obtained in this fashion had a nitrogen content of 9.5-9.7%, contained 84-90% by weight of methionine and showed a specific rotation $[\alpha]_D^{25}$ (c = 2.5 in DMSO) of -11.8 to -13.2° (range observed with 3 independent preparations).

2. Polymerization degree of oligomethionine — The proton-NMR spectrum of oligomethionine in dimethylsulfoxide was compared with that of the corresponding monomer, H-Met-OEt (Figure 1). The intensities of the thioether protons (b) and the ester methyl protons (a) in the spectrum of the polymer were established as b/a = 10 (intensity per proton). This suggested that the repetitive unit was 10 times repeated in an average chain.

Attempts to establish the number average chain length by chemical techniques, such as dinitrophenylation or ninhydrin reaction, did not give reproducible results, probably due to incomplete conversion of the amino groups. In contrast, highly reproducible results were obtained when we applied the ninhydrin reaction to the water-soluble sulfoxide- and sulfone derivatives of the polymer. Fully water-soluble sulfoxide derivative was obtained in quantitative yield by treatment of oligomethionine with a mixture of DMSO/HCl/HAc[6].

An equally soluble sulfone derivative was prepared by treating the polymer with H_2O_2 (3 M) in 0.2 N NaOH. For the sulfoxide derivative we deduced an average chain length of 7.20 residues and for the sulfone derivative 7.80 residues (standard error 0.05 residues).

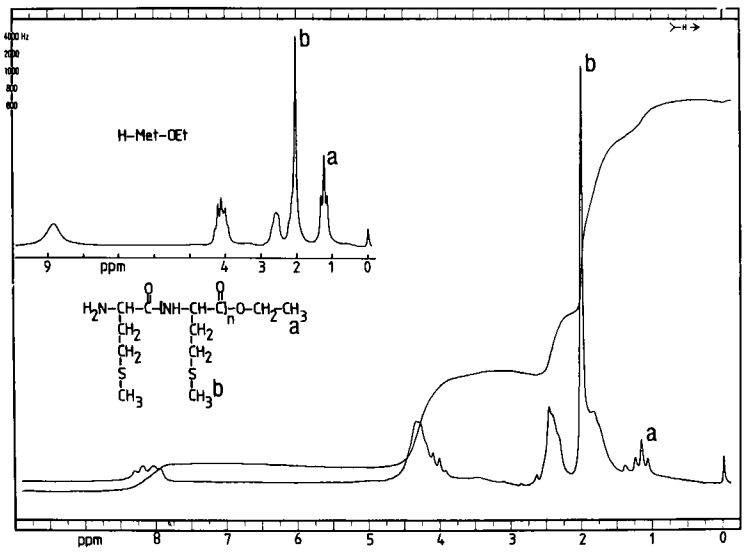

Fig. 1. Proton-NMR spectra of H-Met-OEt and oligomethionine in DMSO-d6; 80 MHz; 36°C

3. Molecular heterogeneity — Gel-filtration on Sephadex G-25 fine in DMSO of oligomethionine synthesized in 2 M citrate did not permit to resolve the product any further than in a major peak (eluted at about 1.3 void volumes) and a shoulder. In contrast, both sulfoxide- and sulfone derivatives gave, on the same gel but in water as the solvent, a second small peak composed of (a) lower species. Whereas the major peak contained 75% of the total radioactivity, the minor peak contained the remaining 25%. Paper electrophoresis at pH 2.0 revealed a major cathodic spot on top of which was a minor spot. Electrophoresis at pH 6.5 however showed 2 distinct spots the first of which was near the origin. This substance was shown to be the free carboxyl form of the polymer whereas the second product with cathodic mobility corresponded to the ester derivative of the same polymer. This spot disappeared for material subjected to alkaline hydrolysis in favor of that for the substance at the origin.

When an aqueous solution of the sulfoxide polymer was passed through a column of Dowex 1-X2 resin in the acetate form, the esterified component was first to emerge from this column in pure form. From the proton-NMR spectrum of this peptide, a chain length of 8.3 residues was obtained by comparing ester methyl proton intensities with that of the side chain protons in the repetitive unit.

This result, suggesting that the predominant species was an octamer, was reasonably close to the av. polymerization degree as obtained by the ninhydrin method. The proton-NMR spectrum of crude oligomethionine as shown in Figure 1 had therefore to be considered as the spectrum of mainly two species with a free and an esterified carboxyl.

Conclusions

Enzymatic polymerization of amino acid esters gives rise to oligomers of 6-10 residues in chain length. The predominant species in the water insoluble polymer of H-Met-OEt, obtained by papain in 2 M citric acid buffer, was an octamer. High concentrations of citrate favor high polymer yields and homodispersity of the product.

Reliable estimation of average chain length and heterodispersity of water-insoluble oligoamino acids depends on the possibility to prepare soluble derivatives. This was achieved in the case of oligomethionine by partial or complete oxidation of the polymer to their respective sulfoxide- or sulfone derivatives.

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SEMISYNTHESIS OF AN ANTIBODY FRAGMENT: QUANTITATION AND CORRECTION OF DELETION SEQUENCES

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The chemical synthesis of a complete antibody is, at present, beyond the scope of solid phase synthetic techniques. Givol and coworkers have shown, however, that IgA myeloma proteins may be cleaved to yield a fragment (F_v) consisting of a noncovalent complex of the heavy and light chain variable regions. Each variable domain is comprised of approximately 115 amino acid residues and is a suitable, if difficult, target for chemical synthesis. The light chain variable region (V_L) from MOPC-315 has been synthesized and combined with a heavy chain variable region (V_H) derived from the same protein.[1] The semisynthetic F_v, like the parent antibody, binds the DNP hapten.

Two major difficulties must be overcome before the solid phase synthesis of V_L fragments is routine. First, low yields of protected peptide obtained from synthesis must be increased. Loss of protein during synthesis is due to acidolysis of the bond which anchors the growing peptide to the solid support. This occurs during deprotection of the α-amino group. As reported previously, use of 6 N HCl-dioxane, rather than 50% TfaOH-CH_2Cl_2, for deprotection reduces loss of synthetic protein by as much as six fold.[2] Studies with model tetrapeptides predict that about 15% of the 108-residue V_L from antibody 3374 will be lost during a synthesis done with HCl-dioxane. Actual losses are however, between 5-10%. The synthesis was initiated with a support containing 243 μMol valine per gram (34% Cl^- replaced). Completion of the synthesis yields a product containing 50 μMol protein per gram (32% Cl^- replaced). Solid phase sequence analysis confirms that there are 48.4 μMol N-terminal alanine per gram of product.

A more quantitative addition of amino acid residues to the V_L is obtained when HCl-dioxane rather than TfaOH-CH_2Cl_2 is used for deprotection. Figure 1 shows incorporation of residues into the growing chain as judged by amino acid analysis. After addition of 21 residues, incorporation of glutamine is only 58% of that obtained early in the

synthesis when 50% TfaOH is used. An almost identical decrease in incorporation is obtained when neat TfaOH is used. In contrast, when HCl-dioxane is used incorporation remains quantitative throughout the synthesis. The report that residual TfaOH in the resin truncates peptide chains during coupling and leads to diminished incorporation, explains these results.[3]

For the routine synthesis of V_L fragments, increases in homogeneity must also be effected. Homogeneity is measured by preview.[4] Samples of peptidyl-resin are subjected to solid phase Edman degradation. When an amino acid residue is missing from the synthetic peptide (deletion sequence), the residue one position nearer the C-terminus is observed or "previewed." The quantity of preview is a measure of the number of deletion sequences in a synthesis. The strategy chosen to minimize deletion sequences is to repeat the coupling of an amino acid residue until no more free amino groups can be detected.

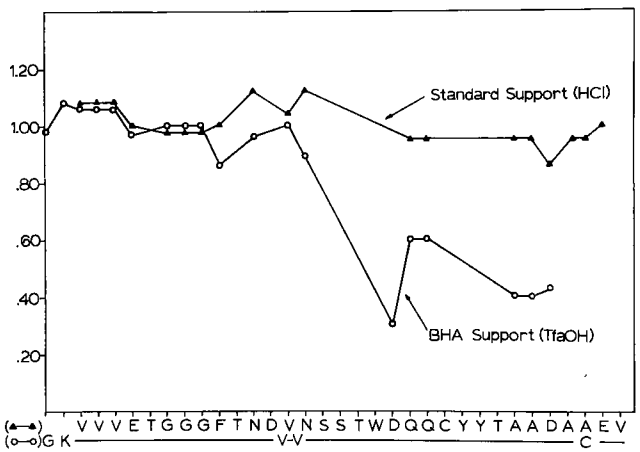

Fig. 1. Incorporation of amino acid residues into V_L fragments when TfaOH-CH_2Cl_2 and HCl-dioxane are used for deprotection.

Sequence analysis of peptidyl-resins indicates substantial preview even after negative ninhydrin tests are obtained.[5] To examine this, three syntheses of 39-residue peptides from the V_L C-terminus have been completed using the following protocols:

A — Repeated coupling with a five fold excess of protected amino acid and DCC until a negative test for free amino groups is obtained.

B — Completion of Protocol A followed by an extra coupling step.

C — Completion of Protocol B followed by reaction with a ten fold excess of acetylimidazole[6] for one hour.

Peptidyl-resins prepared by Protocols A and B contain a total of 19 and 21% preview, respectively (Figure 2). Thus, on average between 0.5-0.6% of the peptide chains do not react during addition of each amino acid residue by these protocols. The extra coupling (B) after a negative ninhydrin test does not diminish the number of unreacted peptide chains. Acetylation (C) does reduce the number of deletion sequences. Presumably acetylimidazole can truncate hindered peptide chains unavailable for reaction with the activated amino acid. Acetylation converts deletion sequences to truncated sequences and thus does not decrease the heterogeneity of the crude product. Truncated sequences are easier to remove from the crude material, however. Either gel filtration or chromatography on a column which will bind a handle attached to the N-terminus of the V_L will effect the separation.

No.	Residue	Protocol A	Protocol B	Protocol C
	T			
70	E	3.3	5.6	1.1
	F			
	T	5.0		1.3
	L			
	T	8.4		3.0
	I			
	S			
	D			
	L			
	E			
80	Asb	12	9.5	4.9
	A			
	D			
	A			
	A			
	T			
	Y	4.9	1.4	0.9
	C			
	Q			
90	S			
	Y			
	S	5.6	6.4	1.4
	I			
	S			
	S			
	A	5.2	10	4.5
	F			
	G			
100	G			
	G	1.4	0.7	1.4
	T	2.0	1.1	1.4
	E			
	V			
	V			
	V	—	—	—
Cumulative Preview		19.2%	20.6%	10.8%

Fig. 2. Heterogeneity of 3374 V_L measured by solid phase sequence analysis.

Synthesis of 3374-V_L by protocols A and C has been completed. A total of 67% preview is obtained with Protocol A and only 20% preview with protocol C. Use of acetylimidazole markedly reduces the deletion sequences in the synthetic product.

Acknowledgement

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THE SEMISYNTHESIS OF SOME STRUCTURAL ANALOGS OF CYTOCHROME *C*

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Introduction

Recently, I reported[1] the development of a semisynthetic route to cytochrome *c* analogs using only natural fragments obtained by CNBr cleavage. The advantages of such a strategy have been discussed by Offord.[2] Of the methods of carboxyl protection given in reference,[1] I find that satisfactory results are more easily obtained by the use of the anisyl diazoalkane.

I have repeated the synthesis of acetimidino [Hse^{65}] cytochrome *c*, and used the anisyl route to prepare acetimidino [Hse^{65},ε-Cbz Lys^{81}] cytochrome *c* and [Hse^{65},o-fluoro Phe^{82}] cytochrome *c*.

I have also developed general conditions for fragment coupling by aminolysis of the homoserine lactone ring. The [$Hse^{65,80}$, Met^{81}] and [$Hse^{65,79a}$] derivatives of acetimidino cytochrome *c* have been prepared. All these derivatives have been assayed in the depleted mitochondria system of Jacobs and Sanadi.[3]

Experimental and Results

The strategy for the semisynthesis of the cytochrome *c* analogs [Hse^{65}], [Hse^{65},$FPhe^{82}$], and [Hse^{65},Lys^{81}] was essentially as described in previous publications,[1,4] although I have made a number of improvements on the published semisynthesis. The full reaction scheme, incorporating these modifications, which are underlined, is shown in Figure 1. The reaction scheme for the synthesis of the [$Hse^{65,80}$,Met^{81}] and [$Hse^{65,79a}$] analogs of acetimidino cytochrome *c* is also included in Figure 1, but involves noticeably fewer steps than the other route.

In this latter scheme, unlike other examples of peptide coupling by aminolysis of homoserine lactone[5] that depend for their success on specific interaction of the peptides, reaction occurs under anhydrous basic conditions and is catalysed by 1-hydroxybenztriazole. I have found

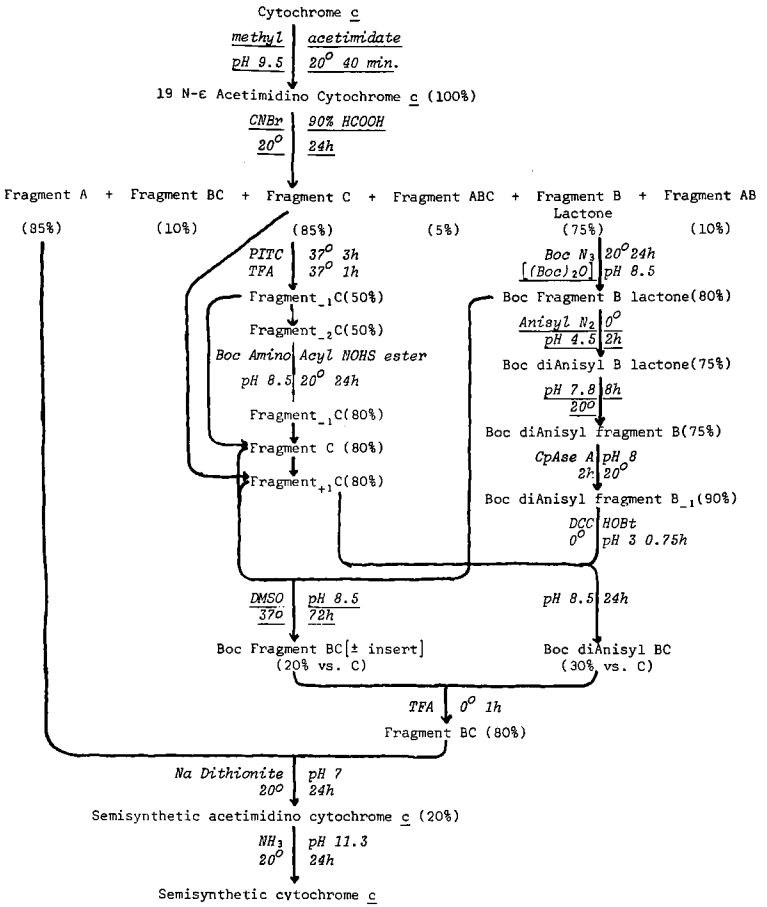

Fig. 1. Approaches to the semisynthesis of cytochrome *c*.

these conditions to be optimal for this coupling reaction in extensive trials.[6]

Approximate optimal recoveries for each step are shown in the two schemes. At the final stage, the natural fragment gives a 60% coupling yield and the semisynthetic of natural sequence, 20-30%. The analogs of fragment BC tested so far give yields of 1-2%.

Coupling products at both the BC and complete protein stages were authenticated by amino acid analysis.

The depleted mitochondria assay[3] was performed as described previously,[1] and the results for all these derivatives are shown in Tables I and II (representing two different mitochondrial preparations), and oxygen uptake (μmol.min^{-1} is expressed as (i) an initial velocity and (ii)

velocity when added cytochrome *c* equals 5 nmoles/ml mitochondrial suspension (this is the concentration of endogenous cytochrome *c* before the mitochondria are depleted).

Table I. Depleted Mitochondria (Preparation I) Assay with Various Cytochrome *c* Derivatives

Derivative	Initial velocity	V_5 nmoles/ml
Resynthesised acetim[Hse^{65}] cyt. c	6.2	8.2
Semisynthetic acetim[Hse^{65}] cyt. c	5.8 (94%)	5.8 (71%)
Acetim. [$Hse^{65,79a}$] cyt. c	1.5 (24%)	1.5 (18%)
Acetim. [$Hse^{65,80}$,Met^{81}] cyt. c	0.95(15%)	1.8 (22%)

Table II. Depleted Mitochondria (Preparation II) Assay with Various Cytochrome *c* Derivatives

Derivative	Initial velocity	V_5 nmoles/ml
Resynthesised acetim[Hse^{65}] cyt. c	20.0	16.3
Acetim[$FPhe^{82}$] cyt. c	4.4 (22%)	4.8 (29%)
Acetim[Lys^{81}] cyt. c	3.6 (18%)	4.6 (28%)
Acetim[$Hse^{65,80}$,Met^{81}] cyt. c	1.65(8%)	3.9 (23%)
Acetim Fragment A	0.15(<1%)	0.7 (4%)

Conclusions

The semisynthetic method is capable of yielding a range of cytochrome *c* analogs in quantities sufficient for study of their properties in a biological system. The semisynthetic [Hse^{65}] analog shows similar biological properties to the natural analog made by the method of Corradin and Harbury.[5]

All other analogs show a reduced ability to stimulate oxygen uptake in mitochondria. Definite conclusions on the reason in each case must await determinations of activity in subsets of the electron transport system and checks on redox potentials and the integrity of tertiary structure.

Tentatively, though, I would suggest that the low activity and loss of low-spin spectral characteristics in the [$Hse^{65,79a}$] and [$Hse^{65,80}$,Met^{81}] analogs imply that displacement of the normal sixth ligand by one residue prevents co-ordination of methionine sulphur to iron. Examination of the 3-dimensional structure of cytochrome *c* shows that

attempts to achieve such co-ordination are likely to put severe strain on the structure.

The reduced activity of the [Hse^{65},FPhe^{82}] analog may indicate the importance of the non-polar bonding to the haem pocket and in particular that of this invariant residue. Similarly, the effect of ϵ-Cbz-Lys at residue 81 (normally Ile or Val) may be due to the polarity of the urethane linkage, or the bulky aromatic group, preventing appropriate hydrophobic interaction at the haem edge, although it is possible that the bulk of the substituent reduces the ease of binding of oxidase or reductase. It should again be stressed that these suggested explanations for the behaviour of the analogs can at this stage only be tentative.

The low activity of analogs parallels the low coupling yields obtained at the final stage of the semisynthesis. This implies that coupling is as dependent on the assumption of the normal conformation of the components of the complex, as has been suggested,[5] as activity is on the precise native conformation of the semi-synthetic protein.

Acknowledgement

I thank the Medical Research Council for financial support.

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SEMISYNTHETIC ANALOGS OF CYTOCHROME *C* PREPARED BY THE NONCOVALENT ASSOCIATION OF MODIFIED TRYPTIC FRAGMENTS

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Introduction

Eukaryotic cytochromes *c* are electron transfer proteins which act as components of the membrane-associated mitochondrial respiratory chain. They are small, basic proteins consisting of a single polypeptide chain wrapped around a covalently attached haem group.[1] The products of the tryptic digestion of acetimidylated horse heart cytochrome *c* (peptide H1-38, corresponding to residues 1-38 with a covalently attached haem and the peptide corresponding to residues 39-104) may be recombined to form a biologically active noncovalent complex exhibiting physical properties similar to those of the native protein.[2,3] Several workers have described the properties of the complex formed between the unprotected peptides (H1-38) and (39-104)[4,5] and similar noncovalent complexes have been obtained by the association of other fragments derived from cytochrome *c*.[6,7]

The association of modified tryptic fragments has been used to prepare semisynthetic analogs of cytochrome *c*.[2,3] This paper reports the properties of the complex formed between N^{ϵ}-acetimidyl-(H1-37) and N^{ϵ}-acetimidyl-(38-104).

Methods

The fragments of acetimidylated cytochrome *c* (haem-containing peptides N^{ϵ}-acetimidyl-(H1-38) and N^{ϵ}-acetimidyl-(H1-37) and non-haem peptides N^{ϵ}-acetimidyl-(39-104) and N^{ϵ}-acetimidyl-(40-104)) were prepared as described.[2,3]

N^{ϵ}-Acetimidyl-(38-104) was prepared by coupling N^{α}-Boc-L-arginine to N^{ϵ}-acetimidyl-(39-104). The product of the coupling was

deprotected with anhydrous trifluoroacetic acid and the desired peptide was purified by cation exchange chromatography.

Noncovalent complexes (Figure 1) were prepared by mixing haem-containing and nonhaem peptides in a 1:1 molar ratio and were characterized without purification.

The ability of cytochrome *c* analogs to restore succinate oxidation to cytochrome *c*-depleted mitochondria[8] was determined as described.[2]

Results

The tryptic fragments and modified peptides used in this work were homogenous by cation exchange chromatography and had the expected amino acid compositions and free amino groups (determined by dansylation).

The noncovalent complexes (Figure 1) exhibited uv and visible absorption spectra that were essentially identical to those of native cytochrome *c*. The ability of these cytochrome *c* analogs to restore succinate oxidation to cytochrome *c*-depleted mitochondria is shown in Figure 1.

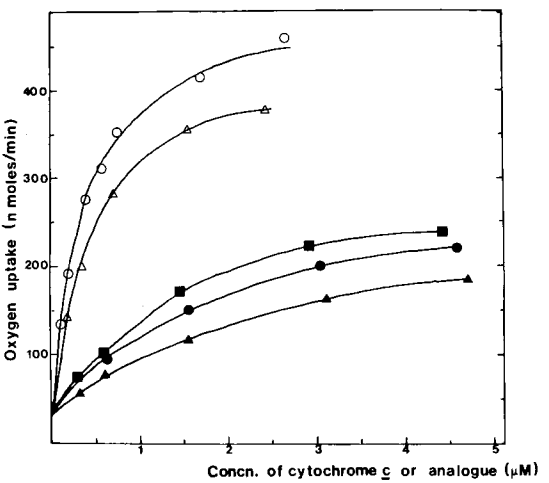

Fig. 1. Restoration of succinate oxidation in cytochrome *c*-depleted mitochondria by o, cytochrome *c*; △, N^{ϵ}-acetimidyl-(H1-37) (38-104); ■, N^{ϵ}-acetimidyl-(H1-38) (39-104); ●, N^{ϵ}-acetimidyl-(H1-37) (39-104) and ▲, N^{ϵ}-acetimidyl-(H1-37) (40-104). The assays were performed in a total volume of 5.1ml at 25°C as described.[2]

Discussion

The association of modified tryptic fragments of acetimidylated cytochrome *c* has been used to prepare several analogs of the protein modified at positions 38 and 39.[2,3] The noncovalent complex N^{ϵ}-acetimidyl-(H1-38) (39-104) and complexes in which residues at the break in the polypeptide chain have been removed (N^{ϵ}-acetimidyl-(H1-38) (40-104), N^{ϵ}-acetimidyl-(H1-37) (39-104) and N^{ϵ}-acetimidyl-(H1-37) (40-104)) exhibit similar physical properties to those of intact cytochrome *c*

and thus have similar structures[2,3] (and see[5]). Their biological activities are lower than that of cytochrome *c* (Figure 1[2]) and this may be the result of the flexibility of the polypeptide chain in the region of the cleaved peptide bond.[4]

The analog of cytochrome *c* in which the polypeptide chain is broken between residues 37 and 38 (prepared by the combination of *N*ϵ-acetimidyl-(H1-37) and N^{ϵ}-acetimidyl-(38-104)) was found to have a higher biological activity than N^{ϵ}-acetimidyl-(H1-38) (39-104) (Figure 1). An intact peptide bond between residues 38 and 39 thus appears to be more important for the biological activity of cytochrome *c* than an intact bond between residues 37 and 38. In intact cytochrome *c* there is a hydrogen bond between the amide nitrogen atom of Trp-59 and the carbonyl oxygen of Arg-38[9] (both of these residues are invariant in eukaryotic cytochromes *c).* If this hydrogen bond is present in the complex N^{ϵ}-acetimidyl-(H1-37) (38-104), it will reduce the flexibility of the polypeptide chain in the N-terminal region of the nonhaem peptide resulting in a complex that closely resembles the native protein. The combination of haem-containing peptide (H1-37) with analogs of (38-104) thus provides a route to the production of semisynthetic analogs of cytochrome *c* for studying the role of residue 38 in the structure and activity of the protein.

Acknowledgements

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SEMISYNTHESIS OF HUMAN INSULIN: TRYPSIN-CATALYZED REPLACEMENT OF ALANINE B30 BY THREONINE IN PORCINE INSULIN

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Introduction

Human insulin differs from porcine insulin by a single amino acid (Thr instead of Ala) at the C-terminal residue of the B-chain. Inouye et al.[1] have established a procedure for semisynthesis of human insulin, in which trypsin is used as a catalyst for the coupling of desoctapeptide-(B23-B30)-insulin with a synthetic octapeptide corresponding to positions B23-B30 of human insulin. The enzymatic method has many advantages over chemical methods,[2,3] especially better yields and simple operation. Here we report an alternative route for the semisynthesis, in which trypsin can catalyze the replacement of alanine-B30 by threonine in porcine insulin.

Results

Desalanine-B30-insulin (DAI) was obtained by digestion of porcine insulin with carboxypeptidase A in the presence of 0.1 M NH_4HCO_3[4]. DAI, so obtained, was treated with 1,2-cyclohexanedione to produce [1,2-dihydroxycyclohex-1,2-ylene arginine (DHCH-Arg)]-DAI according to the method of Patthy and Smith.[5] Trypsin-catalyzed coupling of [DHCH-Arg-B22]-DAI and Thr-OBu^t was quantitatively determined as follows. After the reaction mixture was treated with 1 M NH_2OH (pH 7) for one night at 28° C, the ratio of [Thr-OBu^t-B30]-insulin produced to the remaining DAI was determined using with a reverse-phase LC system based on their peak areas and intensity factors (for conditions, see Figure 1). A preliminary experiment indicated that

coupling is best performed with a large excess of the amine component (Thr-OBu^t) in the presence of high concentrations of organic cosolvents. Their significance in coupling has been described in previous papers.[1,6,7]

Preparative-scale coupling was done as follows. To a 2.1 ml solution of [DHCH-Arg-B22]-DAI (105 mg, 5 mM) and Thr-OBu^t (395 mg, 0.5 M) in a mixture of dimethylformamide and ethanol (1/1 by volume) was added 1.4 ml of 0.5 M borate buffer containing TPCK-trypsin (8.2 mg, 0.1 mM), final pH 6.5, and the reaction mixture was kept at 37° C for one night. LC showed that 71% of the [DHCH-Arg-B22]-DAI had been converted to the desired product. The reaction mixture was treated over night at 28° C with 1 M NH_2OH (pH 7.0), applied to a column (3.2 x 41 cm) of Sephadex G50 (super fine), and eluted with 0.5 M acetic acid. The fraction corresponding to insulin or its derivatives was lyophilized (87 mg) and loaded on to a column of DEAE-Sephadex A25 (2 x 20 cm) which had been equilibrated with 0.01 M Tris buffer (pH 7.4) and 7 M urea. A linear Na^+ gradient (to 0.3 M NaCl) was begun at 4° C according to the method of Bromer and Chance.[8] The first peak (43 mg) corresponded to [Thr-OBu^t-B30]-insulin (Figures 1, 2) and the second one (25 mg) to DAI and/or insulin (Figures 1 and/or 2).

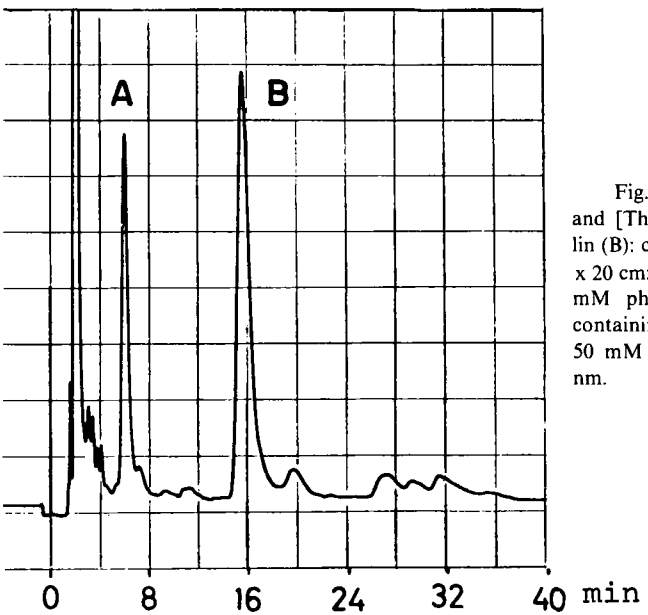

Fig. 1. LC of porcine DAI (A) and [Thr-OBu^t-B30]-porcine insulin (B): column, Nucleosil 5 C_{18}, 0.4 x 20 cm; eluant, 30.5% CH_3CN in 5 mM phosphate buffer (pH 3.0) containing 5 mM n-$BuSO_3Na$ and 50 mM Na_2SO_4; detection at 220 nm.

[Thr-OBu^t-B30]-insulin was treated for deprotection with trifluoroacetic acid in the presence of anisole. The yield was 50% (41 mg) based on the assumption that the purity of DAI used as the starting material was 78%, which had been deduced from its amino acid composition. This semisynthetic human insulin was homogenous according to LC[1] and polyacrylamide gel electrophoresis (Figure 2). Amino acid analysis (theoretical values in parentheses) gave: Lys 1.00 (1), His 1.91 (2), Arg 0.95 (1), Asp 3.21 (3), Thr 2.94 (3), Ser 2.97 (3), Glu 7.35 (7), Pro 1.23 (1), Gly 4.29 (4), Ala 1.26 (1), $CySO_3H$ 5.94 (6), Val 3.86 (4), Ile 1.55 (2), Leu 6.53 (6), Tyr 4.08 (4), and Phe 3.22 (3). In the bioassay for hypoglycemic activity, no significant difference was observed between the semisynthetic human insulin and bovine insulin.

A similar preparative scale experiment was done using DAI prepared from bovine insulin under the conditions described above ([E] = 0.3 mM). LC showed that 77% of [DHCH-Arg-B22]-DAI had been converted into the product. The product was treated as above, and pure semisynthetic [Thr-B30]-bovine insulin was obtained in 51% yield.

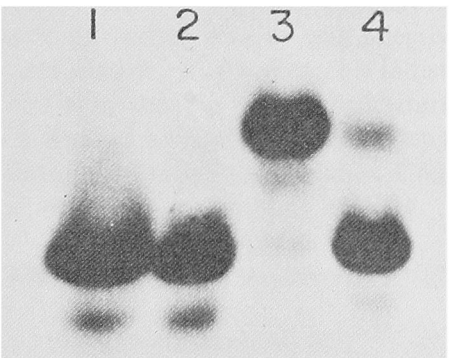

Fig. 2. Polyacrylamide gel electrophoresis of porcine insulins (pH 8.0, 10% gel): 1, native insulin; 2, DAI; 3, [Thr-OBu^t-B30]-insulin; 4, semisynthetic human insulin.

Conclusion

Enzyme catalysis is significant in the semisynthesis of biologically active products, (when) either one or both component(s), starting from natural material(s), is(are) of considerably high molecular weight. Functional groups in high molecular weight starting materials must not be blocked for enzymatic methods but for chemical ones.

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NH_2-TERMINAL MYOGLOBIN SEMISYNTHESIS

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The structural significance of the NH_2-terminal residues of myoglobin is suggested by their highly conserved nature[1] and striking similarity with the physiologically important hemoglobin NH_2-terminals.[2] Experimental assessment of their role can be best obtained through studies involving semisynthetic variants. The production of these myoglobin derivatives is dependent on the ability to selectively and reversibly protect the ϵ-amino groups, to remove nondestructively the first residue, and to reincorporate the desired amino acid. Having achieved the synthesis and isolation of N^{ϵ}_{19}-acetimidomyoglobin,[3] in sizable yield, a chemical procedure was developed in which the NH_2-terminal valine residue was selectively removed and reincorporated to yield a semisynthetic molecule indistinguishable from the virgin protein.[4] The shortcoming of this procedure lies in the diminished number of derivatives which can be made due to the low synthetic yield of the des-Val[1], N^{ϵ}_{19}-acetimidomyoglobin. Consequently this study was undertaken in an effort to maximize its yield.

Treatment of Acetimidomyoglobin with Isothiocyanates

In order to prevent binding of the isothiocyanates to the ferri heme all couplings were conducted after heme removal or conversion to the carboxyferro derivative. Coupling was accomplished under various conditions by the following general procedure. A 2% protein solution was equilibrated at the desired temperature and its pH adjusted to the reaction pH with 0.1*N* NaOH. Varying amounts of isothiocyanate were added with stirring under N_2 and reacted for a specified time interval. Quantitation of reaction was effected through automated NH_2-terminal sequence analysis and/or comparison of electrophoretic profiles before and after coupling.[3,4] Denaturing effects of the isothiocyanates upon the secondary structure of the protein were assessed through circular dichroism measurements before and after reaction.[4]

Optimization of Isothiocyanate Coupling

Attempts to achieve quantitative coupling of isothiocyanate to a protein such as myoglobin which is susceptible to irreversible denaturation in nonaqueous media[5] makes the choice of reagent crucial. Whereas the use of PhNCS was prohibited by its limited solubility in water and aqueous methanol mixtures,[6] 3-sulfo-PhNCS and CH_3NCS were found to be suitable. Although quantitative coupling to the NH_2-terminal of N^{ϵ}_{19}-acetimidoapomyoglobin was obtainable through reaction with two 20-fold molar excess additions of 3-sulfo-PhNCS, the inability to reconstitute the holoprotein in high yield after CF_3COOH cleavage and heme reincorporation[4] led us to investigate other coupling possibilities. Increasing excesses of 3-sulfo-PhNCS were found to lead to increased reductions in the molar ellipticity and yields of synthetic renaturable protein, whereas CH_3NCS treatment was found to be considerably less perturbing (Figure 1). Consequently all coupling conditions were evaluated with respect to extent of reaction and minimization of denaturation.

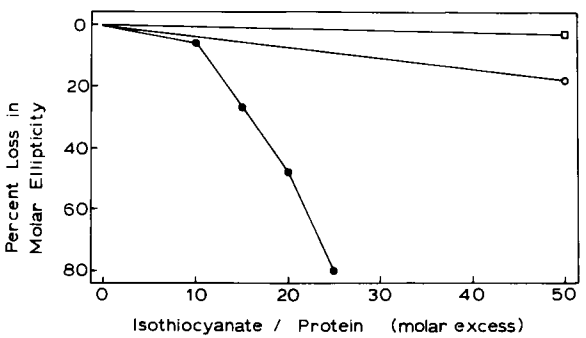

Fig. 1. Percent loss in molar ellipticity at 210 nm versus molar ratio of isothiocyanate to protein. —●—, 3-sulfo-PhNCS/acetimidoapomyoglobin; —O—, CH_3NCS/acetimidoapomyoglobin; —□—, CH_3NCS/(CO-Fe^{+2})-acetimidomyoglobin.

As shown in Table I 3-sulfo-PhNCS proved to be considerably more reactive than CH_3NCS. Although the CH_3NCS couplings proceeded with minimal secondary effects (Figure 1) and responded with greater reactivity to increases in molar excess, pH, and temperature of reaction (Table I), no one set of conditions yielded greater than 85% coupling. Since less than quantitative reaction undesirably necessitates masking of the unreacted protein or its removal prior to CF_3COOH-cleavage, our

Table I. NH_2-Terminal Coupling Yield of the Reaction of Isothiocyanates with N^{ϵ}_{19}-Acetimidomyoglobins

	% Coupling Yield
Reactions of Acetimidoapomyoglobin	
with CH_3NCS	
pH 6.0, 35°, 50-fold excess, 3h	34
pH 7.0, 35°, 50-fold excess, 3h	85
with 3-sulfo-PhNCS	
pH 6.0, 35°, 20-fold excess, 2h,	92
additional 20-fold excess, 2h	96
Reactions of Carboxyferroacetimidomyoglobin	
with CH_3NCS	
pH 7.0, 35°, 25-fold excess, 2h	50
pH 8.0, 35°, 25-fold excess, 2h	59
pH 8.0, 35°, 50-fold excess, 2h	67
pH 8.0, 45°, 50-fold excess, 2h	78
pH 8.0, 35°, 30-fold excess, 1.5h	
additional 20-fold excess, 1.5h	84
with 3-sulfo-PhNCS	
pH 8.0, 45°, 5-fold excess, 2h	100
pH 8.0, 35°, 10-fold excess, 2h	100
pH 8.0, 25°, 5-fold excess, 2h	100

attention was focused upon the 3-sulfo-PhNCS. Where the minimal molar excess of 3-sulfo-PhNCS required for quantitative reaction with the acetimidoapomyoglobin caused extensive denaturation (Table I, Figure 1), coupling to the holoprotein under milder conditions (Table I) was quantitative and structurally unperturbing. This results from the highly increased solubility and stability of the holoprotein in the pH range 7.0-9.0 as compared to the apoprotein. Clearly as the reaction pH is lowered from that of the α-amino pK of ~7.2 the amount of reagent necessary to achieve quantitative coupling rises sharply.

Prior to removal of the 3-sulfo-PhNHCS-valine residue in CF_3COOH it was necessary to remove the heme. Where all attempts at removal in the carboxyferro-form were unsuccessful, specific oxidation of the heme iron with ferricyanide followed by addition of sodium fluoride to a concentration of 10 mM and extraction with butanone was highly effective. Immediate lyophilization subsequent to CF_3COOH-cleavage, reincorporation of the heme and purification produced the des-Val^1, N^{ϵ}_{19}-acetimidomyoglobin in 65% yield.

Semisynthesis of myoglobin through derivatization of the apoprotein has met with limited success.[7] Our experience with isothiocyanate couplings to the acetimidoapomyoglobin has revealed a much increased susceptibility to denaturation and aggregation making purification extremely difficult. Consequently, retention of the heme in all permissible steps simplifies the task of synthesis.

Acknowledgements

This work was supported by PHS Grant HL-14680. This is the 113th paper in a series dealing with coordination complexes and catalytic properties of proteins and related substances.

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APPROACH TO THE SEMISYNTHESIS OF THE BOVINE TRYPSIN-KALLIKREIN INHIBITOR (KUNITZ)

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Introduction

The trypsin-kallikrein inhibitor isolated from bovine organs[1] (BTI) is a single chain protein of 58 amino acid residues containing three disulfide bridges. The amino acid sequence of BTI (Figure 1) has been elucidated[2] and the three dimensional structure has been determined to high resolution by X-ray crystallography.[3] The fully reduced inhibitor, which is virtually inactive, may be refolded by air oxidation with nearly complete recovery of the inhibiting capacity.[4]

Chemical syntheses of the inhibitor have been achieved by fragment condensation on a polymer support,[5] solid phase fragment coupling[6] and solid phase synthesis.[7] Intriguing semisyntheses of analogs of BTI have also been carried out.

Dyckes et al.[8] described the preparation of a fully active 52-homoserine-BTI and Jering and Tschesche[9] succeeded in the enzymatic replacement of lysine 15 by arginine, phenylalanine, and tryptophan in the inhibitor reactive site.

This paper reports attempts to achieve partial synthesis of analogs of the BTI molecule.

Results and Discussion

The present work was stimulated by the finding of Jering and Tschesche[10] who succeeded in preparing the active derivative of BTI in which the reactive site peptide bond Lys 15-Ala 16 is cleaved (BTI*). Bearing in mind that guanidination of the four ϵ-amino groups of lysine residues and subsequent removal of the amino-terminal Arg-Pro-Asp sequence does not affect the BTI inhibitory capacity,[11] a scheme was

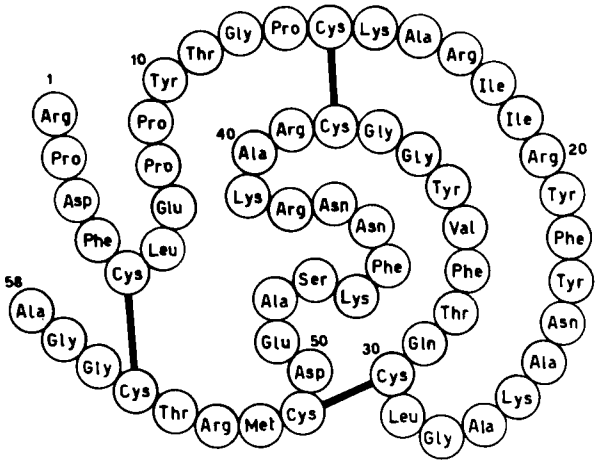

Fig. 1. Primary structure of BTI

designed for the preparation of a semisynthetic des(1-3)-inhibitor in which three out of four lysine residues are replaced with homoarginine residues. The starting material in our semisynthetic approach is the active site-cleaved inhibitor.[10]

Preliminary experiments indicated that the guanidinated virgin inhibitor (4-Guan-BTI)[11] may be reduced with sodium borohydride, in 8 M urea. Air oxidation of the reduced 4-Guan-BTI (5.8±0.15 thiol groups per molecule) in 0.02 M sodium phosphate buffer, pH 8, gave a renatured protein possessing more than 90% of the native BTI inhibitory activity. The reaction of BTI* with O-methyl isourea (pH 10.5, 6 days at 5°, 250-fold molar excess with respect to each amino group) is accompanied by total loss of inhibitory activity against trypsin and converted essentially all lysine residues to homoarginine residues yielding the tetra-guanidinated modified inhibitor (4-Guan-BTI*). The α-amino functions of the N-terminal arginine and of the alanine residue in position 16 should not be significantly affected by the modification reaction.

4-Guan-BTI* consists of two peptide chains corresponding to the sequences 1-15 (T-peptide) and 16-58 (T-protein) of BTI in which lysine residues are replaced by homoarginine residues.

The two peptide chains are linked through the disulfide bridges Cys 5-Cys 55 and Cys 14-Cys 38. The α-amino groups of Arg 1 and Ala 16 remain the only accessible sites of coupling with a carboxyl-activated suitably protected peptide. Spectroscopic data[12] support the idea, which is also evident from the X-ray crystallographic model,[3] that in the

modified inhibitor the chain from Ala 16 to Arg 20 lies on the surface of the molecule and is fully exposed to the solvent.

The synthesis, in solution, of the protected dodecapeptide hydrazide corresponding to the sequence 4-15 of BTI, proceeded via coupling three subunits corresponding to the sequences 4-6, 7-11, and 12-15 employing the azide procedure. Classical carboxyl activation, carboxyl and amino protection and deblocking procedures were used during the synthesis.

Preliminary coupling experiments have been carried out by reacting, through the azide procedure, the tetrapeptide acetyl-Gly-Pro-Cys(Acm)-Lys(Tfa)-N_2H_3, corresponding to the sequence 12-15 of BTI, with 4-Guan-BTI* in 0.05 M sodium borate buffer, pH 8.5. The amino acid composition of the isolated material indicated that both the α-amino group of Arg 1 and Ala 16 have been, at least partially, acylated. This result supports the idea behind the approach used for the semisynthesis of BTI analogs.

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FORMATION OF ION-TRANSPORTING CHANNELS BY ANALOGS OF GRAMICIDIN A

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Introduction

The cation-permeable channel formed by the linear pentadecapeptide gramicidin A is one of the best-studied model channels (for a recent survey of the literature, see Bamberg *at al.*[1]). Gramicidin A is a neutral peptide with the structure HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2$ CH_2OH. According to Urry[2,3] the channel is formed by head-to-head (formyl end to formyl end) association of two gramicidin monomers. In Urry's model the channel is represented by a β-helix with the hydrophobic amino-acid residues lying on the periphery and with a 0.4 nm wide hydrophilic pore running along the axis of the helix. Experiments with chemical derivatives of gramicidin A strongly support the head-to-head model and virtually exclude other dimeric structures, such as double-stranded helices and tail-to-tail or head-to-tail dimers.[4–6]

Chemical derivatives of gramicidin A offer the possibility of studying the relationship between peptide structure and properties the ion-transporting channel.[7–11] In this communication we describe two kinds of chemical analogs of gramicidin A: a series of derivatives in which the amino-terminal formyl group has been replaced by $-CO(CH_2)_n$ COOH, and gramicidin analogs with modified amino-acid sequences.

Dicarboxylic-Acid Analogs of Gramicidin A

According to Urry's model, the two halves of the dimer are

connected by six hydrogen bonds, two of which are provided by the terminal formyl groups. Consistent with this view is the finding that desformyl-gramicidin is virtually inactive[2] and that *N*-acetyl gramicidin forms channels with greatly reduced lifetime.[11] In that N-acetyl derivative the replacement of the hydrogen atom in the formyl group by the bulkier methyl group apparently interferes with hydrogen-bond formation. In view of these findings chemical modifications at the coupling site of the two half-channels seem particularly valuable for a more detailed understanding of the channel formation process. For this reason a series of gramicidin analogs was prepared in which the N-terminal -CHO group is replaced by $-CO-(CH_2)_n-COOH$. These dicarboxylic-acid derivatives offer additional possibilities for hydrogen bond formation. It has already been shown that the succinyl derivative (n = 2) is able to form conducting channels.[10]

The dicarboxylic-acid derivatives were prepared from commercial gramicidin.[6] For the study of the membrane activity of these compounds black films[12] were formed from synthetic lipids, such as dioleoyllecithin, in aqueous phase. In the presence of small amounts of gramicidin the electrical conductance of the black film shows discrete, stepwise fluctuations which result from the formation and the disappearance of single gramicidin channels.[13]

Similar fluctuations were observed with the dicarboxylic derivatives of gramicidin at $pH < 5$ where the carboxyl group is predominantly in the protonated form. From records of such conductance fluctuations the conductance and the mean lifetime of the channel could be obtained.

In Table I the dependence of channel conductance Λ on NaCl concentration is shown for the different analogs and is compared with previous results for gramicidin A. All analogs have a lower channel conductance than gramicidin A, the reduction in Λ being largest for n = 2.

In order to study the ion selectivity of the different analogs, the single channel conductance was measured in 1 M solutions of NaCl, KCl and CsCl. As seen from Table I, there is a pronounced and nonmonotonous influence of chain length of the carboxylic-acid residue on ion selectivity. Whereas gramicidin A has a rather low ion selectivity, substitution of the N-terminal -CHO group by $-CO-(CH_2)_3-COOH$ or $-CO-(CH_2)_4-COOH$ results in a threefold increase in the Cs^+/Na^+ selectivity ratio.

These experiments have shown that the N-terminal formyl groups of gramicidin A may be replaced by carboxylic-acid residues of different chain-lengths without impairing the ability to form ion channels. The replacement of the -CHO group at the N-terminus by a $-CO(CH_2)_n$

Table I. Ion Selectivity of the Dicarboxylic Acid Analogs of Gramicidin A.

compound	$\frac{\Lambda(NaCl)}{pS}$	$\frac{\Lambda(KCl)}{\Lambda(NaCl)}$	$\frac{\Lambda(CsCl)}{\Lambda(NaCl)}$
gramicidin A (G)	25	2.0	3.6
N-succinyl-G (n=2)	2.8	2.2	4.5
N-glutaryl-G (n=3)	3	3	10
N-adipyl-G (n=4)	3.8	1.6	10.5
N-pimelyl-G (n=5)	~9	1.2	2
O-pyromellityl-G	18	1.9	4.5

Membranes made from monoolein/n-hexadecane. The amino-terminal -CHO group of gramicidin A has been replaced by $-CO(CH_2)_nCOOH$. Λ is the single-channel conductance measured at a salt concentration of 1 M. The temperature was 25°C, the pH was maintained at 4.5 with 10^{-4}M citric acid. The applied voltage was 100mV. The data for gramicidin A were taken from Ref. 8 and the data for O-pyromellitylgramicidin from Ref. 14.

COOH residue offers additional possibilities for the formation of hydrogen bonds. From inspection of molecular models two different head-to-head connected dimeric structures seem feasible. In the first structure, the $-CO(CH_2)_nCOOH$ residue is continuous with the peptide chain in the helix and increases the length of the helix by about one fourth of a turn (for n = 2 and n = 3). In this case the carbonyl oxygen of the -COOH group accepts an intramolecular hydrogen bond from the amide group of residue 5 (D-Val), whereas the intermolecular hydrogen bonds are 4 → CO′, 2 → 2′, COOH → 4′ (CO stands for the carbonyl group which is linked to the terminal amino group). Thus, the same number (six) of intermolecular hydrogen bonds are formed as in the head-to-head associated of gramicidin A (6 → CO′, 4 → 2′, 2 → 4′). Although this model seems feasible for n = 2 and n = 3, it leads to considerable distortion of the helical structure for $n \geq 4$. Furthermore, the model cannot explain the much higher stability of the dimer formed by the analog with n = 3 as compared with n = 2.[6]

The second possibility consists in a head-to-head associated dimer with the same hydrogen-bond structure as in the gramicidin A channel, but with the two $-(CH_2)_nCOOH$ residues lying outside the helix. In Urry's model of the gramicidin A channel, the two terminal -CHO groups are located adjacent to each other and the C-H bonds of the -CHO groups are directed outward from the helix. If these two hydrogen atoms are

replaced by $-(CH_2)_nCOOH$, then the two $-(CH_2)_nCOOH$ residues lie side by side and protrude outward from the helix. It can easily be seen from molecular models that under these conditions conformations of the alkyl chains in the $-(CH_2)_nCOOH$ residues exist such that the two carboxyl groups may form two hydrogen bonds with each other for $n \geq 3$. On the other hand, only one hydrogen bond seems possible for $n = 2$. We therefore propose that the long channel-lifetime of the analog with $n = 3$ results from the formation of a pair of H-bonds between the carboxyl residues. Consistent with this interpretation is the finding that the methyl ester of the analog has a much lower channel stability.[6]

Analogs with Modified Amino-Acid Sequence

Gramicidin A contains five different amino acids (valine, glycine, alanine, leucine, tryptophan). It was an open question whether the particular amino-acid sequence of gramicidin A is necessary for membrane activity or whether analogs with modified amino acid composition are also able to form ion channels. For this purpose the following two gramicidin analogs were synthetized:

$HCO\text{-}NH\text{-}L\text{-}Trp\text{-}Gly\text{-}(L\text{-}Trp\text{-}D\text{-}Leu)_6\text{-}L\text{-}Trp\text{-}CO\text{-}NHCH_2CH_2OH$
(gramicidin A-1)

$HCO\text{-}NH\text{-}(L\text{-}Trp\text{-}D\text{-}Leu)_7\text{-}L\text{-}Trp\text{-}CO\text{-}NHCH_2CH_2OH$
(gramicidin A-2).

Gramicidin A-2 contains only tryptophan and leucine as amino acids, in gramicidin A-1 one residue of D-leucine is replaced by glycine. The main reason for choosing alternating sequences of aromatic and aliphatic amino acids instead of a sequence, such as $(L\text{-}Leu\text{-}D\text{-}Leu)_n$, was the expected better solubility of analogs with aromatic side chains. The glycine-containing peptide was studied since glycine, present in all natural analogs of the linear gramicidins, may play a special role in the channel forming process.

It was found that both analogs are able to form cation selective channels in artificial bilayer membranes. The conductance fluctuations observed at low peptide concentration have a similar appearance as those observed with gramicidin A but have a somewhat smaller amplitude. The mean lifetime of the gramicidin A-1 channel is about half the value found for gramicidin under comparable conditions.

Measurements of the membrane potential in the presence of a salt concentration difference across the membrane show that the channels formed by gramicidins A-1 and A-2 are virtually impermeable to Cl^- ions.

The channel conductances Λ measured in alkali chloride solutions (and in NH_4Cl) may therefore be used to determine the specificity sequence for monovalent cations.

Table II. Single-Channel Conductance Λ of Gramicidin A-1.

Ion	Λ	$\frac{\Lambda(A-1)}{\Lambda(A)}$	$\left(\frac{\Lambda_i}{\Lambda_K}\right)_{A-1}$	$\left(\frac{\Lambda_i}{\Lambda_K}\right)_{A}$
Li^+	4.6	0.71	0.36	0.12
Na^+	5.4	0.22	0.42	0.56
K^+	13	0.26	1.0	1.0
Rb^+	30	-	2.3	1.6
Cs^+	40	0.44	3.1	1.6
NH_4^+	43	-	3.4	1.9

1 M solutions of different monovalent cations. Glycerylmonooleate/n-hexadecane membranes, T = 25°C. The applied voltage was 100 mV. $\Lambda(A-1)/\Lambda(A)$ is the ratio of the channel conductances of gramicidin A-1 and A, measured under identical conditions. The values of $\Lambda(A)$ for Na^+ K^+, Cs^+ have been taken from Ref. 8 (Λ_i/Λ_K) is the specificity ratio, referred to K^+; the values of (Λ_i/Λ_K) (gramicidin A) have been taken from data obtained with 0.5 M salt solutions.[13]

In Table II the specificity of the gramicidin A-1 channel (referred to K^+) is given as the ratio Λ_i/Λ_K; for comparison, data for gramicidin A are also shown in Table II. It is seen that for all cations studied so far the gramicidin A-1 channel has a lower conductance than the gramicidin A channel; moreover, the specificity ratio Λ_i/Λ_K is different for both channels.

To a first approximation, the conductance of a channel is proportional to the product of the probability that the channel is occupied by an ion, times the transport rate of the ion in the channel. Both the probability of occupancy and the transport rate depend on the shape of the potential-energy profile of the ion inside the channel, which in turn is determined by the interaction energy of the ion with the ligand groups (carbonyl groups of the peptide). Based on conformational energy calculations, Urry[2] has proposed that the ligand system of the channel accommodates to some extent to the size of the permeating ion. According to Urry[2] the inner diameter of the channel is about 0.4 nm. The extent to which the channel may contract locally to accommodate e.g. a Na^+ ion with a diameter of 0.2 nm depends on the flexibility of the channel

structure. A possible explanation for the reduced conductance of the channels formed by gramicidins A-1 and A-2 therefore consists in the assumption that the bulky tryptophan residues make the channel more rigid. The effect of a rigid channel-structure on the conductance should be the larger the greater the difference between ion diameter and channel diameter is. In fact, the ratio $\Lambda(A\text{-}1)/\Lambda(A)$ decreases with decreasing ion diameter $2r_+$ from Cs^+ ($2r_+$ = 0.34 nm) to Na^+ ($2r_+$ = 0.20 nm). The behaviour of the Li^+ ion, however, cannot be explained by this hypothesis. Possibly the lithium ion with a diameter of only 0.16 nm retains to a greater extent its hydration shell when it enters the channel. Another factor which may influence the permeation rate of ions in the channel is the dipolar potential of the amino-acid residues. Thus, the dipolar moments of the tryptophyl residues may influence the electrostatic potential inside the channel, thereby changing the energy required to transfer an ion from water to the channel. While these considerations are necessarily speculative, they nevertheless suggest further possibilities for studies on the relationship between structure and transport properties of ion channels.

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CONFORMATION OF THE GRAMICIDIN A CHANNEL IN LIPID VESICLES: A ^{13}C AND ^{19}F NUCLEAR MAGNETIC RESONANCE STUDY

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Introduction

Gramicidin A is a linear pentadecapeptide antibiotic which facilitates the diffusion of small monovalent cations across membranes by forming dimer transmembrane channels. Because there is evidence that the gramicidin in phosphatidylcholine vesicles is in the channel conformation,[1,2] spectroscopic studies on gramicidins in this system have yielded the first detailed conformational information on an ion-selective transmembrane channel.

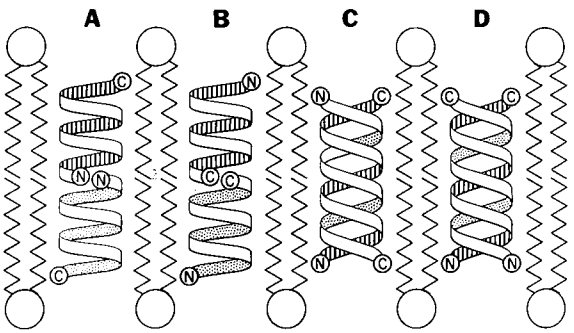

Fig. 1. Proposed Models of the Gramicidin Channel

Four classes of models have been proposed for the conformation of the dimer gramicidin transmembrane channel (Figure 1). Model A is the N-terminal to N-terminal II_6(L,D) helical dimer model proposed by Urry *et al.*[3,4] More recently, Bradley *et al.*[5] have considered the possibility of forming a C-terminal to C-terminal dimer (Figure 1B) of their original II_6(L,D) helix. Model C is the antiparallel-β double helix proposed by Veatch *et al.*[6] as one of the dimer conformations found for gramicidin in

nonpolar organic solvents.[6,7] Model D is a parallel-β double-helix with both N-termini at one end of the channel. A distinctive feature of both double-helical models is the presence of *both* the N- and C-termini at the surfaces of the membrane (Figure 1C,D). For the N-terminal to N-terminal dimer (Figure 1A) *only* the C-termini are on the surfaces, and for the C-terminal to C-terminal dimer (Figure 1B) *only* the N-termini are on the surface.

We have incorporated specific ^{13}C and ^{19}F nuclei at both the N- and C-termini and have carried out nmr experiments to determine the relative accessibility of these ^{13}C and ^{19}F nuclei to paramagnetic nmr probes. Two of these probes are localized in the aqueous solution—manganous ion (Mn^{++}) and thulium ion (Tm^{+++})—and the third is localized in the membrane interior—a nitroxide spin label covalently attached near the end of a stearic acid chain of a phosphatidylcholine molecule. We have measured the upfield change in chemical shift induced by Tm^{+++} and the enhancement in spin-lattice relaxation rate ($1/T_1$-$1/T_{1o}$) for the other paramagnetic probes. These effects are expected to be relatively short range (~5A). ^{13}C was incorporated at the N-terminus with no chemical change in ^{13}C(formyl-Val_1)-des(formyl-Val_1)-gramicidin,O-acetyl-(^{13}C-methyl). The ^{19}F analog was (formyl-^{19}F-Phe_1)-des(formyl-Val_1)-gramicidin, O-^{19}F-benzoyl.

Results and Discussion

Table I summarizes the results of these experiments. For each nmr probe the effects on the dimyristoylphosphatidylcholine carbons provide a reference frame for viewing the gramicidin results:

(1) The water-soluble thulium ion causes a large change in chemical shift for the choline methyls, but little or no change for the fatty acid carbons even near the carbonyl end. The ratio of gramicidin C-terminal label change to N-terminal label change is 3.5 for ^{13}C and 10 for ^{19}F.

(2) The water-soluble manganous ion induces a T_1 rate enhancement for the fatty acid carbonyl that is no less than that for the choline methyls; while carbons 2 and 3 also have large rate enhancements, low values are obtained at the distal end of the chain. The ratio of gramicidin C-terminal rate enhancement to N-terminal rate enhancement is 3-4 for ^{13}C and 20 for ^{19}F.

(3) Phosphatidylcholine labeled with a nitroxide spin label near the distal end of one chain induces T_1 rate enhancements in the distal 12 carbons of the fatty acid chain with little or no rate enhancement for the

Table I. Summary of NMR Data

	Tm^{+++} chemical shift (ppm)	Mn^{++} T_1 rate enhance. (sec^{-1})	Lipid spin label T_1 rate enhance. (sec^{-1})
^{13}C phosphatidylcholine			
Choline methyl	$\geq$ 3.7	13	0.1
Fatty acid carbons:			
C_1	0.2	14	0.2
C_2	0.2	9	0.2
C_3	0.4	3	1.1
C_4-C_{11}	0.4	0.7	0.8
C_{12}	0.2	0.1	0.6
C_{13}	0.2	0.3	0.6
C_{14}	$\equiv$0.0	0.3	0.8
^{13}C gramicidin			
C-terminal:			
O-acetyl methyl	2.1	1.9	0.0
O-acetyl carbonyl	2.1		
N-terminal:			
Val_1 methyl	0.6	0.6	1.9
formyl carbonyl	0.6	0.5	0.8
^{19}F gramicidin			
C-terminal:			
O-^{19}F benzoyl	0.80	23	3
N-terminal:			
^{19}F-Phe_1	0.08	1	9

carbonyl and the choline head group. The gramicidin N-terminal valine methyls have the highest rate enhancement of any carbons in the experiment, while the C-terminal acetyl methyl has zero rate enhancement. The gramicidin ^{19}F N-terminal rate enhancement is 3-fold larger than the C-terminal rate enhancement.

The results with Tm^{+++} and Mn^{++} demonstrate the C-terminus is accessible near the surface of the membrane, but that the N-terminus is not. The lipid spin label results indicate that the N-terminus is accessible deep in the membrane, but the C-terminus is not. These results exclude the C-terminal to C-terminal helical dimer (Figure 1B) and the antiparallel-β and parallel-β double helices (Figure 1C,D) and strongly favor the N-terminal to N-terminal helical dimer (Figure 1A) as the major conformation of the gramicidin channel in phosphatidylcholine vesicles.

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ALAMETHICIN: PURIFICATION, CHARACTERIZATION, CONFORMATIONAL AND SYNTHETIC STUDIES

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The structure of the peptide antibiotic alamethicin, which induces voltage-dependent conductance changes when added to artificial lipid bilayers, has been the subject of repeated investigations and revisions. The primary structure was first reported to be a cyclic octadecapeptide containing seven aminoisobutyric acid (Aib) residues by Payne *et al.*[1] which was subsequently confirmed by Ovchinnikov *et al.*[2] and is shown in Figure 1A. Martin and Williams[3] revised the structure to be linear with the addition of acetyl-Aib as the N-terminus and phenylalaninol (Phol) as the C-terminus as shown in Figure 1B which was supported independently by Jung *et al.*[4] Both the cyclic structure and the linear structure proposed have been prepared synthetically in this laboratory[5] and found to be deficient in membrane activity. Gisin *et al.*[6] independently prepared the linear structure proposed by Martin and Williams and showed the ionophoric activity to be less than 10% that of the native compound.

Rinehart *et al.*[7] had investigated the structure of alamethicin and other peptaibophol antibiotics[8,9] by a combination of techniques emphasizing both electron impact and field desorption high resolution mass spectrometry. Serious questions concerning the linkage of residues 18-20 had been raised and the structure shown as Figure 1C with Glu^{γ} Gln^{γ} Phol was proposed based on preliminary data. This structure was also prepared synthetically in this laboratory and showed about 10% membrane activity in comparison with standard alamethicin.[10] Gisin *et al.*[11] prepared another variant of the possible linkages of residues 18-20 based on the determination of the pKa of the native standard compared with the synthetic structure proposed by Martin and Williams (Figure 1B). This compound 1D has the sequence Glu^{α} Gln^{α} Phol for residues 18-20 and was prepared by fragment condensation on a polymer support, but showed only 20-30% membrane activity.

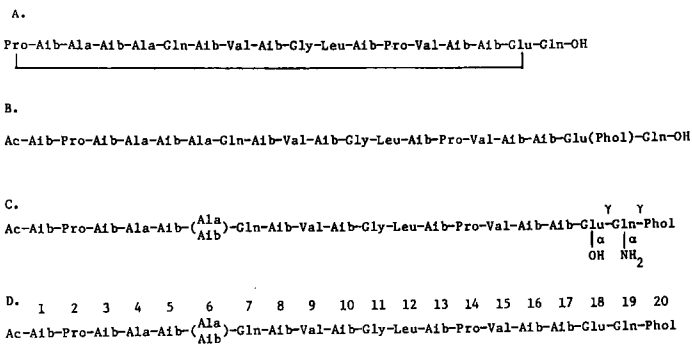

Fig. 1. Various structures proposed for alamethicin.

Based on the low activity of their tentative structure 1C and the somewhat higher activity of the synthetic peptide prepared by Gisin *et al.*, Rinehart *et al.* reinvestigated alamethicin and found independent evidence[7] supporting the alpha-linked Glu-Gln-Phol as proposed by Gisin *et al.* This structure 1D has been prepared in our laboratory by solution fragment condensation.[12] Initially, four major peptide fragments of 1D, Boc-Pro-Aib-Ala-Aib-OBzl (Fragment I), Boc-Ala-Gln-Aib-Val-Aib-Gly-OBzl (Fragment II), Boc-Leu-Aib-Pro-Val-Aib-Aib-OBzl (Fragment III) and Boc-Glu(OBzl)-Gln-Phol (Fragment IV) were synthesized by solution techniques. Coupling reactions were usually carried out in dimethylformamide with dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBT) as coupling agents. The fragment condensation scheme employed (Figure 2) shows the initial coupling of the fragments I and II after the necessary deprotection steps to yield the decapeptide (residues 2 through 11). The decapeptide was then deprotected and coupled to the amino deprotected hexapeptide (fragment III) to give the hexadecapeptide (residues 2 through 17) which was then coupled to fragment IV after appropriate deprotection steps. Acetylaminoisobutyric acid was added in the final step to yield the benzyl ester of 1D which was then catalytically hydrogenated to give 1D. The product was purified by high pressure liquid chromatography to homogeneity in order to determine its activity precisely. In our hands, this peptide showed variable activity compared with native alamethicin depending on the type of artificial bilayers and almost no activity in an antibiotic screen as shown in Table I. This is in contrast to 60-80% antibiotic activity reported by Gisin *et al.*[11] against *B. subtilis* in liquid cultures.

Table I. Antimicrobial Spectrum for the Major Component of Alamethicin and the Synthetic Product.

	Upjohn std.	major isolate component	synthetic
K. pneumoniae	10	0	0
E. coli	trace	0	0
P. vulgaris	8	0	0
S. aureus	14	0	0
S. pyogenes	22	0	11
P. oxalicum	12 hazy	0	9 hazy
S. faecalis	15 hazy	0	0

The samples were dissolved in a minimal amount of DMF and then made up to 1 mg/ml solutions with pH 7.85 Tris buffer, 0.1 *M*, 20 μg of sample was applied per 1/4 inch disc. Numbers refer to size (mm) of inhibited zone.

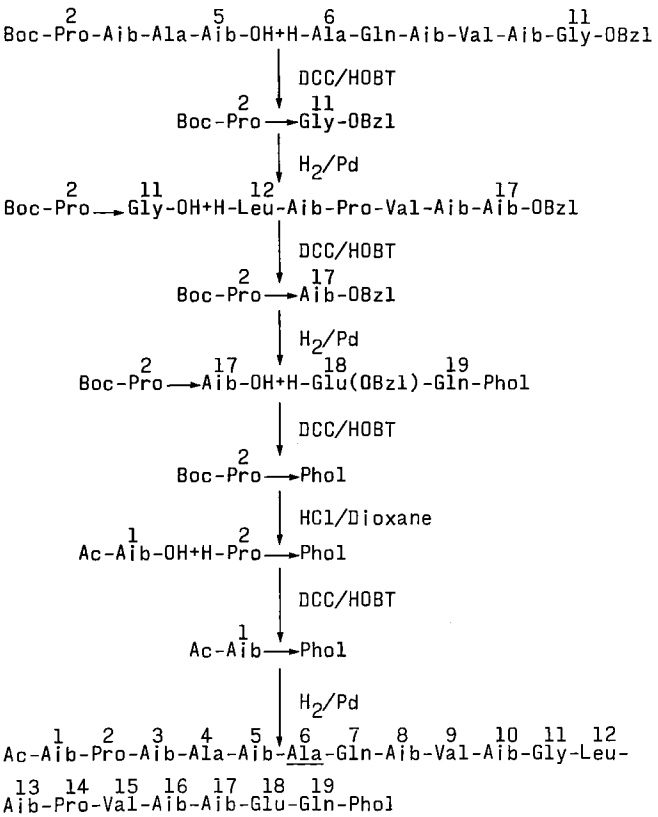

Fig. 2. Fragment condensation scheme for synthesis of alamethicin.

These discrepancies prompted us to compare the native standard with our synthetic material on the HPLC system we had developed. Figure 3 shows the HPLC of the natural product obtained from Upjohn.

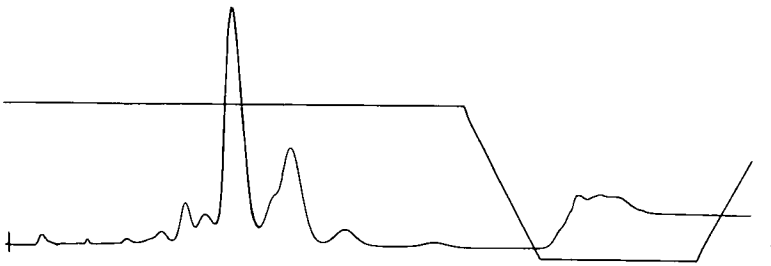

Fig. 3. HPLC chromatogram of Upjohn standard alamethicin on a μBondapak C-18 column. Upper trace shows isocratic elution (49% Mobile Phase B) followed by steep gradient to 100% mobile phase B and return to isocratic elution. Absorption monitored at 210 nm; mobile phase B, THF: CH_3CN: mobile phase A (7:1:2); mobile phase A, $(Et_3NH)_3PO_4$ buffer (0.25 N, pH 3.5).

It is clearly evident that there are minimum of twelve components as opposed to the two different components (either Aib or Ala for residue 6[7] or Gln-NH_2 and Gln for terminal Gln in structure I B[3]) reported by others. Purification of the major component of alamethicin which corresponds in elution position to our synthetic material gave a product with essentially identical amino acid composition, identical diminished antibiotic activity and similar levels of membrane activity when compared with the synthetic standard.[13] Preliminary investigation of the major component by high resolution field desorption mass spectrometry[14] suggests that it is identical in mass to the structure proposed for alamethicin by both Gisin *et al.*[10] and Rinehart *et al.*[7]

These results, although still preliminary, indicate that the properties of the compound designated alamethicin in the literature are due to a complex mixture whose major component does not appear responsible, at least quantitatively, for the activities described either as an antibiotic or when added to artificial lipid bilayers. Work is in progress to isolate and characterize each of the components apparent in the HPLC analysis. Whether all the activities described for alamethicin reside in a single component or in some combination remains to be determined.

Both our preliminary data on the major component and reports of activity in synthetic peptides with alamethicin-like sequences suggest that these molecules possess significant membrane activity. The mechanism would appear dependent on the conformation which is significantly effected by the Aib residues. Models[15,16] of the conformation of alamethicin have been proposed based on the original cyclic structure of

Payne *et al.*[1] as well as for the linear structure of suzukacillin[17] which is similar to that of alamethicin. These models all share a common α-helical structure for the N-terminal segment of the molecule (approximately residues 2-8). Theoretical calculations[18] of the conformational effects of Aib residues show two symmetrical minima (ϕ,ψ = -55, -40 or 55, 40) situated between the dihedral angles associated with either the α-helix $\phi,\psi = \pm(57,47)$ or the 3_{10} helix $\phi,\psi = \pm(60,30)$.

Crystal structures of peptides containing Aib residues show remarkable agreement with theoretical predictions (Figure 4). The crystal structure of protected synthetic fragments corresponding to residues 1-4[19], 2-5[20], 12-14[21], and 10-11[22] of alamethicin have been determined as well as the structure of a pentamer of Aib[23]. In another case, however, Aib occurs in a structure reported for the cyclic tetrapeptide dehydrochlamydocin;[24] not surprisingly, the observed conformation of Aib is significantly different. The 15 to 24 degree variations from 180° of the ω torsion angles is indicative of the amount of strain in this cyclic tetrapeptide. Overlap in sequence occurs in the two linear tetrapeptides (Z-Aib-Pro-Aib-Ala-OMe) comprising residues 1-4 of alamethicin and Boc-Pro-Aib-Ala-Aib-OBz comprising residues 2-5. Although the first Aib residues adopts the expected conformation, Aib-3 in 1-4 is somewhat removed from the minimum energy position and in fact is close to the (ϕ,ψ) value of Ala-4 observed in peptide 2-5. This suggests that the third residue in the "helix" must deviate from the ideal ϕ,ψ value in order to maintain the intramolecular hydrogen bonding scheme. Two values are observed for the Aib corresponding to residue 3 of alamethicin. In the study of Shamala *et al.*[19], a value of (-72,-11) was found, while (-48,-41) was observed by Smith *et al.*[20] This suggests that the local environment -Pro-Aib-Ala, which is identical in the two cases, does not restrict the Aib residue to a single conformational choice. The torsional rotations assumed must, therefore, be influenced by longer range intramolecular interactions and intermolecular forces, such as hydrogen bonding. The structure of the pentamer of Aib which assumes a 3_{10} helix offers additional support as molecules with both right- and left-handed conformations are observed. The total of sixteen experimental observations are compared with the calculated values previously published[18] (Figure 4). With the exception of the Aib residue in dihydrochlamydocin, all of the values cluster near the two symmetrical minima (ϕ,ψ = -55,-40 or 55,40) previously calculated. Only one (ϕ = -72, ψ = -11) of these latter fifteen values lies outside the 0.5 kcal above the minimum contour and within the 1.0 kcal contour line. This agreement

lends strong experimental support to the validity of the Kitaygorodsky parameterization used. Pletnev *et al.*[25] calculated a favorable C_7^{ax} conformation ($\phi = -64$, $\psi = 71$ and the symmetrical conformer) especially in non-polar media which Burgess and Leach[26] also found by PCILO calculations, but which they concluded was at least 5 kcal/mole less stable than the helical conformers from empirical calculations. Marraud and Neel[27] have interpreted infrared spectra of acetylaminoisobutyric acid methylamide in CCl_4 to indicate a predominance of C_5 ($\phi = \psi = 180$) and C_7 ($\phi,\psi = -70,70$ or $70,-70$) conformers. The C_7 conformer would be similar to the conformation of the Aib seen in dihydrochlamydocin, but is not within the allowed region according to the Kitaygorodsky potential (greater than 2 kcal/mole above the potential minima).[18] A relatively small increase (15°) in ϕ and ψ from the C_7 values would allow them to enter a region considered allowed and this may be the conformer which is attributed to C_7 by Marraud and Neel[27] although it is also likely that other conformers may be capable of hydrogen bonding and contribute the appropriate IR bands which they have observed. The fact that none of the fifteen Aib residues observed in linear peptides assume a value near the C_7 conformation nor at the α-helix conformation favored by the model builders probably indicates that these conformers are not favored, and minor differences in parameterization of the potential functions used by the different groups may explain the difference in their predictions, especially since these compounds are sterically hindered and sensitive to choice of van der Waals parameters. The structure of the tripeptide, Boc-Leu-Aib-Pro-OH[21], and the published tetrapeptide, Z-Aib-Pro-Aib-Ala-OMe[19], provide two examples of Aib residues preceding a proline. While one might be tempted to suggest that the negative values of ϕ and ψ might be precluded by the succeeding N-methyl residue, theoretical calculations (Moore and Marshall, in preparation) suggest only a slight preference for the positive values of ϕ and ψ for Aib residues preceding a proline and both combinations are observed experimentally. Systematic calculation[21] of the possible conformers of acetyl-Aib-Ala-Aib methylamide at 11.25° increments gave four conformers with 0.5 kcal/mole of the minimum as shown in Table II. Conformer R,L-A is very similar to the crystal structure observed for the fragment-Aib-Ala-Aib of the tetrapeptide averaging only 8° deviation from the observed structure. This further supports the conclusion that the primary determinants of conformation in these molecules are intramolecular with crystal packing forces selecting between energetically similar conformers. It should, therefore, be feasible to apply the constraints introduced by Aib residues

Table II. Calculated Conformations of Ac-Aib-Ala-Aib-NH-CH_3

Conformer	ϕ_1	ψ_1	ϕ_2	ψ_2	ϕ_3	ψ_3	kcal/mole above minima
R,R	-51	-37	-67	-43	-56	-32	0
L,L	47	34	49	54	50	43	0.2
R,L-A	-57	-49	-79	-30	46	41	0.4
R,L-B	-57	-49	-109	58	46	41	0.4
Crystal Structure of Boc-Pro-Aib-Ala-Aib-OBzl.							
-Aib-Ala-Aib-	-48	-41	-92	-14	48	42	

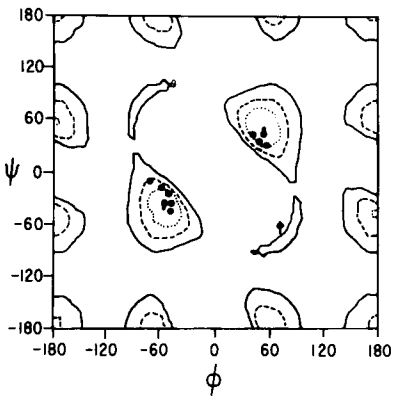

Fig. 4. Potential energy plot of Ac-Aib-NH-CH_3 compared with crystal structure observations for Aib (o). Contours drawn at 0.5, 1.0 and 2.0 kcal/mole above the potential minima. Value outside contours at $\phi = 72$, $\psi = -64$ from cyclic tetrapeptide dihydrochlamydocin.

to limit the possible conformations available to the alamethicin molecule. In addition, other antibiotics such as antiamoebin[8] and emerimicin[9] have been shown to contain Aib residues as do suzukacillin[28] and trichotoxin.[29] The unique properties associated with alpha-methyl substitution have been recognized by natural selection and have resulted in this class of compounds with unique membrane properties.

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STRUCTURAL REQUIREMENTS FOR PORE FORMATION IN ALAMETHICIN AND ANALOGS

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The formation of voltage dependent ion conducting pores in bilayer lipid membranes by alamethicin[1] seems to involve conformational changes of the molecule. The peptide molecules which were added to the aqueous solution change their conformation by lengthening of their helix[2] during contact with the hydrophobic membrane region. It is concluded that a formation of preaggregates occurs out of which pore induction starts. Single pore experiments suggest that the pores adopt different conductance states by uptake or release of monomers.

In order to elucidate the relationship between structure and membrane modifying properties for this type of antibiotics, we started investigations in three ways. Firstly, we studied the conformational change of alamethicin and its natural analogs suzukacillin A and trichotoxin A 40 as caused by the transfer from aqueous to lipophilic media.[2-4] Secondly, voltage-jump current-relaxation experiments were carried out on membranes modified by alamethicin and its analogs.[1,5,6] In addition, these antibiotics were investigated with respect to their hemolytic and conformational behaviour.[7-10] Thirdly, we synthesized by conventional fragment condensation an ion conducting and lytically active nonadecapeptide analog of conformationally designed, modified sequence[11,12] (Figure 1). Very recently we elucidated the complete sequences of the trichotoxin A 40 analog (Figure 1) using selective cleavage conditions and preparative isolation of pure N-terminal dodecapeptide and C-terminal hexapeptide partial sequences.

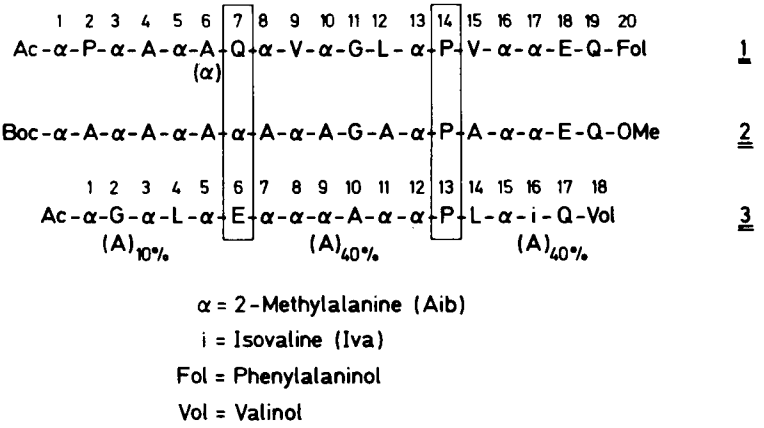

Fig. 1. Sequences of alamethicin 1[15–18], a membrane modifying synthetic nonadecapeptide 2[11,12] and trichotoxin A-40 3[13,14] (single letter abbreviations: Biochemistry 7 (1968) 2703).

Hemolytic activity on erythrocytes[7] may be used as screening test within this class of peptide antibiotics. All analogs exhibiting pore forming activities in lipid membranes are also lytically active. Differences in hemolytic activity of the natural, modified, and synthetic polypeptide analogs are observed. Thus, the lytic activity of the trichotoxin A 40 series increases in this order depending on Glu^6 side chain modification (Figure 2): trichotoxin ($-COO^-$) < hydrazide ($-CONHNH_3^+$) < amide ($-CONH_2$) < trichotoxin ester ($-COOCH_3$). The same behavior is found upon modification of alamethicin at Glu^{18}: alamethicin F-30 ($-COO^-$) < alamethicin F-50 ($-CONH_2$)[7] < alamethicin F-30 ester ($-COOCH_3$). The Glu^{18}-benzyl ester of the synthetic nonadecapeptide[11,12] is more active than the peptide with free Glu^{18}-γ-carboxyl group. Thus, hemolysing properties are exhibited by these analogs, the synthetic nonadecapeptide (fully protected and with free Glu^{18}-γ-COOH), the following peptides of unknown sequences: trichotoxin A-50 analog,[19] trichotoxin A-55[19] with about 50% isovaline residues, and the suzukacillin analog. Similar results are obtained on bilayer membranes using the method of current voltage characteristics.[1,5,6]

The following partial sequences were found to be inactive in both lysis and bilayer experiments: N-terminal dodecapeptide of trichotoxin A-40, Ac-Aib-Gly-Aib-L-Leu-Aib-Glu-Aib-Aib-Aib-Ala-Aib-Aib-OH[13], the C-terminal hexapeptides $^+H_2$-L-Pro-Leu-Aib-D-Iva(Aib)-L-Gln-Valol[14], the synthetic decapeptide Boc-(Aib-L-Ala-)$_5$-OH(-OMe)[3,12], and the hexadecapeptide Boc-(Aib-L-Ala)$_5$-Pro-Ala-Aib-Aib-Glu-Gln-

OMe[12]. These results suggest as minimum requirement for activity a chain length of 18 residues with large variability in particular regions of defined conformation. The lytic action is diminished in the case of the more polar analogs regardless whether the charged side chain is located at the C-terminus (alamethicin F-30) or within the helical region of the peptides (trichotoxin A-40).

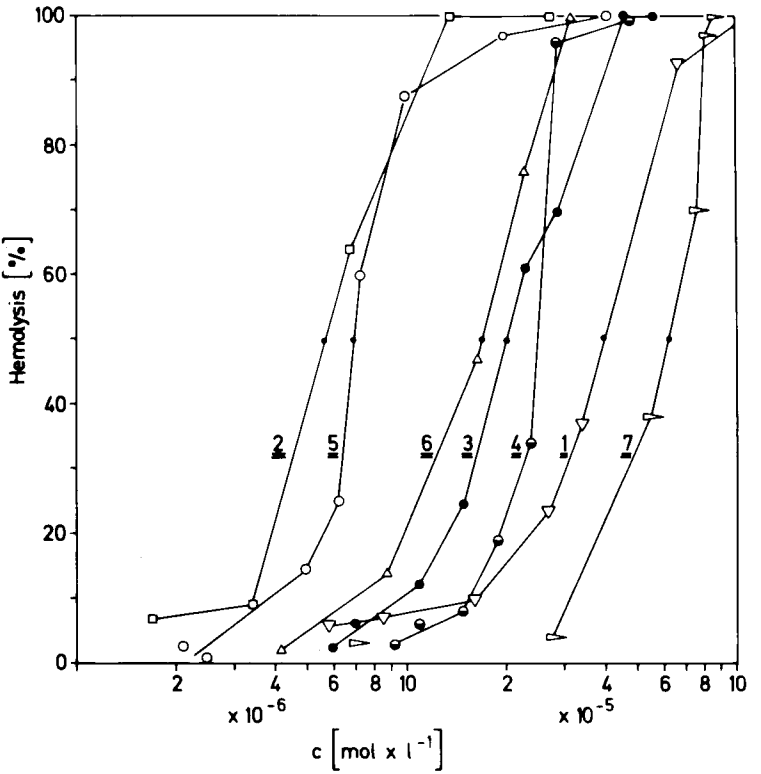

Fig. 2. Concentration dependence of lytic activities of membrane modifying peptides on erythrocytes: trichotoxin A-40 (1), its methylester (2) and hydrazide (3), trichotoxin A-50 (4), alamethicin F-50 (5), synthetic nonadecapeptide (6), and sodium dodecylsulfate (7) (experimental conditions, see Ref. 7).

In bilayer experiments at low antibiotic concentrations the situation is more complex. We have to distinguish between two modes of membrane modifying activity:

1. activity in the form of resolvable, stable ($\tau > 1$ ms) pores,
2. activity in the form of voltage-dependent pore formation (non-resolvable fluctuations) which finally leads to membrane lysis.

Stable pores are formed by alamethicin and suzukacillin (mode 1), whereas trichotoxin A-40 and the synthetic nonadecapeptide induce a voltage-dependent conductance in the form of non-resolvable bursts at low conductance levels (mode 2). Our trichotoxin probes of highest purity isolated by an optimized procedure and purified by countercurrent distribution differ in their pore forming properties from the sample reported earlier[6] in that resolvable pores are no longer observed. Spikes and bursts are recorded, however, and all these trichotoxin probes still exhibit the same lytic activities[7] on both natural and artificial membranes. Surprisingly, the pores are stabilized by the addition of mixtures of partial hydrolysates of trichotoxin A 40[13] (mainly fragments 5-8, Figure 3). The stabilizing effect may be explained by a reduction in the number of collisions and/or by slower movement of pore forming peptide molecules in the bilayer. Experiments with mixtures containing alamethicin and the synthetic nonadecapeptide or the mixture of partial hydrolysates of trichotoxin A 40 showed a similar increase of the mean life time of the alamethicin pores.

The addition of the helical decapeptide Boc-(Aib-L-Ala)$_5$-OH increases the lytic activities in both hemolysis and bilayer experiments. Supposedly resolvable pores are formed only by specific intermolecular arrangements, the Gln^7 residue in the helical region of alamethicin and the Glu^6 residue in trichotoxin molecules may be important for this kind of helix interactions within the bilayer. Thus, the requirements for stable single pore formation may be fulfilled by the introduction of a Gln^6 residue into the synthetic nonadecapeptide.

Our earlier results[2,3,9,10] from conformational analyses using mainly ^{13}C NMR and CD may be refined by the availability of many partial sequences of natural[13,14] and synthetic[4,11,12] origin both free of analogs. For example, CD spectra in the trichotoxin series (Figure 3) clearly indicate the location of the helix turns within the N-terminal undecapeptide region. The CD spectrum of the helical synthetic decapeptide Boc-(Aib-L-Ala)$_5$-OH is similar to that of the trichotoxin undecapeptide (Figure 3). The C-termini of trichotoxin A-40, $^+H_2$-L-Pro-Leu-Aib-D-Iva(Aib)-L-Gln-Valol, exhibit almost exactly the same CD curves as the corresponding synthetic C-terminal hexapeptide, Boc-L-Pro-Ala-Aib-Aib-Glu(OBzl)-Gln-OMe[12]. Conformational analysis based on three different evaluation methods of the CD spectra revealed the following α-helix contents: Boc-(Aib-L-Ala)$_5$-OMe 28% and comparable to that of the hexadecapeptide Boc-(Aib-L-Ala)$_5$-Pro-Ala-Aib-Aib-Glu(OBzl)-Gln-OMe 24%, nonadecapeptide 44% and higher than

alamethicin, 35%. This alamethicin value is similar to that of the dodecapeptide Boc-(Aib-L-Ala)$_6$-OPOE[4] (33%) and corresponds in the mean to 6-7 residues in helical turns. The N-terminus of the helix can be shielded from polar interactions by formation of a β-turn involving hydrogen bridges of Ac-Aib-Pro (or Gly). The C-terminal hexapeptides have predominantly unordered conformations with no evidence of β-turns, which on the basis of CD seem to occur only in the short Aib tri-to pentapeptides.[20,21]

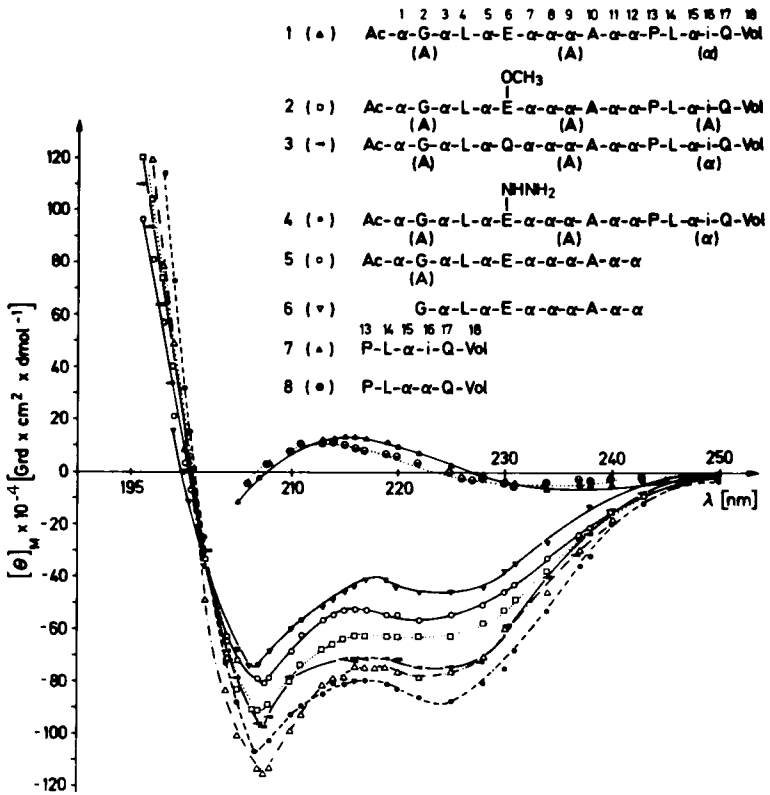

Fig. 3. Circular dichroism spectra of trichotoxin A-40 (1), its methylester (2), amide (3) and hydrazide (4), and of the partial sequences N-acetyl-dodecapeptide (5), undecapeptide (6), and two hexapeptides (7,8); Conditions: c = 5 x 10^{-4} mol/l in methanol; T = 25°C. One letter notations, see Figure 1.

We pointed out earlier, that the Gly^{11}-Ala-Aib^{13} region may be responsible for lengthening the helix when the peptide enters the hydrocarbon layer.[3,7] It is noteworthy that the glycine residue is found in

trichotoxin A-40 in the N-terminal portion of the helix replacing a residue of proline in alamethicin. The synthetic hexadecapeptide lacking these three residues is inactive. The C-terminal aminoalcohols L-phenylalaninol or L-valinol and the N-acetyl group are obviously nonessential residues.

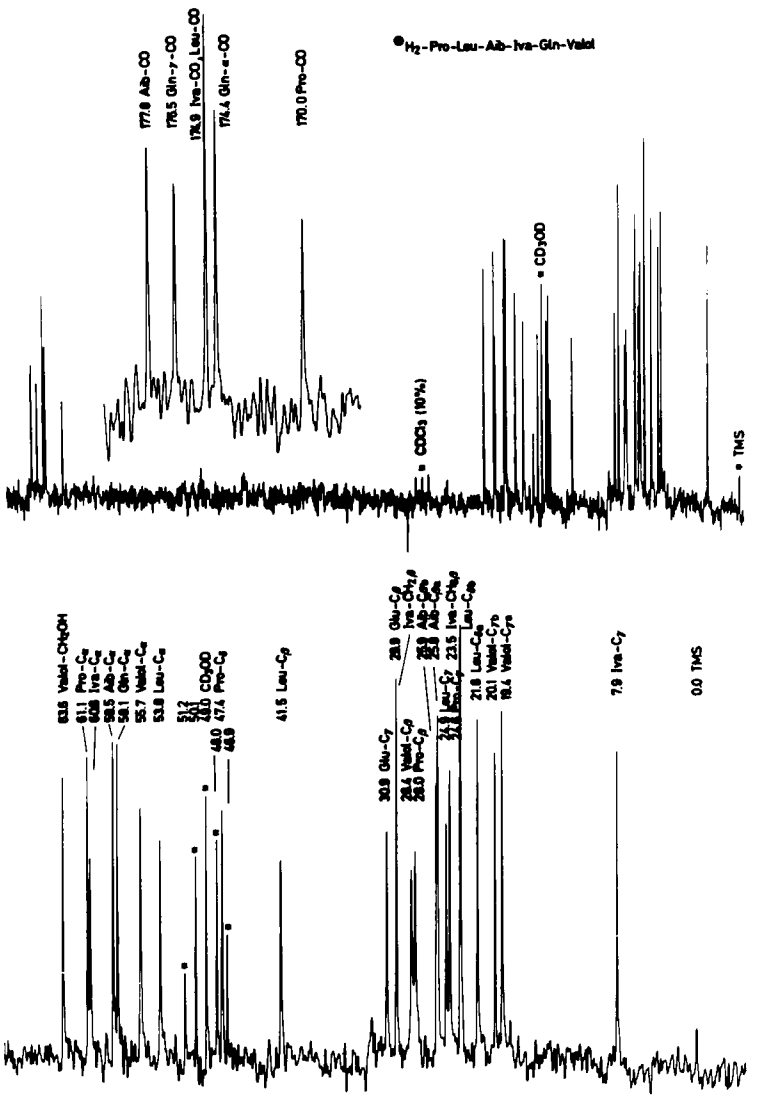

Fig. 4. ^{13}C NMR spectrum of $^{+}H_2$-Pro-Leu-Aib-Iva-Gln-Valol isolated from partial hydrolysate of trichotoxin A-40[13]. Conditions: c = 6 mg/0.5 ml $^{12}CD_3OD$/$^{12}CDCl_3$ (9:1, v/v), 25° C, 60000 scans, 20.115 MHz.

Our cleavage and purification methods[13,14,19] allow the preparation of partial sequences of natural origin in amounts and states of purity for spectroscopic and synthetic purposes. This is illustrated by the ^{13}C NMR spectrum of one of the two C-termini of trichotoxin A-40 (Figure 4).

pH-Dependent spectra were recorded for various model peptides with alternating Aib-Ala sequences in order to find out the maximum pH-induced shift differences for the Aib residues in different sequential positions (Figure 5).

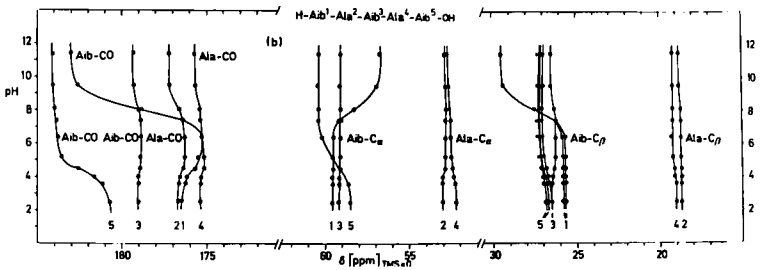

Fig. 5. pH-Dependence of the ^{13}C NMR spectrum of H-Aib-Ala-Aib-Ala-Aib-OH.

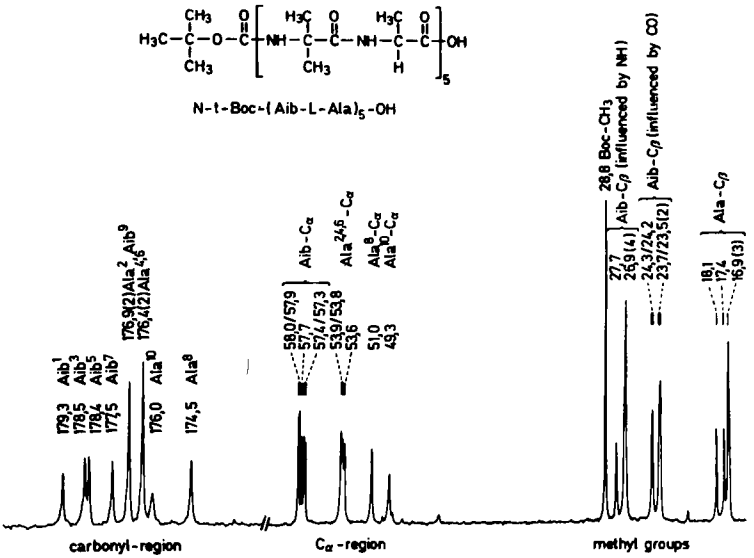

Fig. 6. Assignments in the ^{13}C NMR spectrum of the decapeptide Boc-(Aib-Ala)$_5$-OH in $^{12}CD_3OD$.

Conformational effects on the ^{13}C chemical shifts of Aib residues are illustrated for the helical decapeptide Boc-(Aib-L-Ala)$_5$-OH (Figure 6). The spectrum confirms very clearly our earlier assignments of the alamethicin[2], trichotoxin A-40[10], and suzukacillin A[9] spectra with respect to helical Aib residues exhibiting a magnetic nonequivalence of up to 4 ppm. Nonhelical Aib methyl groups differ by less than 1.5 ppm (Figures 4 and 5).

Temperature-dependent ^{13}C NMR studies on trichotoxin A-40 and the N-acetyl-dodecapeptides suggest, that one of the two Ala residues (-Aib^9-Ala^{10}- or -Ala^9-Ala^{10}-, cf. Figure 1) is helical and the other not. In alamethicin[2] both alanines (Ala^4 and Ala^6) have values typical for helical conformation [cf. Figure 6: Cα 53.6 — 53.9 ppm and Cβ 16.9 ppm for 3 helical alanines in (Aib-Ala)$_5$].

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FURTHER DATA ON THE STRUCTURE-FUNCTION RELATIONSHIP OF PEPTIDE IONOPHORES

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The interest in ionophore-mediated carrier and channel transmembrane transport of alkali metal ions appears to be unabated. In this report the results are described of our recent studies on the structure-property relationship in the series of valinomycin and gramicidin A peptide antibiotics, classic representatives of the carrier and channel type ionophores.

Regarding the former series the Val→Ala, Hyi→Lac, and Lac→Hyi replacements have been studied with respect to their effects on the solution stability and the formation-dissociation rates of the potassium complexes, and also the membrane behavior of the resultant valinomycin analogs.[1] However, the consequences of augmenting the size of the amino acid side chains, i.e. of making them bulkier than isopropyl, still remained to be ascertained. To this end we synthesized and investigated analogs III and IV.[2,3] Compound III, called isoleucinomycin differs from valinomicin (I) in the substitution of all six L- and D-valine residues by isoleucine residues of the same configuration, whereas IV differs also in the size of the ring.

Cyclo[-(D-Val-Lac-Val-D-Hyi)$_n$-]

I n=3 Valinomycin II n=4 Hexadeca-valinomycin

Cyclo[-(D-Ile-Lac-Ile-D-Hyi)$_n$-]

III n=3 Isoleucine IV n=4 Hexadeca-isoleucinomycin

Lac=lactic acid, Hyi=α-hydroxyisovaleric acid

The spatial structure of the free and complexed depsipeptides was investigated by IR, CD, and NMR techniques, the complex stabilities, conductimetrically,[4] and the ionophorous behavior, by the extraction method.[5] It can be seen from Table I, that the stability of the complexes of III and IV is very similar to that of I and II. The spectral data revealed a

Table I. Stability Constants and Stokes' Radii of the Alkali Metal Ion Complexes of Valinomycin and Its Analogs (96% aqueous ethanol, 25°C)

Compound	Stability constant, M^{-1}			Stokes' radius, Å		
	K^+	Rb^+	Cs^+	K^+	Rb^+	Cs^+
I	$1.3 \cdot 10^5$	$2.4 \cdot 10^5$	$6.3 \cdot 10^4$	4.23	4.01	3.92
II[2]	50–100	-	500	-	-	-
III	$1.8 \cdot 10^5$	$0.8 \cdot 10^5$	$4.2 \cdot 10^4$	4.60	4.31	4.18
IV	<50	100	10^3	-	-	-

close analogy in the spatial characteristics of the respective depsipeptides and their complexes.[3] This was to be expected; essentially the only structural difference between the isoleucinomycin and hexadeca-isoleucinomycin complexes from the corresponding valinomycin and hexadeca-valinomycin complexes is the presence, respectively, of six and eight additional methyl groups (Val→Ile) on the molecular periphery of the complex. Such an addition should, however, affect the ionophoric behavior of the compound, and, indeed, on the one hand, the additional methyls decreased somewhat the mobility of the cation complexes, which in the case of III manifested itself in higher values for the Stokes' radii (Table I), and on the other, increased their lipophilicity with concomitant increase in their ability to transfer metal ions from the aqueous to the organic phase. The latest conclusion follows from the results of the extraction experiments represented in Figure 1 and Table II. While retaining the valinomycin order of selectivity $Rb^+ \geqslant K^+ > Cs^+ \gg Na^+$, isoleucinomycin extracted more alkali metal ion into methylene chloride, correspondingly giving higher values for the bulk extraction constant.

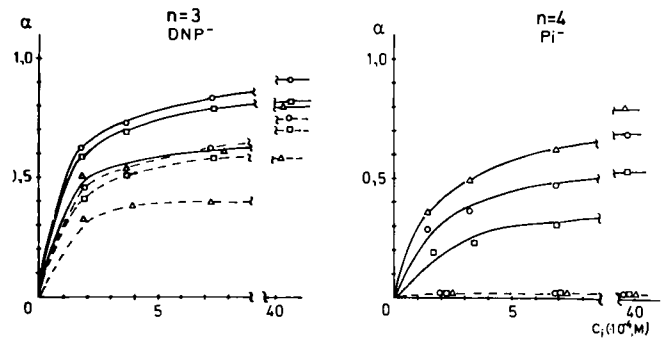


Fig. 1. Extraction of alkali metal ions into dichloromethane by (on the left) valinomycin (— — —), isoleucinomycin (——); (on the right) hexadeca-valinomycin (— — —) and hexadeca-isoleucinomycin (——) □ K^+, ○ Rb^+, △ Cs^+

A still greater increase in the degree of extraction is observed in the case of the cyclohexadepsipeptide IV with respect to II. Whereas II manifests a very weak alkali metal ion extracting capacity even for a large excess of salt (the degree of extraction did not exceed 5% with a 40-fold salt excess), the degree of Cs^+ extraction by IV under the same conditions attains a value of 80%. In keeping with the increased size of the molecular cavity, the ion selectivity shifts to larger ions: $Cs^+ > Rb^+ > K^+$.

Bearing in mind reports of the ability of II to induce the permeability of lipid bilayers to organic cations[6], we studied the extraction of such cations by hexadeca-isoleucinomycin. It can be seen from Table II, that the latter proved to be an efficient carrier of such bulky cations as tetramethylammonium, guanidinium, and acetylcholine. In this respect IV markedly surpasses valinomycin and isoleucinomycin, and what is particularly noteworthy, contrary to I and III a high selectivity against all alkali metal cations is observable here. The results obtained clearly demonstrate the possibility of deriving new ionophores from valinomycin with valuable properties absent in the naturally occurring antibiotic.

Table II. Extraction of Monovalent Picrate (Pi) Solutions by Methylene Chloride Containing 10^{-4} M Valinomycin or Its Analog (25°C)

Compound	Extraction constant, $K^a_{w/o}$						
	K^+	Rb^+	Cs^+	NH_4^+	Me_4N^+	Guanidinium	Acetylcholine
I	430[b]	530[b]	150[b]	670	-	34	57
III	1700[b]	2300[b]	510[b]	1400	-	71	62
IV	40	120	270	10	550	270	1450

[a] $$K_{w/o} = \frac{[(\text{ionophore}\cdot\text{cation})^+]_{org} \cdot [\text{anion}^-]_{org}}{[\text{cation}^+]_{water} \cdot [\text{anion}^-]_{water} \cdot [\text{ionophore}]_{org}}$$

[b] 2,4-Dinitrophenolates (DNP)

The problem of the spatial structure of gramicidin A has now been attracting the attention of researchers for several years. It is known that gramicidin A forms four dimers in solution, interconverting with each other and with the monomer. In nonpolar media equilibration takes place very slowly, and this has been exploited to isolate the dimers in the individual states and determine their spectral characteristics.[7,8] For instance, the respective CD curves taken from the paper by Veatch *et al.*[7] are shown in Figure 2.

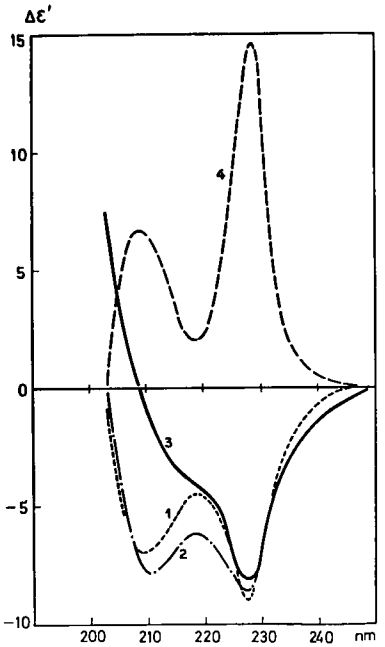


Fig. 2. CD curves, gramicidin A individual 1-4 dimeric species

Urry *et al.*[9] and Ramachandran and Chandrasekharan[10] have independently proposed a helical structure for gramicidin A known as the π_{LD} helix. Two molecules of the antibiotic associating in a "head to head" fashion form a $\overrightarrow{\pi_{LD}}\,\overleftarrow{\pi_{LD}}$-helix. Urry *et al.*[9] considered four types of such helices differing in the number of residues per turn. Of these the $\pi_{LD}^{6.3}$-helix (with 6.3 residues per turn) with an axial cavity diameter of ~4A has a length of ~30A, approximately equal to the thickness of the hydrocarbon part of the lipid bilayer.

Veatch *et al.*[7] proposed another dimer model according to which the peptide chains of the antibiotic form a double helix stabilized by 28 - 30 intermolecular hydrogen bonds. Chain orientation can here be parallel ($\downarrow\downarrow\pi\pi$) and antiparallel ($\uparrow\downarrow\pi\pi$), whereas the axial cavity diameter and the dimer length are approximately the same as the $\overrightarrow{\pi_{LD}}\,\overleftarrow{\pi_{LD}}$-helix.

At the 15th European Peptide Symposium the results of investigating shortened gramicidin A analogs by means of which the individual species 1 + 2, 3 and 4 of the antibiotic could be modeled were reported.[11] An IR spectral study of gramicidin A species 3 and of the gramicidin analogs in dioxan were recorded. The resonance splitting of the amide I vibration frequency for all the earlier dimer models was then calculated. By comparing the calculated and observed IR spectra it was shown that species 1-4 of gramicidin A are not conformationally individual, but are

equilibrium mixtures of predominantly $\uparrow\downarrow\pi\pi_{LD}$ helices and lesser amounts of $\overrightarrow{\pi_{LD}}\ \overleftarrow{\pi_{LD}}$ dimers (Table 3).[11,12]

Table III. Conformation of Gramicidin A Dimers in Dioxan

Species	Helicity	Type of helices and their mole fractions
1	Left-handed	$0.75\uparrow\downarrow\pi\pi^{7.2}_{LD} + 0.15\overrightarrow{\pi^{4.4}_{LD}}\ \overleftarrow{\pi^{4.4}_{LD}} + 0.10\overrightarrow{\pi^{6.3}_{LD}}\ \overleftarrow{\pi^{6.3}_{LD}}$
2	Left-handed	$0.75\uparrow\downarrow\pi\pi^{7.2}_{LD} + 0.15\overrightarrow{\pi^{4.4}_{LD}}\ \overleftarrow{\pi^{4.4}_{LD}} + 0.10\overrightarrow{\pi^{6.3}_{LD}}\ \overleftarrow{\pi^{6.3}_{LD}}$
3	Left-handed	$0.75\uparrow\downarrow\pi\pi^{5.6}_{LD} + 0.15\overrightarrow{\pi^{4.4}_{LD}}\ \overleftarrow{\pi^{4.4}_{LD}} + 0.10\overrightarrow{\pi^{6.3}_{LD}}\ \overleftarrow{\pi^{6.3}_{LD}}$
4	Left-handed	$0.75\uparrow\downarrow\pi\pi^{5.6}_{LD} + 0.15\overrightarrow{\pi^{4.4}_{LD}}\ \overleftarrow{\pi^{4.4}_{LD}} + 0.10\overrightarrow{\pi^{6.3}_{LD}}\ \overleftarrow{\pi^{6.3}_{LD}}$

On the other hand in a recent study, Bamberg *et al.*[13] investigating gramicidin A analogs with charged groups on the C- or N-terminus, and also an analog with covalently bridged N-termini, arrived at the conclusion that the ion-conducting species are the $\overrightarrow{\pi_{LD}}\ \overleftarrow{\pi_{LD}}$-dimers. Apparently in the case of gramicidin A we encounter a rare case of a major conformational rearrangement taking place on passing from a nonpolar medium to a membrane environment.

In order to simplify the conformational equilibrium and thereby to facilitate analysis of the relationship between the spectral parameters, spatial structure, and membrane-affecting activity of gramicidin A and its analogs, we synthesized and investigated a new series of analogs in which the end groups of the monomers were covalently bridged to form "head to head", "head to tail" and "tail to tail" dimers (Figure 3). Derivatives of the first type were obtained by interaction of desformylgramicidin A with various activated derivatives of dicarboxylic acids. Good results were obtained on using the specially prepared dipentafluorophenyl esters PfpOOC - $(CH_2)_n$ - COOPfp where n = 2,3,4,6, or 7. In the case of the poorly soluble succinyl- and glutaryl-bis-N,N'-desformylgramicidins A a two stage synthesis using succinic and glutaric anhydrides proved to be the most convenient. In the first step the corresponding gramicidyl-carboxylic acid was obtained and a second desformylgramicidin A molecule was then added by carbodiimide procedure. "Head to tail" derivatives were also prepared with the aid of cyclic anhydrides that in the first stage acylated the ethanolamide (EA) hydroxyl of gramicidin A; the resultant monogramicidyl esters were then condensed with desformyl-gramicidin A. For the "head to head" derivatives treatment of gramicidin

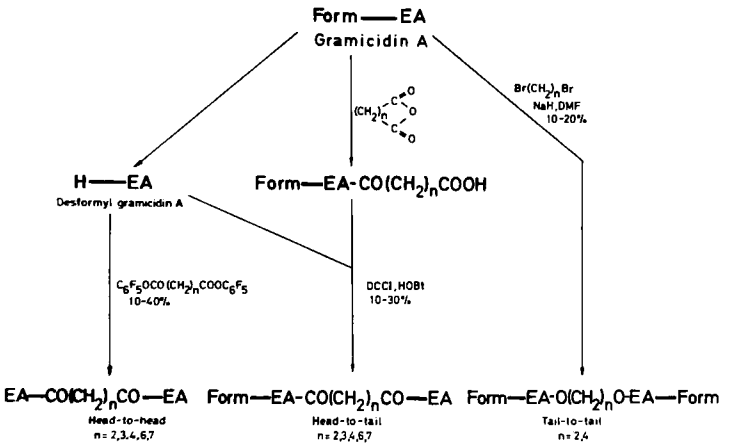


Fig. 3. Synthesis of bis-gramicidin A derivatives

A with sodium hydride in dimethylformamide yielded the corresponding alcoholate which was then condensed with 1,2-dibromoethane or 1,4-dibromobutane. All bis-derivatives were separated from ionogenic contaminants on ion exchangers and were further purified by preparative thin layer chromatography.

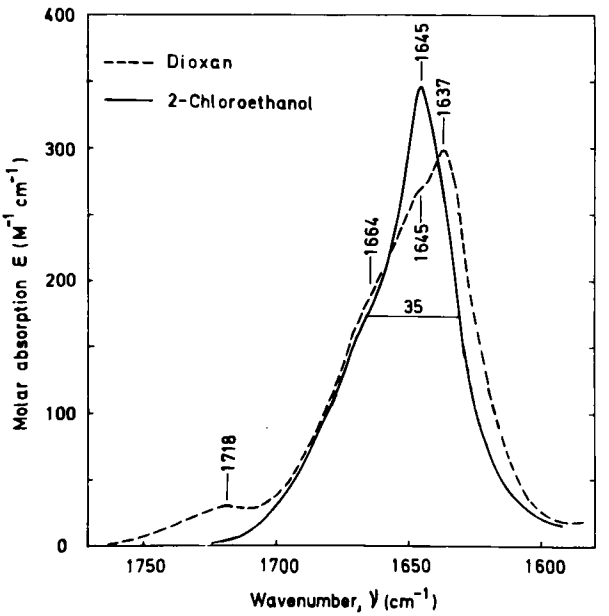
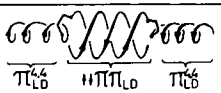
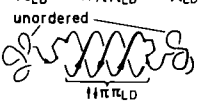
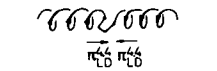


Fig. 4. IR spectra of succinyl-bis-N,N'-desformylgramicidin A

Measurements were made of the amide I IR spectral region of succinyl-bis-N,N'-desformylgramicidin A in dioxan, ethanol, methanol, and 2-chloroethanol (Figure 4, Table IV). The aforementioned calculation showed that the frequency 1637 cm^{-1} corresponds to a $\uparrow\downarrow\pi\pi_{LD}^{5.6}$ or $\uparrow\downarrow\pi\pi_{LD}^{7.2}$ helix, whereas the frequency 1645 cm^{-1} corresponds to the $\overrightarrow{\pi_{LD}}$ $\overleftarrow{\pi_{LD}}$ dimer; a shoulder at 1664 cm^{-1} corresponds to a small fraction of random structure, whereas the 1718 cm^{-1} band is due to the free formamide carbonyl. It thus follows that in dioxan, ethanol and methanol there are $\uparrow\downarrow\pi\pi_{LD}$ helical regions, which could only be possible by intermolecular association. The presence of intermolecular associate was confirmed by the strong concentrational dependence of the CD spectra. No association occurs at 10^{-4}M in 2-chloroethanol, and the bis-derivative has the structure $\overrightarrow{\pi_{LD}}$ $\overleftarrow{\pi_{LD}}$. The results, schematically represented in Table IV show that in no case are there signs of a $\uparrow\downarrow\pi\pi$ helix, proof of the earlier drawn conclusion as to the absence of such forms in solution[11,12] and in the membrane.[13]

Table IV. IR Data on bis-Gramicidins A, EA——$OC(CH_2)_nCO$——EA

Solvent	Amide I frequency, cm^{-1}		Conformation
	n=2	n=4	
Dioxan	1636, 1645, 1664sh, 1718	1635, 1645sh	
Ethanol	1636, 1660	-	
Methanol			
2-Chloroethanol	1645, 1664sh	1644, 1664sh	

A number of bis-derivatives of gramicidin A were investigated on lipid bilayers under single channel conditions. It was found (Table V) that the conductivity of single channels formed by "head to head" type derivatives is of approximately the same value for all these channels, equalling ~24 pS (1 pS=10^{-12} ohm^{-1}). Under the same conditions the conductivity of a single gramicidin A channel is 30.2 pS. The lifetime of a single channel of a bis-analog is at least 20 - 25 fold that of a gramicidin channel. The minimum concentration required for the formation of a single channel in the case of the "head to head" succinyl or adipyl-bridged bis-analogs is by four orders of magnitude less than in the case of gramicidin A (10^{-16}M and 10^{-12}M, respectively). The azelaylic derivative

Table V. Single Channel Parameters of Membranes Modified by Gramicidin A and Its bis-Derivatives

Compound	Single channel conductance (pS)	Mean channel life-time (sec)
Form—EA (gramicidin A)	30.2	<0.5
EA—$CO(CH_2)_2CO$—EA	24.2	12.6
EA—$CO(CH_2)_4CO$—EA	24.6	10.1
EA—$CO(CH_2)_7CO$—EA	~24	~9
Form—EA-$CO(CH_2)_2CO$—EA	24.3	9.7
	30.3	<0.5

[a] Membranes formed from 2% (w/v) glycerol mono-oleate/cholesterol=2/1 (mole ratio) solution in n-octane. Electrolyte, 0.5M KCl, V=100 mv, T=21±0.5°C

forms a single channel only at a concentration of 10^{-10}M, and, moreover, destabilizes the membrane, i.e. acts as a detergent.

The shift in the peak of the channel conductivity amplitude from 30.2 pS in gramicidin A to 24 pS in the bis-derivative, is apparently due to the appearance of an additional barrier in the energy profile of the channel, localized in the region of the bridge. Shortening of the lifetime with increase in the number of methylenes in the bridge can be accounted for by the increased mobility of the monomer units.

With analogs of the "head to tail" type, a channel of long half-life and one of short half-life are formed, that are similar in conductivity to that of gramicidin. We attribute its appearance to the formation in the membrane of intermolecular $\pi_{LD}^{\rightarrow}$ $\pi_{LD}^{\leftarrow}$-helical associates, whereas the channel of long half-life must be formed by a $\uparrow\downarrow\pi\pi_{LD}$ or $\pi_{LD}^{\rightarrow}$ $\pi_{LD}^{\rightarrow}$ helix. In any case the results obtained lead to the conclusion that in the gramicidin A series the ion conducting channels can be formed by polypeptide structures differing considerably with respect to conformation. A strict condition for the formation of a channel is evidently the existence of an axial molecular cavity and conformity of the channel length to the thickness of the membrane. Hopefully studies of this kind will aid in the understanding of the behavior of more complex protein channels, such as the proton channel of bacteriorhodopsin[14] whose primary structure and folding topography have been recently elucidated by Ovchinnikov *et al.*[15]

In conclusion it should be pointed out that there is as yet no firm proof that the function of gramicidin A in the producer organism is that of an ion channel. To the contrary, there are reports that the primary act

in the functioning of gramicidin A is inhibition of RNA polymerase (binding to DNA?) with subsequent development of sporulation.[16] In that case we might have expected higher specificity in the functioning of the antibiotic. In particular, it might be possible that the active structure here is that of the double helix predominant in solution.

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SOME BINDING PROPERTIES OF THE PEPTIDE BACKBONE INFERRED FROM STUDIES OF A NEUTRAL NON-CYCLIC CARRIER HAVING IMIDE LIGANDS†

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Introduction

We have studied ion permeation in lipid bilayers mediated by the imide-containing, neutral, noncyclic Li^+-selective complexone ETH149* (Figure 1), developed by Simon *et al.* as a Li^+-selective carrier for solvent-polymer membrane electrodes,[1] and have found it to be remarkably versatile, capable of acting not only as a simple carrier of monovalent cations[2–4] but also as a simple carrier of monovalent anions.[2–5] It can also act as a symport carrier of charged complexes of cations together with anions.[4,6] The various permeant complexes can bear charges of +1, –1, –2, or +2.

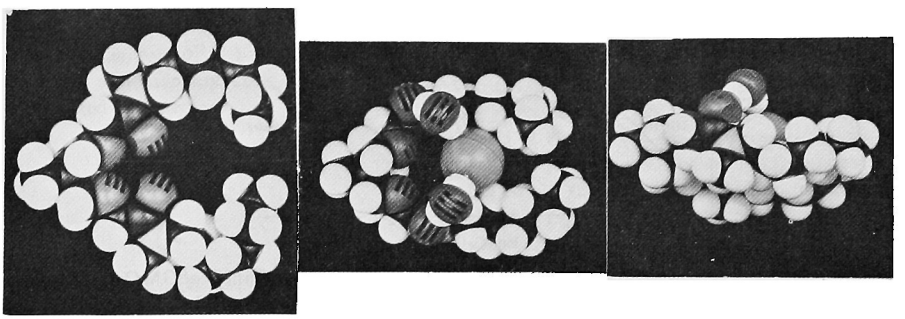

Fig. 1. CPK model of the Li^+-selective complexone ETH149. Top and side views of the postulated 1:1 complex with Br^- show how two H_2O molecules could be interposed between the carbonyl oxygens and Br^-.

†Supported by NSF (PCM 7620605) and by USPHS (GM 24749)

*N,N'-diheptyl-N,N',5,5-tetramethyl-3, 7-dioxanonan diamide, the code name ETH149 given by Simon *et al.*[1]

These multiple ion-carrying abilities imply the ability of a molecule with uncharged imide ligands to bind ions selectively and therefore make it a prototype for interactions of cations and anions with the backbone (as opposed to the charged residues) of proteins.

Selective Permeation of Monovalent Cations in Lipid Bilayers — To characterize the stoichiometry and composition of the permeant complexes of monovalent cations we have combined measurements of zero-current conductances and dilution potentials in single salts. To characterize the selectivity further we have also measured the membrane potentials in salt mixtures.[3,4]

The dependence of membrane conductance of glyceryl monooleate/decane (GMO) membranes on carrier and salt concentrations is illustrated in Figures 2 and 3 for the sulphate salts of the indicated cations. In Figure 2 the slopes of 1 and 2 show that the conductance depends on the first power of carrier concentration at low concentrations and on the 2nd power for the higher concentrations. The corresponding dependence on salt concentration in Figure 3 is first power over the entire concentration range. Combining these conductance data with dilution-potential measurements (not illustrated here),[4] one can infer that the predominant permeating species is usually the 2:1 carrier:cation complex, a 1:1 carrier:cation complex also existing at low concentrations.

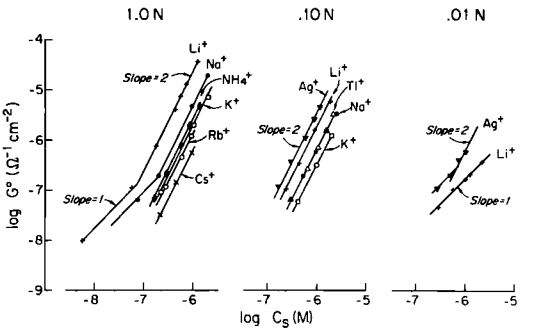

Fig. 2. First and second power dependences of zero-current conductances of GMO membranes on carrier concentration, for the Li^+-selective carrier and the sulphate salts of the indicated cations.

The selectivity among monovalent cations can be assessed from the membrane potentials in salt mixtures (dilution-potentials show the sulphate anion to be impermeant), as illustrated in Figure 4 for the 2:1 species. The points are experimental, the curves are theoretical, drawn to the usual carrier equation for cations,[7] with the permeability ratios which

give the following selectivity sequence: $Ag^+(2.7)>Li^+(1.0)>Tl^+(.39)>Na^+(.22)>NH_4^+(.11)>K^+(.09)>Rb(.06)>Cs^+(.03)$. These ratios are in good agreement with the ratios of conductances illustrated in Figures 2 and 3,[4] as expected[7,8] for carrier systems in the "equilibrium domain", where the interfacial reactions are at equilibrium and selectivities are directly related to ion-binding equilibria. Consistent with the equilibrium domain character of this carrier, these bilayer selectivities are similar to those reported in bulk electrodes.[1] Note that the experimental points (Figure 4) fall exactly on the theoretical curves and do not show the voltage-dependent deviations typical of carriers with kinetic limitations on unloading.[9,10] This provides, in agreement with the "hyperbolic" current-voltage relationship behavior we observed,[4,5] further evidence that this carrier is indeed in the "equilibrium domain". Interestingly, although Li^+-selective among the alkali ions, it is most selective to Ag^+ among the monovalent cations investigated.

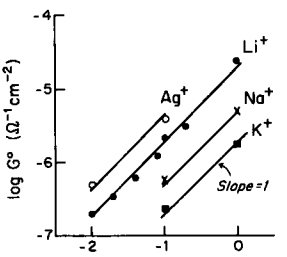

Fig. 3. First power dependence of zero-current conductance of GMO membranes on salt concentrations, for 10^{-6}M Li^+-selective carrier and sulphate salts of the indicated cations.

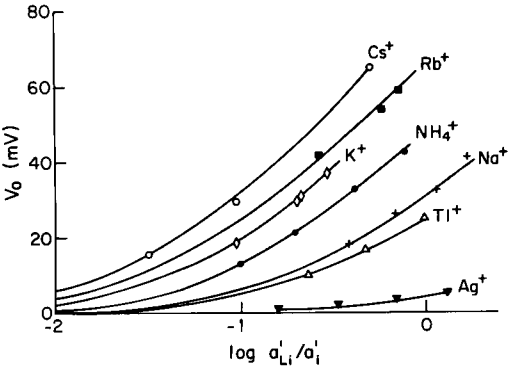

Fig. 4. Membrane potentials in mixtures of Li^+ and the indicated cations, at 10^{-6}M Li^+-carrier. Li^+ was added to initially symmetrical 1.0N sulphate salts (excepting .1 for Tl^+ and .1 $AgNO_3$ for Ag^+). Theoretical curves for permeation by 2:1 carrier:cation complexes were drawn according to the Goldman-Hodgkin-Katz equation for the permeability ratios listed in the text.

The Ligand Groups Involved in the Cationic Selectivity — For cation-selective complexones, both ether and carbonyl oxygens have been well established as cationic ligands.[11] The carbonyl oxygens of the polypeptide backbone are generally accepted as the selective binding sites for cations[13] not in small part because of the specific carrier:cation complexes formed with the backbone of such peptide and peptide-like carriers as valinomycin.[11-13] Therefore, in a structure with imides and

ethers, like our carrier (see Figure 1), either of its two ligand types or both could be involved in binding the cations.

The high Ag^+ selectivity observed for the tertiary amide-containing ETH149 molecule (and, interestingly, also in the fully hydrogen bonded neutral channel-forming polypeptide gramicidin A[14]) is in contrast to the low Ag^+ selectivity observed with the ether (and ester) containing nactin carriers.[11] This strongly suggests that the amide oxygens are involved here as cationic ligands, especially for the smaller alkali cations. A supporting argument comes from the observed high NH_4^+ selectivity, indicative of a tetrahedral array of the cationic ligands.[11] CPK models of the 2:1 carrier:cation complexes indicate that of the two types of oxygen ligands the amide-oxygens are better positioned in the molecule for surrounding the cation in a tetrahedral array.

Further evidence for the imide carbonyl being an important ligand comes from the high sensitivity of the selectivity, among the smaller alkali cations, to the nature of the *N*-imide substituents in a series of structurally related noncyclic molecules.[15,16] For example, replacing the aliphatic *N*-imide substituents of the ETH149 by benzyl residues results in a selectivity shift to a sequence of: $Na^+ > K^+ > Rb^+ > Cs^+ > Li^+$ both in bulk membranes[15,16] and in bilayers.[17]

We therefore suggest that the imide oxygens are primary in complexing the smaller cations, with participation of the ethers likely in complexing the larger cations which can accommodate additional ligands.

Selective Membrane Permeation of Monovalent Anions in Lipid Bilayers — The dependences of zero-current conductances of GMO and PE membranes on carrier and salt concentrations are illustrated in Figures 5 and 6 for Ca^{2+} and Mg^{2+} salts of the indicated monovalent anions. The increase of conductance with increasing salt and carrier concentrations is characterized either by a 1st power dependence on salt together with a first power dependence on carrier or by a 2nd power dependence on both salt and carrier, implying 1:1 and 2:2 carrier:ion complexes as the sole membrane-permeating species. To identify the ions participating in the complex, dilution-potential data must be used. We confine the results presented here to those concentrations where only anions participate in these complexes. This is illustrated by the example in Figure 7 for the observed potentials (points) in comparison with theoretical limits for the dilution-potentials (indicated in the figure), expected for the various possible ions being carried in these 1:1 and 2:2 complexes.

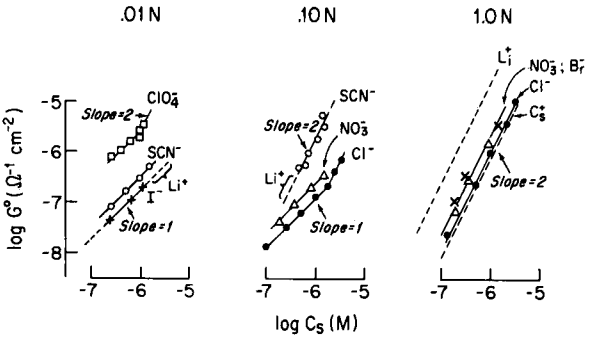

Fig. 5. First and second power dependences of the conductance of GMO membranes on the concentration of the Li^+-carrier (ETH 149), for the indicated anions, at three salt concentrations. The accompanying cations were Ca^{2+} or Mg^{2+}. Dashed lines indicate conductance for Li_2SO_4.

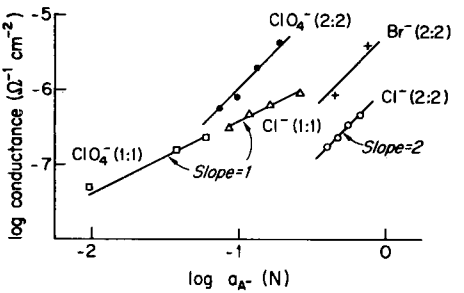

Fig. 6. First and second power dependence of the conductance on anion activity. Numbers in parenthesis are the corresponding carrier:ion stoichiometries: ClO_4 (1:1) in PE membranes, 1.6×10^{-6}M carrier. ClO_4 (2:2) in PE membranes, 3.7×10^{-6}M carrier. Cl^- (1:1) in GMO membranes 1.8×10^{-6}M carrier. Cl^- (2:2) in GMO membranes, 6.4×10^{-7}M carrier. Br^- (2:2) in GMO membranes 2×10^{-6}M carrier.

The possible compositions of the 1:1 carrier:ion complex can be represented as XS^- and MS^{2+} (S-denoting the neutral carrier, X^--a monovalent anion and M^{2+}-a divalent cation). Inserting these respective net charges of –1 and +2 and the total ion stoichiometry of 1 into the dilution potential equation presented in Figure 7, gives theoretical slopes of –58.5 and +29.3 for XS^- and MS^{2+}, respectively. As can be seen from comparison of these slopes and the observed potentials (left hand side) there is an unambiguous agreement between the data and the expectations for a 1:1 carrier:anion complex.

The realistic* possibilities for the composition of the 2:2 carrier:ion complex can be represented as MXS_2^+ (i.e. an ion-pair) and a less likely pair of two monovalent anions $X_2S_2^{2-}$. Using these respective net charges

*We consider unrealistic the possiblity of a high valence species $M_2S_2^{4+}$.

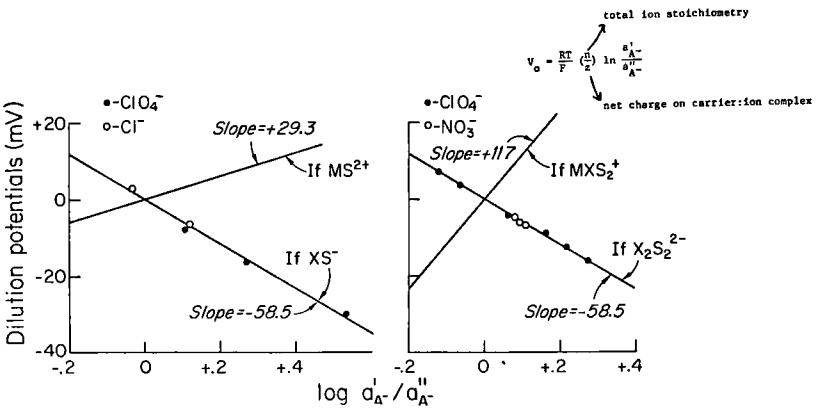

Fig. 7. Proof that the complexes are negatively charged. Dilution potential measurements verifying the sign of the charge on the carrier:ion complex. Left: 1:1 complexes, ClO_4^- in PE membranes, Cl^- in GMO membranes. Right: 2:2 complexes; ClO_4^- in PE membranes, NO_3^- in GMO membranes. Theoretical slopes drawn to the indicated equation for total ion stoichiometry of one and two, respectively.

of +1 and –2 and the total ion stoichiometry of 2 in the dilution potential equation gives theoretical slopes of +117 and –58.5 for MXS^+ and $X_2S_2^{2-}$ respectively. As can be seen from Figure 7 (right), the data surprisingly agree with the anionic theoretical plot, i.e. the membrane permeating species is a 2:2 carrier:anion complex,[4] a unique case of a divalent species permeating the bilayer in a doubly charged state.

As to selectivity among the anions, we have already observed an indication of selectivity with the different levels of conductance observed for various salts with a common cation and different anions (Figures 5 and 6). To pursue further the selectivity we have measured potentials in salt mixtures (not illustrated here)[4] and found the permeability ratios to be in good agreement with the ratios of conductances illustrated in Figures 5 and 6, not only within each stoichiometry—which is expected of equilibrium domain carriers—but also between stoichiometries, as can be seen from the following sequences: $ClO_4^-(240) > SCN^-(34) \geqslant I^-(28) > NO_3^-(2.0) > Cl^-(1.0) > F^-(.5)$ and for the 1:1 species, and $ClO_4^-(260) > I^-(43) > SCN^-(40) > NO_3^-(1.7) \geqslant Br^-(1.5) > Cl^-(1.0) > Ac^-(.15)$ for the 2:2 complexes.

The Molecular Factors Involved in the Anionic Selectivity of the Li^+-Carrier — Binding to neutral backbones with H-bond donating abilities (via -NH groups unengaged in internal H-bonding) is easy to visualize as the mechanism for favorable anionic interactions with the backbone, but binding of anions to the peptide unit of fully H-bonded backbones is less intuitively obvious. However, selective anion binding to the peptide unit, in structures unable to supply H-bonding, does exist, as exemplified not

only by our findings with the present (ETH149) carrier, but also with the non H-bond-donating imide Na^+-complexone[17] as well as in the anionic interactions found[18] for the presumably fully H-bonded gramicidin channel; therefore, anion affinity should exist within the N-C=O region of an amide, whether it is a primary, secondary or tertiary one.

The N-C=O link is a "π-electron system"; its chemical reactivity due mainly to these π electrons.[19] The distribution of the charge of the four mobile π electrons, two from the π C-O bond and the nitrogen lone pair, deduced by Pullman *et al.*[19] from molecular orbital calculations places partial positive-π-charges of +.141 on the nitrogen and +.256 on the carbon, making the N-C link a di-positive center, to be found in typical π-electron systems such as the ester C-O links of carboxylic and phosphoric esters. This distribution of the amide's mobile π electrons is similar to the traditional resonance configuration of $N^+=C-O^{-20}$ in placing the partial negative charge on the carbonyl-oxygen, but quite different regarding the balancing positive charge, spreading it over the N-C link (not merely at the nitrogens as in the traditional configuration) and making the N-C bond the anion-affinity region of the N-C=O system.

To evaluate the contribution of this anion-affinity to the molecular factors involved in the anionic selectivity of our tertiary-amide carrier (which has no intrinsic H-bond donating ability) three major properties should be considered: Are the interactions of anions with tertiary amides substantial at all? Are they ion-specific? Are they substantial enough to account alone for the anionic selectivity of this carrier?

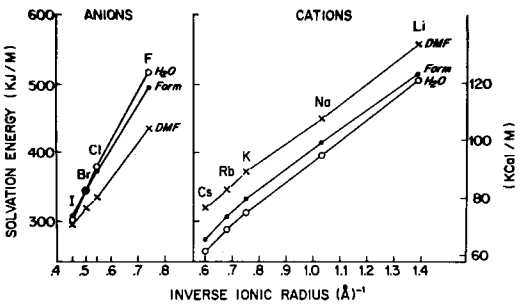

Fig. 8. Solvation energies in amide solvents. Data is from Somsen's thermochemical studies[25], separated for anions and cations using the Halliwell and Nyborg[26] convention.

A qualitative attempt to answer these questions is possible by evaluating the solvation energies of anions and cations in amide and imide solvents. We have plotted in Figure 8 the solvation energies of the halide and alkali ions, separately, in two amide solvents: dimethylform-

amide (DMF; tertiary) and formamide (FA; primary), as function of inverse ionic radius. From I^- to F^-, the anionic solvation energies are seen to be sizable, within the range of the cationic* solvation energies. I^-, the weakest among the halides in H-bonding, is solvated by DMF, a tertiary amide like our carrier, almost as well as by formamide and water, with solvation energies of the order of 75 KCal/Mole, showing the interaction of an anion with the tertiary amide to be considerable, as has also been suggested by Schleich *et al.*[21] for the tertiary nitrogen of proline. On the other hand, the solvation energies of F^-, a strongly H-bonding anion, in the hydrogen-bond donating solvent formamide and in water, are roughly 10 KCal/Mole higher than the corresponding solvation of F^- in DMF, indicating H-bonding to have a substantial contribution to the solvation energy.

A clear demonstration of ion-specificity in the solvation of anions (and of cations) in the amide solvents already apparent in Figure 8, can be seen in Figure 9, where the ionic selectivities of the two amide solvents (i.e. the difference between the enthalpies of solvation of an ion in an amide solvent and in water) are compared to each other and to the ionic selectivities of two tertiaryamide compounds: The Li^+-carrier (ETH149) and a polymer (poly-l-pro-II). (Note that while in all four systems the anionic selectivities are monotonic functions of ion-size, such is not the case for the cationic selectivities, the non-monotonic nature of the cations being discussed elsewhere.[11])

So far, we have considered the anionic solvations only. To assess whether the anionic interactions with a pure tertiary amide (i.e. interactions with the N-C dipositive center) are substantial enough to account alone for the anionic selectivity of our carrier (ETH149), one has to consider the anionic vs. cationic selectivities of the systems represented in Figure 9, and at first glance that comparison is rather disappointing. Both the tertiaryamide carrier (ETH149) and the tertiaryamide polymer (poly-l-pro-II) have *comparable* anion vs. cation selectivities. In contrast, DMF, which should be an exact model for these two imide structures, exhibits *low* anionic vs. cation selectivity, indicating that the anion affinity to the N-C bond, although contributory, is unlikely to account alone for the anionic selectivity of our carrier. However, considering formamide as a system that can supply an anion with both favorable N-C interactions *and* with H-bonding, the increase in anionic vs. cationic selectivity upon replacing DMF by formamide as the solvent, indicates that H-bonding can provide the additional stabilization needed for the anion to have selectivities comparable to those of the cations.

*The analysis of the cationic solvation energies has been discussed elsewhere.[11]

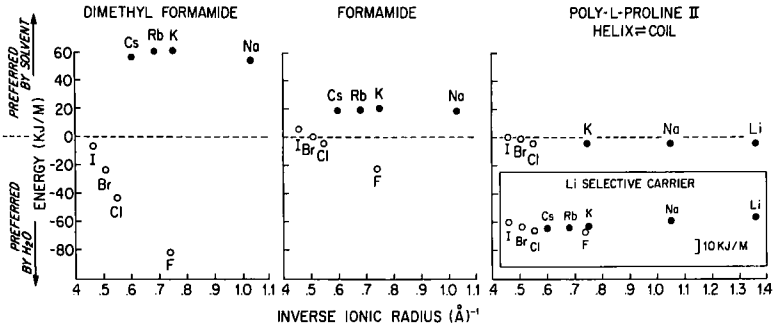

Fig. 9. Cationic and anionic selectivities of amide solvents, poly-l-proline-II and the Li^+-selective carrier (ETH149). Ions preferred by the solvent, backbone or carrier are above the dashed horizontal line and those rejected (i.e. preferred by water) are below. For the Li^+-carrier it was not possible to assess an absolute preference but only relative values between cations and anions.

Since our imide-containing carrier has no intrinsic H-bond donating ability the H-bonding needed would have to come from a source outside the imide-carrier, which could be included in the imide-anion complex and would supply the needed H-bonding. A water molecule seems likely since water is present in the media where the ionic interactions with the tertiary amide systems — excepting the non-aqueous DMF solvent — take place and is known to be strongly hydrogen-bonded to amide-carbonyl-oxygens.[22] We therefore propose that a water molecule interposed between the imide-carbonyl-oxygen and the anion, provides the latter with the needed H-bond stabilization, giving the carbonyl-oxygen indirectly a favorable role in the anion binding while screening the anion from the partial negative charge of the oxygen.

Two such water molecules, each interposed between a carbonyl-oxygen and the anion, can be seen in the CPK model of our suggested structure for the 1:1 carrier:anion complex, presented in Figure 1 (top view), with Br^- as the complexed anion. The anion is suggested to occupy a "cavity" formed with the imide groups facing each other, providing the favorable N-C interactions in addition to the H-bonding of the interposed water molecules discussed above. Additional water molecules can be interposed between the anions and the ethers, screening the latter.

As to possible structures for that unusual case, the 2:2 carrier:anion complex, a stable complex demands maximum possible separation of the two negative charges; and comparison of the top and side views of the 1:1 complex (presented in Figure 1) provides some insight as to how this can be achieved. Suggesting the 2:2 complex to be a dimer of two 1:1 species and not a rearrangement, a back to back stacking would provide a far

better charge separation than a face to face stacking would, while satisfying the affinity of the heptyl chains to each other. It is clear from the side view in Figure 1, that such a back to back stacking would not be too sensitive to the identity of the complexed anion. Since this is an equilibrium domain carrier (i.e. selectivities are directly related to ion binding equilibria), in such a back to back dimerization, where the stacking is not strongly ion-specific, the selectivities of the 2:2 species should continue to be governed by the ion-specific binding equilibria of the 1:1 complexes. This prediction is in agreement with the practically identical permeability ratios observed for both stoichiometries.

A Test of the Predictions for our Model Compound (ETH149) with Peptide Carriers — Anionic membrane permeation, mediated by a cyclic peptide carrier would be an important test of our suggestion that an interposed water molecule is necessary for the binding of anions to the peptide backbone in regions lacking H-bond donating abilities. No such anionic permeation has been reported, moreover it would not be expected for small carriers like valinomycin, which do not have room in the center for a water molecule together with an anion (although it might exist for sandwiches). On the other hand, with cyclic peptide carriers possessing larger cavities, anionic permeation might be expected.

We have tested two cyclic depsipeptides:* Hexadecavalinomycin, which is a tetramer of the repeating unit of valinomycin — i.e. cyclo[DValLLacLValDHyIv]$_4$[23] and an imide-ligand-containing analogue — cyclo[DValLProLValDHyIv]$_4$ (both these molecules were synthesized by Ivanov's group at the Shemyakin Institute), with cavities assumed to be large enough to include both an ion and a water molecule.

Preliminary data indicating that these molecules can carry anions are given in Figures 10 and 11 for perchlorate. The first power dependence of zero-current conductance of PE membranes on carrier concentrations, for the imide-containing carrier *c*[DValLProLValDHy Iv]$_4$ (code named L-Pro-16-HDVal) in the presence of $Mg(ClO_4)_2$, illustrated in Figure 10, indicates that a charged membrane-permeating species has been formed and that this species is a monocarrier complex and not a sandwich. The corresponding dilution-potentials, illustrated in Figure 11 are anionic, demonstrating the perchlorate and not magnesium is the transported ion. We have data of a similar type for hexadecavalinomycin.

*Strictly speaking, the depsipeptides, with alternating ester and peptide bonds are not purely peptide-structured carriers, but the ester units are enough peptide-like for these carriers to be relevant models for testing the anion-binding predictions of the study with ETH149.

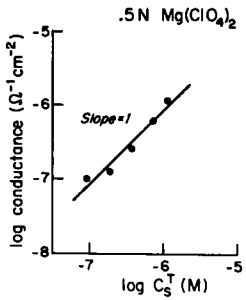

Fig. 10. First power dependence of zero-current conductance of PE membranes on c[DValLPro LValDHyIv]$_4$ concentration in .5N $Mg(ClO_4)_2$.

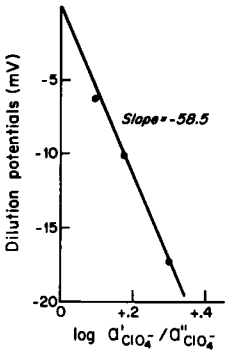

Fig. 11. Dilution-potentials verifying that the charge of the 1:1 complex of ClO_4^- with c[DValLPro LValDHyIv]$_4$ is -1. Theoretical plot is drawn to the equation indicated in Fig. 7, for a total ion stoichiometry of one.

Both these 16-membered depsipeptide carriers (and the smaller valinomycin) are H-bonded internally,[13] therefore similar to the noncyclic carrier (ETH149) in having no H-bond donating ability. Of the two, the proline-containing analogue seems a better model than hexadecavalinomycin to test the predictions of our study with the noncyclic carrier; since, while the latter differs from valinomycin in cavity-size only, c[DValLProLValDHyIv]$_4$ differs in both cavity size and in having tertiary amides replace some of the ester links of valinomycin and hexadecavalinomycin.

It is possible to evaluate separately the effects of cavity size and the effects of imide vs. ester ligands on anionic permeation and selectivity, by a comparative study of anionic vs. cationic selectivities of a series of valinomycin analogues. An example is illustrated in Figure 12, where we have compared the ratios of zero-current conductances of perchlorate to potassium for valinomycin, hexadecavalinomycin, [DValLProLValDHy Iv]$_4$ and our noncyclic carrier (ETH149). Considering first valinomycin and hexadecavalinomycin, identical in their repeating unit and differing only in cavity size, the smaller-sized valinomycin shows extreme preference for K^+ over ClO_4^-, while the larger-sized hexadecavalinomycin although still K^+-selective shows a decrease in the extent of the K^+ over ClO_4^- preference. Examining next the effects of imides vs. esters, the imide-containing [DValLProLValDHyIv]$_4$ shows an increase of the ClO_4^-

to K^+ preference relative to the ester-containing similar-sized hexadecavalinomycin, to the point of crossing over to ClO_4^- being the preferred ion. This ClO_4^- selectivity over K^+ increases considerably for the noncyclic carrier which has both imide groups and an "open cavity".

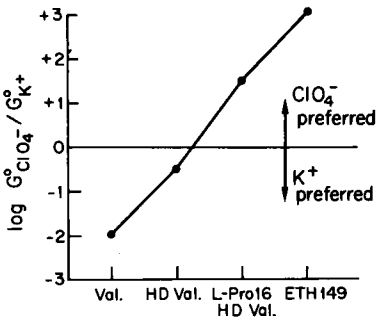

Fig. 12. Ratios of zero-current conductances of perchlorate to potassium, for valinomycin (Val), hexadecavalinomycin (HDVal), *c*[DValLProLValDHyIv]$_4$ (L-Pro-16-HDVal) and the Li^+-carrier (ETH149); ClO_4^- conductances were measured in PE membranes, K^+ conductances were measured in GMO or GDO membranes and "normalized" to PE membranes, assuming $G^o_{PE}/G^o_{GDO\ GMO}$ for K^+ is 10^{-3}.[27]

It should be borne in mind that these data are only preliminary where the depsipeptides are concerned, pointing the way to further studies of ClO_4^- and additional anions, since in contrast to the noncyclic carrier, the conductance-voltage behavior of perchlorate in these cyclic carrier systems (not presented here) shows the existence of kinetic limitations, differing from one carrier to the other. Therefore, a comprehensive analysis of anionic vs. cationic selectivities awaits not only the additional data of permeability ratios but also elucidating the conductance-voltage relationships.

Ion Binding Properties of Neutral Polypeptide Backbones Inferred from Bilayer Studies with Peptide and Peptide-like Carriers —

Although the carriers we have studied are strictly models only for polypeptide backbones that are fully H-bonded internally (or proline rich), the essential role of H-bond stabilization for the binding of anions, obvious now from both the data and the analysis of ion solvation in amides, allows us to generalize to backbones with no restrictions as to their H-bond donating abilities. Neutral polypeptide backbones can bind anions and cations selectively and independently, the cationic-affinity due to favorable interactions with the amide-carbonyl-oxygen, the anionic-affinity due to both favorable interactions with the N-C link and to H-bonding. In those regions of the backbone unable to supply H-bonding, a water molecule interposed between the amide-carbonyl-oxygen and the anion will donate to the latter the necessary H-bonds (this might involve competition between the cation and the anion—via the water molecule—on the carbonyl-oxygen).

We propose, therefore that the system we have studied in bilayers is a relevant model for the effects of "neutral salts",[24] observed in solution, on enzymes and proteins greatly varying in composition and structure. Our studies suggesting these effects, ranging from stabilization to complete disruption of the native protein conformation, to be attributed to direct independent and selective binding of both anions and cations to their backbones and postulating a major role for water molecules in these interactions.

Summary and Conclusions

When sulphate is the anion, the Li^+-selective complexone, ETH149 can act in bilayer membranes as a pure equilibrium domain carrier of monovalent cations forming 1:1 and 2:1 membrane permeating carrier:cation complexes with the selectivity sequence of $Ag^+ > Li^+ > Tl^+ > Na^+ > NH_4^+ > K^+ > Rb^+ > Cs^+$ for the dominant 2:1 species.

With Ca^{2+} or Mg^{2+} salts this molecule can also act as a selective equilibrium domain carrier of monovalent anions, forming 1:1 and 2:2 carrier:anion membrane permeating complexes, with the selectivity sequence of $ClO_4^- > SCN^- > I^- > Br^- > NO_3^- > Cl^- > F^- > Ac^-$ found for both stoichiometries.

The selectivities of the cations and the anions in this system are comparable; the selectivity sequence of the 2:1 cationic and the 2:2 anionic species being: $ClO_4^- > Ag^+ > SCN^- > I^+ > Li^+ > Tl^+ > Na^+ > NH_4^+ > K^+ > Rb^+ > Cs^+ > Br^- > NO_3^- > Cl^- > Cs^+ > F^- > Ac^-$ at 1N (it is salt-concentration dependent).

We argue that the tertiary-amide carbonyl oxygens serve as cationic ligands, especially for the smaller cations, with participation of the ethers in liganding the larger cations. For the anions we propose that the ligands are not only the positively charged regions of the N-C bond but also postulate that H-bonding via a water molecule interposed between the carbonyl oxygen and the anion provides important additional binding energy.

This postulate implies that cyclic peptide carriers having no H-bond donating ability but cavities large enough to contain both an ion and a water molecule should have the potential of being anionic carriers; we tested this with several large valinomycin analogues. We found the ester-ligand-containing depsipeptide and an imide-ligand-providing proline analogue both to be anionic (perchlorate) carriers. Comparison of the conductance ratios of perchlorate to potassium of these two carriers,

valinomycin and the Li^+-carrier (ETH149) shows increasing anion vs. cation selectivity with replacement of esters by tertiary-amide residues and with increasing cavity size.

This verifies our postulate and we therefore suggest that the interactions of cations, anions and water molecules with the polypeptide backbones of proteins and enzymes can be directly attributed to selective binding of the ions, individually and independently, to the peptide unit.

Acknowledgements

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ANTAMANIDE: ALKALI METAL ION COMPLEXATION, CHANNEL FORMATION, AND EFFECTS OF POLARITY OF SOLVENT

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Introduction

Antamanide[1] is a cyclic decapeptide, isolated from the poisonous mushroom *Amanita phalloides,* with the following sequence (all L-):

$$\begin{array}{ccccc} Pro^{8}\text{-}Phe^{9}\text{-}Phe^{10}\text{-}Val^{1}\text{-}Pro^{2} \\ | \qquad\qquad\qquad\qquad | \qquad\qquad (AA) \\ Pro^{7}\text{-}Phe^{6}\text{-}Phe^{5}\text{-}Ala^{4}\text{-}Pro^{3} \end{array}$$

It is an antitoxin that counteracts the deadly toxin phalloidin, also found in *Amanita phalloides,* if administered simultaneously or previously to a lethal dose of the toxin. Phalloidin acts upon the membrane of the liver, inducing release of K^+ ions, while antamanide prevents the uptake of phalloidin by the liver. Antamanide has the additional property of complexing with alkali and alkaline earth metal ions, selectively with Na^+ and Ca^{++} rather than K^+. Synthetic analogs of antamanide,[2] such as [Phe^4Val^6]antamanide, also called symmetric antamanide (SAA), that exhibit biological activity, i.e. counteract phalloidin, also complex with Na^+, while those that are biologically inactive, such as [Ala^1]antamanide, do not form complexes. The one exception is the perhydro analog (HAA or HSAA) where the phenyl groups are replaced by cyclohexyl groups. This biologically inactive analog readily forms complexes with Na^+ and also with K^+. Despite the formation of ion complexes and its membrane activity in counteracting phalloidin, antamanide has little effect on cation permeability of model membranes. Perhydroantamanide, however, has significant effect on K^+ and Na^+ penetration through membranes.[3]

With the purpose toward explaining some of the above effects from a physical point of view, the crystal structures of antamanide, some of the analogs, and their alkali metal ion complexes have been determined. The conformations of antamanides in the solid state have exhibited some unusual and unexpected features, not observed previously in other cyclic peptides.

Table I. Summary of Single Crystal X-Ray Diffraction Analyses.

COMPLEXED*	UNCOMPLEXED		
	Channel*		Non-channel†
	Polar	Non-polar	
1) AA·Li^+·CH_3CN	1) SAA	AA	1) SAA
2) SAA·Na^+·EtOH	n-hexane/MeAc	CH_3CN/water	acetone/water
	2) SAA		EtOH/water
	$Ca(NO_3)_2$/CH_3CN/		CH_3CN/water
	acetone		DMSO/water
			Me_4urea/water
			2) HSAA
			n-hexane/MeAc
			CH_3CN

*Complete structure analysis.
†Cell parameters, space group, intensity data only.
AA = antamanide SAA = symmetric antamanide HSAA = perhydro SAA

Results

A summary of the crystals analyzed by x-ray diffraction is shown in Table I. All these crystals are stable only in contact with their mother liquor. Upon drying, the crystal lattices disintegrate. Thus all the x-ray diffraction data are collected from crystals sealed in thin-walled glass capillaries with some mother liquor. Crystals of alkali metal complexes and uncomplexed antamanide, as well as crystals grown from polar and nonpolar solvents, with and without the presence of water have been studied. Thus far, only *two* conformations for antamanide have been found, a folded conformation for the alkali metal complex, Figure 1(a), and an elongated conformation for the uncomplexed antamanide, Figure 1(b). The nature of the solvent, however, dictates the manner in which the peptide molecules aggregate in the crystal. One crystalline form contains continuous channels of large diameter lined with the polar carbonyl groups and filled with solvent, another form has continuous channels of a smaller diameter lined with the lipophilic side chains and filled with solvent, while the third form does not appear to have any channels. Some details of the structures are as follows:

Alkali metal complexes — A cyclic decapeptide is not large enough to encapsulate an alkali metal ion completely in the manner of valinomycin,[4,5] a cyclic dodecapeptide. Rather, in antamanide[6,7,8] a polar cup is formed by the folded backbone in which the Li^+ or Na^+ atom is held by four ligands from the carbonyl oxygens from residues 1, 3, 6 and 8, Figure 1(a). A fifth ligand is donated by a solvent molecule such as CH_3CN, ethanol or acetone. The ligands are near 2.1 A for Li^+ and 2.3 A for Na^+. The environment is not conducive for K^+ complexation since K^+———O ligands usually are nearly 2.7 A, a value that would impose considerable strain upon the size of the cavity. Furthermore, K^+ is usually hexa- or octa- rather than pentacoordinated.

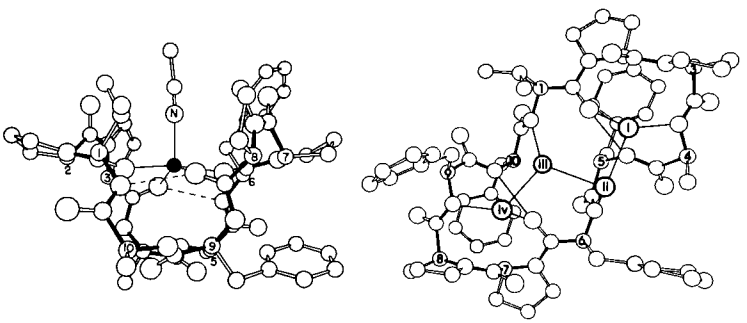

Fig. 1. (a) Conformation of Li^+·antamanide·CH_3CN. Phenyl rings on Phe^5 and Phe^{10} have been omitted for clarity. (b) Conformation of uncomplexed antamanide. Sites labelled i-iv are oxygen atoms from water molecules.

The conformation of the complex is identical for antamanide and the symmetric analog (except, of course, for the side chains on residues 4 and 6) despite different solvents and entirely different packing in their crystallographic lattices. A schematic cross-section of the molecule shown in Figure 2 (compare to Figure 1(a)) shows the regions of the polar interior and lipophilic exterior. The non-polar end of a solvent molecule completes the exterior lipophilic surface, a classic model for membrane permeability. It is reasonable to expect that the Na^+ complex of perhydroantamanide (HAA or HSAA) would have the same conformation as AA or SAA. The four cyclohexyl groups, replacing the four phenyl groups in AA, enhance the lipophilic character of the exterior surface, and most likely account for the ion transport activity.[3]

Water complexes — Antamanide crystallized from a polar mixture of CH_3CN and H_2O[9] and symmetric antamanide crystallized in the absence of water from a non-polar mixture of n-hexane and methylacetate[10] both have the same conformation, Figure 1(b) and

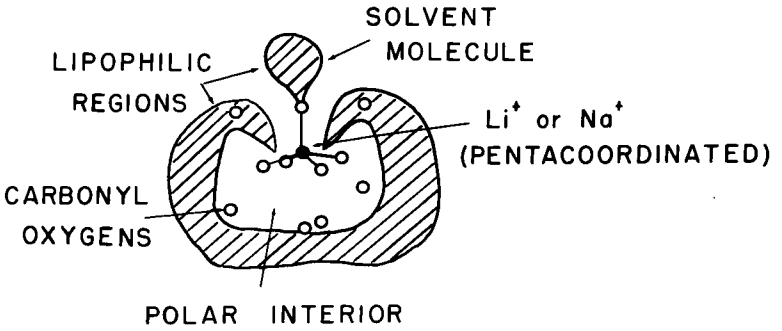

Fig. 2. Schematic cross-section of complexed antamanide, compare to Figure 1(a).

Figure 3. Furthermore, in an attempt to form crystals of a Ca^{++} complex, symmetric antamanide was crystallized from a solvent containing $Ca(NO_3)_2 \cdot 4H_2O$, CH_3CN and acetone.[11] The Ca^{++} complex was not formed and the conformation of the SAA molecule was identical to that in Figure 3. Torsional angles of the two molecules, compared on the ψ,ϕ map in Figure 4 differ slightly only as a consequence of the different side groups on residues 4 and 6, particularly the presence of the bulky side group on Phe^6 in AA and are not influenced by the nature or the polarity of the solvent. A unique feature is the inclusion of water molecules into the interior of the backbone ring as an integral part of the complex. The water complex of symmetric antamanide has three H_2O molecules while four water molecules are accommodated by the somewhat larger cavity in antamanide. The great affinity for water is demonstrated by symmetric antamanide that contains three intrinsic H_2O molecules even though it was carefully dried and crystallized in the absence of water. The water molecules primarily serve as intra annular bridges between NH groups. As a consequence, the cyclic backbone has considerable rigidity, as demonstrated by the low values for the thermal values for the individual atoms as determined from the crystal structure analyses.

The water complexes of AA and SAA do not contain any 3→1 or 4→1 intramolecular hydrogen bonds. However, there is a pair of 5→1 hydrogen bonds: N(5)H···O(1) and N(10)H···O(6). These hydrogen bonds encompass the sequences Pro-Pro-Phe and Pro-Pro-Ala. There is a *cis* amide bond between each pair of Pro residues.

A comparison of the alkali metal complex with the water complex, in Figure 3, shows that the circled areas are very similar in both conformations. From the map of the torsional angles, Figure 5, it can be seen that the two major differences in conformation are ψ_3 (rotation

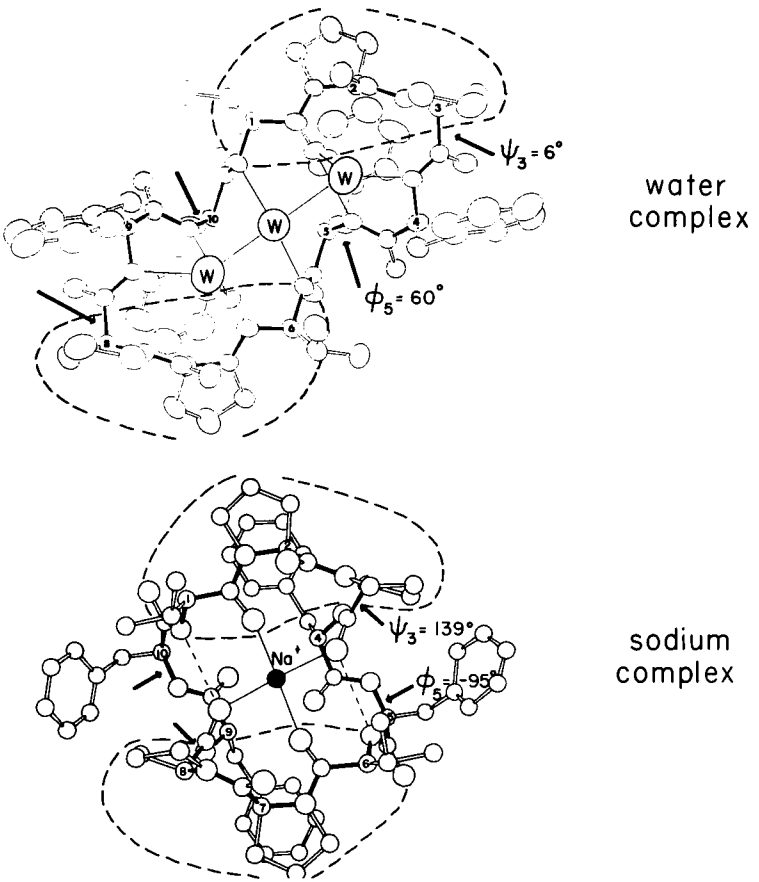

Fig. 3. Comparison of conformations of symmetric antamanide and the Na^+ complex.

about the C_3^{α}-C_3 bond), and ϕ_5 (rotation about the N_5-C_5 bond), and by C^2 symmetry, ψ_8 and ϕ_{10}. All other differences in the torsional angles are relatively minor. A simplistic mechanism for converting the backbone conformation from the water complex to the alkali metal complex would be to grasp C_4^{α} and C_9^{α} and to pivot downward and inward about ψ_3 and ϕ_5 (and by symmetry, ψ_8 and ϕ_{10}). Somehow the phenyl groups on Phe[5] and Phe[10] would have to be threaded through the backbone ring. This type of rotation brings O_3, O_4, O_8 and O_9 which are on the exterior in the water complex to the interior in the alkali metal complex and conversely for N_4, N_5, N_9 and N_{10}. Since antamanide complex formation with Na^+ is a relatively slow process,[12] this type of cumbersome conformational change is not improbable.

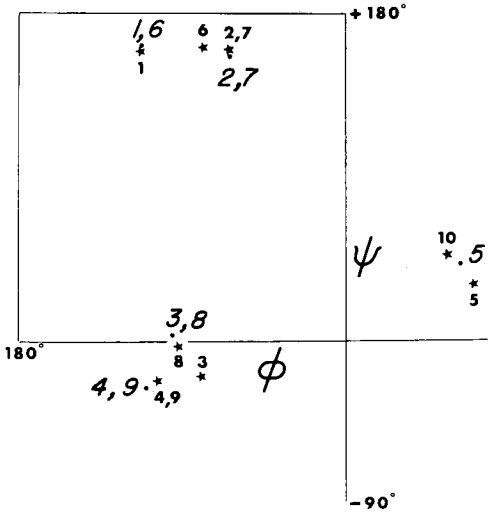

Fig. 4. Comparison of torsional angles $\phi(N_i\text{-}C_i^{\alpha})$ and $\psi(C^{\alpha}\text{-}C_i')$ for antamanide * and [Phe^{4}Val^6]antamanide •.

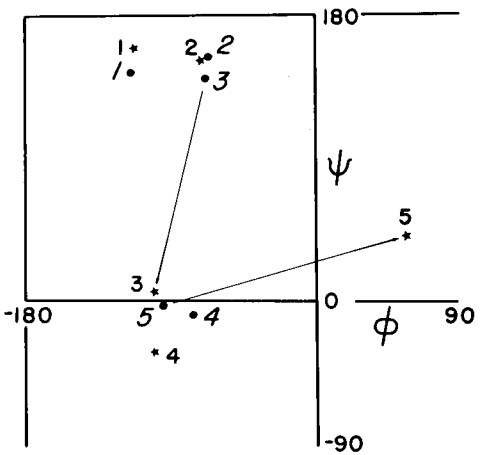

Fig. 5. Comparison of torsional angles ϕ and ψ for uncomplexed antamanide (water complex) * and the Na^+ complex •. Two-fold rotation symmetry has been approximated.

Channel Formation — In crystals of symmetric antamanide grown either from n-hexane/MeAc or from $Ca(NO_3)_2 \cdot 4H_2O$/CH_3CN/acetone, the antamanide molecules stack over each other to form large diameter continuous channels,[10,11] as indicated in the schematic diagram in Figure 6. The lipophilic regions of the peptide molecules are in contact with neighboring peptide molecules (not shown in Figure 6). In spite of the

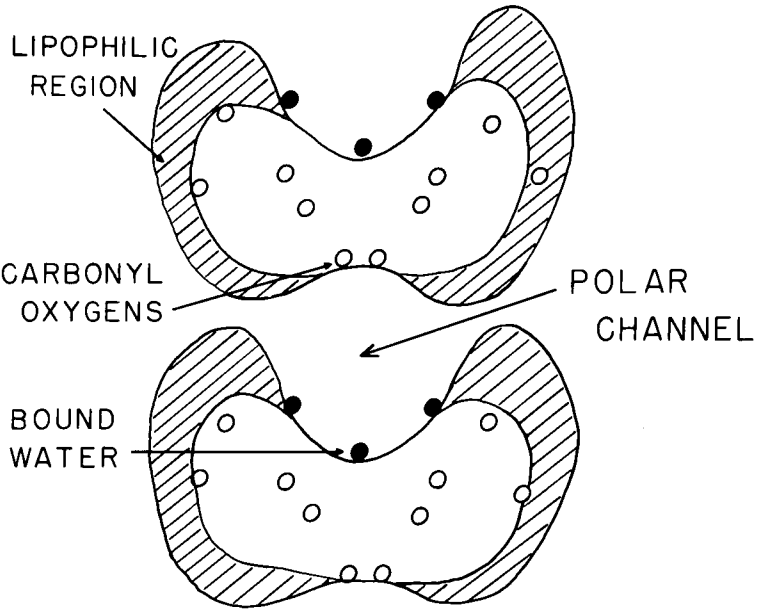

Fig. 6. Schematic diagram of channel formed in the crystal by symmetric antamanide molecules.

polar lining of the channel, in crystals grown from n-hexane/MeAc, the channel is filled with the non-polar solvent in random positions. In crystals from $Ca(NO_3)_2 \cdot 4H_2O/CH_3CN$/acetone, the channel is filled with water molecules in definite crystallographic sites, Figure 7. The water molecules are of two types, "bound" water molecules in the outer layer of the channel that make hydrogen bonds to carbonyl oxygens or amide nitrogens of the peptide, and water molecules in the core that are completely surrounded by other water molecules. Not all water sites in the core can be occupied simultaneously by water molecules, hence the occupation is different and somewhat random along the channel in different unit cells of the crystal. It is conceivable that this structure could be a model for K^+ ion transport through membranes as facilitated by perhydroantamanide.[3] It is unlikely that HAA will make K^+ complexes by encapsulation similar to the Na^+ complexes for the geometric reasons discussed earlier. On the other hand, the dimensions of the inner core of the channel as evidenced by the water-water hydrogen bond distances, are compatible with K^+———O ligands of 2.7 A.

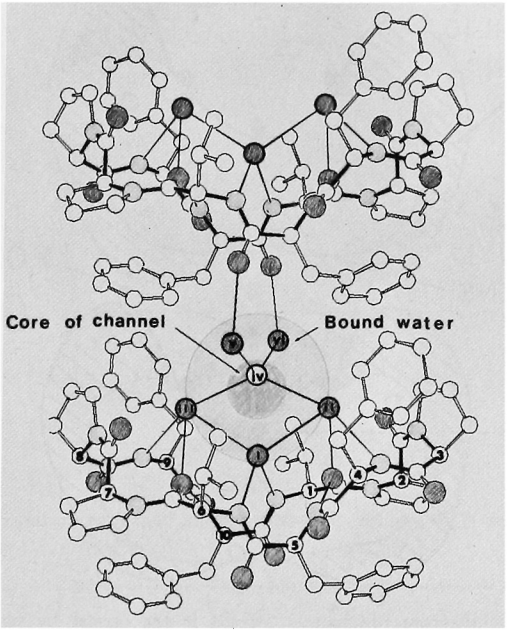

Fig. 7. Channel formed in the crystal by symmetric antamanide (compare to Figure 6). Ten additional water sites in the channel are located in front of and behind the six shown (i-vi).

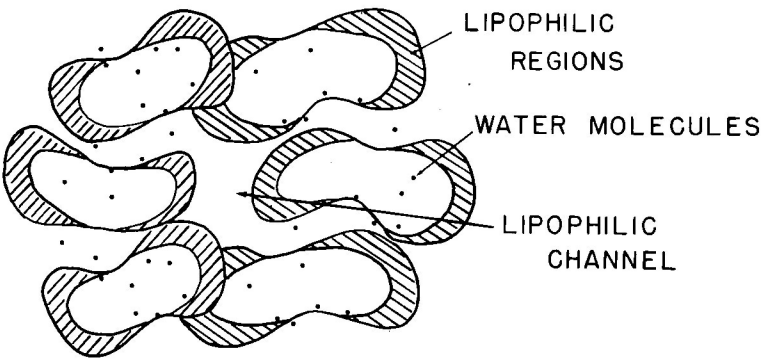

Fig. 8. Packing of natural antamanide in the crystal.

Natural antamanide[9] crystallized from CH_3CN/H_2O, although having the same conformation as the symmetric antamanide just discussed, packs quite differently in the crystal, Figure 8. The molecules do not stack to form polar channels. Rather they have been shifted somewhat to nestle in the depressions of adjacent molecules. Eight molecules of water serve as hydrogen bonding bridges between peptide molecules. However, there are continuous channels in the lattice with a cross-section of 6.1 x 8.0 A. These channels are almost completely surrounded by the methyl, isopropyl, pyrrolidine and phenyl groups of the peptide molecules. No specific sites for the remaining solvent molecules have been found in the channel. Thus four H_2O molecules or one molecule each of H_2O and CH_3CN (based on density measurements) occupy random position in the hydrophobic channel.

Non-Channel Formation — Crystals of symmetric antamanide grown from mixtures of H_2O and one of the following: acetone, ethanol, CH_3CN, DMSO, or tetra methyl urea, all exhibit almost identical cell dimensions and very similar diffraction patterns. Hence, it may be assumed that all these crystals are isomorphous. Their cell parameters a=20.19 A b=21.12 A, c=16.13 A (space group $P2_12_12_1$) are quite different from those of all the crystals already discussed. Since the volume per molecular unit is only 1719 A^3 as compared to 1821 A^3 for the SAA crystallized from $Ca(NO_3)_2 \cdot 4H_2O/CH_3CN$/acetone and 1884 A^3 for the AA crystallized from CH_3CN/H_2O, it seems safe to assume that these crystals do not contain channels.

Perhydrosymmetric antamanide crystallized from n-hexane/MeAc and from CH_3CN also has a diffraction pattern very similar to the SAA discussed immediately above, however, the cell dimension in the *c* direction is longer by 1.0 A. This increase could be accounted for by the extra volume needed for cyclohexane moieties as compared to planar phenyl rings. It appears that most probably HSAA, crystallized in the absence of water, is isostructural with the SAA crystallized in the presence of water. It is not possible to predict at this point, prior to the crystal structure analysis, whether these two structures will yield the same conformation as the water complex in Figure 1(b) or 3.

Discussion

Only two conformations for antamanide and [Phe^4Val^6]antamanide have been found in the crystalline state so far, the alkali metal ion complex and the water complex. The water complex forms independently

of the nature of the solvent, particularly independent of the presence or absence of water. These results are not compatible with data from solution studies by ultrasonic absorption,[12] ultraviolet absorption,[12] proton and nuclear magnetic resonance measurements,[13] and optical-rotatory dispersion,[14] e.g., where several, probably three, conformations in rapid equilibrium are indicated for uncomplexed antamanide, depending upon the nature of the solvent. These may involve only rotations of the side chains or changing the number of hydrogen bonds between carbonyls and the solvent, rather than changes in backbone conformation. Addition of water to antamanide in non-polar solvents causes changes in CD spectra[2] similar to those caused by complexation with Na^+. In an effort to resolve some of these differences, x-ray diffraction analyses are continuing not only on variations in crystallizing antamanide but also diffraction data from antamanide solutions is being analyzed.

Meanwhile, the structure analyses of antamanide crystals have provided possible models for two modes of ion transport, encapsulation and channel formation.

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CATION TRANSPORT BY SYNTHETIC CYCLIC PEPTIDES: APPROACH TO CALCIUM-SELECTIVE TRANSPORT PEPTIDES

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Substances which can perturb the transmembrane cation balance in a predictable manner have wide-ranging uses in the study of cellular processes.[1,2] In order to identify the structural and conformational requirements for binding and transport by proteins of calcium — a factor of universal importance in "cell communication"[3] — we have undertaken the development of peptides which render membranes selectively permeable to calcium.

Cation transport across biological membranes — whether mediated by pores, channels, conformational transitions of membrane proteins, or by mobile carrier molecules — must at some stage involve binding of the transported cation to liganding sites composed of the functional groups typically encountered in protein-like materials. Among calcium-binding proteins for which X-ray crystallographic data are now available (including carp parvalbumin, rabbit skeletal muscle troponin, Concanavalin A, and thermolysin[4]), binding sites invariably consist of six oxygen ligands derived from a combination of side chain Glu or Asp carboxylate groups and peptide carbonyl groups, coordinated to calcium in approximately octahedral geometry.

These structural criteria are met by cyclic octapeptides of the sequence *cyclo*(Glu-Sar-Gly-(N-R)Gly)$_2$ (and Asp analogs) which we have synthesized (e.g., R = n-decyl, n-hexyl, cyclohexyl).[5] Cyclization of the peptide creates a central cavity lined with peptide carbonyl groups and brings Glu (or Asp) side chains into proximity. Electrically neutral complexes with divalent cations (Figure 1) are produced *via* a two protons-for-one-metal exchange mechanism. The problem of imparting membrane solubility to these peptides (and their metal complexes) was

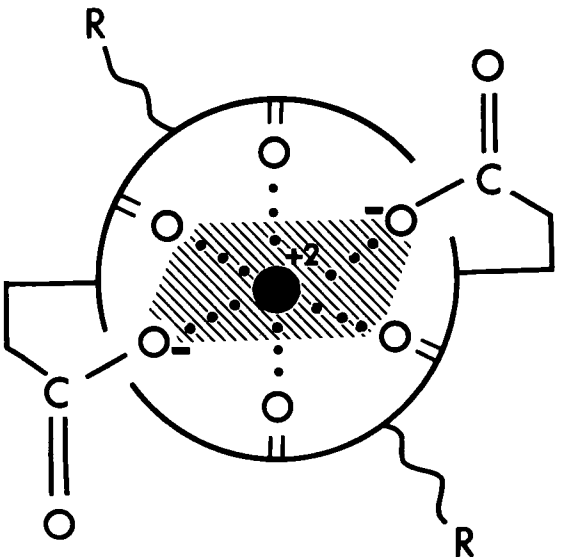

Fig. 1. Schematized representation of the electrically-neutral cyclic octapeptide/divalent metal complex. A second set of four peptide carbonyls (on the opposite face of the molecule) and all peptide nitrogens are omitted. R = N-alkyl chain.

solved through incorporation of the covalently-linked lipophilic "tails" (R).

Cation transport mediated by two synthetic peptides, *cyclo*-(Glu-Sar-Gly-(N-decyl)Gly)$_2$ ("DECYL-2")[5,6] (Figure 1, R = N-decyl), and its aspartic acid analog, *cyclo*(Asp-Sar-Gly-(N-decyl)Gly)$_2$ was monitored in a U-tube apparatus (Pressman cell).[7] These peptides were observed to induce the passive diffusion of Ca^{2+} ions at comparable levels across the organic phase (Figure 2). Net transport of Ca^{2+} was essentially unaffected — as determined from competition experiments — by the presence of equimolar Mg^{2+}, K^+, or Na^+ ions. Ca^{2+} transport by DECYL-2 was not diminished by the presence of 10-fold molar excess $^{22}Na^+$ (data not shown).

Although appreciable transport of Mg^{2+} and K^+ was observed in the absence of Ca^{2+} with both peptides, there was reduced or no transport of Mg^{2+}, K^+, or Na^+ when Ca^{2+} was present. Net Mg^{2+} transport was greater than net Ca^{2+} transport for each cation singly, while Mg^{2+} transport became negligible in the presence of Ca^{2+}; this result suggests that affinity of DECYL-2 for Ca^{2+} is greater than for Mg^{2+}, but that the decomplexation step (i.e., cation release) may be rate-determining. Variation in dimensions of the binding cavity — side chain carboxylate

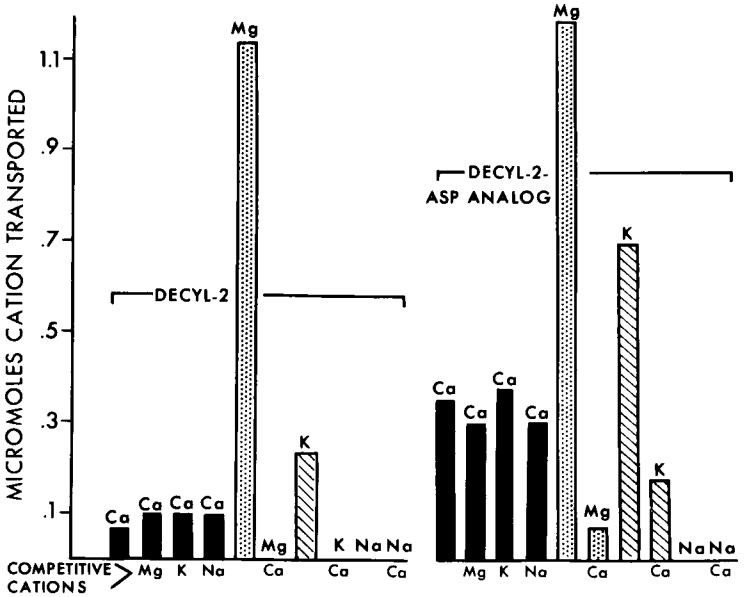

Fig. 2. Cation transport in Pressman cells mediated by *cyclo*-(Glu-Sar-Gly-(N-decyl)Gly)$_2$ and *cyclo*(Asp-Sar-Gly-(N-decyl)Gly)$_2$. A "liquid membrane" phase (25 ml chloroform containing 5 μmoles peptide) separates aqueous phase 1 (5 ml of 20 mM metal chloride (100 μmoles of cation) in 2 mM HEPES buffer at pH 7.5) from aqueous phase 2 (5 ml of 2 mM HEPES at pH 7.5). The organic phase was stirred slowly. After 7 hr, aliquots taken from aqueous phase 2 were analyzed for cation(s) by scintillation counting of radioisotopes and/or by atomic absorption spectroscopy. Cations shown on top of the bars were those assayed; cations given at the bottom, if any, were also present initially in aqueous phase 1 at equimolar levels.

moieties of Asp residues may be positioned closer than Glu carboxylates to the peptide backbone — presumably accounts for the nominal differences in selectivity profile between DECYL-2 and its analog. The overall results indicate that although the synthetic peptides are excellent Mg^{2+}-ionophores, *DECYL-2 is a calcium-specific transport peptide whenever* Ca^{2+} *is present.* Under conditions identical to those in Figure 2, transport mediated by A23187 (a naturally-occurring (non-peptidic) ionophorous antibiotic selective for divalent cations[8]) amounted to *ca.* 0.3 μmoles Ca^{2+}, and less than 0.02 μmoles Mg^{2+}, K^+, or Na^+.

The membrane activity of these peptides, and a number of their analogs, is presently being examined in phospholipid bilayers, mitochondria, and lymphocytes. In one study, preliminary results showed that DECYL-2 could reduce to zero the $^{45}Ca^{2+}$ content of unilamellar phosphatidylcholine vesicles, but considerable nonspecific efflux of Ca^{2+} also occurred at the doses required (ca. 10% w/w

peptide/lipid). This finding suggests that the two linear 10-carbon side chains of the peptide — while effective for membrane solubilization — may impede its transbilayer mobility. This latter property can be investigated through variation of N-alkylglycine residues.

While the relationship between the mode of action of naturally-occurring calcium-transport proteins[9] and peptides such as DECYL-2 remains to be elucidated, the present synthetic peptides may be counted among the most parsimonious structures which can function effectively in calcium binding and transport processes.

Acknowledgement

We thank the Medical Research Council of Canada (under MRC Grant No. 5810 to C.M.D.) for generous support of this work.

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PEPTIDES IN MEMBRANES: STRUCTURE OF A HYDROPHOBIC DIMER IN PHOSPHOLIPID VESICLES

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The conformations of membrane proteins determine their structural and functional roles within biological membranes. (Since the hydrophobic environment of the lipid bilayer is substantially different from the aqueous environment experienced by cytoplasmic proteins, it might be expected that proteins and peptides located within membranes would adopt structures different from those in aqueous solution.) To demonstrate a method by which membrane protein structures may be determined spectroscopically (and to ascertain whether structures adopted by peptides in aqueous and hydrophobic solvents do differ significantly), a hydrophobic oligopeptide, Boc-Pro-Leu-Val-OMe[1], was incorporated into phospholipid vesicles and its structure was determined by nuclear magnetic resonance, infrared and circular dichroism spectroscopy, and vapor pressure osmometry.

The first task was to demonstrate that the peptide was indeed located within the hydrophobic portion of the bilayer. In small sonicated vesicles, because the volume of the lipid bilayer interior is small compared to that of the surrounding aqueous solution, a partition coefficient of at least 10^5 - 10^6 is required in order to assure that greater than 90% of the peptide is located within the bilayer.

The peptide locations were demonstrated by ^{13}C nmr, using the paramagnetic shift reagent, thulium chloride. The thulium ion is located in the aqueous solution surrounding the membrane and does not penetrate the lipid bilayer[2], as evidenced by the lipid head group peaks which are strongly shifted due to their proximity to the probe in the aqueous solution (Table I), while the fatty acid chain peaks are virtually unaffected by the probe (data not shown). Although the peptide backbone peaks are obscured by lipid peaks in the presence of thulium, the upper limit to which they could be shifted is less than 0.2 ppm, clearly small in comparison to the head group shifts. Even the peptide side chains, which might be expected to be more accessible and exposed to the

probe, shift by 0.1 ppm or less (Table I). Clearly, this indicates the peptide is not accessible to the aqueous solution and located near the lipid fatty acid chains. While the exact position within the bilayer cannot be determined (indeed, the peptide is free to translate and rotate and, thus, is probably not localized), it must spend no substantial portion of its time in the hydrophilic region but, rather, is in the hydrophobic region at about the level of the C_2 of the fatty acid chain or lower.

Table I. Effect of Thulium Shift Reagent on Peptide and Lipid Peaks[a]

Assignment	Δ ppm	Assignment	Δ ppm
Lipid:		Peptide:	
Head Group Carbons		C_γ val	0.0
choline methyls	1.0	C_δ leu	0.0
CH_2-O-P	1.6	CH_3BOC	0.1
glycerol-1	0.2	C_β leu	0.1
glycerol-2	0.4	C_δ pro	0.0
glycerol-3	0.4	C_α leu	(<0.2)[b]
CH_2N	1.4	C_α val	(<0.2)[b]

[a] Molar ratio of thulium:peptide = 1:1.

[b] Obscured by lipid peak in presence of thulium. The value reported is the upper limit of possible shift.

Other evidence for the location of the peptide within the bilayer comes from circular dichroism. The spectrum of the peptide in vesicles is similar (λ_{min}, θ) to its spectrum in hexane but totally dissimilar from its spectrum in water[3], suggesting that in vesicles the peptide is located in an environment similar to hydrocarbons (such as the lipid fatty acid chains), not in an aqueous environment.

A Solvent Model System to Simulate the Lipid Bilayer

Since the peptide peaks of the nmr spectra in vesicles were too broad to permit detailed structural studies[3], in order to examine the molecular conformation of the peptide, a solvent system which simulated the environment of the hydrophobic region of the bilayer and permitted isotropic tumbling of the peptide was required. To indicate whether a

solvent was similar to the lipid, the ^{13}C nmr chemical shifts of the peptide peaks in the solvent were compared to the shifts of the peptide peaks in vesicles; in addition, the CD spectra in vesicles and solvents were compared in several cases. The following solvents were examined: hexane, hexadecane, water, chloroform, methanol, and acetone. Peaks which differed by less than 0.1 ppm from the peaks in vesicles were considered to result from virtually identical environments. These results suggest that linear hydrocarbons (hexane and hexadecane) present an environment to the peptide similar to the membrane interior. The other solvents do not. In another study using the peptide gramicidin[4], other organic solvents were similarly examined by CD and/or nmr, and none of these solvents were good models. Thus, since linear hydrocarbons were the only solvents shown to be good model systems, they were used in the following studies of the peptide structure.

The Peptide Forms an Intermolecularly Hydrogen-Bonded Dimer

The valine amide group of the peptide is involved in hydrogen bonding, as determined by the temperature-dependence of the ^1H nmr shifts of its amide proton. While the leucine amide temperature coefficient was $\sim 11 \times 10^{-3}$ ppm/°C, indicating exposure of this amide group to solvent and the absence of peptide-peptide hydrogen bonds, the valine temperature coefficient was $\sim 3 \times 10^{-3}$ ppm/°C, suggesting it is hydrogen bonded. The NH stretching region of the IR spectrum exhibits one-half of the area of the amide absorption band at 3280 cm^{-1} (hydrogen bonded) and half at 3380 cm^{-1} (non-hydrogen bonded), consistent with one hydrogen bonded and one free amide proton.

The Boc carbonyl group is hydrogen bonded, as indicated in the IR spectrum by the C=O stretch at 1689 cm^{-1}, the position for hydrogen bonded Boc, whereas the methyl ester carbonyl and the peptide amide I carbonyl stretches for central and C-terminal residues are located at the non-hydrogen bonded positions of 1730, 1662, and 1653 cm^{-1}, respectively.

That the valine amide and Boc carbonyl groups are intermolecularly hydrogen bonded, forming a dimer or multimer, rather than intramolecularly, forming a β-turn, is indicated by the high concentration-dependence of the ^1H nmr valine amide proton shift. Vapor phase osmometry in spectrograde hexane, using benzophenone as a standard, demonstrated that the effective molecular weight of the

peptide species was ~880 (monomer = 440) over the concentration range used in the nmr, CD, and IR experiments (5×10^{-4} to 10^{-2} *M*), suggesting the species examined was, for the most part, dimer. Dihedral angles were determined from ^{1}H nmr coupling constants[3].

These data, as well as T_1 measurements of molecular motion[3], were then used to construct a model for the peptide conformation. The backbone is restricted to a single conformation, which, as seen in the CPK model (Figure 1) has a very hydrophobic surface.

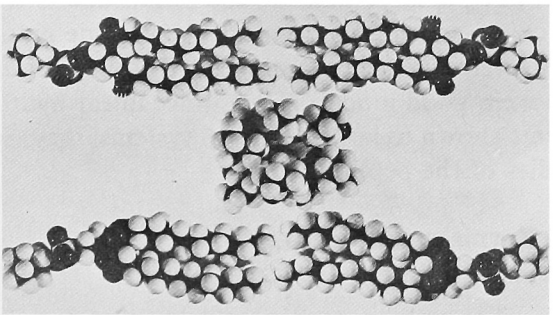

Fig. 1. CPK models of Boc-Pro-Leu-Val-OMe flanked by four dimyristoyl phosphatidyl choline molecules, indicating its relative location within the lipid bilayer. Note the highly hydrophobic surface the peptide presents to the lipid fatty acid tail region.

To conclude, the structure of a hydrophobic peptide has been determined in phospholipid vesicles. Hydrocarbon solution has been proposed as a model system which simulates the environment of the bilayer interior. The methods used in this work may be extended for studies of larger membrane proteins.

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MODELS OF THE *E. COLI* OUTER MEMBRANE AND EXCITABLE MEMBRANE CHANNELS

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In this paper I propose nine stranded β barrel models for the protein structure of three membrane channel systems, the *E. coli* outer membrane,[1] action potential channels of nerve and muscle,[2] and postsynaptic acetylcholine receptors (AChR).[3] These models were derived from and are supported by existing biochemical, electron microscopic, X-ray diffraction, and circular dischrosim data. They are consistent with the kinetics of the opening and closing of the channels and can account for pharmacological effects of many drugs and toxins. When these channels are closed, the center of the β barrel is occupied by a triple stranded coiled-coil as shown in Figures 1 and 2. The grooves between the

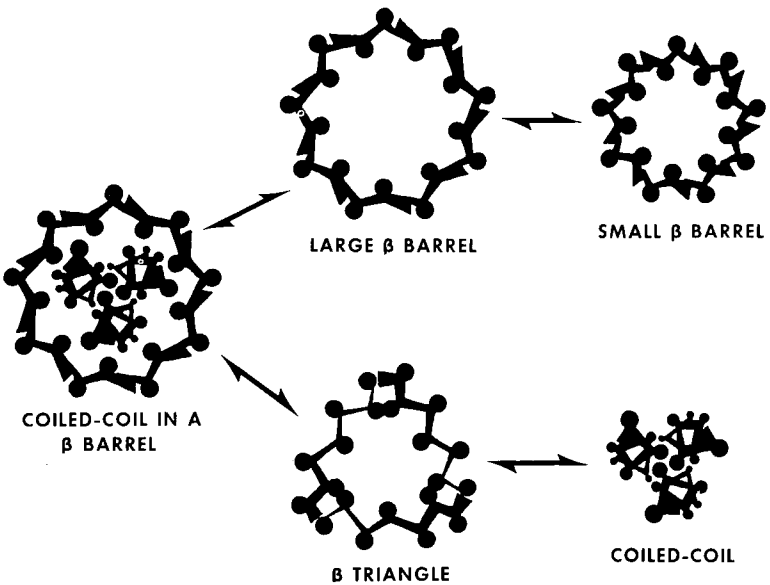

Fig. 1. Cross sectional view of conformations of *E. coli* outer membrane, excitable membrane and postsynaptic membrane proteins. The arrows indicate the conformational changes that occur when channels open and then inactivate. The straight lines connect α carbons and the circles represent side chains.

side chains of the β barrel twist around the barrel in a left handed manner with approximately the same pitch as the super helix of the coiled-coil. This allows the side chains of the coiled-coil to fit between those of the β barrel. The β barrels aggregate in trimers or tetramers (Figure 3).

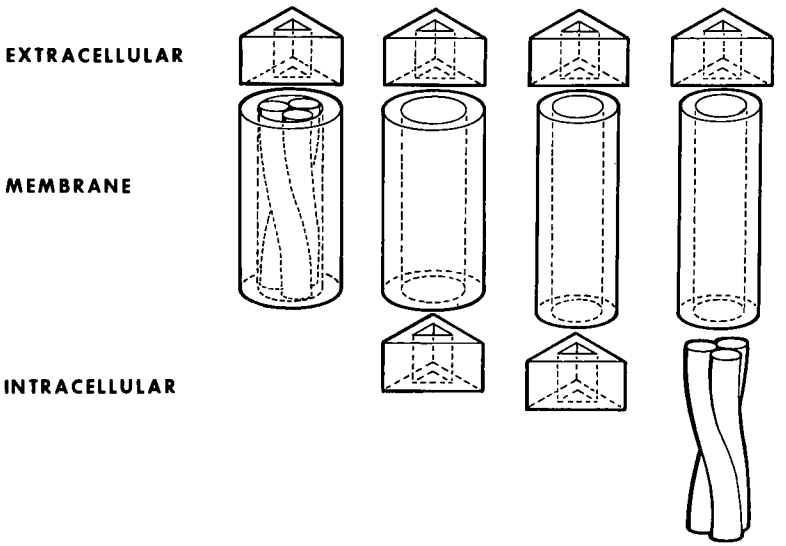

Fig. 2. Side view of the different conformations of individual subunits.

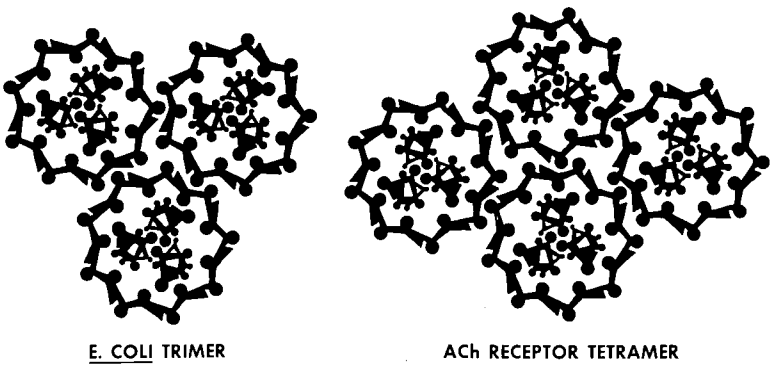

Fig. 3. The aggregation of the β barrels in *E. coli* outer membrane and in postsynaptic membranes. The distance between the centers of the β barrels is 30A.

The Na^+ action potential channel and the AChR have a β triangle on top of the β barrel (Figure 2). The center of the β triangle forms tetrodotoxin and saxitoxin binding sites in Na^+ channels and the top forms the α-bungarotoxin site of AChR. In Na^+ and AChR channels, the protein that forms the coiled-coil can be in one of three conformations: a coiled-coil in the β barrel, a β triangle on the cytoplasmic side of the membrane, and a coiled-coil that extends into the cytoplasm. The conformational changes between these states accounts for activation and inactivation of the channels. Drugs that bind in the β triangle conformation hold the channel in an open but blocked conformation. When the coiled-coil is removed from the β barrel, it can undergo a conformational change to narrower and longer β barrel. Drugs that inhibit this conformational change keep the channels in an open conformation with an altered ion selectivity. Channels inactivate when the coiled-coil extending into the cytoplasm is formed. The gating process can also involve interactions between adjacent β barrels. When the β barrels become smaller, channels permeant to K^+ or Ca^{++} may form between adjacent β barrels. Scorpion toxins and sea anemonia toxins alter Na^+ and K^+ currents by binding between adjacent β triangles that are on the outside of the membrane.

This system is very dynamic and therefore could be used to form many types of membrane channels and pumps. The proposal that the basic structure is found in both *E. coli* and nervous systems suggest that it may be a major building block of biological membranes.

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ONE-STEP SYNTHESIS OF AN ION BINDING PEPTIDE BY CYCLOTRIMERIZATION OF A LACTAM-CONTAINING DIPEPTIDE

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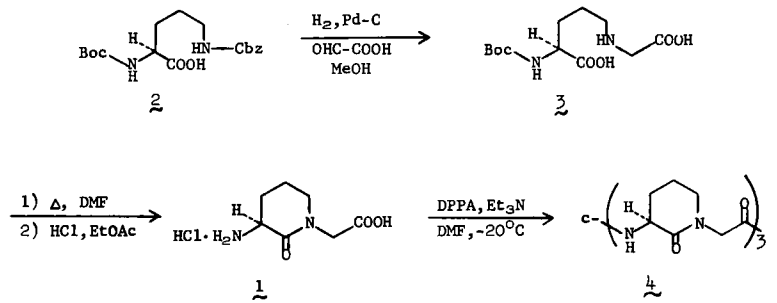
Ion binding cyclic peptides continue to attract considerable attention because of their interesting physical and biological properties.[1] One class of these compounds which has seen extensive study is the 18-membered macrocycles including cyclic hexapeptides and cyclic hexadepsipeptides. These cyclic peptides are usually prepared by synthesis of an appropriate linear sequence followed by cyclization.[2] Several interesting ion binding cyclic hexapeptides have repeating dipeptide units,[3] and an alternate approach to this type of system could be cyclotrimerization of an appropriate dipeptide sequence. This method is normally not a feasible route to cyclic hexapeptides since the most favored cyclization product is expected to be a diketopiperazine.[4] This pathway could be eliminated, however, if the dipeptide amide bond were forced to remain in the trans configuration. With all trans peptide bonds, formation of a cyclic tetrapeptide would also be unlikely. Thus, under proper conditions, the cyclic hexapeptide should be a major product.

The dipeptide chosen to test this hypothesis was *S*-3-amino-2-oxo-1-piperidineacetic acid (1) which may be viewed as a bridged analog of alanylsarcosine. Lactam 1 was prepared stereospecifically in three steps from *Nα*-Boc-*Nδ*-Cbz-L-ornithine (2) as shown in Scheme I. Removal of the side chain protecting group of 2 and *in situ* reductive alkylation of the resultant primary amine with glyoxylic acid was accomplished by hydrogenation in the presence of 10% palladium on carbon catalyst. The reaction was stopped at the point where the monoalkylation product was major, and the diacid 3 was isolated in 54% yield by fractional crystallization from methanol-ether.

Cyclization of compound 3 to the protected dipeptide analog was readily accomplished without carboxyl activation in essentially quantitative yield by warming to 50-55°C in dimethylformamide for 90 minutes. The cyclization also occurs at room temperature, but the rate is

slow ($t_{1/2}\approx 15$ hrs). Cyclization was also observed in methanol, but at a reduced rate. No lactam formation was observed in water at 60° under acidic, basic, or neutral conditions. Removal of the Boc group in a saturated solution of HCl in ethyl acetate at 0°C gave lactam 1 as the hydrochloride salt. Analysis by the method of Manning and Moore[5] (using the *N*-carboxyanhydride of leucine for derivatization) showed this material to be optically pure.



Scheme I. Synthesis of Cyclic Trimer 4

Cyclotrimerization of this lactam at a concentration of 0.1 M using the diphenylphosphoryl azide (DPPA) procedure[6] gave the crystalline cyclic hexapeptide 4 in 25% yield. The cyclic tetramer was also isolated (5% yield), but cyclic dipeptide and tetrapeptide were not detected.

Table I. Physical Properties of New Compounds

Compound	m.p.	$[\alpha]_D^{24}$	R_f [a]
3	150°(dec.)	2.59(c, 0.97, MeOH)	0.40 (A)
Boc-1	113-116°	-22.14 (c, 1.00, MeOH)	0.69 (B)
1	---	-1.84 (c, 0.98, MeOH)	0.35 (C)
4	>290°(dec.)	-220 (c, 1.00, $CHCl_3$)	0.30 (A)

[a]Tlc systems, ethyl acetate: pyridine: acetic acid: water — A, 5:5:1:3; B, 10:5:1:3; C, 5:10:2:6, silica gel plates.

The key compounds in this synthesis have been fully characterized, and some physical properties are given in Table I. In addition, the correct molecular weight for cyclic trimer 4 was found by gel filtration.[6] The proton NMR spectra of compound 4 gave information about its conformation and ion binding properties, and some important chemical shifts and coupling-constants are listed in Table II. Simple spectra

indicative of C_3 symmetry were obtained in chloroform and DMSO, and the large difference in chemical shift between the geminal Cα protons is consistent with a fixed conformation. The H-N-Cα-H coupling constants convert to dihedral angles of 145° (DMSO) and 130° ($CHCl_3$)[7] which on the basis of model building would be consistent with structures with three 1 ←3 hydrogen bonds similar to Conformer S of c-(Pro-Gly)$_3$[3a] or those predicted for c-[Tyr(OBzl)-Gly]$_3$[3b] or c-(Val-Sar)$_3$.[3c] The proposed conformation of 4 in $CDCl_3$ is further supported by the nonequivalence of the two Cβ methylene protons of the lactam ring. The equatorial hydrogen is expected to be deshielded by the amide carbonyl relative to the axial hydrogen. The coupling constants between Cα-H and the two Cβ protons are in agreement with a single chair conformation for the lactam ring.

Table II. NMR Data for Cyclic Trimer 4

Solvent	$\delta_{N\text{-}H}$	$J_{H\text{-}N\text{-}C^{\alpha}\text{-}H}$	$\delta_{C^{\alpha}H_2}$	$J_{C^{\alpha}H_2}$	$\delta_{C^{\beta}H_2}$
d-DMSO	7.70	7 Hz	3.1, 4.9	16 Hz	1.8
$CDCl_3$	7.70	5 Hz	3.15, 4.95	15 Hz	1.6,2.5
$CDCl_3$+Na^+	7.55	10 Hz	3.35, 4.6	16 Hz	2.1

In $CDCl_3$, c-(Ala-Sar)$_3$ appears to have a conformation similar to that of 4, but it exhibits a complex spectrum in DMSO probably consisting of an equilibrium mixture of more than one conformer. The difference between the spectra in DMSO reflects the molecular constraint imposed by the lactam ring in structure 4. The lactam amide bond which corresponds to the Ala-Sar peptide bond is prevented from isomerizing.

Gradual addition of one equivalent of sodium thiocyanate in DMSO to the chloroform solution of the cyclic trimer produced a modification in the conformation characterized by a H-N-Cα-H coupling constant of 10 Hz (dihedral angle of 180°) and the near equivalence of the Cβ methylene protons. A model consistent with these observations has the three lactam carbonyls on one side of the plane of the macrocycle to complex the cation. The remaining three amide carbonyls are in the plane of the macrocycle turned outward.

Transport of cations by cyclic trimer 4 was investigated with a U-tube experiment.[8] The relative rates of transport were Li^+:Na^+:K^+:Ca^{++} = 1:6.2:3.4:2.9. The corresponding rates for c-(Ala-Sar)$_3$ (1:6.6:2.8:0.1)

show it to be relatively less efficient for transport of calcium ion.

In conclusion, we have shown cyclotrimerization to be a useful method for the synthesis of a novel cyclic hexapeptide. The three lactam ring bridges in the macrocycle produce conformational and ion binding properties different from those of the unbridged structure.

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BIOLOGICAL FUNCTION OF GRAMICIDIN A: COMPARISON OF THE EFFECT OF LINEAR GRAMICIDIN A ANALOGS ON MEMBRANE PERMEABILITY, BACTERIAL SPORULATION, AND RNA POLYMERASE

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Introduction

Gramicidin A is a polypeptide antibiotic produced by *Bacillus brevis* at the onset of sporulation with the following sequence: formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-(D-Leu-L-Trp)$_3$-NH(CH$_2$)$_2$OH. A mutant previously isolated by Mukherjee and Paulus[1] does not synthesize gramicidin and does not sporulate properly. However, addition of exogenous gramicidin restores the normal spore phenotype, thereby demonstrating a *biological function* for gramicidin. This raises an important question as to the mechanism by which gramicidin exerts its biological effect. Gramicidin can act as an antibacterial agent by affecting the cation permeability of membranes.[2-4] On the other hand, we have found that the antibiotic can specifically inhibit transcription with purified bacterial RNA polymerase.[5,6] Here we report studies with a series of gramicidin analogs in which the structural specificity of the *in vivo* response of the gramicidin-negative mutant was compared with the ability to affect membrane permeability and to inhibit RNA polymerase.

Results and Discussion

The mutant spores are abnormally temperature sensitive and have only 20% of their normal dipicolinate content. The addition of exogenous gramicidin at the onset of sporulation stimulates mutant dipolicinate production 3-fold and yields temperature-stable spores. For all of the

Table I. Effect of Gramicidin and Related Peptides on Dipicolinate Production, RNA Polymerase, and Bilayer Membranes.

Gramicidin analog	Stimulation of DPA production in *B. brevis* M1 by 11μM peptide (nmoles/ml)[a]	% Inhibition of *B. brevis* RNA polymerase at peptide conc. of 2.7μM	11μM	27μM[b]	Channel forming capacity in black bilayer membrane[c]
Val_1	104	0	100	100	1.0
Cys_1	117	76	80	72	<0.001
S-methyl-Cys_1	98	0	74	91	0.1
N-acetyl-des(formyl)	116	22	99	100	0.03
Des(formyl)	50	100	100	100	<0.001
Des(formyl-Val_1)	16	60	100	92	<0.001
GD(1)[d]	96	45	99	99	0.01
GD(3-6)[d]	153	0	76	88	<0.001
GD(3-8)[d]	20	0	43	87	<0.001
GD(9-10)[d]	138	0	11	85	<0.001
GD(6-11)[d]	18	-10	-29	-27	<0.001
GD(6-13)[d]	16	0	0	0	<0.001
GD(9-14)[d]	22	0	0	0	<0.001

[a]Dipicolinate (DPA) production in the absence of added peptide was 80 nmoles/ml (S.D. = 20 nmoles/ml).

[b]Purified enzyme assayed with bacteriophage φe DNA (0.001 A_{260}/ml) as template.

[c]Conductance relative to gramicidin.

[d]GD(n-m) is a gramicidin analog with residues n to m deleted.

gramicidin analogs so examined, the increase in spore heat stability and the more easily quantified stimulation of dipicolinate production always occurred together.

Natural gramicidin predominantly has an N-terminal Val_1, which is formylated. Semisynthetic substitution $CyCys_1$ (also N-formylated) completely abolishes the channel-forming ability of this gramicidin analog,[4] but increases its inhibition of RNA polymerase (Table I).

Because its ability to stimulate dipicolinate production in the mutant was the same as that of natural gramicidin (Table I), this proves that the biological function does not involve ion transport. In general, while many biologically active gramicidin analogs had no effect on membrane permeability, *all* biologically active peptides were able to inhibit RNA polymerase. *These observations exclude membranes as the site of action of gramicidin during bacterial sporulation but are consistent with the notion that gramicidin functions to control RNA synthesis during the transition from vegetative growth to sporulation.*

For a series of gramicidin analogs[7], deletion of up to six residues in the N-terminal half of the molecule had little or no effect on the RNA polymerase inhibition, while deletion of just one Trp-Leu dipeptide from the C-terminal half decreases RNA polymerase inhibition, while deletion of two dipeptides abolishes it.

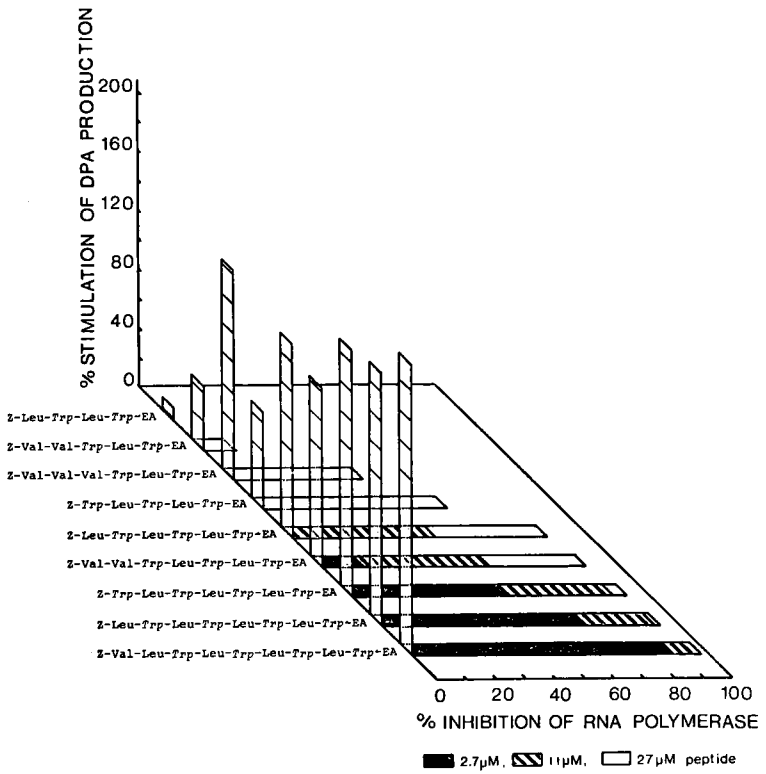

Fig. 1. Effect of short synthetic peptides (11μM) on dipicolinate production by *B. brevis* strain M1 and on RNA polymerase. EA = $NH(CH_2)_2OH$.

For a series of synthetic peptides related to the C-terminal sequence of gramicidin[7] (Figure 1), both the biological activity and the RNA polymerase inhibition increased progressively with the number of amino acid residues and attained a level comparable to that of gramicidin at the heptapeptide stage, containing all four Trp residues. In general, the structure-function studies showed that the eight N-terminal residues have little influence on the *in vitro* inhibition of RNA polymerase, whereas the C-terminal L-Trp-(D-Leu-L-Trp)$_3$ is essential and represents the site of interaction with RNA polymerase. Because gramicidin inhibits RNA polymerase by binding *competitively* with the double stranded DNA template[8], a model for the C-terminal half of gramicidin is proposed which bears a striking resemblance to one chain of a DNA double helix with the tryptophan residues in place of bases.

Acknowledgement

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INCLUSION COMPLEXES OF CYCLOPEPTIDES RELATED TO VALINOMYCIN

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Introduction

The ion-carrier antibiotic, valinomycin (VAL), has obtained much attention because of its high potency and selectivity in transporting K^+, but not Na^+, through lipid membranes.[1] The large number of synthetic analogs of this depsipeptide[2] has substantially increased our understanding of both membrane structure and ion transport mechanisms. The peptide analogs,[3–5] lacking α-hydroxy acid residues entirely, have proven particularly useful, as they exhibit properties that are strikingly different from those of their natural counterpart. For instance, the peptide *cyclo*-(L-Pro-L-Val-D-Ala-D-Val)$_3$ binds the alkali cations 10 times more strongly than VAL but has no effect on membranes.[6,7] Thus, changes in primary structure have lead to properties that were not anticipated. In this contribution a general formula for peptides and depsipeptides suitable for inclusion of cations is proposed. Secondly, the X-ray structure of the PV-Rb^+ complex [PV = proline valinomycin, *cyclo*-(L-Pro-L-Val-D-Pro-D-Val)$_3$] is discussed and thirdly, ion-binding data for PV-Lac [PV-Lac = *cyclo*-(L-Lac-L-Val-D-Pro-D-Val)$_3$; Lac = lactyl] are presented.

General Formula

VAL transports potassium across lipid membranes based on its ability to form, with high selectivity, a hydrophobic inclusion complex with the cation. However, ion-binding, though necessary, is not a sufficient condition for membrane activity. Uncharged peptides and depsipeptides that have been found to bind cations appear to conform to the following general formula: *cyclo*-(L-A-L-B-D-A′-D-B′)$_n$, where A, A′

= α-amino, α-hydroxy or N-alkyl-α-amino acid, B, B′ = α-amino acid and n = 2,3,4. This empirical formula is based on molecular modelling and published structure-activity reports. It should be noted that positions 2 and 4 of the β-turn (Figure 1) must be amino acids as they have to provide the amide N-H for stabilization of the β-bend. Moreover, the L-L-D-D sequence of the residues must start at position 1, so that the backbone reverses direction at position 3 and provides a carbonyl group for coordination of the cation.

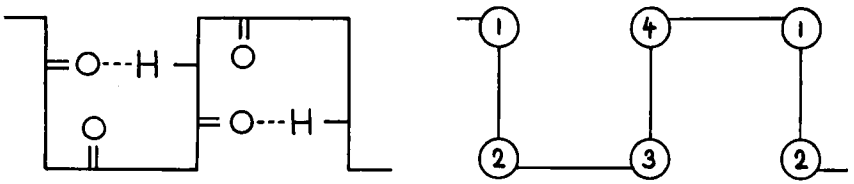

Fig. 1. Schematic representation of 2 β-turns of an ion-binding cyclopeptide and numbering of residues. Non-hydrogen bonded carbonyls point away from us and coordinate to the cation.

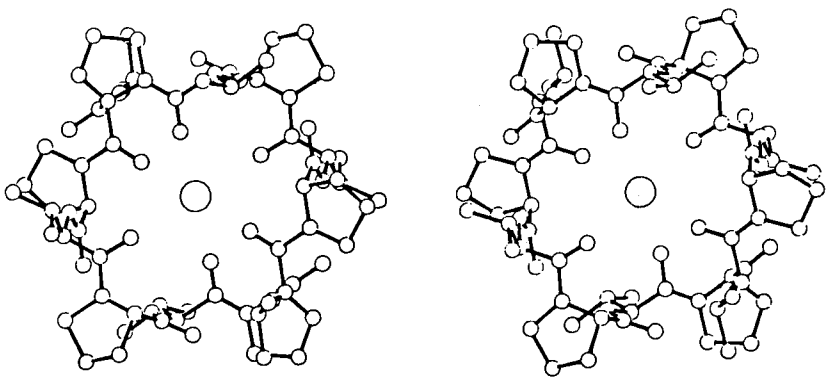

Fig. 2. The structures of the two independent molecules in the PV-Rb^+ picrate crystal are almost identical.

X-Ray Structure

The PV-Rb^+ picrate complex crystallizes in the triclinic system with two each of PV molecules,[3] rubidium cations, and picrate anions in the unit cell.[8] The conformation of the independent PV molecules (Figure 2) is similar to that of the VAL-K^+ complex and to the conformation found in solution.[9] PV binds the cation by providing a centrosymmetric pair of short bonds, a pair of medium bonds, and a pair of longer bonds. This is in contrast to providing six medium length bonds. The averages of the Rb . . . O bonds for the two molecules are 2.892 A and 2.910 A, which is a

difference of about one standard deviation (0.015 A). These lengths are however, significantly longer than 2.636 A to 3.025 A (average 2.833 A, standard deviation 0.011 A) found for a VAL rubidium complex. The C-O . . . Rb bond angles of the two PV molecules have average values of 153.3 and 152.0 degrees, which are within one standard deviation (1.2 deg.). The average length of the six hydrogen bonds is 3.16 A, which is about 0.1 A longer than those found in valinomycin structures and indicates that PV is a slightly larger cage.

Table I. Half Saturation Concentrations, K_{50}[a], of 3 x 10^{-5}M PV-Lac in $CHCl_3$ Equilibrated with Aqueous Picrate Salt Solutions.

Compound	Li^+	Na^+	K^+	Rb^+	Cs^+	NH_4^+	Tl^+
PV-Lac	$2x10^{-2}$	$8x10^{-3}$	$1x10^{-4}$	$6.5x10^{-5}$	$1x10^{-4}$	$2.5x10^{-4}$	$2x10^{-4}$
PV[b]	$2x10^{-4}$	$1.4x10^{-4}$	$2x10^{-7}$	$6x10^{-7}$	$5x10^{-7}$	$8x10^{-7}$	$1x10^{-6}$
VAL[b]	-	$> 10^{-1}$	$2.8x10^{-4}$	$2.2x10^{-4}$	$5x10^{-4}$	$2.8x10^{-3}$	$1.8x10^{-3}$

[a] Aqueous salt concentration (M) that converts 50% of the ion carrier in the organic phase into the cation complex.

[b] Values taken from ref. 6 for comparison.

PV-Lac

PV-Lac is a hybrid between VAL ("top" half) and PV ("bottom" half). The affinities of this compound for monovalent cations are listed in Table I in comparison with VAL and PV. These data were obtained with a two-phase (chloroform-water) extraction assay.[6] For all cations studied, PV-Lac has about a two orders of magnitude lower affinity than PV and binds cations 3-10 times more strongly than VAL. Thus, in this assay PV-Lac assumes a position intermediate between its structural parents, PV and VAL. The ionic selectivity sequence of PV-Lac is $Rb^+ > K^+ \sim Cs^+ \gg Na^+ > Li^+$, thus very similar to that of PV ($K^+ > Rb^+ \sim Cs^+ \gg Na^+ > Li^+$) and of VAL ($Rb^+ \sim K^+ > Cs^+ \gg Na^+$), preferring the large alkali ions over the smaller ones. However, the preference of PV-Lac for potassium over sodium (K_{50Na}/K_{50K}) is only ~80 compared to ~700 δ of PV and >360 of VAL. PV-Lac has also been described by Fonina *et al.*[10]

Acknowledgements

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INTERACTION OF CALCIUM IONS WITH α-ELASTIN

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Introduction

The problem of connective tissue calcification is of great biological relevance. An impressive amount of work has been reported in the literature on the interaction of calcium ions with elastin, a protein component of connective tissue, and with the fragment α-elastin, suitable for solution studies.[1] Spectroscopic investigations on the interaction of the latter peptide with Ca^{++} in organic solvents gave valuable structural information,[2] but did not allow a direct determination of the thermodynamic parameters for the binding process. In the present paper we are evaluating the above mentioned parameters in trifluoroethanol (TFE) by means of equilibrium dialysis, circular dichroism, and calorimetric techniques.

Results and Discussion

Equilibrium dialysis measurements — The binding isotherm at 20°C for the system α-elastin-Ca^{++} in TFE containing 2.5% water (v/v) is reported in Figure 1. It can be seen that the binding to α-elastin rises rapidly to an r value (bound Ca^{++}/protein) of about 0.06 and increases much more slowly after this binding ratio is reached. The Scatchard plot[3] relative to the above measurements is linear within the concentration range we investigated. We can therefore conclude that, under our experimental conditions only one type of independent binding site is present. This conclusion is further supported by the evaluation based on the theoretical treatment proposed by Hill[4] and Schwarz.[5] The absence of cooperativity in our case is indicated by values of the n_H and q parameters very close to unity.

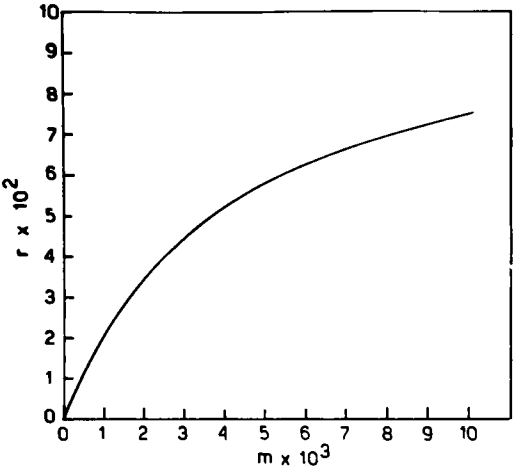

Fig. 1. Binding isotherm at 20°C for the system α- elastin-Ca^{++} in TFE containing 2.5% H_2O (m is the free Ca^{++} conc.)

From a best fit treatment of the binding isotherm, and the above mentioned plots[3–5] we obtain a maximum fraction of binding sites (n) ranging between 0.100 and 0.112, thus indicating that about 10 residues percent can be involved in the complex between α-elastin and Ca^{++}. From the same plots a binding constant K of 250 ± 30 $mole^{-1}$ has been calculated.

Several studies dealing with Ca^{++}-protein complexes indicate the presence of oxygen atoms in the coordination sphere of the metal ion,[6] from carboxylic groups or from the amide group. Most probably carboxylate moieties are not involved in complex formation in our case, since the value of n is substantially higher than the average number (0.025) of such moieties per protein mean residue. Moreover the interaction of α-elastin with Ca^{++} is not affected by the presence of trifluoroacetic acid or by acetylation of the protein.[2,7] It is therefore reasonable to assume that the oxygen of the amide group is involved in the complex.

The low value of the complex formation constant (250) points toward a poorly selective and specific interaction between Ca^{++} and α-elastin. This should not be the case if binding were promoted by the conformational properties of well defined regions of the protein as reported in the literature.[1] Finally, as a consequence of lack of cooperativity the metal ion should be randomly distributed along the peptide chain.

Circular Dichroism Measurements — Addition of Ca^{++} to α-elastin causes a conformational change in the protein as shown by spectroscopic measurements.[2] The observed modification in the CD spectrum has been employed to monitor the binding process, by assuming a linear relationship between the degree of binding and the CD response.[2]

A plot of the ellipticity of α-elastin at 225 nm as a function of the Ca^{++}/peptide ratio is reported in Figure 2. In the same figure the r data are shown for comparison as a function of the Ca/peptide ratio. It is immediately evident that, while the binding process is non-cooperative, the reverse is true for the conformational change, so that a relevant degree of binding has to be achieved in order to promote the structural modification. It is therefore not possible to obtain binding data from CD measurements in our system, unless experiments at different protein concentrations are performed.

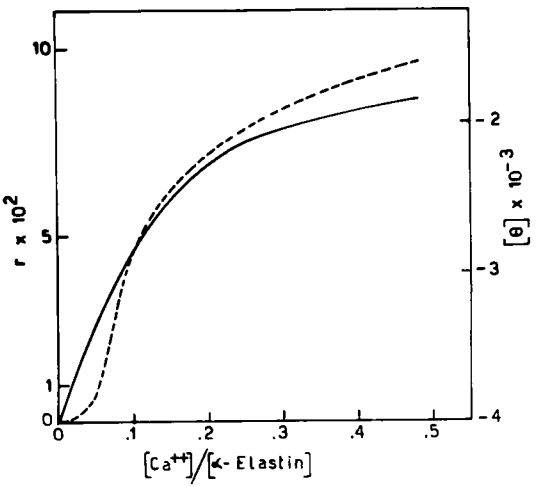

Fig. 2. Plot of r (solid line) and [θ] (dashed line) vs. Ca^{++}/α-elastin ratio in TFE containing 2.5% H_2O.

Calorimetric measurements — As reported in the previous section binding of the metal ion causes a conformational transition at Ca^{++}/peptide values higher than 0.05. Calorimetric data should enable us to calculate both the ΔH of the binding and the enthalpy change due to the conformational transition. In fact the calorimetric response at low Ca/peptide ratios (besides the heat of mixing and friction) is completely due to binding of the metal ion, while at high ratios the additional heat of conformational transition has to be considered.

The binding process presents a positive ΔH change of +1,900 cal/gr.ion Ca^{++}, so that the driving force of the reaction must be entropic in nature. Considering a K value of 250, the net entropy change can be evaluated around +17 e.u. These data suggest that displacement of hydration water around the metal ion should play an important role in the overall binding pattern using TFE as the solvent. As a matter of fact no binding can be observed in water under the same experimental conditions.

A ΔH of +120 cal/residue has been found for the conformational change induced by calcium ions. This low value is in agreement with the CD measurements,[2] which show a relatively small structural change upon binding of the metal ion.

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RATES AND MECHANISMS OF CATION DISSOCIATION FROM CYCLIC PEPTIDES

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Introduction

The peptide, *cyclo*-(L-Pro-L-Val-D-Ala-D-Val)$_3$, (PVAV), is a synthetic analogue of the transport antibiotic valinomycin (VAL). Like its prototype, PVAV binds alkali metal ions and increases their solubility in organic solvents. Although the affinities of PVAV for these ions are generally ten times greater than the corresponding affinities of VAL, PVAV is *in*capable of lowering the electrical resistance of model lipid bilayer membranes.[1] It has been proposed that the latter is a consequence of the slow dissociation of the cation-peptide complex.

In order to measure the rate of this reaction, a method using isotope exchange and ^{1}H-NMR was developed. The technique makes use of the fact that in the complexed form (M^+-PVAV), amide protons do not exchange with solvent deuterons. In the free form (PVAV), however, the rate of isotope exchange is fast. Thus, the only way that protons can be lost from M^+-PVAV is via dissociation into M^+ + PVAV.

Theory

The chemical reactions, shown schematically in Figure 1, lead to the exchange of the i-th peptide proton (^{1}H)$_i$ of M^+-PVAV with solvent deuterium (SOLV-^{2}H). From these reactions the following rate equations (1 and 2) for the loss of protons from M^+-PVAV and PVAV by solvent exchange can be derived:

$$\dot{[MPH]} = -k_{dissoc}[MPH] + k_{assoc}[PH]\,[M^+] - k_{ex}^{mp}[SOLV\,^2H] \qquad (1)$$

$$\dot{[PH]} = -k_{ex}^{p}\,[SOLV\,^2H]\,[PH] - k_{assoc}[M^+]\,[PH] + k_{dissoc}[MPH] \qquad (2)$$

By making the following substitutions and approximations:

$$k_e^{mp} = k_{exch}^{mp} \quad [SOLV\ ^2H]$$

$$k_e^{p} = k_{exch}^{p} \quad [SOLV\ ^2H]$$

$$M^+ = K_{eq}\,[MP]_{tot}/[P]_{tot}$$

$$= (k_{dissoc}/k_{assoc})\,[MP]_{tot}/[P]_{tot}$$

equations (1) and (2) simplify to:

$$\dot{[MPH]} = (k_d\,[MP]_{tot}/[P]_{tot})\,[PH] - (k_d + k_e^{mp})\,[MPH] \qquad (1')$$

$$\dot{[PH]} = (k_e^{p} + k_d\,[MP]_{tot}/[P]_{tot})\,[PH] + k_d\,[MPH] \qquad (2')$$

CASE I: If only K^+-PVAV(^{1}H) is present initially, equations (1′) and (2′) reduce to:

$$[MPH] = k_e^{mp}\,[MP]\ ;\ [PH] \cong 0$$

since $K_{eq} \ll 1$ and $[PH] = [K^+] \cong 0$ Thus

$$[MPH] = [MPH]_0\ EXP(-k_e^{mp}t).$$

Experimentally it is found that k_e^{mp} is on the order of (weeks) and may be ignored in subsequent analysis.

CASE II: If, in addition to K^+-PVAV, an equivalent amount of deuterated free PVAV is added to the reaction mixture so that: $[KP^1H]_0 = [P^2H]_0$ then equations (1′) and (2′) reduce to:

$$\dot{[MPH]} = -k_d\,[MPH] + k_d\,[PH]$$

$$\dot{[PH]} = -(k_e^{p} + k_d)\,[PH] + k_d\,[MPH]$$

Integrating these equations leads to the solutions:

$$[MPH] = C_1^+\ EXP(\Lambda^+ t) + C_1^-(EXP(\Lambda^- t) \qquad (3)$$

$$[PH] = C_2^+\ EXP(\Lambda^+ t) + C_2^-(EXP(\Lambda^- t) \qquad (4)$$

where $\Lambda^{\pm} = -0.5\,(2k_d + k_e^{p}) \pm 0.5\,(4k_d^2 + k_e^{p})$

and $C_1^+ = [KP^1H]_0(k_d + k_e^{p} - \Lambda^+)/(\Lambda^+ - \Lambda^-)$

$$= [KP^1H]_0 - C_1^-$$

$$C_2^+ = [KP^1H]_0(k_d/(\Lambda^+ - \Lambda^-)) = -C_2^-$$

Results

The intensities of the D- and L-Val NH protons in a 1:1 mixture of K^+-PVAV(^{1}H) and PVAV(^{2}H) ([peptide]~2 x 10^{-3}M) in methanol-d_4

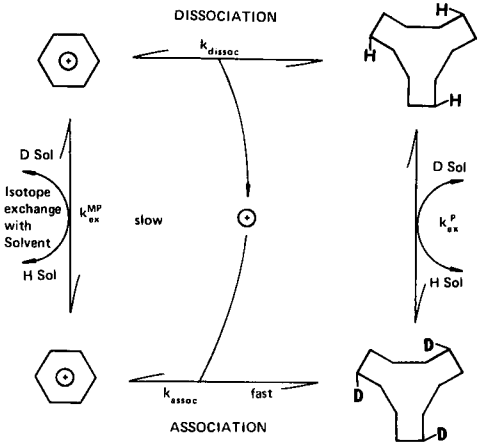

Fig. 1. Reaction scheme leading to the loss of amide ^1H from M^+PVAV and free PVAV

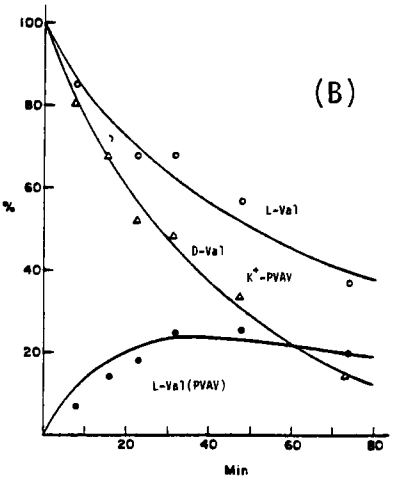

Fig. 2. Least-squares fits of equations (3 and 4) to data.

were measured at different times after mixing. The assignment of these lines to D and L isomers was made using (homo) nuclear Overhauser enhancement (NOE) techniques[3].

When equations (3) and (4) are fit by a least-squares procedure to the experimental intensities (Figure 2) the following (averaged) rate constants were obtained:

$$k_d = 3.7\ (\pm 0.7) \times 10^{-4}\ sec^{-1}$$

$$k^{L\text{-}Val} = 4.6\ (\pm 0.3) \times 10^{-4}\ sec^{-1}$$

$$k^{D\text{-}Val} > 10^{-2}\ sec^{-1}$$

(An independent measurement of the k-values for PVAV alone gives: $k_e^{L\text{-}Val} = (10(\pm 0.8) \times 10^{-4}\ sec^{-1}$ and $k_e^{D\text{-}Val} > 0.7 \times 10^{-2}\ sec^{-1}$).

Discussion

When compared with the K^+-ion dissociation rate for VAL (which was measured under similar conditions and estimated by band-shape analysis to be $10^2 sec^{-1}$); the K^+-dissociation from K^+-PVAV is indeed slow, and lends strong support to the proposal that PVAV cannot function effectively as a membrane-bound ion carrier because of its slow dissociation kinetics.[1,2]

The large difference between the isotopic exchange rates for the D- and L-Val NHs of free PVAV also suggests there is a preferred pathway

for the capture and release of cations from the peptide namely from the D-Val-L-Pro side of the ring. The slow rate of exchange for the L-Val NHs suggests that they remain solvent-shielded or intramolecularly H-bonded upon cation release, while the faster exchange of the D-Val NHs implies a greater degree of exposure to the solvent. A comparison of molecular models as well as torsional angles, ϕ, estimated from J_{NC_α} coupling constants[3] indicates that upon dissociation, the bracelet form of the complex (Figure 3A) opens into a "basket" (Figure 3B). In the bracelet form all six Val NHs are intramolecularly H-bonded but in the basket form the H-bonds to the D-Val NHs break and an enlarged opening for the exit and entrance of cations is created on the D-Val-L-Pro side of the macrocyclic ring.

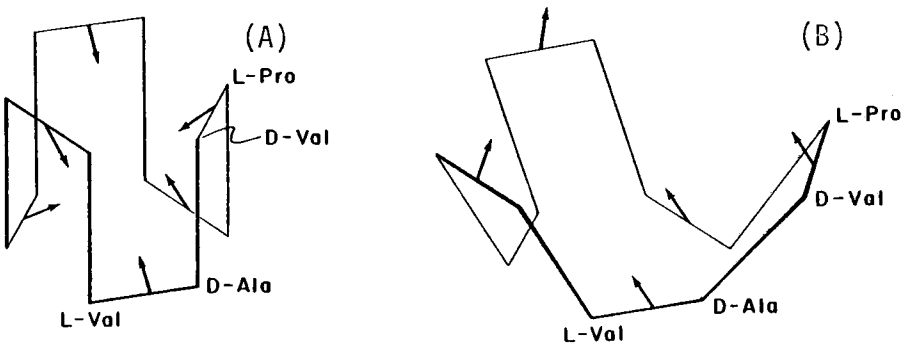

Fig. 3. (A) Bracelet and (B) Basket forms of K^+-PVAV and PVAV respectively.

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SEQUENCE ANALYSIS OF THE ALAMETHICIN ANALOG ANTIBIOTIC TRICHOTOXIN A-40

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The membrane modifying peptide antibiotics trichotoxin A-40 and A-50 can be isolated from the dry mycelium of the trichoderma viride strain NRRL 5242. An improved isolation procedure includes extraction by dichloromethane, Sephadex LH-20 chromatography in methanol and counter-current distribution in 2-butanol/0.015 M ammonium acetate (pH = 8.9) for the separation of A-40 and A-50 components. Selective cleavage conditions for trichotoxin A-40 using anhydrous trifluoroacetic acid yielded three N-acetylated dodecapeptides and two N-prolyl-hexapeptides ending with valinol (Figure 1). These fragments were uniform after fractionation on a silica gel column (chloroform/methanol/water/acetic acid 65:25:4:3) followed by countercurrent distribution (dodecapeptides: 2-butanol/ethylacetate/methanol/0.3% ammonium acetate 3:5:2:3; hexapeptides: 1-butanol/ethyl acetate/ammonium acetate (pH = 4.6) 2:4.5:5).

The sequences of the fragments (Figure 2) were determined via gas liquid chromatography-mass spectrometry (GLC-MS) of trifluoro-acetylated peptide methylesters of partial hydrolysates and solid phase Edman degradation; furthermore field desorption mass spectrometry (FDMS), ^{13}C NMR, and CD data were recorded. Chiral phase gas chromatography and GLC of diastereomeric derivatives established the L-configuration of Ala, Leu, Glu, Pro, Val and the D-enantiomer of isovaline (Iva).

By solid-phase Edman degradation Aib peptides up to 10 residues can be determined unequivocally despite the particularly strong increase in overlap (Figure 3).

Comparison of MS of low temperature derivatized Gln-Valol with

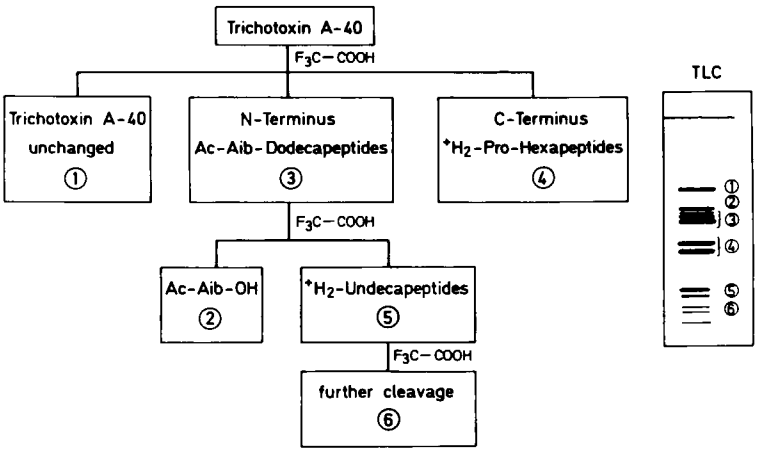

Fig. 1. Scheme of the trifluoroacetolysis of trichotoxin A-40 (3 h 15 min, 37°C) and TLC of cleavage products in chloroform/methanol/water/acetic acid 65:25:4:3 on silica gel.

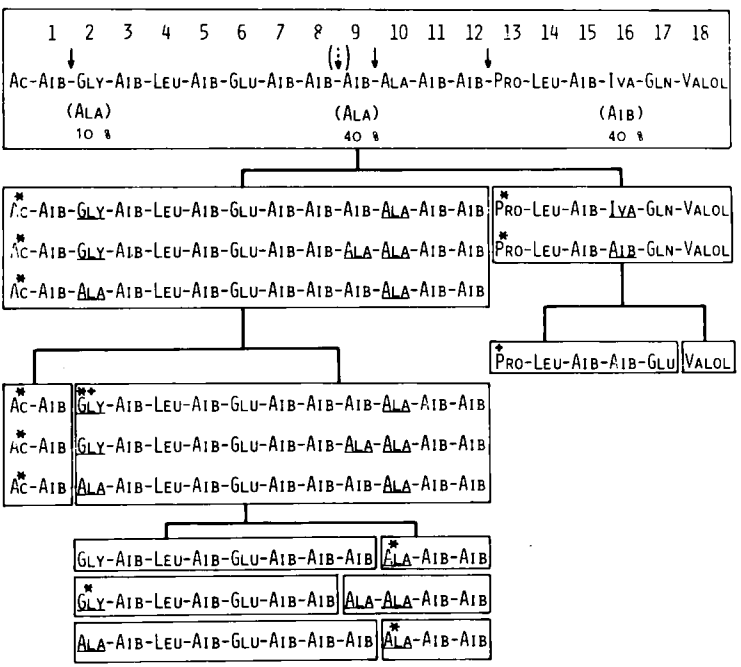

Fig. 2. Sequences obtained after trifluoroacetolysis of trichotoxin A-40 and separation according to Figure 1 (*GLC-MS of trifluoroacetylated peptide methylesters; +solid-phase Edman degradation via pentafluorophenyl- and phenylhydantoin derivatives (selective cleavage of valinol with dioxan/6 n HCl); order of cleavage: 12-13 > 1-2 > 9-10 > or 8-9; % amino acid exchange based on amount of isolated peptides and on TLC).

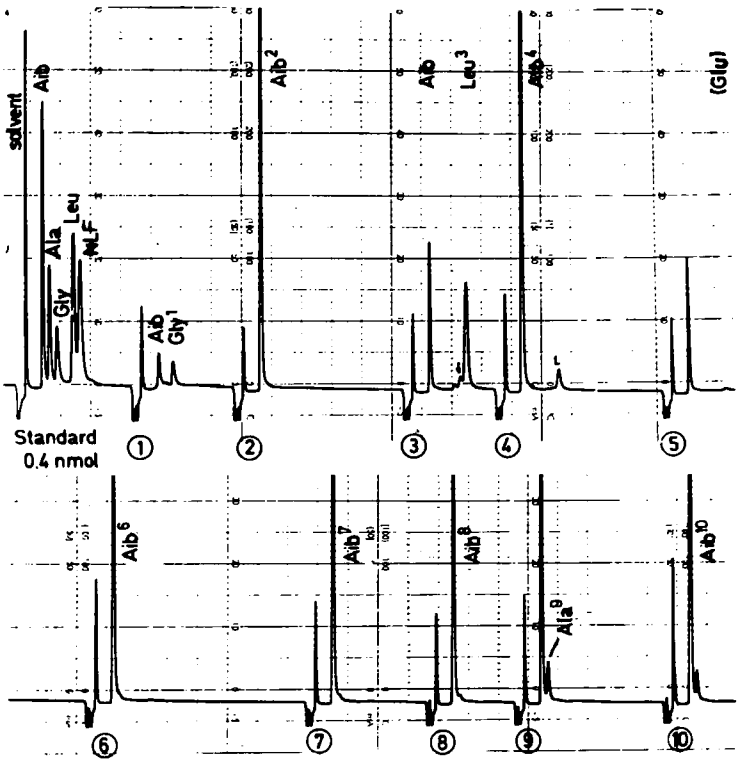

Fig. 3. GLC of PTH derivatives from solid phase Edman degradation of one of the undecapeptides (see Figure 2).

that of trichotoxin A-40 indicates the α-linkage (Figure 4). Rearrangements can be ruled out under these conditions, but esterification at 100°C leads to transpeptidation.

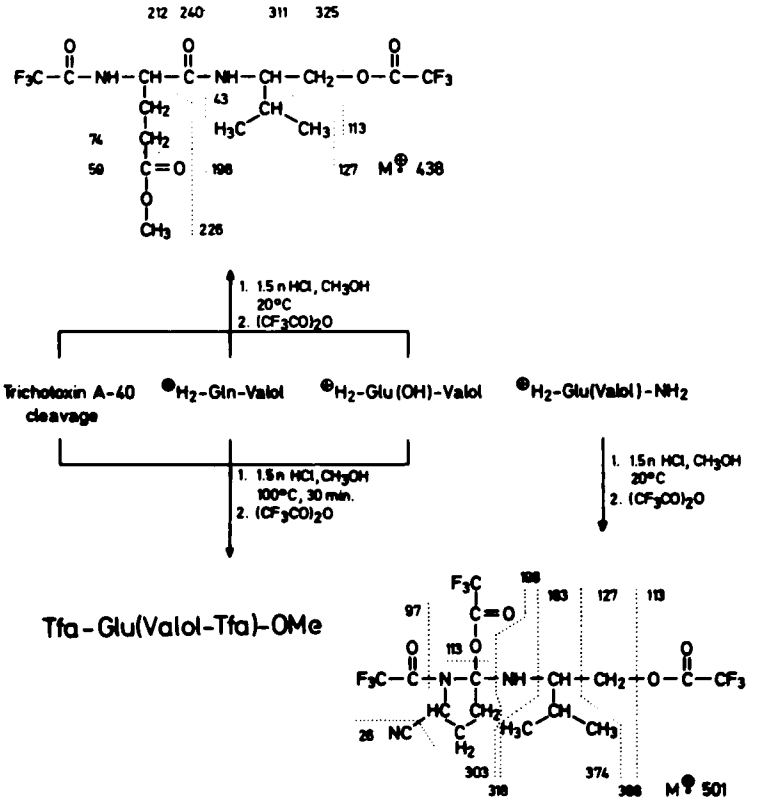

Fig. 4. Derivatization and MS of the C-terminus of trichotoxin A-40 and models.

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THE SYNTHESIS OF ALAMETHICIN-FRAGMENTS BY FOUR COMPONENT CONDENSATION

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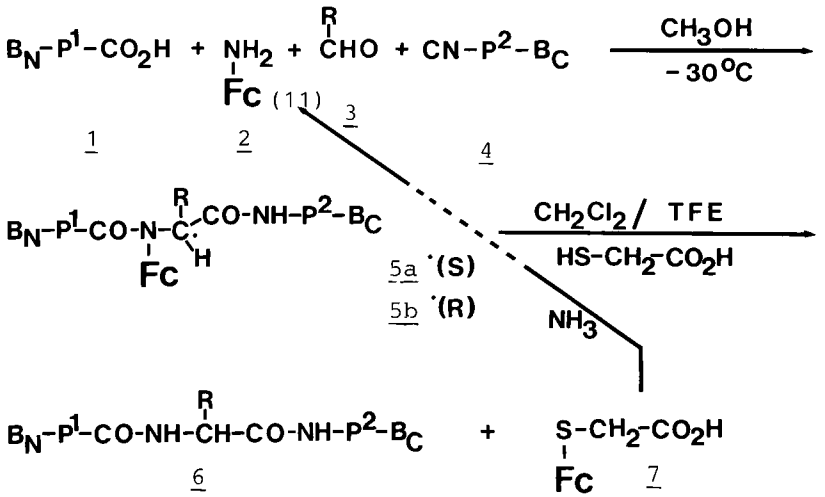
Alamethicin, an extracellular peptide from the fungus *Trichoderma viride,* has two aspects of interest. Besides antibiotic and strong lysing properties on membranes of Ehrlich ascites carcinoma cells and on erythrocytes, alamethicin, from the physiological point of view, has gained importance for its membrane activity.[1,2] In artificial lipid bilayers as models for biological membranes it induces a voltage-dependent ionic conductance. From the chemical point of view, the high amount of the unusual aminoacid α-methylalanin was the most interesting feature.[3] The aminoacid sequence and the difficulties which arose during the conventional synthesis[4,5,6] of this polypeptide (because of steric hindrance) led us to undertake the synthesis of alamethicin fragments by Four Component Condensation (4 CC).



Scheme 1

The development of methods for the synthesis of peptides by stereoselective 4 CC[7] led to new ways to prepare chirally pure chemical compounds in high yield by asymmetric syntheses. The Four Component Condensation now used (Scheme 1)[8,9] starts with a so-called "productively" stereoselective synthesis affording a high yield of the desired product *5a*, accompanied by a minor amount of a stereoisomeric by-product *5b*. The latter is converted into an easily removable non-isomeric compound by a subsequent reaction with so-called "destructive" stereoselectivity. In general, only small losses of the main product occur during this step. The efficiency of such asymmetric syntheses is enhanced by the use of chiral templates which can be recycled.

The isonitriles used for the synthesis of the following fragments were synthesized by the phosgene method described in reference 10. New observations were made during the conversion of the condensation products 7 and 8 to dipeptide isonitriles by cleavage of the ferrocenyl-alkyl residue in a one step reaction.

Table I.

	SYNTHESIZED FRAGMENTS (underlined the new aminoacid)	YIELD (%)	ISOMER RATIO AFTER CONDENSATION-STEP
1	BOC-L-Val-Aib-Aib-OCH_3	72	-
2	BOC-Aib-L-Val-Aib-OCH_3	78	97.5:2.5
3	BOC-Gly-L-Leu-Aib-OCH_3	85	96 : 4
4	BOC-Aib-L-Ala-Aib-OCH_3	70	92 : 8
5	BOC-L-Pro-Aib-L-Ala-Aib-OCH_3	56	-
6	BOC-L-Pro-L-Val-Aib-Aib-OCH_3	40	97 : 3
7	Formyl-Aib-Aib-OCH_3	85	-
8	Formyl-L-Ala-Aib-OCH_3	72	94 : 6

Isomer free peptides are obtained by partial acidolysis[9] and subsequent total cleavage of the chiral template. The isomer purity of the peptide fragments so synthesized was determined by thin-layer chromatographic and HPLC analysis and by comparison with conventionally synthesized peptides.

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11. Fc = 1-ferrocenyl-2-methyl-propylamine

CHEMOTACTIC PEPTIDES AS PROBES OF MOLECULAR EVENTS IN LEUKOCYTE CHEMOTAXIS

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Chemotaxis, the directional migration of cells in response to a chemical gradient, is displayed by a variety of prokaryotic and eukaryotic cells including bacteria[1], the cellular slime molds[2] and leukocytes [3-6]. Leukocytes respond to factors derived from complement[4], materials elaborated by bacteria[5,6], certain lipids[7] and well-characterized N-formylated oligopeptides which appear to be related to bacterial factors[8]. These peptides have been particularly useful in studying biochemical events in leukocyte chemotaxis, and we shall describe here some recent findings obtained with these compounds.

Materials and Methods

Materials — Labelled and unlabelled synthetic peptide attractants and antagonists were obtained, in part, under NIDR Contract DE-

52477. They contained L-amino acids unless otherwise indicated and were prepared using both solid and classical procedures in acccordance with previously described methods[9-11]. Other peptides, such as Boc-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe were prepared as previously described[12].

Complement-derived leukoattractant was prepared according to previously described procedures[4].

Proteolytic inhibitors L-(1-Tosylamido-2-phenyl) ethylchloromethyl ketone (TPCK) and *N*-α-p-Tosyl-L-lysylchloromethyl ketone (TLCK) were obtained from Sigma Chemical Company. The ionophore, A23187, was obtained from Eli Lilly and Co. and phorbol myristate acetate from Consolidated Midland Corporation (Brewster, NY). Cytochalasin B was obtained from Calbiochem, while Mepacrine, a phospholipase A_2 inhibitor, was obtained from Dr. Seymour Heisler, Laval University, Quebec, Canada.

Protein carboxylmethylase and a preparation containing protein methylesterase activity were both obtained according to Gagnon[14]. Myeloperoxidase was obtained from granulocytes according to the method of Clark[15,16].

Methods — Rabbit peritoneal exudate neutrophils were obtained as previously described[17]. Human neutrophils were obtained as previously described[13]. Chemotaxis was measured in modified Boyden chambers using micropore (5μm average pore diameter) filters[18].

Binding of CHONle-Leu-[^{3}H]-PheOH or CHOMet-Leu-[^{3}-H]-PheOH to neutrophils or cell components was carried out and determined as previously described[11]. Protein carboxylmethylation was determined according to O'Dea *et al*[19], and protein methylesterase according to Gagnon[14]. Phospholipase A_2 activity was assayed in whole cells by measuring the release of [^{3}H] labelled arachidonic acid from cells that had been previously labelled with this compound[20].

Lysosomal enzyme release (lysozyme, β-glucuronidase) was measured as described earlier[13].

Results

Agonist-Receptor Interaction — Previous structure activity studies[9] have resulted in the synthesis of highly potent chemoattractants and have contributed to characterization of the receptor. This work has been extended and is reported in detail elsewhere in this volume. Here we present examples of our probing the receptor with the aid of chemotactic peptides.

Evidence for stereoselectivity in the binding of peptides to neutrophils has already been adduced[11]. We have extended these observations in the present work (Table I). The all *L* tripeptide, CHOMet-Leu-PheOH, is about three orders of magnitude more effective in both binding and chemotactic potency than the *D*-Leu diastereoisomer. Similarly, the all *L* Boc-Phe-Leu-Phe-Leu-Phe is about ten fold more active in the binding assay ($ED_{50} \sim 7 \times 10^{-8}$)M than the all *D* enantiomer. The results indicate, therefore, that the receptor is *stereoselective* in its interaction with peptides.

Table I. Effects of Diastereoisomeric Peptides upon Neutrophil Chemotaxis and Binding of [^{3}H]-CHONle-Leu-PheOH to Neutrophil Receptor.

Peptide	Specific Binding[a,b] ID_{50} (M)	Chemotactic Activity ED_{50} (M)
CHO-L-Met-L-Leu-L-PheOH	3.8×10^{-10}	7.6×10^{-11}
CHO-L-Met-D-Leu-L-PheOH	8×10^{-8}	6×10^{-8}
Boc-L-Phe-L-Leu-L-Phe-L-Leu-L-PheOH	6.8×10^{-8}	1.6×10^{-5}[c]
Boc-D-Phe-D-Leu-D-Phe-D-Leu-D-PheOH	3.7×10^{-7}	1.8×10^{-5}[c]

Standard conditions, as described in Materials and Methods, were employed for both chemotaxis and binding.

[a] These values were obtained from dose-inhibition plots of varying concentrations of peptides in the presence of the labelled ligand.

[b] Values are means of triplicate measurements that varied less than 10%. Three experiments were performed.

[c] These values are for the *inhibition* exerted by the *antagonists (Boc* compounds) upon chemotaxis in response to a maximally stimulating concentration of *agonist* (CHO-L-Met-L-Leu-L-PheOH).

Efforts to identify functional groups required for binding peptide to receptor led to the finding that sulfhydryl groups were involved (Table II). p-Mercuribenzoate (PMB) treatment of receptor preparations caused a 90 per cent decrease in binding, which was largely restored by subsequent treatment with dithiothreitol. Since the sulfhydryl reagent PMB is not structurally related to the peptide agonist, it is likely that it exerts its effect by allosterically altering the conformation of the binding site. Other receptors have been shown to require intact sulfhydryl groups for activity[21].

Table II. Effect of Sulfhydryl Reagents upon Specific Binding of [^{3}H]-CHO-Nle-Leu-PheOH to Particulate Preparations of PMN Receptors.

Pretreatment[a]	Per Cent of Control Binding[c]
None	100
p-mercuribenzoate, 10^{-5}M	8
p-mercuribenzoate, 10^{-5}M followed by dithiothreitol, 10^{-4}M	70
Dithiothreitol, 10^{-4}M	75
p-mercuribenzoate, 10^{-5}M[b] under assay conditions	96

[a] All pretreatments were carried out at 25° for 40 minutes and the reagents removed before the binding assay.

[b] The reagent was present only during the binding assay (1 hr at 0°).

[c] Values are means of triplicate assays that varied less than 10%. Four experiments were performed.

Post Receptor Events in Chemotaxis — Protein carboxylmethylation has been shown to be involved in the signal transduction phase of bacterial chemotaxis[22]. O'Dea *et al.*[19] observed that methylation of protein carboxyl groups was stimulated in neutrophils in response to peptide chemoattractants. Since these changes were rapid and transient (1 min), and in light of the recent demonstration of the involvement of a protein methylesterase in bacterial chemotaxis[23], the participation of the latter enzyme in neutrophil chemotaxis was studied. This enzyme was found to exist in the cytosolic fraction of neutrophils. Peptide chemoattractants were found to stimulate the hydrolysis of protein methylesters (Figure 1). In this experiment, cells previously incubated for 30 min in the presence of [^{3}H-methyl]-methionine (1mCi/30 ml of cells at 5 x 10^6 cells/ml) at 37° to produce labelled methyl esters were subjected to a 'chase' procedure by subsequent incubation in 10 mM methionine (unlabelled) with or without CHOMet-Leu-PheOH (10nM). Hydrolysis of labelled protein methylesters was stimulated within 10 min after the addition of chemoattractant. In other experiments this response was found to be specific for compounds which exhibited chemotactic activity and showed a dose-dependent relationship that correlated well with the concentration of peptide which produced maximal chemotaxis.

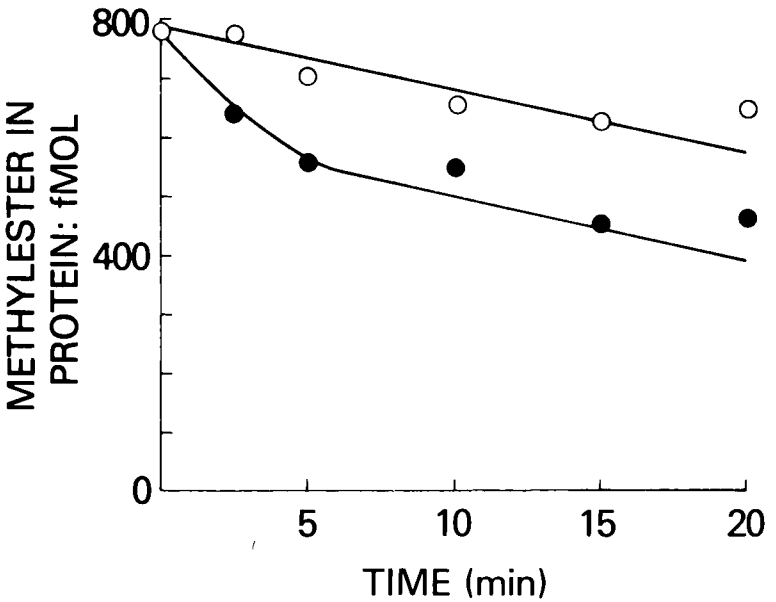

Fig. 1. Stimulated release of ^{3}H-methanol from neutrophils previously labelled with (^{3}H-methyl)methionine. Attractant (10 nM CHOMet-Leu-PheOH, •—•) was added at 0 time or not (o—o) and protein-bound ^{3}H-methyl determined. Values are averages of duplicate determinations S.E.M. ⩽ 10 per cent.

In other studies we determined the effect of peptide attractants upon neutrophil membrane phospholipase since it had been shown that such enzymatic activity was stimulated in a number of agonist-cell interactions[20]. A stimulated release of labelled arachidonate was observed after the addition of attractant in cells that had been previously labelled with ^{14}C-arachidonate (Figure 2). There was a concomitant disappearance from the cells of ^{3}H-methyl-containing phospholipid, presumably lysolecithin, during this period. These changes were maximal after 10 min.

These results indicate that increased turnover of protein methylesters and phospholipids could play a role in neutrophil chemotaxis. It is conceivable that these reactions occur as part of the signalling process between the activated receptor and the cell motility apparatus, since their inhibition by methylation antagonists (deazaadenosine) did not affect binding of chemotactic peptides to cell receptors[24].

Modulation of Chemotaxis — One possible mechanism by which cells regulate their chemotactic response might be through the rapid inactivation of chemoattractants. Others have suggested that this occurs through hydrolysis of the peptide attractants[25], but it might also result

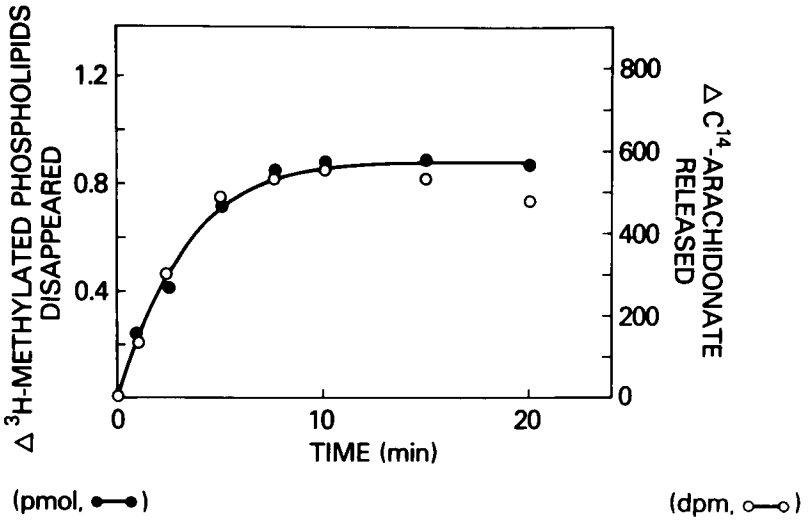

Fig. 2. The stimulatory effect of CHOMet-Leu-PheOH on the release of [^{14}C] arachidonic acid and the disappearance of [^{3}H] methyl group from phospholipids. Neutrophils were preincubated with 20 μM [methyl-^{3}H] L-methionine (2 μCi/nmol) and 0.4 μCi/ml [^{14}C] arachidonic acid (55.5 μCi/μmol) for 30 min at 37°. After washing, the cells were 'chased' with 1 mM unlabelled methionine and 0.2 mg/ml unlabelled arachidonic acid. The reaction was started by the addition of 5 ml of 10^{-6}M CHOMet-Leu-PheOH to 0.5 ml of cell suspension (5 x 10^{6} cells) and terminated by the addition of 1 ml of cold Gey's buffer. After centrifugation at 6000 x g for 10 min, the supernatant (2 ml) was measured for the release of [^{14}C] arachidonic acid (o—o). the precipitate containing the lipid fraction was washed with 0.5 ml of 10% TCA, and the [^{3}H] methyl labelled phospholipids were measured as described in Methods (●—●).

from oxidation processes generated by the myeloperoxidase system of neutrophils. This enzyme has been shown to inactivate certain chemoattractants[51]. The myeloperoxidase system was found to inactivate peptide chemoattractants containing methionine (Table III), but not those without this amino acid (CHOPhe-Leu-Phe-Leu-PheOH, CHONle-Leu-PheOH). This result was confirmed by studying the effect of exogenously added myeloperoxidase upon subsequent binding to receptor of both Met-containing (including C5a) and Met-lacking peptide attractants (Figure 3). It is evident that the enzymatic pretreatment markedly reduces the binding of CHOMet-Leu-PheOH to cells at peptide levels of 0.1, 1 and 10 μM, whereas the same treatment had very little effect upon the binding of CHONle-Leu-PheOH. Although these results were obtained with purified enzyme preparations, it is likely that these reactions also occur in the intact cell, since chemoattractants stimulate myeloperoxidase activity[15].

Table III. Effect of the Myloperoxidase System (MPO) on Various Chemoattractants[a].

Chemoattractant	Inactivation (Percent)
C5a	94.4 ± 1.8 (17)[b,c]
CHO-Met-Leu-PheOH	98.3 ± 4.4 (15)[c]
CHO-Met-Leu-Phe-LysOH	75.6 ± 10.0 (6)[c]
CHO-Norleu-Leu-PheOH	0.1 ± 9.2 (7)
CHO-Phe-Leu-Phe-Leu-PheOH	10.9 ± 8.0 (10)

[a] The reaction mixture contained 0.8 ml of C5a, 50 pmoles of CHO-Met-Leu-PheOH, CHO-Met-Leu-Phe-LysOH, or CHO-Nle-Leu-PheOH or 0.5 nmoles of CHO-Phe-Leu-Phe-Leu-PheOH. With C5a, the reaction was performed in 1.0 ml of 40 mM sodium phosphate buffer, pH 7.0 and with the oligopeptides 0.5 ml of 20 mM sodium phosphate buffer, pH 7.0. The MPO system components employed were as follows: MPO — 16 mU/ml; H_2O_2 — 10 μM; NaCl — 0.1 M. Samples were incubated at 37°C for 15 minutes.

[b] Mean ± S.E. (number of experiments).

[c] $p < 0.001$ vs control, others not significant.

[d] Leu-residues are of the D-configuration.

Another mechanism by which the neutrophil could regulate its chemotactic responsiveness is through induced exocytosis. It has been shown that the amount of binding of CHOMet-Leu-[^{3}H]PheOH to cells is dependent upon the extent of secretion of lysosomal enzymes which the cells have undergone (Figure 4)[13]. With minimal secretion, there is enhancement of binding, while vigorous secretion results in a marked decrease in attractant binding[13]. Some insight into the significance of these findings may be obtained from studies[26] on the relative amounts of binding of labelled peptide attractant to subcellular components of cells (Table IV). It is evident that in addition to the membrane fraction, the specific granules bind appreciable amounts of the peptide. These granules are mobilized during minimal secretory stimulation, which, after fusion with the cell membrane, could expose additional binding sites for the attractant. Recent studies indicate that the enhanced binding with limited exocytosis reflects increased receptor availability (Fletcher and Gallin, submitted). With vigorous secretion, the azurophil granules are released. Since they contain a variety of hydrolytic enzymes, their effect upon surface receptors or ligand hydrolysis (after fusion with the cell membrane) might be to degrade them.

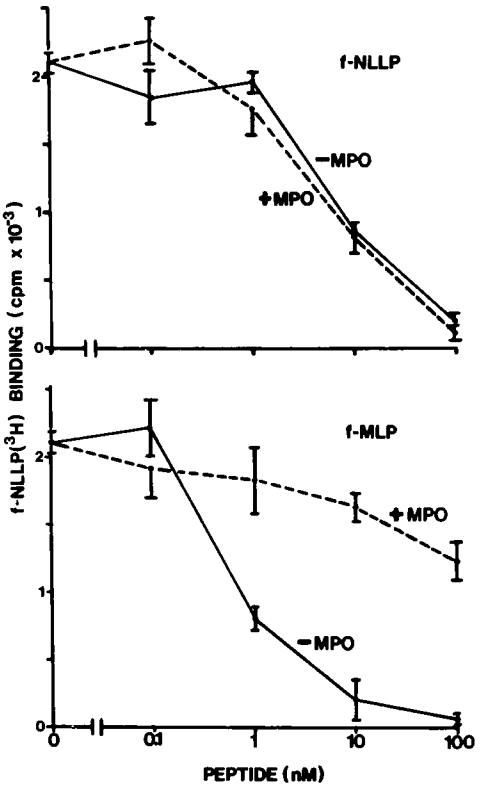

Fig. 3. Effect of the myeloperoxidase (MPO) system on the binding of chemotactic peptides to polymorphonuclear receptors (PMN). The peptides were first exposed to the MPO system as follows: reaction mixtures contained 20 nmoles of CHO-Met-Leu-PheOH or CHO-Nle-Leu-PheOH, 20 mM sodium phosphate buffer pH 7.0 and 0.1 M NaCl in a volume of 0.5 ml; these control samples (-----) were compared with samples which were identical except for the addition of MPO (16 U/ml and 0.1 μM H_2O_2) (—). After incubation at 37° for 15 minutes, the samples were placed in an ice bath, diluted 1:1 in glacial acetic acid, frozen, and lyophilized. The peptides were dissolved in Gey's balanced salt solution and tested in varying concentrations for their ability to compete with the radiolabelled ligand CHO-Nle-Leu-[^{3}H]PheOH for binding to rabbit PMN receptors as described in Methods. Control samples containing the complete MPO system, but no peptide, had no effect on binding. The data presented are the means (± S.E.) of triplicate samples. Upper panel: effect of MPO treatment on binding the [^{3}H] ligand CHO-Nle-Leu-PheOH. Lower panel: effect of MPO treatment on binding of [^{3}H] CHO-Met-Leu-PheOH.

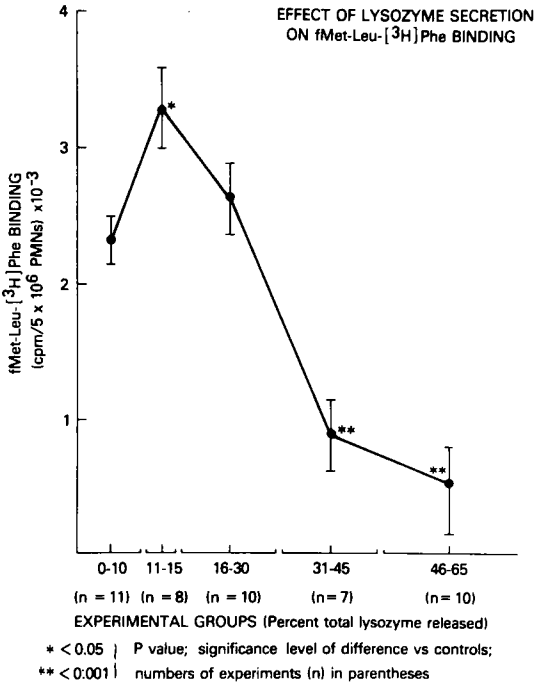

Fig. 4. Composite data relating degranulation by secretagogues to lysosomal enzyme release and CHO-Met-Leu-[^{3}H]PheOH binding. The data were arbitrarily grouped by the per cent total lysozyme release as indicated and the data represent mean ± S.E.M. of the number of experiments shown in parentheses. P values are the significance level of difference from controls (Student's test).

Discussion

Leukocyte chemotaxis can be considered to consist of at least two discrete stages: the interaction of attractant with receptor to generate a signal and the transduction of the signal from the activated receptor to the cell's motility apparatus. The first stage involves the binding of highly specific ligands to a high affinity receptor. Formylation of the N-terminal residue of a tripeptide attractant is required for maximal potency (Freer *et al.*, this volume) and stereoselectivity by the receptor has been demonstrated with agonists containing all *L* residues eliciting the most effective response. The peptide binding site may be regulated allosterically by free sulfhydryl groups.

In the second, or transduction stage, both methylation of protein methylesters and phospholipids appear to be involved. Peptide attractants stimulate protein methyl ester turnover and an increase in phos-

Table IV. Binding of CHO-Met-Leu-[^{3}H]PheOH to Isolated Neutrophil Granule Membranes[a].

	Cell Fraction			
	Azurophil Granules		Specific Granules	Membrane Fraction
Experiment	A	B	C	D
			(% total cpm recovered)	
1	2	8	31	58
2	0	0	30	70
3	6	13	28	53
4	10	8	46	36

[a] 2 x 10^8 human peripheral blood neutrophils (Hypaque-Ficoll) were lysed and then fractionated on sucrose gradients into four fractions. Each fraction was washed three times in Hanks' media, sonicated, and then incubated for 1 hr with CHO-Met-Leu-[^{3}H]PheOH (5 x 10^8 containing 200,000 cpm). Data for each fraction are expressed as per cent total counts per minute recovered from all the fractions within a single experiment. The pooled mean counts per minute per mg protein for each fraction in the four experiments were: Azurophil granules (A) = 3118; (B) = 5175, specific granules (C) = 15,242; cytoplasmic membrane (D) = 28,170.

pholipase A_2 activity. Also, agents which inhibit S-adenosylmethionine-mediated transmethylation depress leukocyte chemotaxis, protein carboxylmethylation and lipid methylation[27]. It is conceivable that methylation processes may play the role of a 'second messenger'. The molecular events in which they participate could contribute to the changes in membrane fluidity and receptor mobility that are characteristic of a variety of agonist-induced cellular functions[20].

Finally, it appears that the leukocyte may be able to regulate its chemotactic responsiveness both by inactivating methionine-containing attractants through oxidation of the sulfur of that residue, or by modulating availability of receptors as a consequence of the extent of exocytosis of its lysosomal granules.

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SYNTHETIC CHEMOTACTIC PEPTIDES AS INITIATORS OF MULTIPLE BIOLOGIC FUNCTIONS OF THE NEUTROPHIL: THE ROLE OF CALCIUM

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Introduction

In the preceding paper, Dr. Schiffmann has described the discovery and development of the formylmethionyl peptides as chemotactic factors for neutrophils and other white blood cells[1]. He has also presented the evidence that these peptides are chemotactic because they interact with a specific receptor on the neutrophil. However, these same oligopeptides are not only chemotactic but induce neutrophils to secrete their granule enzymes and to aggregate. They also cause these same cells to produce superoxide (O_2^-) and the other manifestations of the so-called "respiratory burst." The peptides cause all these functions by interacting at the same receptor responsible for the chemotactic response of the neutrophil. We have summarized elsewhere the evidence for the latter conclusion and will not repeat it here[2].

The question then arises, "How do these simple peptides acting at a single population of receptors cause the neutrophil to do so many different things?" Our tentative answer, developed from work of the last three or so years, is that the chemotactic oligopeptides initiate the manifold biologic responses of the neutrophil by causing an increase in the free Ca^{2+} of their cytoplasm. The nature of the biologic response initiated depends upon the source of the increased free Ca^{2+}, the site in the cytoplasm where the Ca^{2+} is increased, the extent of the increase and the attendant experimental circumstances.

In what follows, we shall present the accumulation of evidence that the chemotactic peptides cause an increase in free cytoplasmic calcium by two processes. In the first process, the peptides induce an increase in the membrane permeability to calcium; this, in the presence of extracellular

calcium, results in an influx of Ca^{2+} into the neutrophil. In the second, they also cause a displacement of calcium from some intracellular membranous source, possibly the plasma membrane. The result of either or both processes is to increase exchangeable cell calcium and, by inference, raise free cytoplasmic calcium. Lastly, we shall present the present indirect and admittedly skimpy evidence that it is the increase in cytoplasmic calcium caused by one or both proceses which is the proximate cause of the induced biologic functions.

Chemotactic Peptides Cause An Increase In Neutrophil Membrane Permeability to Calcium — Gallin and Rosenthal[3] and Boucek and Snyderman[4] demonstrated that the chemotactic peptide fragment of the fifth component of hemolytic complement, C5a, causes an increased influx and efflux of radioactive $^{45}Ca^{2+}$ from human neutrophils. Naccache *et al.* were able to repeat and extend both findings using formylmethionyl-leucyl-phenylalanine, f-Met-Leu-Phe, as the stimulus for rabbit peritoneal neutrophils[5]. In addition, we showed that in the presence of 250 μM external Ca^{2+}, f-Met-Leu-Phe induces an increase in the steady state level of cell associated, radioactive $^{45}Ca^{2+}$. This we attributed to the chemotactic factor causing an increase in the membrane permeability to Ca^{2+} and a consequent influx into the cells when calcium is present in the extracellular medium. The increase in intracellular calcium is seen as an increase in exchangeable Ca^{2+}. In contrast, cells stimulated under conditions of low extracellular calcium showed a transient decrease in their levels of steady state calcium. On the basis of this latter finding, we suggested that the chemotactic factor causes, in addition, a displacement of intracellular Ca^{2+}, possibly from the membrane, into the cytoplasm.

The chemotactic peptides and other chemotactic agents induce appreciable secretion of granule enzymes only in the presence of the fungal metabolite cytochalasin B[6,7]. In addition, the aggregation of neutrophils and the formation of O_2- by the chemotactic peptides is also greatly enhanced by cytochalasin B.[8,9] We therefore tested the effect of cytochalasin B on the influx of $^{45}Ca^{2+}$ into the neutrophil and showed that cytochalasin B present at the same time as f-Met-Leu-Phe increased at least 10 fold the rate and extent of the influx of ^{45}Ca into the neutrophil compared to f-Met-Leu-Phe alone[10]. Cytochalasin B correspondingly enhanced the increase in steady state level of ^{45}Ca induced by f-Met-Leu-Phe. Moreover, we found a linear relation between the increased uptake of Ca^{2+} or increase in steady level of $^{45}Ca^{2+}$ and the secretion of granule enzymes induced by f-Met-Leu-Phe in the presence of cytochalasin B. However, as yet we have been unable to obtain conclusive evidence that

the linear relationship is not a result of the neutrophil degranulation rather than a cause, as we would like to believe[11].

A difficulty in all of the above studies, our own included, was that no attempts were made to concomitantly measure total cell calcium. This means that the observed changes in calcium fluxes just described can have several different interpretations. In an effort to decrease the ambiguities, Dr. Richard Petroski, then a postdoctoral fellow in our laboratory, recently undertook a complete and systematic investigation of the effects of f-Met-Leu-Phe on the levels of total and exchangeable calcium in rabbit neutrophils[11,12]. Concomitant determination of both the radioactive and the total cell calcium of unstimulated or stimulated neutrophils allows the calculation of the specific activity of the cell radioactive calcium. At steady state, the intracellular exchangeable calcium is equal to the total calcium times the ratio of the specific activity of the cellular calcium to the specific activity of the extracellular calcium[12].

Dr. Petroski found that rabbits incubated in buffer lacking added calcium lost one third of their total cell calcium. The total cell calcium does not change when neutrophils are equilibrated in buffers with concentrations of calcium varying between 50 to 1,000 μM. The chemotactic peptide, f-Met-Leu-Phe has no statistically measurable effect on the level of total calcium in the neutrophil whether the cell calcium is measured one, three or five minutes after stimulation with varying concentrations of f-Met-Leu-Phe.

In confirmation of previous work, f-Met-Leu-Phe greatly affects the steady state level of radioactive calcium, $^{45}Ca^{2+}$. In the absence of added extracellular calcium, f-Met-Leu-Phe induces only a transient loss of steady state $^{45}Ca^{2+}$ no matter how high the concentration of f-Met-Leu-Phe used as a stimulant. In the presence of greater than 50 μM extracellular calcium, low concentrations of chemotactic peptide induced a loss of steady state $^{45}Ca^{2+}$, whereas higher concentrations increased steady state levels. In all instances, the changes in steady state levels were transient. Concomitant measurements of total calcium enabled these changes to be expressed as $^{45}Ca^{2+}$ specific activity. As already explained, in the absence of a change in total cell calcium the specific activity reflects the level of exchangeable calcium. It was thus possible to show that sufficiently high concentrations of f-Met-Leu-Phe cause a doubling or more of the exchangable calcium of neutrophils incubated in buffers containing more than 50 μM Ca^{2+}.

Thus, the studies we have summarized provide evidence that chemotactic factors affect calcium transport and translocation in the neutro-

phils in two distinct ways. First, they induce a dose dependent increase in the membrane permeability to calcium. The evidence for this is: 1) They increase the uptake of radioactive calcium into the neutrophils. 2) They increase the level of the cells' steady state $^{45}Ca^{2+}$. 3) They increase the specific activity of the cells' $^{45}Ca^{2+}$. The increase depends on the presence of external calcium. 4) They increase the exchangeable calcium of the cell.

Secondly, they also induce a dose dependent displacement of intracellular calcium. The evidence for this, reviewed so far, is: 1) The chemotactic factors cause an efflux of radioactive calcium. 2) They cause a decrease in steady state radioactive calcium detectable in the absence of extracellular calcium or in the presence of extracellular calcium when the concentration of chemotactic peptide is sufficiently low. In other words, the displacement of bound intracellular calcium is detectable only under circumstances when it is not swamped out by the influx of calcium induced by the increased membrane permeability to calcium also caused by the chemotactic peptide. 3) The chemotactic factors, under the same circumstances, cause a decrease in the calcium specific radioactivity. The explanation for this is that the chemotactic factor induces a displacement of nonlabeled bound, i.e. non-exchangeable calcium, possibly from the membrane. This Ca^{2+} dilutes the specific activity of the free calcium. It also increases the intracellular level of free calcium; this increases the activity of the putative, outward directed calcium pump resulting in a net loss of radioactivity in the cell. The resulting exchange of radioactive calcium from the exterior restores the cells' radioactivity to the original value.

As is clear, the evidence for the chemotactic factor causing a displacement of intracellular bound calcium, although consistent and unequivocal as far as it goes, is, nevertheless, indirect. In attempting to provide more direct evidence, Dr. Paul Naccache has recently studied the effect of chemotactic factors on the fluorescence of chlorotetracycline loaded cells[13]. Chlorotetracycline binds to the hydrophobic elements of cell membranes and when chelated with calcium shows a characteristic fluorescence. As recently reported, the fluorescence of chlorotetracycline loaded cells is decreased in a concentration dependent manner both by f-Met-Leu-Phe and by C5a. Moreover, the decrease in fluorescence occurs in the absence of extracellular calcium as well as in its presence.

These and other results of the same study provide powerful support for the notion that chemotactic factors do indeed cause a displacement of intracellular bound calcium and also indicated that the calcium comes from some membrane bound source. Moreover, the displacement and

the resulting increase in free intracellular calcium occur in the absence of extracellular calcium, in fact, even when the cells are suspended in EDTA. Thus, the work with $^{45}Ca^{2+}$, just reviewed, as well as the study with chlorotetracycline, make evident that chemotactic factors can provide increased levels of intracellular calcium to the neutrophil.

Evidence That Increased Levels of Cytoplasmic Calcium Are Required For The Responses of The Neutrophil to Chemotactic Peptides — Granted that the chemotactic peptides can induce rises in the levels of neutrophil cytoplasmic calcium, what is the evidence that the elevation of this cation is responsible for causing the various biologic functions of the cell? Moreover, even if the latter is true, how does the increase in the single cation cause the cell to respond so differently?

The evidence that the elevation in cytoplasmic calcium is responsible for the various functions is of several kinds. Like the evidence for the rise itself, it is still indirect and likely to remain so until we know more than we do now what the nature is of the various biochemical sequences responsible for the different biologic responses. External calcium is required for optimal granule enzyme secretion[6], aggregation[15], and O_2-formation[9,14] induced by f-Met-Leu-Phe. However, using a chemotaxis chamber devised by Dr. Sally Zigmond, Mr. Wayne Marasco has recently shown, in confirmation of Dr. Zigmond[16], that neither Ca^{2+} nor Mg^{2+} is required for neutrophils to orient in a gradient of f-Met-Leu-Phe[17]. He has further demonstrated that Ca^{2+} is also not required for movement in the Zigmond chamber in response to the chemotactic peptide, although Mg^{2+} is[17]. The lack of requirement for external calcium means that either an increase in free cytoplasmic Ca^{2+} is not involved in orientation and movement, or that the mobilization of intra- and not extracellular calcium is responsible. As will be described, the evidence accumulated so far is consonant with the mobilization of intracellular Ca^{2+} being required for cell locomotion, and the mobilization of extracellular Ca^{2+} for cell responses of aggregation, enzyme secretion and O_2-formation.

A second type of evidence comes from the ability of A23187, an ionophore specific for divalent ions, to induce neutrophils to aggregate[15], secrete granule enzymes[6], and form O_2- anion[9]. In each instance, the induced secretion requires the presence of calcium in the medium. Mr. Marasco has recently shown, in unpublished work that a gradient of A23187 in a Zigmond chamber is chemotactic, i.e. causes neutrophils to orient and also stimulates their locomotion.

There are other bits of miscellaneous evidence[14,18] for the involvement of calcium in the various neutrophil functions. Moreover, there is a

hierarchy of sensitivities of the different kinds of response to f-Met-Leu-Phe and the other chemotactic peptides; stimulated locomotion can be a more sensitive response to receptor occupancy than induced secretion, aggregation or O_2- formation[2,6,15,19]. Correspondingly, the displacement of membrane calcium is a more sensitive response to receptor occupancy than is the raised influx of calcium due to increases in membrane permeability to that ion. It is also probable that triggering of the various neutrophil functions by intracellular calcium differs both in the levels needed and where the calcium must be located. Thus, the differences in the sensitivity of response of the various biologic functions probably reflect the source of the rise in the calcium, the intracellular localization of the raised calcium, and differing qualitative and quantitative requirements for the ion.

Whatever the present uncertainties, the work we have reviewed is one illustration of the progress made possible in understanding the complex responses of a very important cell by the availability of simple peptides of known structure.

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STRUCTURAL REQUIREMENTS FOR SYNTHETIC PEPTIDE CHEMOATTRACTANTS AND ANTAGONISTS

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There are numerous substances which reportedly have the ability to stimulate directed migration (i.e. chemotaxis) of motile cells[1]. Until recently, however, none of these substances was available in a homogeneous form and therefore their utility in studying the phenomenon of chemotaxis was limited. The discovery by Schiffmann *et al.*[2] that N^{α}-formyl-methionine and some formylated Met-X dipeptides have chemotactic activity led the way to the development of the extremely potent chemoattractant (ED_{50} chemotaxis — 7×10^{-11} M) N^{α}-formyl-methionyl-leucyl-phenylalanine (CHO-Met-Leu-Phe-OH)[3]. Recently antagonists of CHO-Met-Leu-Phe-OH have also been described[4]. The following constitutes a brief summary of the structural requirements in both the agonist and antagonist series.

1. The formyl group is essential and unique since the free amine (II), the acetyl derivative (III), and the desamino analog (IV) are several orders of magnitude less active than the parent peptide (I).

2. The sidechain in position one also confers specificity. As one increases sidechain length in a linear aliphatic series (V thru VII) there is increasing biological activity. A similar effect is seen in the sulfur containing sidechains (I, IX, and X). The presence of sulfur in the sidechain generally increases activity versus its aliphatic counterpart (compare I, V and II; IX and XI). An exception is seen when comparing VII and X. Branched sidechains are, in general, not well tolerated (XII and XIII) although CHO-Ile-Leu-PheOH (XIV) exhibited surprisingly good biological activity.

Compound #	Structure	Chemotaxis* ED_{50} (agonists) or ID_{50} (antagonists) (M)
I	CHO.Met.Leu.Phe.OH	agonists 7.0×10^{-11}
II	Met.Leu.Phe.OH	6.7×10^{-7}
III	Ac.Met.Leu.Phe.OH	2.0×10^{-7}
IV	desamino.Met.Leu.Phe.OH	1.9×10^{-7}
V	CHO.Gly.Leu.Phe.OH	4.3×10^{-6}
VI	CHO.Abu.Leu.Phe.OH	3.8×10^{-7}
VII	CHO.Nva.Leu.Phe.OH	5.8×10^{-9}
VIII	CHO.Nle.Leu.Phe.OH	6.6×10^{-10}
IX	CHO.Hep.Leu.Phe.OH	3.3×10^{-10}
X	CHO.Cys(Me).Leu.Phe.OH	2.4×10^{-8}
XI	CHO.Eth.Leu.Phe.OH	4.3×10^{-10}
XII	CHO.Phe.Leu.Phe.OH	1.5×10^{-7}
XIII	CHO.Leu.Leu.Phe.OH	1.2×10^{-8}
XIV	CHO.Ile.Leu.Phe.OH	2.6×10^{-10}
XV	CHO.Met.Leu.Glu.OH	1.3×10^{-6}
XVI	CHO.Met.Leu.Arg.OH	3.6×10^{-7}
XVII	CHO.Met.Leu.Leu.OH	4.8×10^{-8}
XVIII	CHO.Met.Leu.Ala.Phe.OH	1.9×10^{-9}
XIX	CHO.Met.Ala.Leu.Phe.OH	5.3×10^{-9}
XX	CHO.Met.Leu.Pea	1.9×10^{-7}
XXI	CHO.Met.Leu.Phe.OBzl	4.0×10^{-11}
XXII	CBz.Phe.Met.OH	antagonists $> 10^{-4}$
XXIII	CBz.Met.Leu.Phe.OH	weak agonist
XXIV	MeO.Met.Leu.Phe.OH	weak agonist
XXV	Boc.Met.Leu.Phe.OH	6.4×10^{-7}
XXVI	Boc.Phe.Leu.Phe.Leu.Phe.OH	2.6×10^{-7}

*Chemotaxis measured as described in Ref. 3.

Compounds I through XXI are agonists, XXII through XXVI display antagonist properties.

Abu = aminobutyric; Nva = norvaline; Nle = norleucine; Hep = amino heptanoic; Pea = β-phenethylamine; Eth= ethionine

3. Phenylalanine in position 3 is required. Substitution by acidic (XV), basic (XVI), or even neutral (XVII) residues severely reduces biological activity. Similarly, insertion of Ala spacers (XVIII and XIX) in the tripeptide sequence reduces activity, suggesting that the position of the residues relative to each other is also important.

4. The role of the carboxyl group is unclear. Elimination of this functional group (XX) results in over a 1000 fold reduction in activity. In contrast, neutralization of this group by formation of a benzyl ester (XXI) has no significant effect on biological activity.

5. The structural requirements for antagonism are also unclear at the present time. The first reported antagonist contained the CBZ blocking group (XXII). Subsequently other urethan type groups were incorporated into the most active tripeptide sequence (XXIII through XXV). Only the Boc derivative was an effective antagonist, the others were weak agonists. The most active antagonist reported to date is Boc-Phe-D-Leu-Phe-D-Leu-PheOH[4].

All of the above strongly suggest that these peptides are reacting with a specific receptor. This has been confirmed by radioreceptor assay[5] using a tritium labeled analog[6].

Acknowledgments

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SYNTHETIC TETRAPEPTIDES AS CHEMOATTRACTANTS FOR HUMAN EOSINOPHIL LEUKOCYTES: STRUCTURE-FUNCTION RELATIONSHIPS AMONG VARIOUS H-Val-Gly-Ser-Glu-OH ANALOGS

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Introduction

There are a range of structurally diverse principles that preferentially induce migration of eosinophil leukocytes[1]. The tetrapeptides of the eosinophil chemotactic factor of anaphylaxis (ECF-A), derived from human lung, are the only naturally occurring peptides which have the capacity to enhance eosinophil migration, as assessed in Boyden micropore filter chambers[2,3]. One of the tetrapeptides of ECF-A, H-Val-Gly-Ser-Glu-OH, and a series of analogs were prepared by standard solid-phase methods[4], and their availability permitted a systematic and detailed analysis of the structural requirements necessary for the induction of cell migration. All peptides were homogeneous by thin-layer chromatography and displayed the expected amino acid analyses.

Results and Discussion

The migratory responses of human eosinophils to H-Val-Gly-Ser-Glu-OH occurred in two concentration ranges: (i) 10^{-4} to 10^{-5} M and (ii) 3 x 10^{-7} to 3 x 10^{-8} M. Fourteen analogs (Table I) were compared to the parent molecule in a dose-response manner encompassing these two ranges. The five asterisked analogs enhanced eosinophil migration in a manner comparable to the parent tetrapeptide. Formylation or removal of the amino group or substitution of a D-Val[1], Ala[1], or Ala[3] had no appreciable effect on the activity of the parent molecule. Thus, neither

the presence of the free amino group or the seryl hydroxyl group nor the chirality of the valyl residue are required for the induction of eosinophil migration.

Table I. H-Val-Gly-Ser-Glu-OH Analogs

Amino Terminal Modifications	D-Amino Acid Substitutions
*Desamino-Val-Gly-Ser-Glu-OH	*H-D-Val-Gly-Ser-Glu-OH
*f-Val-Gly-Ser-Glu-OH	H-Val-Gly-Ser-D-Glu-OH
N-Ac-Val-Gly-Ser-Glu-OH	
	Substitutions
Carboxyl Terminal Modifications	*H-Ala-Gly-Ser-Glu-OH
H-Val-Gly-Ser-Glu-OCH_3	H-Val-Gly-Ser-Asp-OH
H-Val-Gly-Ser-Glu-NH_2	H-Val-Gly-Ser-Gln-OH
	*H-Val-Gly-Ala-Glu-OH
Amino and Carboxyl Terminal Modifications	H-Val-Pro-Ser-Glu-OH
N-Ac-Val-Gly-Ser-Glu-NH_2	
	Substitution and Carboxyl Terminal Modification
	H-Val-Gly-Ser-Gln-NH_2

In contrast, the other nine analogs displayed a specific enhancement or diminution of cell migration relative to the parent molecule in either the high or low dose-response ranges (Table II):

Table II. Structural Modifications of H-Val-Gly-Ser-Glu-OH Leading to Functional Differences in the Migratory Response of Human Eosinophil Leukocytes.

High Dose-Response Range		Low Dose-Response Range	
Activity Decrease	Activity Increase	Activity Decrease	Activity Increase
Methyl esterification of α-carboxyl	Substitution of Gln^4	Acetylation of α-amino	Methyl esterification or amidation of α-carboxyl
Substitution of D-Glu^4 or Asp^4		Substitution of Asp^4	Acetylation of α-amino and amidation of α-carboxyl
Substitution of Gln^4 and amidation of α-carboxyl		Substitution of Gln^4 and amidation of α-carboxyl	Substitution of Pro^2

1. methyl esterification but not amidation of the α-carboxyl, Gln^4 substitution with α-carboxyl amidation, or D-Glu^4 or Asp^4 substitution decreased cell migration toward the high dose concentrations.

2. Gln^4 substitution resulted in an increased high dose migratory response.

3. acetylation of the α-amino group decreased the migratory response in the low dose range, whereas formylation or removal of the amino group had no effect.

4. Gln^4 substitution with α-carboxyl amidation or Asp^4 substitution decreased cell migration in the low dose range.

5. methyl esterification or amidation of the α-carboxyl group increased the low dose migratory response and so did simultaneously blocking the amino group and the glutamyl α-carboxyl group.

6. substitution of a Pro^2 also increased the migration in the low dose range.

In summary, (i) the nature of the carboxyl terminal residue and the state of its α-carboxyl group appear to be the dominant functionally critical elements (Table II). (ii) the enhancement of cell migration in response to the Pro^2 analog and the known increased frequency of occurrence of proline at the $i + 1^{th}$ residue of β-turns[5] suggests that the design of additional analogs with this conformational feature may lead to still more potent analogs. (iii) Since the functional capacity of the α-carboxyl modified analogs is dependent on concentration, it is possible that both high and low affinity binding sites may exist. Because opposite functional effects were observed at these two concentration zones, these two putative receptors may regulate different migratory responses.

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STRUCTURAL AND FUNCTIONAL SIMILARITIES AMONG HORMONES AND ACTIVE PEPTIDES FROM DISTANTLY RELATED EUKARYOTES

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Vertebrates and Arthropods

Among the peptides from vertebrates and arthropods that are considered to be similar in structure and function are the bradykinin-related peptides and the bombinin—melittin-related peptides.[1-4] The hypotensive peptide bradykinin is found in mammalian plasma and some secreted fluids. Related peptides with similar activities are found in turtle plasma, amphibian cutaneous gland secretions, and hymenopteran venom. Selected peptides in the alignment all possess a highly conserved nonapeptide sequence.[1,2,4] Amphibian cutaneous gland secretions and hymenopteran venom contain another group of related peptides.[1-3] European *Bombina* species produce hemolytic and antibiotic peptides that may be important for controlling microorganisms on the skin and deterring predators.[5] Bee melittins are detergents with cytolytic, antibiotic, and kinin-like activities.[6] The α and ϵ amine groups (concentrated in the C-t hexapeptide) are responsible for cytolysis.[7]

We find that some arthropod peptide neurohormones may be related to vertebrate hormones.[4] Among those produced by the eyestalk sinus gland of prawns is the distal-retinal-pigment hormone, which causes migration of distal and reflecting retinal pigment granules into the proximal end of the pigment cells surrounding the crystalline cones and tracts of the ommatidia.[8] There is 69% similarity between the sequences of the prawn peptide and the vertebrate melanotropins.[4] The latter are thought to be released from the precursor in the pituitary pars intermedia; no function in mammals is known, but the peptides cause melanin dispersion in the skin of lower vertebrates. A second peptide from prawn eyestalks, the red-pigment-concentrating hormone, stimulates pigment granule concentration in red (and black) hypodermal chromatophores. The amidated C-t Trp is essential for activity. The locust adipokinetic hormone, released from corpora cardiaca cells after the beginning of

flight, causes release of diglycerides from the fat body into the hemolymph and then stimulates the flight muscles to use these diglycerides as an energy source. It is structurally homologous to the prawn pigment-concentrating hormone and induces pigment concentration in prawns, while the prawn hormone has lipid-mobilizing activity in locusts.[8] Both are structurally similar to the N-t region of vertebrate glucagon-related peptides, which contains residues involved in both receptor binding and specific activity.[4] Glucagon is involved in blood sugar homeostasis and intestinal VIP stimulates secretion of pancreatic juice and affects blood pressure; the latter is also found in the brain.[9]

Vertebrates and Molluscs

The substance P—eledoisin-related group of peptides possesses a hydrophobic pentapeptide at or near the amidated (except enkephalin) carboxyl end, preceded by a hydrophilic region.[1,4] Mammalian substance P, enkephalins, and cholecystokinin-pancreozymin (CP) are present both in intestinal or gastric mucosa and the central nervous system. The amphibian peptides, caerulein, kassinin, uperolein, physalaemin, and phyllomedusin, are present in skin and cutaneous glands; cephalopod venom, secreted by paired glands, contains eledoisin.[9,10] CP (and caerulein pharmacologically) stimulates secretion of certain pancreatic enzymes, affects gastric juice secretion, and produces gall bladder contraction; its nonsulfated C-t octapeptide and Leu-enkephalin in the brain may regulate food intake.[9,11] The enkephalins also act as natural analgesics in the brain. Substance P, eledoisin, and the amphibian peptides can lower blood pressure, produce contraction of extravascular smooth muscle, and stimulate digestive gland secretions. Eledoisin also produces paralysis. The C-t region of mammalian gastrin could be included with this group if a 7-residue segment were excluded.[1-4] Gastrin-like activity has been demonstrated in all vertebrate classes and also in some gastropod molluscs.[9]

Vertebrates and Fungi

We have found that some of the newly sequenced fungal mating hormones appear to be related to vertebrate sex hormones. There is 80% similarity between gonadoliberin from the mammalian hypothalamus and the N-t 9 residues of baker's yeast mating hormone α1:[2,4]

gonadoliberin, mammals <Q H W S Y G L R P G-NH_2
mating hormone α1, baker's yeast W H W — L Q L K P G Q P M Y

There is over 70% similarity between the N-t 11 residues of some gonadotropin α chains,[1,3,12] from the vertebrate adenohypophysis, and the smut fungus hormone rhodotorucine A:[13]

lutropin α chain, bovine F P DGE F T M Q G C . . . .
gonadotropin α chain, carp Y P RND M N N F G C . . . .
rhodotorucine A, smut fungus Y P E I S W T R N G C-FARNESYL

This N-t region of gonadotropin α chain may be involved in receptor binding.[4] Gonadoliberin stimulates adenohypophyseal secretion of gonadotropins, which in turn stimulate gonadal tissues to secrete steroid sex hormones.[3] In ascomycetes and basidiomycetes, the mating process is a fusion of sexually differentiated haploid cells to form diploid zygotes. The sex hormones are low MW peptides, lipids, or lipopeptides, sex- and species-specific, and act at low concentrations to induce formation of conjugation tubes by cells of the opposite mating type.[4,13] It is of interest that the active sesquiterpene moiety of the basidiomycete hormone is structurally related to the vertebrate sex steroids.

An Evolutionary Time Scale

The evolutionary tree of selected mitochondrial cytochrome c sequences provides a relative time scale for the divergence of the eukaryote groups featured in the seven alignments. It is slightly modified from the tree of Figure 3 in Reference 4. As cytochrome c is a very conserved protein (mutation acceptance rate of 2.2 PAMs/100 my), it provides an outline of eukaryote evolution over an extensive period of time. Furthermore, this array of sequence information is not available for any other protein. Mutation acceptance rates have also been calculated for some of the peptide hormone families represented in these alignments; all values are low enough that one would be able to recognize homologs from any eukaryotes. Several approximate divergence times are indicated: mammals and birds, 300 mya; higher vertebrates and bony fishes, 400 mya; vertebrates and arthropods-molluscs, 700 mya; animals and fungi, 1000 mya.

Summary

Peptides in each alignment group share: 1) structural features, including essential moieties; 2) physiological functions and/or

pharmacological activities; and 3) for animals, similarity of cell or tissue origin. Although most peptides are too short to treat statistically, the combination of >50% sequence similarity with those of tissue location and function makes these relationships probable.

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FUNCTIONAL AND EVOLUTIONARY CROSSOVER IN PEPTIDE HORMONES: LRH AND ACTH

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Introduction

A close structural similarity exists between luteinizing hormone releasing hormone (LRH) and adrenocorticotropic hormone (ACTH).[1] This homology suggests that LRH may have been derived in evolution from the "active core" of ACTH by transposition of DNA coding for the tripeptide Glu-His-Phe (nine nucleotides) by approximately one turn of DNA double helix. The hydrated DNA helix contains ten nucleotides per turn:

<Glu-His-Trp-*Ser-Tyr-Gly-Leu*-Arg-Pro-Gly-NH_2 [LRH]
Ser-Tyr-Ser-Met-Glu-His-Phe ———————— Arg-Trp-Gly [ACTH(1-10)]

LRH and ACTH share not only structural features, but some functional similarities as well. LRH and some fragments of ACTH have been reported to show similar effects on behavioral modifications in the rat.[2] Intracerebral injection of ACTH or certain fragments brings about sexual excitation in mammals,[3] a function which could be attributed to hormones involved in sexual regulation. Endogenous opioid peptides, biosynthesized coordinately with ACTH, apparently play a role in mating behavior.[4] Peptides related to ACTH and endorphin may be the "yang and yin" of sexual behavior. The mating pheromone of *Saccharomyces cerevisiae* (the "α-factor") is analogous to both LRH and ACTH.

Subsequent to our recognition of these homologies, two reports appeared, one dealing with the structural similarities between LRH, α-MSH and the Saccharomyces α-factor,[5] and the other suggesting analogies among LRH and three yeast sex factors.[6] However, neither of them mentioned the analogy between Ser-Tyr-Ser-Met of ACTH and Ser-Tyr-Gly-Leu of LRH. Recognition of this similarity was one of the

pivotal points in the proposed hypothesis of the primordial role of ACTH in reproduction and the evolution of LRH.[1]

It was of interest to prepare peptides having structures intermediate between LRH and ACTH and study their biological activities. The peptides listed in Table I were synthesized by standard methods of solid phase synthesis[7] and examined for LRH-like (agonist and antagonist) and ACTH-like (steroidogenic and lipolytic) activities.

Table I. Biological Activities of Synthetic Peptides.

Peptide	Activity (in vitro)		
	Steroidogenic[a]	Lipolytic[b]	LRH[c]
1. ACTH(1-10)-NH_2	0.58 @ 620	540	
2. [DPhe^7]-ACTH(1-10)-NH_2	0.55 @ 220	490	
3. LRH	NA	112	1
4. [DPhe^6,Met^7,Trp^9]-LRH	0.8 @ 10	NA	10^{-5}
5. [DPhe^{2,6},Met^7,Trp^9]-LRH	NA	700	Weak Antag.
6. [Trp(Nps)^3]-LRH	NA	100	10^{-4}

[a]Corticosterone (nmoles) released @ peptide concentrations (nM) in rat cortical cell cultures.[8] [b]Fatty acids (nmoles/h) released from rabbit fat cells.[9] [c]Relative LRH agonist and antagonist activities in the rat hemi-pituitary assay.[10] NA = not active.

Results and Discussion

LRH Activities: The results shown in Table I indicate that the presumed agonist analog, [DPhe^6,Met^7,Trp^9]-LRH, has only little LRH agonist activity. Since Leu^7 of LRH agonists can be replaced by Met with no reduction in activity,[11] substitution of Pro^9 by Trp causes the loss in potency. The corresponding presumed antagonist, [DPhe^{2,6},Met^7,Trp^9]-LRH, showed very weak inhibitory action compared to the potent inhibitor, [DPhe^{2,6}]-LRH[12]. It is reported that [Trp(Nps)^9]-ACTH has very little lipolytic activity but retains the full intrinsic steroidogenic activity of ACTH, and that it acts as an inhibitor of ACTH-mediated lipolysis.[13] [Trp(Nps)^3]-LRH, prepared with the hope of attaining a similar separation of LRH activities, did not show significant activity as an agonist or antagonist.

ACTH Activities: ACTH(1-10)-NH_2 and [DPhe^7]-ACTH(1-10)-NH_2 were approximately equipotent in releasing corticosterone from rat

cortical cells and fatty acids from rabbit fat cells. LRH showed moderate lipolytic activity but no steroidogenic activity. Thus during the presumed evolution some lipolytic activity of ACTH has been retained in LRH whereas the steroidogenic potency has completely vanished. In [DPhe^6,Met^7,Trp^9]-LRH the steroidogenic activity of ACTH reappears; indeed, it is about 60 times that of ACTH(1-10)-NH_2. In [DPhe^2,DPhe^6, Met^7,Trp^9]-LRH we see the selective enhancement of lipolytic activity. More interesting than the mere return of ACTH activities in these two peptides is the clear separation of steroidogenic and lipolytic activities. These two peptides differ by only one amino acid, having either His or DPhe at position 2 (corresponding to His^6 in ACTH). This may indicate indirectly that the His residue in ACTH is the key for expression of steroidogenic intrinsic activity. This is very intriguing because the His residue at position 2 in LRH plays the crucial role in gonadotropin releasing activity. Its deletion or replacement with D amino acids leads to analogs that are antagonists of LRH action. Specificity in the expression of hormonal activities of both LRH and ACTH appears to be centered around these histidine residues.

The biological activities discussed above and the close structural relationship between LRH and ACTH strongly suggest that an ACTH-like peptide may have been the evolutionary precursor of LRH. Once a useful structure is developed in nature it may be put to use time and again, with modifications to confer functional and target organ specificity upon the product. Some substantiation of this prediction may appear in the fact that sequences closely related to the active core of ACTH are present in a number of peptides and proteins of diversified origin exhibiting a wide array of functions.[1,14]

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STUDIES ON THE PURIFICATION AND ACTION OF AN OOCYTE MATURATION INHIBITOR ISOLATED FROM PORCINE FOLLICULAR FLUID

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Introduction

The mammalian oocyte remains in the arrested dictyate state of the first meiotic division from shortly before or at the time of birth until immediately prior to ovulation. Under the influence of the preovulatory surge of luteinizing hormone (LH) the oocyte in the large preovulatory follicle continues its first meiotic division which consists of breakdown of the germinal vesicle (nuclear membrane), chromosomal condensation and separation of homologous chromosomes, and extrusion of the first polar body.[1] Shortly thereafter a mature fertilizable oocyte is ovulated. The meiotic process is resumed again at the time of fertilization immediately after sperm penetration (Figure 1). In order for an oocyte to be fertilizable it must complete the first meiotic division (Figure 2). The intrafollicular factors which keep the oocyte in the immature dictyate state from birth until ovulation — which may be as long as 40 years in the human — are unknown. When immature oocytes from medium sized or large follicles of human,[2] porcine,[2,3] rabbit,[4] or rat[1] are cultured in vitro they resume meiosis spontaneously. Subsequently, it was demonstrated first by Chang in the rabbit[5] and later in the pig by Tsafriri and Channing,[6] in the hamster by Gwatkin and Andersen,[7] in the human by Hillensjö et al.,[8] and in the rat by Tsafriri et al.,[9] that addition to the culture medium of follicular fluid from small or medium sized follicles prevents the spontaneous resumption of meiosis. This observation led to the concept that an oocyte maturation inhibitor (OMI) is present in follicular fluid and that the preovulatory surge of LH overcomes its

action. The OMI activity of follicular fluid is destroyed by trypsin,[10] but it is stable to treatment at 60° for 20 minutes.[10] The current status of the purification of the OMI from porcine follicular fluid is summarized herein.

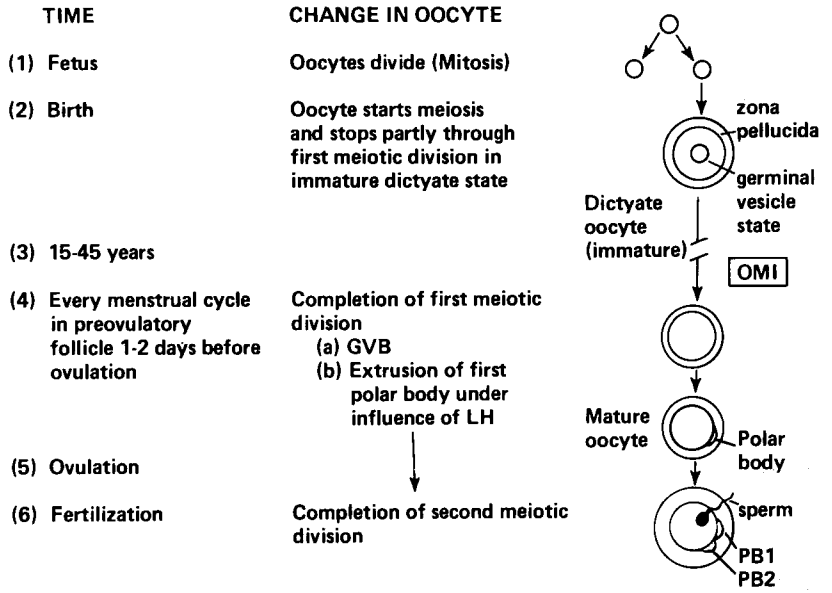

Fig. 1. Diagrammatic scheme of the temporal events involved in human oocyte maturation.

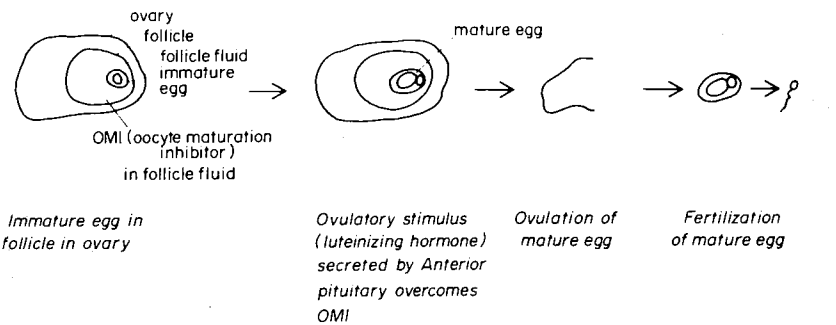

Fig. 2. Scheme of oocyte maturation and fertilization of the oocyte by sperm and concept of inhibition of oocyte maturation by a follicular oocyte maturation inhibitor (OMI).

Materials and Methods

Bioassay of OMI — The OMI activity of various fractions of porcine follicular fluid was detected by a bioassay employing cultured cumulus-

enclosed oocytes either from pigs[10] or from immature rats treated with pregnant mares' serum gonadotropin (PMSG).[9] Porcine cumulus-enclosed oocytes were harvested from medium sized follicles and cultured for 44-48 hours in a modified medium 199 containing 25 mM Hepes bicarbonate buffer, 15% porcine serum, 10 mIU/ml insulin, 2.5 mM L-lactate, and 0.03 mM pyruvate. At the end of the culture period the oocytes were fixed in ethanol-acetic acid (3:1), stained with aceto-orcein, and evaluated microscopically for stage of meiosis. Mature oocytes were those which exhibited germinal vesicle breakdown. Under conditions employed here about 70% of matured pig oocytes had a polar body. Degenerated oocytes were less than 10% of the total. Maturation data in the presence of an inhibitory fraction was expressed as % inhibition of oocyte maturation:[11]

$$\% \text{ Inhibition} = \frac{100 \times (\% \text{ Maturation in control} - \% \text{ Maturation in inhibitory fraction})}{\% \text{ Maturation control}}$$

A unit of OMI was defined as that amount of inhibitor which could inhibit oocyte maturation by 50%. Dose response curves for various fractions of OMI obtained after Amicon PM10 filtration as well as Sephadex G-25 column chromatography have been published elsewhere.[11]

In the case of rat oocytes the oocytes were examined for maturation after 6 hours of culture in the presence of inhibitor and assessed for germinal vesicle breakdown using Nomarski interference optics.[9] A dose response curve using the rat oocyte culture is shown in Figure 4. In some instances cumulus cell progesterone was estimated by radioimmunoassay as detailed previously.[12]

Collection of porcine follicular fluid and purification procedures — Figure 3 summarizes our current purification scheme. Porcine follicular fluid was collected from small (1-2 mm), medium (3-5 mm) and large (6-12 mm) porcine follicles from ovaries collected at a local meat packing plant as outlined previously.[10] The fluid was centrifuged at 1000 x g to remove granulosa cells and frozen at -35° until adequate quantities (500 to 1500 ml) were accumulated. The fluid was pooled and filtered through an Amicon PM10 membrane, and the filtrate lyophilized to dryness. The bulk of the residue was dissolved in water (10 ml/500 ml starting follicular fluid) and centrifuged at 100,000 x g for 1 hour to remove insoluble material. An aliquot was applied to a column of Sephadex G-25 (5 x 70

cm) and the column eluted with 0.01 M NH_4HCO_3 (pH 8).[11] The material eluted from the Sephadex G-25 column in the region of OMI activity was pooled and applied to a column of CM-Sephadex equilibrated with 0.01 M NH_4HCO_3 (pH 8). The column was eluted with 0.01 M NH_4HCO_3 buffer.

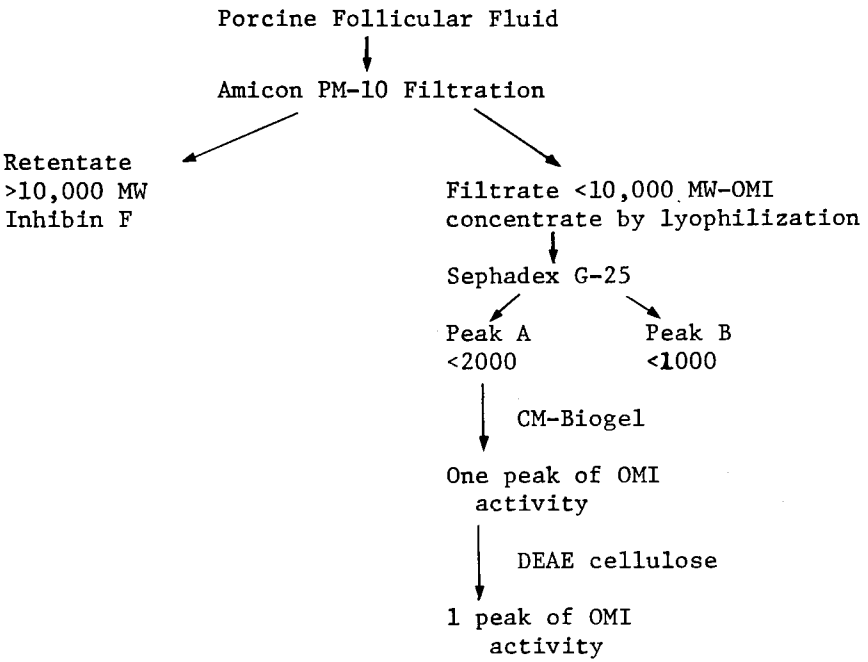

Fig. 3. Purification scheme for oocyte maturation inhibitor isolated from porcine follicular fluid.

Subsequently, the region containing OMI activity was applied to a column of DEAE-Sephadex (1 x 50 cm) which had been equilibrated with 0.01 M NH_4HCO_3 (pH 8). A portion of the CM-Sephadex OMI-fraction was loaded on the resin and the column was eluted, in succession, with 0.01 M NH_4HCO_3, a gradient of 0.01 M NH_4HCO_3 to 0.1 M NH_4HCO_3, and finally 0.5 M NH_4HCO_3. The peptide content of the eluates was determined by the fluorescamine method[13] using β-MSH as a standard as detailed previously.[11]

At each step in the purification the recovery of OMI activity in a pooled fraction was estimated by construction of a dose response curve.

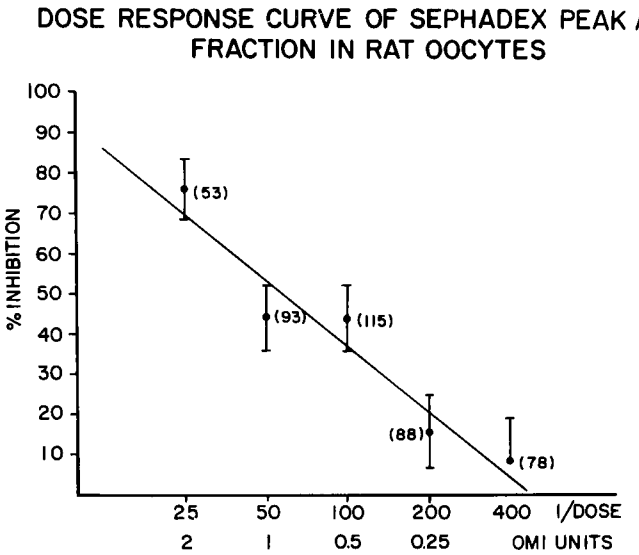

Fig. 4. Dose response curve for effects of various doses of OMI preparation (Sephadex Peak A) on maturation of rat oocytes. The numbers of oocytes are given in parentheses. The bars indicate ± S.E.M.

Results and Discussion

As summarized in Table I, it is evident that considerable purification is achieved during the procedure, amounting to 125 fold when compared to the Amicon PM10 filtrate. Virtually all the OMI activity resides in the <10,000 molecular weight fraction (PM10 filtrate) of porcine follicular fluid.[10] As shown in Figure 5, chromatography of the PM10 filtrate on Sephadex G-25 yields 2 peaks of OMI activity, one (Peak A) corresponding to a molecular weight of less than 2000 and a second (Peak B) corresponding to a molecular weight of less than 1000.[11] Material eluted in both peak regions inhibited cumulus cell progesterone secretion (Figure 6) in addition to inhibiting oocyte maturation.[12] Both inhibitory actions of Sephadex Peak A and Peak B were reversed if the inhibitory fraction was removed at 20-24 hours and fresh culture medium added.[11,12]

Chromatography of Peak A on CM-Sephadex gave the elution diagram shown in Figure 7. All of the activity was either eluted with the solvent front or was only very slightly retarded. About 50% of the fluorescamine positive peptide applied to the resin was eluted only by washing the resin with 0.5 M NH_4HCO_3 (not shown).

Fractionation of the CM-Sephadex fraction on DEAE-Sephadex gave the elution diagram shown in Figure 8. Fractions 48-61 contained

Table I. Purification of Oocyte Maturation Inhibitor from Porcine Follicular Fluid.

Fraction	Volume (ml)	Peptide* (mg/ml)	Units/mg	Total Units	Fold* Purification
FF1	900			1,800**	-
Amicon PM-10 Filtrate	24.5	105	0.48	1,225	1
Sephadex G-25 Peak A	20	43.4	1.38	1,100	2.9
CM-Sephadex	15	29	5.17	2,200***	10.8
DEAE-Sephadex (on 1,100 units of CM-Sephadex fraction)	2	1.67	60	200	125

* Based on peptide determination by the fluorescamine method (Udenfriend et al., 1972, Ref. 13) using β-MSH as a standard.

** Estimated, 1:1 dilution inhibited maturation by approx. 50%.

*** The observed increase in activity probably due to removal of a stimulator by purification.

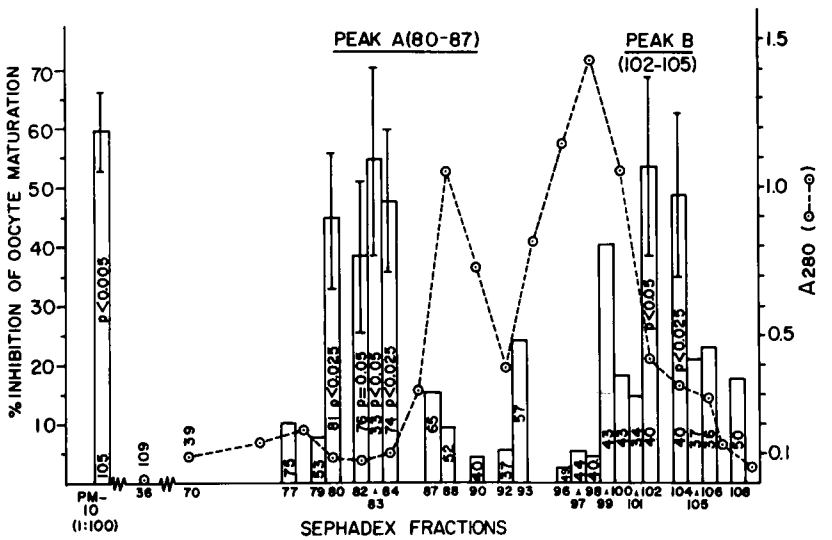

Fig. 5. Fractionation of PM10 concentrate by chromatography on Sephadex G-25. The absorbance at 280 nm (------) was determined on the original fractions (15.7 ml). Fractions were then lyophilized, redissolved in 2 ml H_2O and the inhibitory activity assayed at a dilution of (1:100) as described in Materials and Methods. The numbers in the lower parts of the bars are the numbers of oocytes for each test. The bars show ± SEM. Inhibition values without error bars were not significantly different from controls. (Taken from Stone et al., (1978) Ref. 11, with permission).

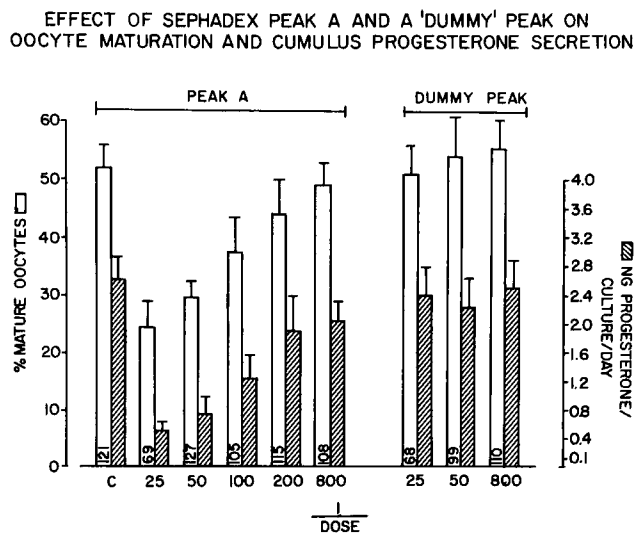

Fig. 6. Dose-response of Sephadex Peak A and of a "dummy" peak on oocyte maturation and cumulus progesterone secretion (mean ± S.E.). Peak A in 1:100 dilution produced significant ($p<0.05$ or 0.01) inhibition of oocyte maturation, and in 1:25 to 1:200 dilution, a significant ($p<0.01$) inhibition of progesterone secretion. The total number of oocytes examined is indicated (bars). Between 5 to 10 cultures per group were analyzed for progesterone. No significant effects were found using the "dummy" peak. (Taken from Hillensjö et al., (1979, Ref. 12) with permission.)

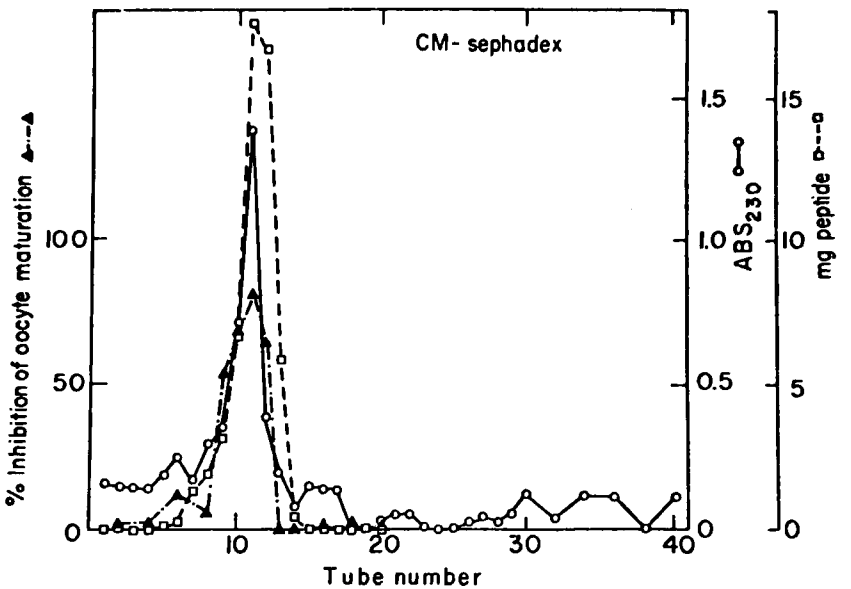

Fig. 7. Chromatography of Peak A of Sephadex G-25 on a CM-Sephadex column. OMI activity was assayed in cultured rat oocytes.

the bulk of the OMI activity (200 units). In order to detect low levels of activity the remaining fractions were divided into groups of 10 to 15 and aliquots from each fraction in a group were pooled for the OMI analysis. In this manner we found about 10 units of activity in fractions (13-26), and 5 units were eluted from the column after changing the salt to 0.5 M NH_4HCO_3 (not shown in the Figure). Although a 10-fold increase in specific activity was obtained in this step, only about 20% of the activity was recovered.

Earlier we found[11] that paper electrophoresis of Peak A in formic-acetic acids at pH 1.9 moved the OMI activity a short distance from the origin with only a two-fold purification and with considerable smearing. It is possible that this step can be inserted successfully at a later stage. We also plan to investigate both high performance liquid chromatography and partition chromatography.

An oocyte maturation inhibitor has great potential as a contraceptive. Its effects are reversible,[11] an essential prerequisite for any contraceptive agent. We hope that OMI has only one significant target tissue (the oocyte) and that it does not interfere with other reproductive processes such as the cyclic secretion of estrogen and the secretion of progesterone. The effects of OMI on the male reproductive system have not been tested. OMI must have an effect in vivo in order to rank as a contraceptive. If it is not active in vivo because of a short half life it is possible that other agents may be given which act upon the ovary to stimulate OMI production. An increase in OMI could keep ovulated oocytes in an immature, unfertilizable state. Alternatively, orally active chemical derivatives of OMI could be synthesized. Further progress depends, however, on isolation of a pure OMI, determination of the amino acid sequence and eventually synthesis in quantities sufficient for animal and eventually human testing.

Summary

Partial purification of an oocyte maturation inhibitor (OMI) has been achieved using porcine follicular fluid as a starting material and utilizing filtration on an Amicon PM10 membrane, gel filtration on Sephadex G-25, and ion exchange chromatography on CM-Sephadex and DEAE-Sephadex. The OMI activity was bioassayed in cultures employing cumulus-enclosed porcine or rat oocytes.

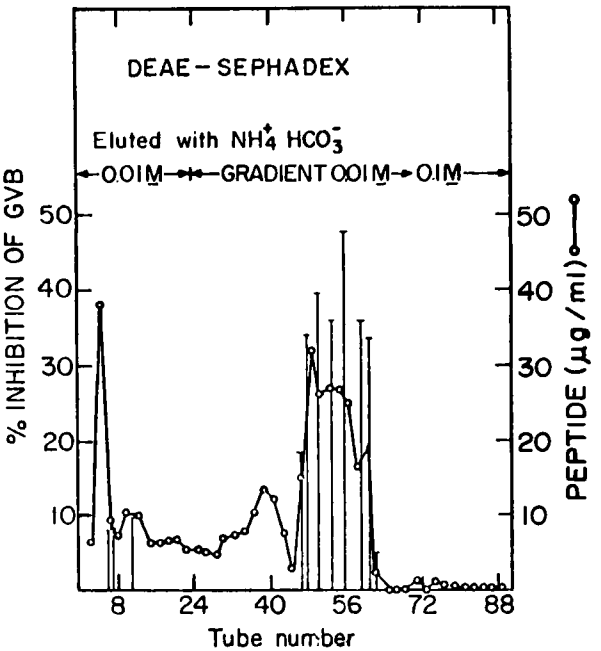

Fig. 8. Chromatography of the CM-Sephadex fraction on DEAE-Sephadex. Vertical bars show percent inhibition of germinal vesicle breakdown. O———O, peptide content by fluorescamine method.

Acknowledgements

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LH-RH ANTAGONISTS WITH POTENT ANTIOVULATORY ACTIVITY

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Among the principal approaches which we have been examining for increasing the gonadotropin-release inhibiting activity of competitive antagonists of the LH-RH decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) has been the substitution of D-Lys for Gly in position 6 and the use of its ϵ-amino group as a peptide branching[1] and dimerization point.[2] In this way, analogs with much increased antiovulatory activity have been obtained which appear to be more effective as a result of their ability to bind to two receptor sites simultaneously. One of the most active compounds in this series was the isophthaloyl dimer[2] of [D-Phe^2,D-Trp^3,D-Lys^6]-LH-RH which gave significant blockade of ovulation in the rat at a single dose of 0.5 mg, an improvement over the best of the monomeric analogs of that time, such as [D-Phe^2,D-Trp^3,D-Phe^6]-LH-RH, which were fully active in the 1.5 mg range.

Several ways were envisaged for improving activity still further, including the replacement of D-Lys by D-*p*-NH_2-Phe since aromatic D-amino acids in position 6 are more effective than those with alkyl side-chains for improving binding affinity. Thus, [N$^{\phi}$-(pGlu-D-Phe-D-Trp-Ser-Tyr)-D-Phe^2,D-Trp^3,D-*p*-NH_2-Phe^6]-LH-RH, a branched chain analog, was active at one half the dose of its D-Lys^6-counterpart.

Attempts to prepare dimers via the aromatic amino group on D-*p*-NH_2-Phe were unsuccessful because of lack of reactivity towards dicarboxylic acid active esters and it was necessary to introduce a more reactive amino group by derivatization of the para-position. A route (Figure 1) was devised to accomplish this within the framework of a standard solid-phase synthesis on benzhydrylamine resin. N^{α}-Boc-N$^{\phi}$-

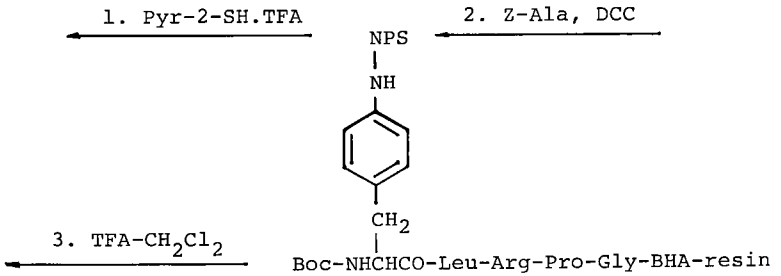

Fig. 1. Synthesis of N-Ala-D-p-NH_2-Phe-peptides

NPS-D-*p*-NH_2-Phe was prepared by catalytic reduction of N^{α}-Boc-D-*p*-NO_2-Phe followed by reaction with NPS-Cl under standard conditions. After incorporation of the amino acid in position 6, the NPS group was selectively removed in the presence of the α-Boc group using 2-thiopyridone trifluoroacetate, a reagent described by Fries et al. elsewhere in this volume. *Z*-Ala was then coupled to the *p*-NH_2-group by regular carbodiimide-mediated reaction, the Boc group removed, and the synthesis completed in the normal manner. HF cleavage of the peptide from the resin simultaneously liberated the α-amino group on Ala which was readily reactive for dimerization purposes.

Table I. Dimeric (N-Ala-D-*p*-NH_2-Phe)[6]-Antagonists — Blockade of Ovulation in the Rat.

Peptide	Dose (mg)	% Blockade of Ovulation
D-Phe[2],D-Trp[3],N-Ala-D-p-NH_2-Phe[6]-LH-RH (a)	1	20
D-pGlu[1],D-Phe[2],D-Trp[3],N-Ala-D-p-NH_2-Phe[6]-LH-RH (b)	0.5	30
Isophthaloyl-dimer of (a)	0.25	40
	0.125	0
Isophthaloyl-dimer of (b)	0.25	80
	0.125	33

The inhibitory activities (Table I) of the isophthaloyl dimers were not as high as anticipated, approximately twice as high as the analogous D-Lys[6]-dimers.[2] In this series of analogs, the D-pGlu[1]-modification[3] had a far less beneficial effect than it did when incorporated in less active monomeric analogs.[2,3]

In parallel with the polymer studies, structure-activity work was

continued on position 2, particularly the effects of various substituents on the benzene ring of the D-Phe residue generally present in the potent antagonists. We had previously shown[4] that the presence of *p*-NO_2- and *p*-NH_2-groups had little effect on the inhibitory activity of [D-Phe^2,D-Trp^3,D-Phe^6]-LH-RH and that incorporation of D-F_5Phe in this position severely diminished activity. Beattie et al.[5] reported modest increase in inhibitory activity with [D-*p*-F-Phe^2,D-Ala^6]-LH-RH, however, this observation had not been carried through to some of the new series of inhibitors.

Table II. Antagonists Containing D-pGlu^1 and D-p-halogeno-Phe^2 — Blockade of Ovulation in the Rat.

Peptide	Dose (mg)	% Blockade of Ovulation
D-pGlu^1,D-Phe^2,D-Trp^3,D-Lys^6-LH-RH	1.5	100
	0.5	25
Isophthaloyl-dimer "	0.25	100
	0.125	30
D-pGlu^1,D-p-Cl-Phe^2,D-Trp^3,D-Lys^6-LH-RH	1	60
Isophthaloyl-dimer "	0.25	100
	0.125	53
D-pGlu^1,D-p-F-Phe^2,D-Trp^3,D-Lys^6-LH-RH	1	60
D-pGlu^1,D-p-Br-Phe^2,D-Trp^3,D-Lys^6-LH-RH	1	30
D-pGlu^1,D-p-Cl-Phe^2,D-Trp^3,D-Trp^6-LH-RH	0.25	30

The effects of D-*p*-F-, D-*p*-Cl-, and D-*p*-Br-Phe in the second position of several antagonists were examined (Table II). Although the results were not too impressive, the p-Cl-peptides, both dimers and monomers, had slightly improved activities. The D-*p*-F-Phe^2, followed by the D-*p*-Br-Phe^2-modification, was less effective.

In addition to the D-pGlu^1-modification, Channabasavaiah and Stewart have reported[6] that acylated D-amino acids, including D-Phe, in

position 1 also give excellent results. [D-Phe^1,D-Phe^2,D-Trp^3,D-Trp^6]-LH-RH was prepared and, as expected in the nonacylated peptide, had no activity at moderate doses. Suprisingly, replacement of D-Phe by D-*p*-Cl-Phe in position 2 of this peptide dramatically increased antiovulatory activity (Table III) so that almost complete blockade of ovulation was obtained with 0.25 mg. The D-*p*-Br-Phe^2-peptide was less active and the F-derivative inactive at the same dose. This was the reverse of the situation observed with D-pGlu in position 1 (Table II). Acetylation of the free amino-terminus improves activity still further so that [N-Ac-D-Phe^1,D-*p*-Cl-Phe^2,D-Trp^3,D-Trp^6]-LH-RH gave good blockade of ovulation at an unprecedentedly low dose of 62 μg when injected in propylene glycol-saline solution. When administered as a suspension in corn oil, high blockade was observed down to 15 μg possibly because of prolongation of release and activity in that medium. Comparing the activity of the chloro-peptide with [N-Ac-D-Phe^1,D-Phe^2,D-Trp^3,D-Trp^6]-LH-RH^6, it is clear that the chloro-modification increases inhibitory activity roughly 10-fold. The contrasting results exhibited by D-*p*-Cl-Phe^2-analogs of the D-pGlu^1- and D-Phe^1-series suggest that the improved results with the latter are due to interactions between aromatic side-chains. Whether these are primarily steric or electronic in character remains to be clarified by examining the effects of various substituents in various places on both aromatic rings in studies presently in progress.

Table III. D-Phe^1-Antagonists — Blockade of Ovulation in the Rat.

Peptide	Dose (mg)	% Blockade of Ovulation
D-Phe^1,D-Phe^2,D-Trp^3,D-Phe^6-LH-RH	1	0
D-Phe^1,D-p-Cl-Phe^2,D-Trp^3,D-Phe^6-LH-RH	0.25	82
D-Phe^1,D-p-Br-Phe^2,D-Trp^3,D-Phe^6-LH-RH	0.25	50
D-Phe^1,D-p-F-Phe^2,D-Trp^3,D-Phe^6-LH-RH	0.25	10
Ac-D-Phe^1,D-p-Cl-Phe^2,D-Trp^{3,6}-LH-RH	0.062	100
	0.031	64

Acknowledgements

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LRF AGONISTS AND FERTILITY REGULATION IN THE MALE

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The decapeptide LRF, *p*Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2[1,2] is the key mediator in the neuroregulation of the secretion of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). In addition to its role as a hypothalamic hypophysiotropic hormone, this peptide can modify sexual behavior[3,4] and is distributed in and exhibits electrophysiologic effects in various neural regions. The essential function of LRF in the regulation of gonadotropin secretion and in reproductive processes has been demonstrated by the marked reductions in plasma gonadotropin and sex steroid levels, gonadal atrophy and inhibition of gametogenesis observed following active or passive immunoneutralization of LRF.

In order to study and exploit the actions of LRF, synthetic analogs of this peptide have been developed for use as investigational and therapeutic tools. Analogs which are more potent, longer acting, more selective or which behave as antagonists have been particularly sought. Furthermore the consideration of the biological activities of the hundreds of analogs synthesized has led to an appreciation of the structural requirements for biological activity. It is recognized that the interpretation of structure/function data is complicated by multiple factors as the biological activities of analogs can reflect variations in absorption, distribution, rates of metabolism, receptor affinity, or intrinsic activity. In an attempt to define the determinants of an analog's activity, a variety of biological tests have been employed. The effects of peptides on the secretion of gonadotropins *in vitro* and *in vivo* as well as in radioligand membrane binding assays have been studied. In addition the *in vivo* distribution and rates of degradation *in vitro* have been examined for select analogs. Finally, since the biologic activities of an analog could reflect either an involvement of the altered region in the action or metabolism of the peptide or might be secondary to changes in intramolecular associations or conformation, a variety of physical and chemical studies are carried out in order to investigate the three-dimensional structure of the peptides.

The relative potencies (with 95% confidence limits) of several analogs to stimulate the secretion of LH by primary anterior pituitary cell cultures[5,6] are shown in Table I. This highly quantitative *in vitro* system minimizes but does not eliminate differences due to degradation. Replacement of Gly[6] by D-Ala was shown by our group[7] to increase potency ca. four fold as does the C-terminal modification described by Fujino *et al.*[8]. The combination of these two modifications results in analogs whose potencies are multiplicative. The most potent LRF agonists have aromatic D amino acids such as D-Phe, D-Trp, or imidazole benzyl-D-His in position 6 and Pro[9]-NEt at the C-terminus[9,10,11]. *In vitro* [D-Trp[6],Pro[9]-NEt]-LRF is ca. 140 times more potent[9] than LRF while the recently developed [imBzlD-His[6],Pro[9]-NEt]-LRF is over 200 times more potent.[12] Another alteration that slightly increases potency in some analogs involves *Nα* methylation of Leu in position seven.[13] The triply modified analog, [D-Ala[6],*Nα*MeLeu[7],Pro[9]-NEt]-LRF is ca. 30 times more potent than LRF.[9,10]

Table I. Biological Activities of LRF Agonists

	IN VITRO BIOASSAY	RADIOLIGAND MEMBRANE BINDING ASSAY
pGlu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5] -Gly[6] - Leu[7]-Arg[8]-Pro[9]-Gly[10]-NH_2	1	1
pGlu - His - Trp - Ser - Tyr -Gly - Leu -Arg -Pro[9]-NEt	4	-
pGlu - His - Trp - Ser - Tyr D-Ala[6] - Leu -Arg -Pro -Gly -NH_2	4	4
pGlu - His - Trp - Ser - Tyr- D-Ala[6] - Leu -Arg -Pro[9]-NEt	14	8
pGlu - His - Trp - Ser - Tyr- D-Ala[6]- MeLeu[7]-Arg -Pro[9]-NEt	31	12
pGlu - His - Trp - Ser - Tyr- D-Trp[6] - Leu -Arg -Pro -Gly -NH_2	36	10
pGlu - His - Trp - Ser - Tyr- D-Trp[6] - Leu -Arg -Pro[9]-NEt	144	15
pGlu - His - Trp - Ser - Tyr- D-His[6](Bzl)-Leu -Arg -Pro[9]-NEt	210	30

Also shown in Table I are the potencies relative to LRF to compete for the binding of tritiated LRF to high affinity binding sites in rat pituitary membrane homogenates.[14,15] Similar rank orders in the binding assay and in the secretion assay are observed with the more biologically active agonists exhibiting the higher potencies in the binding assay, suggestive of those peptides having higher affinities for the LRF receptor. However, the actual potencies relative to LRF are higher in the bioassay than in the radioligand binding assay. Perhaps the increased

hydrophobicity of these analogs gives them an advantage in the intact cell but not in the membrane preparations. It is also possible that relative resistance to degradation contributes to the higher potencies seen with intact cells during a 4-hour incubation at 37°C. Various groups have reported that several of the more potent agonists are degraded at lower rates than is LRF.[9,10,17,18] The analog [D-Ala^6,NαMeLeu^7,Pro^9-NEt]-LRF appears to be completely stable during incubations in tissue extracts.[9]

Consistent with their *in vitro* activities and reported resistance to degradation, the more potent LRF agonists exhibit high potency and prolonged duration of action *in vivo* in experimental animals and human beings.[11,18,19,20] Based upon integrated plasma gonadotropin levels, these agonists, often referred to as superagonists, such as [D-Leu^6,Pro^9-NEt]-LRF, [D-Trp^6,Pro^9-NEt]-LRF and [imBzl-D-His^6,Pro^9-NEt]-LRF are hundreds of times more effective to stimulate LH and FSH *in vivo* than is LRF in the rat.[10,11] The superagonists are effective when given by various routes, including intranasally and orally.[10,11]

While the acute administration of superagonists by various routes is associated with increases in gonadotropins and sex steroid levels as well as the induction of ovulation,[11] the long term administration of superagonists, however, decreases reproductive functions in males and females. This report deals exclusively with the antigonadal effects of LRF agonists in the male. As illustrated in Figure 1, the daily delivery of a superagonist to male rats results in dose related decreases in testes, seminal vessicle and prostate weights, testosterone and prolactin.[10,20] The analog [D-Trp^6,Pro^9-NEt]-LRF is more than 1000 times more potent than LRF[22] to exhibit these effects (Figure 1). In other studies, the injection of as little as 1 ng of this peptide per day reduces seminal vessicle weights and 10 ng per day significantly lowers testosterone production.[21] These effects can be observed within 3 days of the initial injection.

Morphologically there is evidence for considerable inhibition of spermatogenesis[23] which can also be observed as soon as 3 days after beginning of treatment,[21] at which time in one study,[21] tubular diameter was already reduced and only 20% of tubules showed evidence of mitotic activity. After 5 days of treatment most tubules were empty; membranes were disrupted and polynucleated cells were present. Thus both Leydig cells which produce testosterone and sperm producing tubular elements are suppressed by exposure to superagonists.

The mechanisms whereby superagonists inhibit steroidogenesis probably involve desensitization at both the pituitary and gonadal levels.

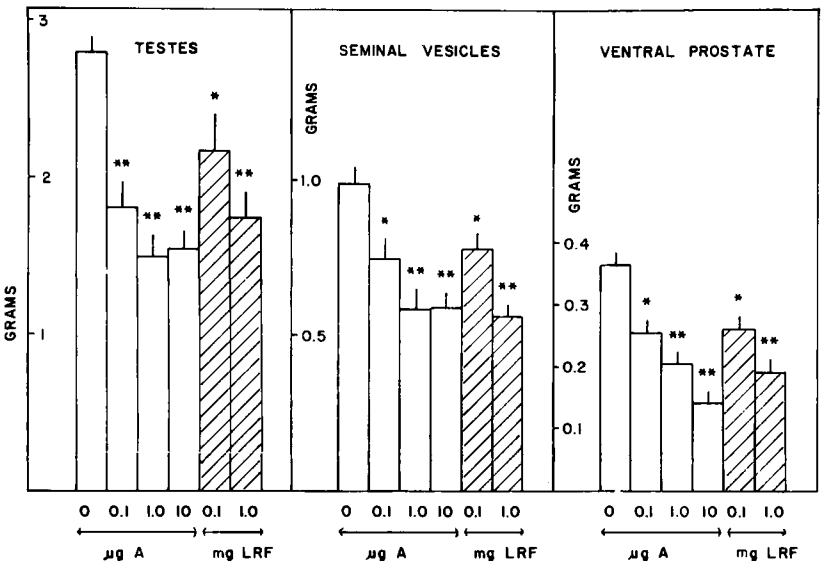

Fig. 1. Comparison of the effects of increasing doses of A and of LRF given for 7 days on reproductive organ weights in intact male rats. Each bar represents the mean ± SEM of 6 animals. *, $p \leq 0.05$; **, $p \leq 0.01$.

The initial injection of a superagonist induces a prolonged (up to 5 hours) elevation of gonadotropin levels. However, the gonadotropin secretory responses to subsequent injections of LRF or a superagonist are blunted considerably, for at least 72 hours following the first dose of superagonist.[21] When given daily for 9 days (Figure 2), the acute LH and testosterone responses on days 2 through 9 are much smaller than on day 1.

Knobil and associates[24] have shown that the pulsatile delivery of LRF (6 minutes per hour) to castrated rhesus monkeys with hypothalamic lesions results in a physiological-type pulsatile pattern of gonadotropin secretion which could be maintained chronically. However, when LRF was delivered continuously, the LH secretory rates as well as integrated amounts produced over given time periods were drastically reduced.

Recent clinical studies of Kelch and Crowley and their associates have shown improvements in gonadotropin and testosterone production in hypogonadal males following the delivery of LRF in a pulsatile manner to hypogonadotropic hypogonadal males.[25,26] Crowley and colleagues[27] similarly found that gonadotropin and testosterone levels and testicular size in such subjects could be elevated by the administration of [D-

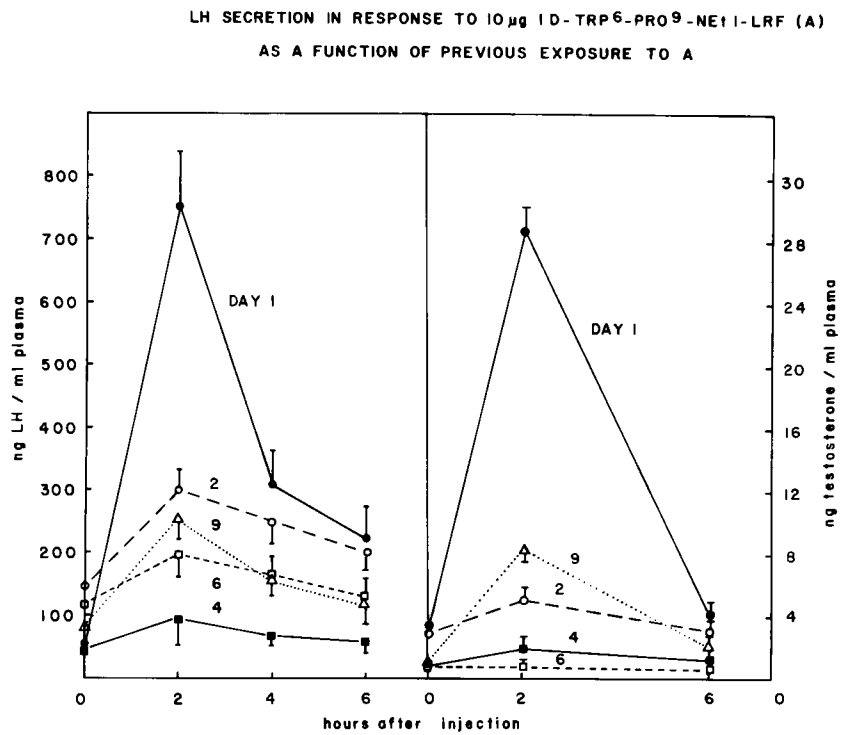

Fig. 2. Effect of chronic administration of 5 μg A on daily changes in plasma LH and T levels (intact rats). Length of peptide administration. ●———●, 1 d; ○———○, 2 d; ■———■, 4 d; □———□, 6 d; △———△, 8 d. Each point represents the mean ± SEM of 6 rats.

Trp^6,Pro^9-NEt]-LRF every other day. When the peptide was given every day, the improvements were reversed.

Thus it appears that the pituitary may become refractory to LRF and its agonists during prolonged exposure. This desensitization may involve changes in receptor numbers or binding kinetics or might involve post-receptor mechanisms.

Several laboratories have demonstrated that the administration of high doses of gonadotropins (LH or hCG, human chorionic gonadotropin) or of LRF agonists results in prolonged loss of gonadal LH receptors.[23,28-30] Furthermore, since the Leydig cells' steroidogenic responses to hCG as well as other stimuli such as dibutyryl 3'5'cyclic AMP are also blunted, post-receptor mechanisms are probably also affected.[31]

The administration of high levels of exogenous gonadotropin can, however, reverse the effects of superagonists on testosterone production

as demonstrated by the high weights of the testosterone-dependent organs (such as the seminal vesicles) of rats receiving [D-Trp^6,Pro^9-NEt]-LRF and the gonadotropins, hCG and pregnant mare's serum gonadotropin (PMSG) (Table II). Thus, the high amounts of gonadotropins secreted acutely in response to LRF agonists are not the exclusive reason for the inhibition of steroidogenesis induced by superagonists. We have proposed that a combination of pituitary and Leydig cell desensitization conspires to produce the fall in testosterone production.[15,21] The administration of a superagonist results in a 4-6 hour elevation in LH followed by an ensuing period with non-detectable LH levels. The Leydig cells, desensitized as a consequence of the initial burst of LH, would be subjected to long periods of low LH levels during a time when sensitivity to LH was minimal.

Table II. Effects of Hormonal Replacement on Reproductive Organ Weights

Treatment	N	Testes Weights	Seminal Vesicle Weights	Spermatogenesis Histologic Evidence
Intact Rats				
Control	6	3.734 ± 0.102	0.907 ± 0.073	Normal
5 μg A	6	2.456 ± 0.175 **	0.605 ± 0.051 **	Inhibited
30 IU hCG + 30 IU PMSG	6	2.293 ± 0.235 **	1.462 ± 0.149 **	Inhibited
3 mg TP	6	3.033 ± 0.209 *	1.870 ± 0.141 **	Normal
A + hCG + PMSG	6	2.758 ± 0.176 **	1.298 ± 0.096 *	Inhibited
A + hCG + PMSG + TP	6	2.987 ± 0.213 *	1.419 ± 0.103 **	Inhibited
Control	6	2.878 ± 0.103	0.717 ± 0.074	Normal
5 μg A	6	1.839 ± 0.069 **	0.442 ± 0.033 **	Inhibited
3 mg TP[(c)]	6	2.312 ± 0.060 *	1.578 ± 0.110 **	Normal
A + TP	6	2.414 ± 0.141 *	1.028 ± 0.059 **	Inhibited
Hypophysectomized Rats				
Control	5	1.252 ± 0.104	0.082 ± 0.008	Present
5 μg A	6	1.386 ± 0.155	0.087 ± 0.008	Present
3 mg TP	6	2.428 ± 0.113 **	1.392 ± 0.097 **	Normal
A + TP	8	2.555 ± 0.121 **	1.237 ± 0.105 **	Normal

The disruption in tubular structure and spermatogenesis might in part be due to the fall in testosterone production. However, it is noteworthy that while gonadotropins restore testosterone production in superagonist treated male rats, gonadotropins do not reverse or prevent damage to seminiferous tubules. In fact, gonadotropins themselves can inhibit spermatogenesis whether or not superagonists are co-administered.[21] Consistent with a possible role of gonadotropins in permitting or mediating tubular damage in superagonist treated rats is the observation (Table II) that superagonists do not alter the tubular morphology of testosterone treated hypophysectomized rats but cause tubular disruption in testosterone treated rats with intact pituitary glands.

It may be relevant that superagonist treated rats exhibit increased LH to FSH ratios, since after 2 or 3 daily injections, the acute FSH secretory response is abolished while the acute LH response (though blunted) is maintained (Figure 3). This results in markedly elevated LH/FSH ratios for several hours following each additional injection of a superagonist.[32]

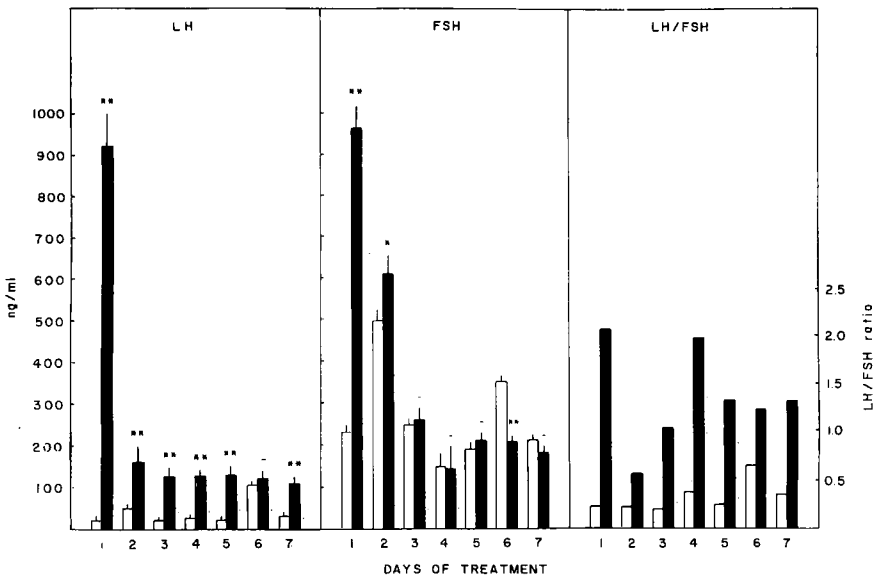

Fig. 3. Hormonal patterns of male rats during daily treatment with 5 μg A during a 7-day period. Each bar represents the mean ± SEM of 5 rats. Open bars correspond to pre-injection values; closed bars correspond to post-injection (2 hours) levels. ** –, $p > 0.05$; **, $p \leq 0.01$.

Catt and associates[31] have shown that testes from hCG treated male rats exhibit specific steroidogenic defects *in vitro* at the 17,20 desmolase and earlier steps. We have demonstrated the presence of similar enzymatic blocks in incubated Leydig cells of rats treated with the superagonist, [D-Trp^6,Pro^9-NEt]-LRF.[33] Following the intense gonadotropin stimulation induced by the initial injections of a superagonist, intermediates such as progesterone (Po) continue to be synthesized even though the development of blocks of specific enzymes progressively suppresses testosterone secretion (Figure 4). Eventually, through mechanisms that are not defined, production of Po and other precursors are also inhibited. When superagonists are administered

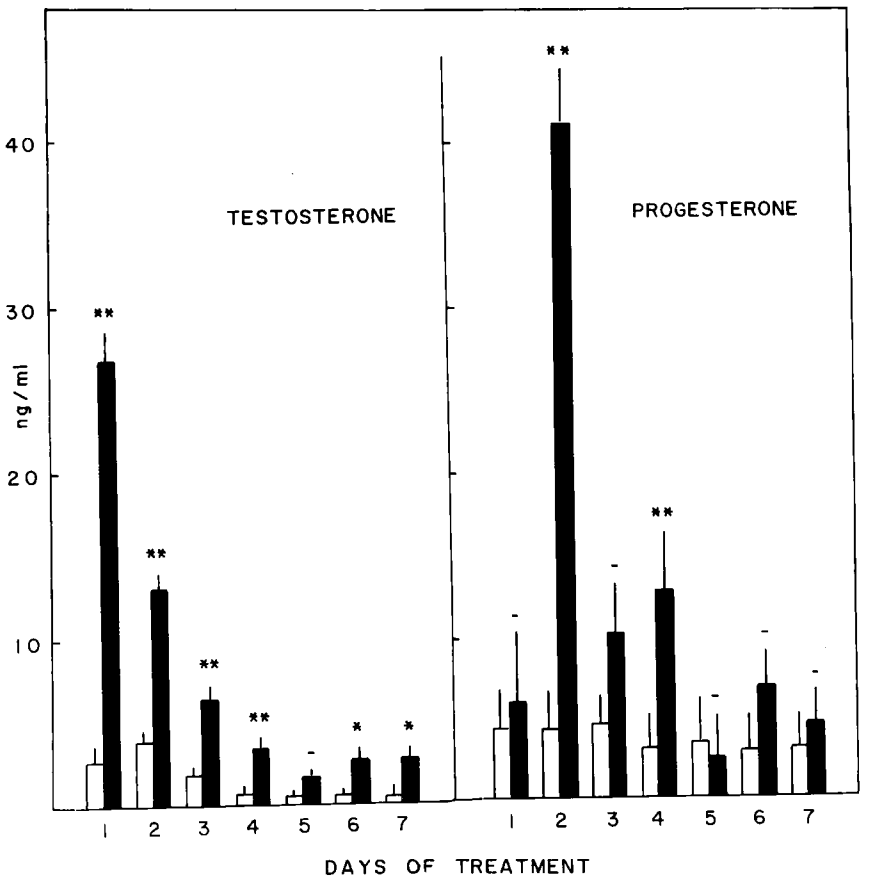

Fig. 4. Testosterone and progesterone levels of male rats during daily treatment with 5 μg A during a 7-day period. Each bar represents the mean ± SEM of 5 rats. Open bars correspond to pre-injection values; closed bars correspond to post-injection (2 hours) levels. ** –, $p > 0.05$; **, $p \leq 0.01$.

intermittently, every second, third, or fourth day (Figure 5), high levels of Po are attained with each injection. We have noted that an intermittent administration schedule produced the greater disruption of tubular morphology.[21]

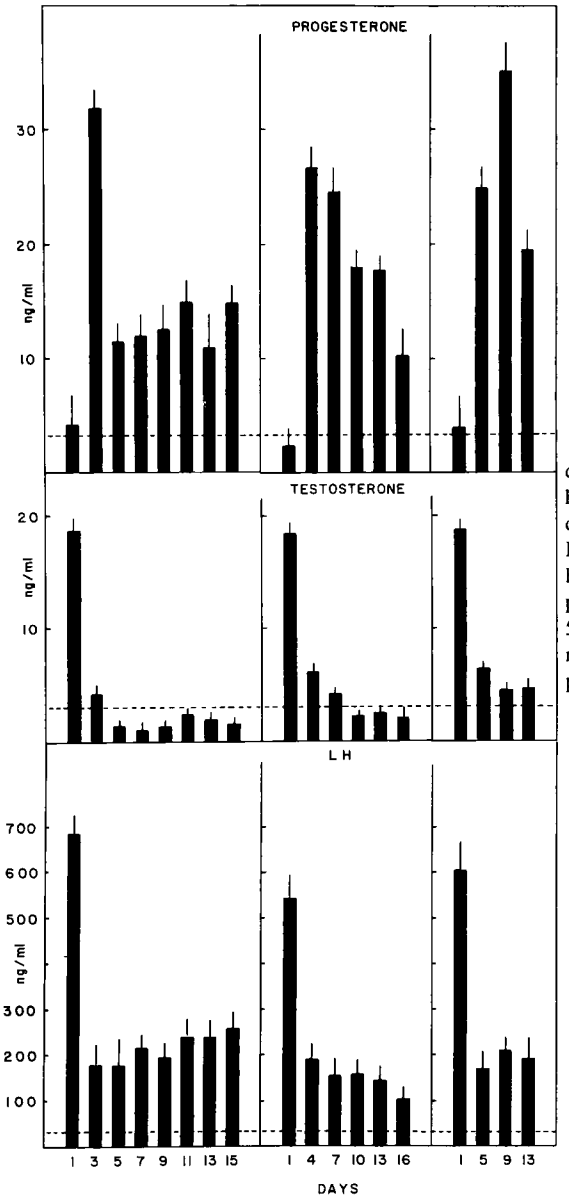

Fig. 5. Plasma LH, testosterone and progesterone levels of rats having received repeated injections of 5 μg A under various regimens. Blood samples were obtained 2 hours after each injection. Each point represents the mean ± SEM of 5 rats. The dotted lines indicate the mean levels of LH, testosterone and progesterone in control animals.

Since Po has been reported both to have deleterious effects on seminiferous tubules of male rats,[34] and yet to maintain qualitatively normal spermatogenesis in hypophysectomized animals,[35,36] the role played by the abnormal elevation of Po is not defined. However, the intratesticular levels of Po in these studies are probably astronomical and may well, in part, mediate the deleterious effects of gonadotropins and LRF superagonists on seminiferous tubules.

The failure of exogenous testosterone to prevent the effects of superagonists on tubular morphology might be partially explained by an inhibitory effect of high intratesticular progesterone. It is plausible that a regimen of superagonist plus testosterone or another androgen might be used to maintain secondary male sex characteristics in the presence of an inhibition of spermatogenesis.

The relative rates of recovery of Leydig cell and spermatogenic functions might provide another clue to the development of a superagonist-based male contraceptive. Within three to five days following a one-week treatment of male rats with a superagonist, plasma testosterone levels are usually comparable to untreated controls.[32] In the recovery experiment shown in Figure 6, although seminal vesicle weights and testosterone levels have recovered to control levels within one week after cessation of a seven day superagonist treatment, testes weights and tubular structure were not completely restored by one month. It may be possible within periodic administration (e.g. 1 week/month) of superagonists to produce an infertile state with minimal effects on secondary sex characteristics.[15,21] As described earlier, the periodic treatment regimen may involve an intermittent (every 2-4 days) treatment of superagonists with or without co-administration of androgens.

In summary, the inhibition of Leydig cell steroidogenesis by superagonists may be caused by desensitization at both gonadal and pituitary levels. The superagonist mediated inhibition of testosterone production can be overcome by exogenous gonadotropins. The disruption of tubular structure and spermatogenesis is pituitary dependent and may be related to supernormal production of progesterone. Though additional studies are needed to assess the precise roles played by the non-physiological secretions of LH, FSH, testosterone and progesterone in the anti-reproductive effects of LRF superagonists, these peptides are providing important investigational and perhaps therapeutic tools. The application of these agents to the regulation of fertility is promising.

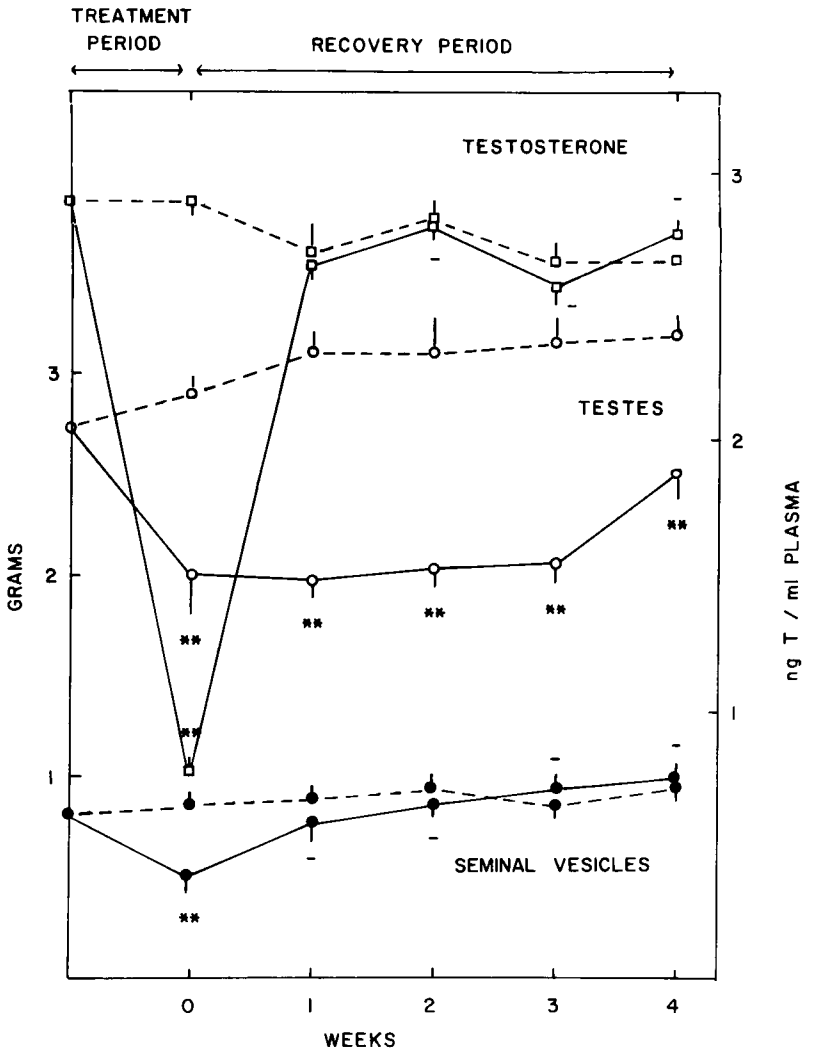

Fig. 6. Effect of 7 days treatment with 5 μg A on reproductive organ weights and plasma T levels as a function of time after cessation of treatment (intact rats). ○ = testes; ● = seminal vesicles; □ = T; — — — — represents control animals; —— represents animals having received 5 μg A. Each point represents the mean ± SEM of 6 intact male rats. -, $p > 0.05$; **, $p \leq 0.01$.

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SYNTHESIS AND EFFECTS ON PROLACTIN OF THR-SER-LYS

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Introduction

In 1978, Orts *et al.*[1] reported the sequence of the bovine pineal antireproductive peptide threonylseryllysine (Thr-Ser-Lys). We have synthesized this peptide in order to compare its HPLC properties, and its effects on reproduction, with those of the bovine pineal prolactin regulating substance(s) (PPRS) presently being studied in our laboratory.[2]

Materials and Methods

Gel filtration: Chromatography was carried out on a 1.5 x 80 cm column of Sephadex G-10 eluted with 0.2 M acetic acid. Fractions (4.0 ml) were collected and their absorbance at 220 nm determined.

Paper chromatography: Ascending paper chromatography was carried out using butanol:ethanol:glacial acetic acid: H_2O (4:1:1:1) as solvent. Analytical chromatograms used Whatman #1 while preparative runs were made on Whatman #3MM paper. The peptide was visualized using ninhydrin.

High pressure liquid chromatography (HPLC): Chromatographic procedures employed a μBondapak C_{18} column (0.39 x 30 cm) eluted isocratically at a flow rate of 2.0 ml/min. The two solvent systems used were 10^{-4} M acetic acid and 60/40 acetonitrile/H_2O.

Mass spectral analysis was carried out using a Finnigan 330. The peptide was prepared for mass spectral analysis using the *N*-acylation permethylation procedure of Leclercq and Desiderio.[3] Spectra were obtained using direct probe techniques.

Synthesis: Synthesis was carried out by the solid phase method using 3.0 g (1.11 mmol) of *N*α-Boc-*N*ϵ-Cbz-Lysine-O-Resin substituted with Lys to an extent of 0.37 mmol/g resin. The *tert*-butyloxycarbonyl (Boc) group was used for the protection of all α-amino groups. In addition to

the Cbz protection of the ϵ-amino group of Lys, the side chain hydroxyl groups of Thr and Ser were *O*-benzyl protected. The synthesis program for coupling amino acids was similar to that used for other syntheses in our laboratory.[4] Following the synthesis, 3.4 g of *Nα*-Boc-Thr(Bzl)-Ser(Bzl)-Lys(Cbz)-O-Resin was obtained. Cleavage of the peptide from the resin was achieved by stirring a mixture of 1.7 g of the peptide-resin, 2.0 ml of anisole, and 18 ml of anhydrous HF (freshly distilled from CoF_3) for 1 hour at 0°C. The HF was removed *in vacuo* and the resin was washed with ethyl acetate (3 x 20 ml). The peptide was extracted into 0.2 M acetic acid (3 x 40 ml), and the combined aqueous solution lyophilized to give a white powder (310 mg). The lyophilizate was redissolved in 1.0 ml 0.2 M acetic acid, chromatographed on Sephadex G-10 and the fractions pooled (Figure 1).

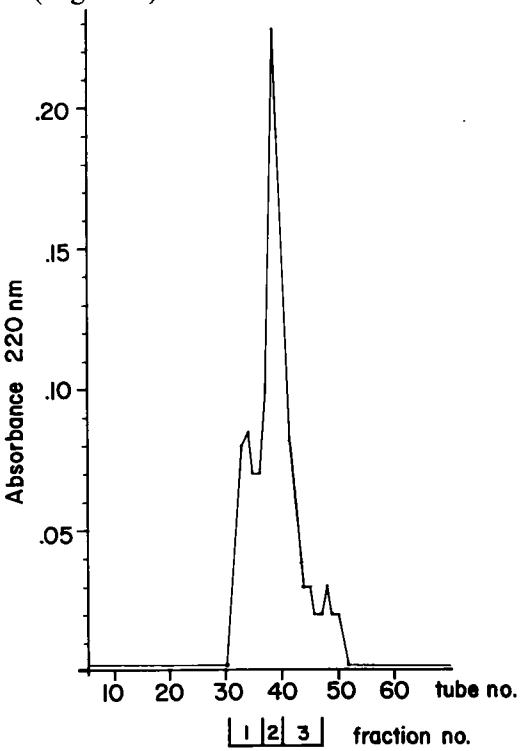

Fig. 1. Gel filtration on Sephadex G-10 (1.5 x 92 cm) of the reaction product following peptide synthesis. The eluant, 0.2 M acetic acid, was flowing at 60 ml/h; absorbance at 220 nm.

Analytical paper chromatography was performed using 1% of each pooled fraction. Fraction F_1 had one ninhydrin-positive spot at R_f 0.13 as well as the minor spot remaining at the origin. Initially, preparative paper

chromatography was chosen for final purification. A 10 mg sample from F_3 was dissolved in 50% methanol/H_2O. The entire solution was placed on a 10 x 22 cm strip of Whatman 3MM paper and developed with the solvent system described. A band, R_f 0.13 at the center, was visualized when the edges of the chromatogram were developed with ninhydrin. The area between 1.3 and 2.8 cm above the origin was cut and eluted with 4 ml of 0.2 M acetic acid. After lyophilization 4 mg (44% based on starting *Nα*-Boc-*Nε*-Cbz-Lysine-O-Resin) of fine white platelets was isolated. Rechromatography on paper showed one spot with an R_f at 0.13. Standard amino acid analysis showed that the peptide contained serine, threonine and lysine in a 1:1:1 ratio.

The homogeniety of the purified peptide was verified by HPLC using two solvent systems, 10^{-4} M acetic acid (Figure 2a) and acetonitrile/H_2O (Figure 2b). It was found that the acetonitrile/H_2O system could be used for purification of the G-10 fractions (0.5 mg per run) thus eliminating the need for paper chromatography.

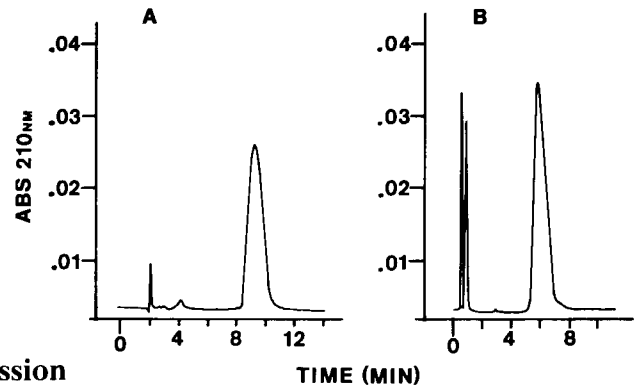

Fig. 2. HPLC of purified Thr-Ser-Lys. (ca. 4 μg) on μBondapak C_{18} 0.39 x 30 cm, 2.0 ml/in, 0.1 AFS at 210
a) eluant 10^{-4} M HoAc
b) eluant 60/40 acetonitrile/water.

Results and Discussion

Using solid phase synthesis techniques, Thr-Ser-Lys was synthesized as described. The peptide was cleaved from the resin by liquid HF and purified by gel filtration on Sephadex G-10 followed by preparative paper chromatography. The purity of the sample was checked by amino acid analysis, paper chromatography and HPLC. The HPLC results (Figure 2) indicated the peptide was homogeneous.

The spectrum shown in Figure 3 was obtained by direct probe mass spectral analysis of the *N*-acylated permethylated[3] peptide. The fragmentation scheme illustrated in Figure 3 depicts the ions expected for simple cleavage of the peptide backbone (sequence ions). The ions at m/e 144, 172, 287, 457, 485 are consistent N to C terminal sequence ions assigned to the peptide. The ion found at m/e 229, confirming the C

terminal lysine, and the ion at m/e 372 are the result of fragmentation from the C to N terminal sequence of the peptide. Other significant ions of the spectrum can be assigned to structures resulting from known fragmentation and/or rearrangements of the aforementioned sequence ions, giving further structural corroboration. The molecular ion, m/e 516, offers the final unequivocal evidence that the structure of the synthetic product isolated by paper chromatography is Thr-Ser-Lys.

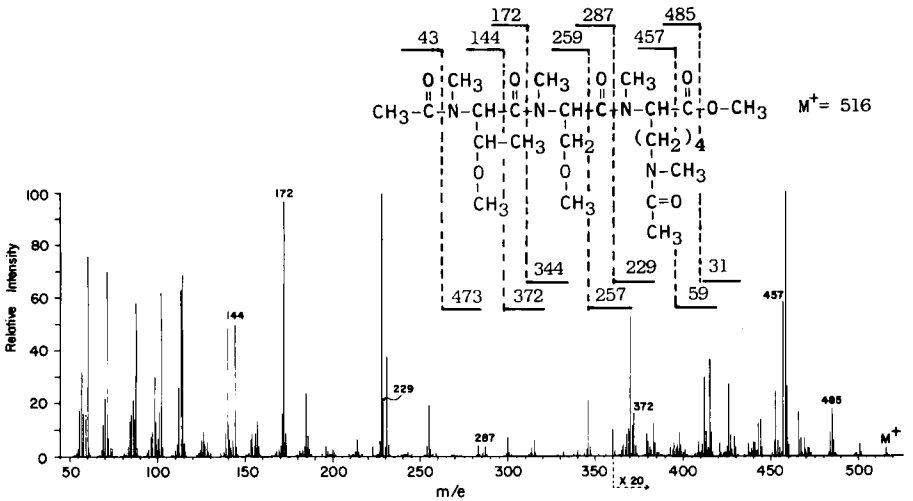

Fig. 3. Mass spectrum of *N*-acylated-*N*, *O*-permethylated Thr-Ser-Lys methyl ester.

Conclusion

Biological testing now in progress indicates Thr-Ser-Lys has a dose-related response for blocking compensatory ovarian hypertrophy in mice. Effects on serum prolactin levels and the onset of puberty in rats have also been observed. The effect on prolactin is consistent with that of our semipurified PPRS. However, the molecules differ in both size and chromatographic properties.

Acknowledgement

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SYNTHETIC MODELS OF CONCEPTUAL PRO-RELEASING HORMONES

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Introduction

The cleavage of pro-glucagon to glucagon, bovine proinsulin to insulin, large gastrin to gastrin, β-lipotropin to β-melanotropin and to β-endorphin exemplify an -AA^1-AA^2- peptide structure which liberates active hormones. Frequently, -AA^1-AA^2- represents a "double basic pair" consisting of Lys and Arg in four possible variations.[1]

It is conceivable that TRH and LHRH are derived from pro-hormones possessing an -AA-AA-Gln- sequence which is cleaved between -AA-Gln- to yield an H-Gln intermediate in a similar way as large gastrin is converted to gastrin. The H-Gln intermediate then cyclizes to the <Glu- peptide.

To investigate this possible mechanism for the N-terminal region of TRH and LHRH, the analogs, [Val-Lys-Lys-Gln]^1-TRH, [Val-Lys-Lys-Gln]^1-LHRH and [Lys-Lys-Gln]^1-LHRH were synthesized by the solid phase method and tested *in vivo* and *in vitro* for the release of TSH, LH and FSH.

Methods

The peptides were synthesized by an automated solid-phase procedure using the BHA resin and a program as described.[2] Purification was performed by gel-filtration and partition chromatography.

The following peptides were synthesized: I [Val-Lys-Lys-Gln]^1-TRH; II [Val-Lys-Lys-Gln]^1-LHRH; III [Lys-Lys-Gln]^1-LHRH.

HPLC was used to prove the absence of TRH and LHRH as possible contaminates. A μ Bondapak C_{18} column, and the solvent system, 50%

acetonitrile and 50% 10 mM aqueous ammonium acetate, adjusted to pH 5, was used for elution. Due to their basicity, the peptides did not elute in this system, but LHRH and TRH did. By comparison of V_R and peak height of synthetic LHRH and TRH, the analogs contained no TRH and LHRH that could explain the activity. Using the same column, and the solvent system 20% isopropanol and 80% of an aqueous 0.1 M solution of KH_2PO_4 (pH 4.3), the analogs eluted as single peaks.

Bioassays were conducted essentially as described.

Results

[Val-Lys-Lys-Gln]^1-TRH (I) at a dose of 270 ng/ml released ($p<0.001$) TSH *in vitro*. Pituitaries from 20-day old female rats were used (2/ml beaker). The differential TSH, ng/ml, was 5,823 ± 607. For a comparison, the differential TSH from synthetic TRH was 18,450 ± 2,030 ($p<0.001$). There was a given dose-response, since 90 ng/ml of this analog gave a differential TSH of 1,609 ± 490 ($p<0.001$). In other words, at a dose of *ca.* 300-fold that of TRH, [Val-Lys-Lys-Gln]^1-TRH released ($p<0.001$) *ca.* one-third as much TSH as did TRH, *in vitro*.

[Val-Lys-Lys-Gln]^1-LHRH (II) at a dose of 10-120 ng/ml did not release LH or FSH, *in vitro*. However, [Lys-Lys-Gln]^1-LHRH (III) at a dose of 1 μg/ml released 269 ± 32 ng/LH/ml (differential value; $p<0.001$). Correspondingly, the differential release of FSH was 5,592 ± 804 ($p<0.001$). In other words, for comparable activity of [Lys-Lys-Gln]^1-LHRH to release LH and FSH in comparison with synthetic LHRH, about a 1,000-fold dosage of the synthetic model was required in comparison with synthetic LHRH. There was no significant difference in the activity of this model for the release of LH and FSH, *in vitro*.

The data, *in vivo,* on the activity of the two synthetic models of LHRH are most interesting. As control, synthetic LHRH at a level of 0.1 μg/rat gave a differential value of 39.0 ± 6.0 for LH in ng/ml ($p<0.001$). [Val-Lys-Lys-Gln]^1-LHRH at a level of 50.0 μg/rat gave a differential value of 23.0 ± 6.0 for released LH ($p<0.001$). In other words, at a 500-fold level, this model released about 50% of the LH released by LHRH. From the following data, it is evident that [Lys-Lys-Gln]^1-LHRH was the more effective of the two models. The control level of LH from 0.1 μg/rat of LHRH was 12.0 ± 0.8 ($p<0.001$). At a dose of 50.0 μg/rat, the latter dodecapeptide model released ($p<0.001$) the same amount, 12.0 ± 0.9, of LH as did LHRH. In other words, at a 500-fold level, [Lys-Lys-Gln]^1-LHRH released LH as effectively as did LHRH.

In adult male chimpanzees, both [Val-Lys-Lys-Gln]^1-LHRH and [Lys-Lys-Gln]^1-LHRH released LH after both 15 and 30-minute intervals, when the dose of the model peptides was 1,000 μg in comparison with 3 μg of LHRH, and when the degree of release of LH from the model and that of LHRH were comparable. In other words, these two model peptides at a level of about a 300-fold that of LHRH released as much LH as LHRH in the adult male chimpanzee.

The time-response activity of LHRH and [Val-Lys-Lys-Gln]^1-LHRH for the release of LH were compared in adult male rats. LH measured after 15 and 30-minute intervals from the action of LHRH were identical, but the level measured after 60 minutes had decreased. The same time-response for the release of LH was observed for [Val-Lys-Lys-Gln]^1-LHRH. In other words, at 150-fold the dose of LHRH, iv., in the rat the level of LH in the medium was about one-third to one-half that over the 15-30 minute period as observed for LHRH.

Discussion

These data, particularly the *in vivo* data, on these three synthetic models of conceptual "pro-hormones" of TRH and LHRH support the concept that both TRH and LHRH could be derived from pro-hormones by cleavage between a "double basic pair" and Gln. The resulting [Gln]^1-TRH and [Gln]^1-LHRH could then cyclize, either enzymically or non-enzymically, *in vivo,* to TRH and LHRH, respectively.

Since the molecular weights of the conceptual pro-TRH and pro-LHRH would be expected to be considerably larger than that of the synthetic models used in this study, it is not disappointing but encouraging that the releasing activities of these synthetic models are as potent as they are although they are less active than TRH and LHRH, respectively.

These data on synthetic models of conceptual pro-hormones of TRH and LHRH are seemingly in agreement with the study by Millar *et al.*[3] in 1977 on higher molecular weight immunoreactive species of the luteinizing hormone-releasing hormone as possible precursors of the hormone. These investigators found that separation of extracts of sheep hypothalami on Sephadex G-25 gave three peaks which exhibited luteinizing hormone-releasing hormone immunoreactivity. One of these peaks corresponded in elution volume to the known luteinizing hormone-releasing hormone. Material represented by the other two peaks, I and II, which eluted from G-25 before LHRH appeared to be of higher

molecular weights. Digestion of I with trypsin resulted in an increase in total immunoreactivity and the production of an entity with the same elution volume as II. Tryptic digestion of II yielded a small amount of an immunoreactive peptide which eluted as did the luteinizing hormone-releasing hormone. The authors concluded that the increase in total immunoreactivity, when peptide I was subjected to limited trypsin digestion, supported their concept that I may be a precursor or a pro-hormone of LHRH.

Our data on the three synthetic models of conceptual pro-hormones of TRH and LHRH in conjunction with the data by Millar *et al.*[3] indicate that a pro-hormone of TRH and of LHRH may have one of the following generic molecular structures, respectively:
PRO-TRH: [Peptide-Lys-Lys-Gln]^{1}-TRH, [Peptide-Arg-Arg-Gln]^{1}-TRH, [Peptide-Lys-Arg-Gln]^{1}-TRH, and [Peptide-Arg-Lys-Gln]^{1}-TRH.
PRO-LHRH: [Peptide-Lys-Lys-Gln]^{1}-LHRH, [Peptide-Arg-Arg-Gln]^{1}-LHRH, [Peptide-Lys-Arg-Gln]^{1}-LHRH, and [Peptide-Arg-Lys-Gln]^{1}-LHRH.

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NEW POTENT AGONIST AND ANTAGONIST ANALOGS OF LUTEINIZING HORMONE RELEASING HORMONE

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Introduction

Research on luteinizing hormone releasing hormone (LRH) in recent years has led to the discovery of highly potent agonists and antagonists. Both these classes of compounds are being investigated as potential contraceptives, antagonists as ovulation inhibitors and super-agonists as post-coital agents which interfere with implantation or maintenance of pregnancy. Even though such compounds have been effective in some experimental animals, for application in humans, agents with higher potency are needed. We report here biological activities of analogs of LRH prepared with the hope of obtaining analogs more potent than those known currently. These analogs were synthesized by the Merrifield solid phase method,[1,2] purified by countercurrent distribution (CCD) or chromatography and characterized by tlc, HPLC and amino acid analysis. The details of syntheses have either been published[3] or will be reported elsewhere[4] (see Table I).

Results and Discussion

The first competitive inhibitor of LRH, prepared by the Salk group,[5] was [des-His^2]-LRH. It was later found that replacement of His^2 by D-Phe led to a better inhibitor and that the potency could be enhanced by substituting a D-amino acid for Gly^6 (see Rivier *et al.*[6] for a recent review). Therefore, initially, we prepared peptides related to [DPhe^2,DLys^6]-LRH and [DPhe^2,DPhe^6]-LRH. These included dimers (nos. 6-8), a cyclic analog (no. 15), and derivatives bearing hydrophobic and alkylating moieties (nos. 1-5). However, these analogs did not show

Table I. Biological Activities of Synthetic LRH Analogs.

Analog[a]	In Vitro Activity[b]			In Vivo Activity[c]	
	Agonist	Anti-LH	Anti-FSH	Response	Dose (mg)
1. [DPhe^2,DLys(Chlorambucil)^6]-LRH	0.06	NA	NA	10/10	3.0
2. [DPhe^2,DLys(Stearoyl)^6]-LRH	0.001	NA	NA	10/10	2.0
3. [DPhe^2,DLys(4-Phenylbutyryl)^6]-LRH	0.01	NA	NA	10/10	3.0
4. [DPhe^2,DLys(Boc-Gly-Pro$_9$)^6]-LRH				9/10	1.5
5. [DPhe^2,DLys(Stearoyl-Gly-Pro$_9$)]-LRH	0.001	100:1	160:1	9/10	1.5
6. Pentamethylene-bis-([DPhe^2,DLys^6]-LRH) (Position 6 Dimer)	0.001	NA	NA	10/10	1.5
7. Succinyl-bis-([DPhe^2,DLys^6]-LRH) (Position 6 Dimer)				7/10	1.0
8. Succinyl-bis-([des-<Glu^1,DPhe^{2,6}]-LRH) (Position 1 Dimer)	0.001[d]	150:1	166:1	10/10	1.5
9. Hydrazido-bis-([DPhe^{2,6}]-LRH) (Position 10 Dimer)	0.04[d]	166:1	NA	10/10	1.5
10. [DPhe^{2,6}]-LRH-NHNH$_2$	0.001[d]	166:1	166:1	10/10	1.5
11. [DPhe^{2,6}]-LRH-NHCH$_2$CH$_2$NH$_2$	0.001	NA	NA	10/10	1.5
12. [DPhe^{2,6}]-LRH-NHCH$_3$	5.0	NA	NA	10/10	1.5
13. [DPhe^{2,6},DAla-OL^{10}]-LRH	0.1	NA	NA	10/10	1.5
14. [DPhe^{2,6,10}]-LRH	NA	130:1	200:1	8/10	2.0
15. Cyclo(-DPhe-Trp-Ser-Tyr-DPhe-Leu-Arg-Pro-Gly-NHNH-COCH$_2$CH$_2$CO-)	2.0(LH) 0.6(FSH)	NA	NA	10/10	1.5
16. [Acetyl-DTrp^1,DPhe^2,DTrp^{3,6}]-LRH				0/10 1/10 4/10	0.5 0.25 0.1
17. [Acetyl-DPro^1,DPhe^2,DTrp^{3,6}]-LRH				0/10 4/10 5/10	0.5 0.25 0.1
18. [Acetyl-DPhe^1,DPhe^2,DTrp^{3,6}]-LRH				0/10 3/10	0.25 0.1
19. [Acetyl-DAla^1,DPhe^2,DTrp^{3,6}]-LRH	NA	12:1	15:1	0/10 2/10	0.25 0.1
20. [Acetyl-DAla^1,DPhe^2,DThi^{3,6}]-LRH				0/10 1/10	0.25 0.05
21. [Acetyl-DThi^1,DPhe^2,DTrp^{3,6}]-LRH				0/10 1/10 4/10	0.5 0.25 0.1
22. [D-Glu^1,DThi^2,DTrp^{3,6}]-LRH				3/10	0.5
23. [(D-Glu-DThi-DTrp-Ser-Tyr)$_2$-DLys^6]-LRH				7/10	0.5
24. [(-Glu-DAla)^1,DPhe^2,DTrp^{3,6}]-LRH	NA	15:1	20:1	0/10	0.25
25. [(-Glu-Ala)^1,DPhe^2,DTrp^{3,6}]-LRH	NA	50:1	50:1		
26. [Chlorambucil-DPhe^1,DPhe^2,DTrp^{3,6}]-LRH	NA	50:1	50:1	7/10	1.0
27. [Acetyl-DTrp^1,DPhe^2,DTrp^3,DTrp(Nps)^6]-LRH				8/10	0.5

[a]In addition to the standard abbreviations, Ala-OL = alaninol; Thi = β-(2-thienyl)-alanine, Trp(Nps) = 2-(2-nitrophenylsulfenyl)-tryptophan. [b]The *in vitro* assays using the rat hemi-pituitaries were carried out following the published procedure[7]. The agonist activities of peptides are compared to that of LRH = 100. The antagonist activities reported are IDR_{50}s, *i.e.*, the analog:LRH ratio that caused 50% inhibition of the releasing action of LRH. NA = not active. [c]Anti-ovulatory Assay: No. of rats ovulating/no. treated. The animals were given the peptide suspended in corn oil in a single injection at noon on the day of proestrus and autopsied the following day. These *in vivo* assays were provided by the Contraceptive Development Branch, Center for Population Research, NICHD-NIH, through the courtesy of Dr. Marvin Karten. [d]Only FSH was released.

any significant antagonist activity and some were even weak agonists. In some peptides an indication of separation of LH and FSH releasing action was observed. The analogs 8-10 released only FSH, but their potencies were low. The cyclic analog 15, on the other hand, had 2% and 0.6% the potency of LRH in releasing LH and FSH, respectively. These compounds may form the basis for design of LRH analogs with dissociated releasing activities.

Later, we discovered that introduction of an acylated D-amino acid at position 1 yielded analogs with enhanced antiovulatory activities.[3] A compound in this series, [Acetyl-DAla^1,DPhe^2,DThi^{3,6}]-LRH (no. 20) completely blocked ovulation in 9 of 10 rats tested at a single injection dose of 50 μg/rat. This analog and three others, 18,19 and 26, blocked ovulation in all the animals tested at doses of 250 μg, and the peptides 16, 17 and 21 were fully effective at 500 μg doses.

Table II. Agonist Activities of Synthetic LRH Analogs.

	Analog	Activity In Vitro[a]	Activity In Vivo[b]
28.	LRH[<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2]	100	0/10 @ 1.0 mg
29.	[DLys^6]-LRH		5/10 @ 0.25
30.	[DLys(Chlorambucil)^6]-LRH	200[c]	0/10 @ 0.1
			5/10 @ 0.025
31.	[DLys(Stearoyl)^6]-LRH	0.5	
32.	[DLys(4-Phenylbutyryl)^6]-LRH	0.5	
33.	[Cys(Acm)^6]-LRH	0.6	
34.	([Cys^6]-LRH) Disulfide Dimer	10	

[a]See footnote b, Table I. [b]Post-coital contraceptive activity: No. of rats with resorbing fetuses/no. treated. The animals were injected (SC) daily, for 4 days, with the indicated dose of the analog. [c]Release of LH and FSH persisted for more than 3 hr, even though the medium was changed at hourly intervals.

Among LRH agonists prepared, [DLys(Chlorambucil)^6]-LRH (no. 30) was a long-acting (released gonadotrophins for more than 3 hours in the *in vitro* assay) and potent (2 x LRH) agonist. This derivative was also effective as a post-coital contraceptive at lower doses than either LRH or [DLys^6]-LRH.

Acknowledgement

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UNIQUE STRUCTURAL CHARACTERISTICS AND CERTAIN SIMILARITIES IN THE FUNCTIONAL AND BINDING PARTS OF THE HYPOTHALAMIC RELEASING FACTORS

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Introduction

Systematic studies on the structure-activity relationships of various agonistic as well as antagonistic superactive analogs of the hypothalamic peptide hormones were undertaken to obtain a clear insight into the unique structural features which contribute towards the biological expression of the molecule. The importance of these studies relates to the observation of nonspecific effects of these hypothalamic peptide hormones on different classes of anterior pituitary cells as also the specific releasing or inhibiting effects of small neurotransmitter-like molecules such as amino acids and amines directly on the pituitary.

Materials and Methods

Synthetic hormone samples — Synthetic gonadotropin-releasing hormone (Gn-RH, PyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), des-His^2-Gn-RH, the superactive analogs His^2-Me D-Ala^6 and D-Ser^6-des-Gly^{10}-Gn-RH-ethylamide (superactive analog I and II) and others were synthesized by adopting methods described earlier,[1,2] but using His-Me or D-Phe in place of His during the solid phase procedure.[3,4] After purification by countercurrent distribution, the biological potency was evaluated *in vivo* in rats, as reported previously.[3] Clinical evaluation of Gn-RH and superactive analog I, in terms of the extent and duration of human LH and FSH release, was performed essentially according to methods previously reported.[4] The endorgan response was also evaluated by measuring the gonadal steroids as described by Mathur et al.[5]

Hypophyseal stalk portal vessel infusion of the hormones and ultrastructural work on the anterior pituitary, preparation and purification of ^{125}I-Gn-RH and binding studies with pituitary slices or dispersed cell preparations were carried out as reported earlier.[3,6]

Results

Biological potency. — The superactive analogs I and II showed 25 to 30 times more gonadotropin-releasing activity than Gn-RH in rats. The His-Me analog of TRH showed 10 times more activity than TRH itself by the T_3-assay in mice. No side effects were noted following injections of the hormone or its superactive analogs.

Binding studies. — The relative competition of the two superactive analogs of Gn-RH for binding sites on the ^{125}I-Gn-RH-bound ligand was equivalent to that of Gn-RH itself at all optimum concentrations of binding (M X 10^{-10},Gn-RH=33.8, analog I=33.75, analog II=33.65%). Striking parallelism in the kinetics of binding data was indicated by Scatchard and reciprocal plots. Similar experiments with des-His^2-Gn-RH or des-His^2-D-Phe^2-Gn-RH showed that its affinity for the receptor site was the same as that of Gn-RH (33.7 to 33.8% B/F, at M X 10^{-10}).

Ultrastructural observations. — Gn-RH and the superactive analogs I and II caused an increase in granule release from gonadotrophs within one to two minutes postinfusion. However, at three hours, gonadotrophs of Gn-RH infused rats resembled saline infused controls. In contrast, gonadotrophs of superactive analog infused rats were still undergoing extensive granule release,[3,6] even three hours after infusion. In addition, the endoplasmic reticulum was dilated and frequently coalesced into large vacuoles indicating excessive hormone synthesis induced by the analog. Similar ultrastructural findings of increased metabolic activity at the Leydig cell level in rats two hours after administration of Gn-RH or the superactive analog (I) were obtained.[4]

Studies in humans (Males). — Administration of the superactive analog (10 μg) or Gn-RH (100 μg) resulted in a significant elevation of LH, a 10-fold increase for the analog and 3-4-fold rise for Gn-RH, as calculated by the peak areas (Figure 1). The FSH elevation was modest being 53% for Gn-RH and 63% for the analog. However, following the analog, the FSH levels remained elevated throughout the period of our study. Testosterone levels were observed to be significantly elevated at 60 minutes following the superactive analog (10 μg) and at 90 minutes with Gn-RH (100 μg). For the remainder of the study period, these elevated levels were found to persist.

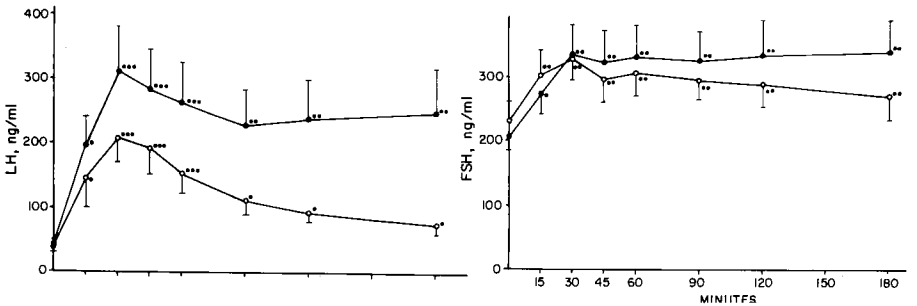

Fig. 1. LH and FSH levels (M ± S.E.) in males following i.v. administration of Gn-RH, 100 μg (open circles) and analog I, 10 μg (dark circles). p-values: .<.05;..<.01;...<.005; N=8.

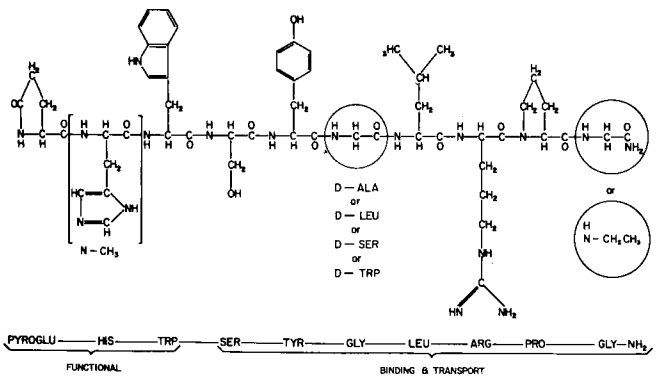

Fig. 2. Structural formula of Gn-RH, showing the functional and binding parts, and the modifications for the analogs.

Discussion

The studies on structure-activity relationships of the hypothalamic peptide hormones have revealed certain common functional features like PyroGlu, His, Pro, and C-terminal amide linkage which are essential for the biological activity of Gn-RH and TRH. Recent work on the characterization of growth hormone-releasing factor (GH-RF) indicates that PyroGlu and His are the first two amino acid moieties in this molecule.[7] The ability of TRH and Gn-RH to elevate the GH levels in

certain acromegalic patients could possibly be explained in the face of this common functional segment.

An examination of the essential structural requirements of Gn-RH based on our studies has shown that the first three amino acids are mainly responsible for the message, or functional part, and the remaining part of the molecule for binding, or address portion of the hormone (Figure 2). The prolonged activity of the two superactive analogs (I and II) of Gn-RH proves the efficacy of careful and well balanced structural modifications of the functional as well as binding parts of the molecule. By eliminating the essential message portion (His^2) or by exchanging it with a functionally negative moiety ($D\text{-}Phe^2$), it is still possible for the rest of the molecule to occupy the receptor sites on the gonadotrophs without communicating any message to the cell. Such structural changes, therefore, lead us to powerful antagonistic analogs. It may also be possible, by using excess amounts of the amino acid moieties which trigger the message to the cell, to bring about the biological expression of the hormone, in a nonspecific fashion, which could easily be distinguished since such small neurotransmitter-like amino acids or amines are required in larger quantities than a releasing or inhibiting factor to show activity. The sequential array of the neuroendocrine and endocrine events, mediated through neurotransmitter and hypothalamic neurons and the pituitary gland result in the release of appropriate hormones in the blood stream and such hormones in turn act on specific target organs, which react with specific metabolic responses. The sequence and precision of such endocrine events are governed by the unique structural features of the particular peptide hormone.

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BRAIN ENDORPHINS: DEVELOPMENTAL, ELECTROPHYSIOLOGICAL, AND BEHAVIORAL ACTIONS

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Introduction

Our recent research on the endorphin-containing neurons of the central nervous system have been directed at two major goals: 1) the possible precursor product relationships between the natural peptides and 2) the source of their behavioral and electrophysiologic actions. As is now well known, the endogenous opioid peptides share pharmacological properties and a common N-terminal sequence of the C-terminal fragment (61-91) of β-lipotropin (β-LPH).[1,2,3] Peptidases present in brain can cleave this fragment, β-endorphin, into the opioids γ-endorphin (61-77 β-LPH), α-endorphin (61-76 β-LPH) and 5-methionine -enkephalin (61-65 β-LPH).[4,5] Nevertheless, both specific radioimmunoassay and immunohistochemical studies (see Figure 1)[6-10] indicate that the endorphin- and enkephalin-containing systems exist in separate neuroanatomical networks in the brain, and that brain systems are separate from those of pituitary. Furthermore, distribution of β-endorphin immunoreactivity in cell bodies and nerve fibers is unchanged in rats after long term hypophysectomy even when the completeness of that procedure is verified by lack of a stress-induced secretion of corticotropin or β-endorphin and by lack of detectable adenohypophysial peptides in scrapings of sella turcica (unpublished studies).

Study of the developmental patterns of these differential distributions could provide better insight into relations between the adult endorphin systems. We have, therefore, investigated both the late prenatal and early postnatal development of the endorphin- and the enkephalin-containing systems in several regions of the rat brain.[11]

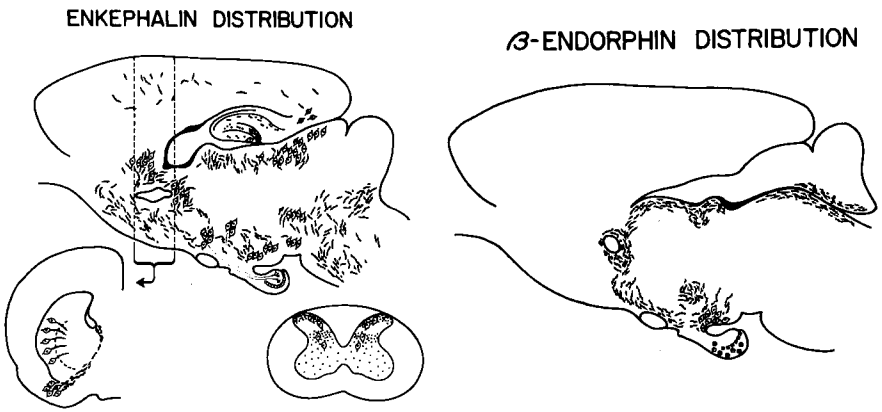

Fig. 1. Schematic representations of the immunohistochemical localization of enkephalin containing neurons and fibers (left) and endorphin immunoreactive cells and fibers (right). Note that all endorphin fibers in brain arise from the neurons in the ventral hypothalamus, while enkephalin fibers arise from many distributed neuronal sources. Possible enkephalin containing circuits are indicated striato-pallidal, amygdala-stria terminalis, hypothalamo-neurophypophysial, and intra-hippocampal connections. Summarized from published and unpublished data.

Developmental Studies

Birth-dated Sprague-Dawley male albino rats and timed-pregnant females (timing accuracy ± 12 hours) were obtained from Zivic-Miller, Pittsburgh, Pa. The postnatal animals were decapitated to remove the brain. In the prenatal experiments, embryos were individually dissected. After decapitation, heads were boiled 3 minutes in Ringer's solution to facilitate subsequent dissection. This procedure makes no difference in total endorphin or enkephalin content.[11] A standardized dissection procedure under microscopic control yielded essentially comparable and reproducible brain anatomical regions in animals from embryonic day 16 to postnatal day 25. Assays, and data expression were as previously reported.[6]

On embryonic day 16, high β-endorphin levels were already found in regions that contain endorphin in the adult: namely diencephalon, preoptic midline telencephalon, and midbrain.[6,17] In contrast, enkephalin distribution at prenatal day 16 does not correlate with that found in the adult. Levels of enkephalin at this prenatal period are much lower in all regions, except the medulla and midbrain. These results are summarized in Table I.

Table I. Regional distribution of endorphin immunoreactivity, enkephalin immunoreactivity, and protein in the 16th embryonic day rat brain.

Brain Region	Endorphin Units* / Tissue	Endorphin % of PN-25 value	Enkephalin Units / Tissue	Enkephalin % of PN-25 value	Protein mg / Tissue	Protein % of PN-25 value
Medulla & Midbrain (M)	0.315±.045	3.38	0.106±.025	1.92	1.70±0.02	6.72
Diencephalon (D)	0.231±.060	1.37	0.012±.001	0.22	1.15±0.04	5.78
Midline Telencephalon (T)	0.266±.052	2.32	0.006±.001	0.08	0.33±0.02	1.72
Striatum (St)	0.086±.007	4.72	0.008±.002	0.11	0.23±0.04	1.64
Ventral Hippocampus (Hc)	0.130±.040	8.66	0.003±.0003	0.30	0.25±0.05	1.97
Cortex & Amygdala (Ct)	0.140±.007	1.64	0.014±.001	0.27	1.81±0.19	4.12

The immunoreactivity levels are expressed in endorphin and enkephalin units as defined in 11, and as the precent of 25 day-old rat brain regions. (PN-25); tissue protein is expressed as mg/tissue piece and as percent of PN-25.

*mean of 3 independent determinations ± standard error of the mean. Each determination was performed at two sample dilutions in duplicate. Each sample was obtained by pooling a brain region from 5 animals; results are expressed per brain region for one animal.

The subsequent regional development of both the endorphin and the enkephalin systems appears to proceed along different time courses. Endorphin content does not increase as rapidly as protein and therefore drops when expressed relative to this standard, although absolute amounts increase progressively (see Figure 2). In contrast, enkephalin concentration increases by virtue of a more rapid absolute increase than the tissue protein and is, therefore, considerably higher than that of endorphin. During the postnatal period from days 6 to 25, both endorphin and enkephalin concentrations increase in all brain regions except the striatum, where apparent endorphin-like immunoreactivity drops until the low values of the adult brain are attained. Between the perinatal and the postnatal epochs the absolute rates of increase of endorphin immunoreactivity show a several-fold increase in the diencephalon and medial telencephalon (Figure 2). The absolute rate of increase of enkephalin immunoreactivity is also higher during the period of postnatal development than during earlier periods.

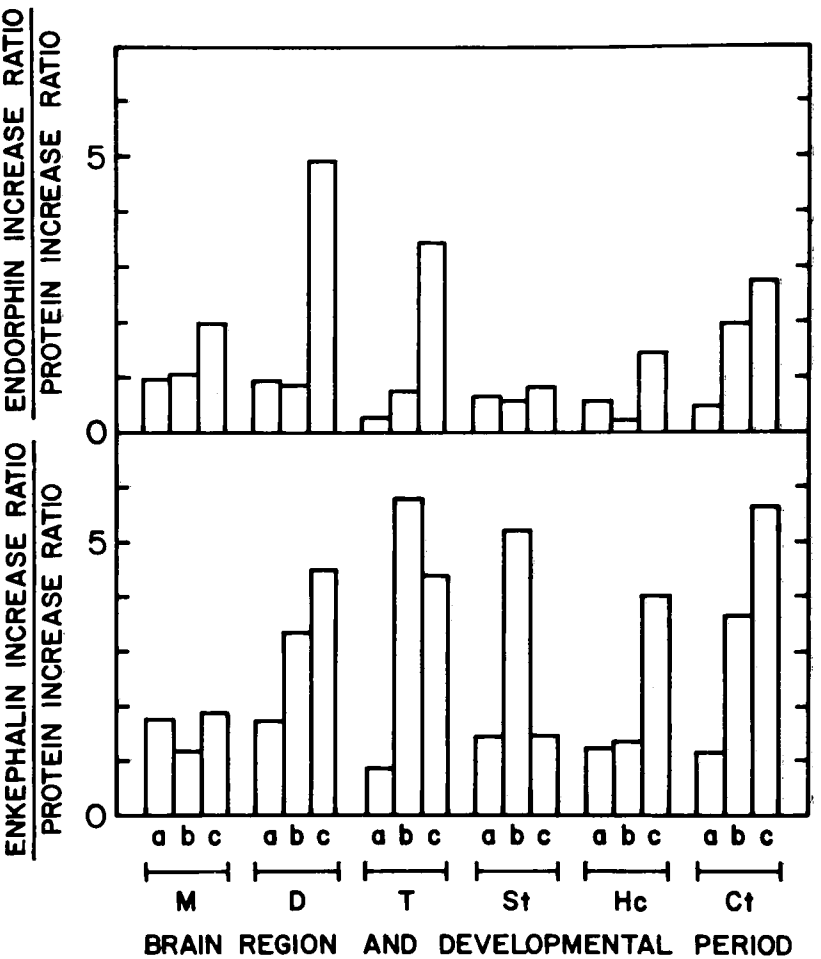

Fig. 2. Regional rates of increase of endorphin and enkephalin immunoreactivity during three early periods of development. The rates of increase (ordinates) were calculated as the total change in mean value of endorphin or enkephalin units during each developmental period, divided by the number of days in that period. The abscissa indicates the six brain regions studied (see Table I) and the rates corresponding to each of three arbitrarily defined developmental periods: *a, Prenatal,* from embryonic day 16 to 20; *b, perinatal,* from embryonic day 20 to postnatal day 6; *c, postnatal,* from postnatal day 6 to 25.

These results indicate that as early as embryonic day 16, the regional distribution of endorphin already reflects the adult pattern. Thus, this system would appear to be among the earliest systems known to exhibit chemical differentiation (also see[12,13]). In contrast, enkephalin immunoreactivity was not detectable in the embryonic day 13 rat brain

and its levels remain low until birth, even in areas which show a rich content in the adult. This suggests that, in general, enkephalin systems differentiate much later than the endorphin systems. Such a temporal pattern would be anticipated for small local interneurons, as most of the enkephalin-containing neurons would appear to be. These small or golgi-type 2 neurons frequently migrate and differentiate after the systems of larger neurons which give rise to forming neural long distances pathways, such as those containing β-endorphin. The relatively high levels of enkephalin in the prenatal medulla-midbrain region, probably reflects the fact that phylogenetically older structures frequently develop earlier than the cephalic end of the neural tube.[3]

When we analyzed our data for possible developmental relationships between the endorphin and the enkephalin containing systems, we could find none. Altogether our data are consistent with the view that the endorphin- and the enkephalin-containing systems are independent from each other, not only in the relatively stable adult brain but also very early during the dynamic developmental process. Nevertheless, the general qualitative observation that enkephalin systems develop after those containing endorphin, with the peculiarity that that endorphin regional concentrations drop as enkephalin ones tend to rise, indicates the possibility that enkephalin-producing peptidase systems could appear early during development differentiating certain apparent endorphin-like systems into enkephalin ones.

Behavioral Effects of Systemically Administered Endorphins

Recent studies by De Wied and associates have shown behavioral effects after subcutaenous administrtion of minute amounts of peptides derived from β-lipotropin (β-LPH).[14] Met^5-enkephalin, alpha-endorphin and beta-endorphin all produced an increased resistance to extinction in aversively motivated tasks.[14] These effects are similar to those previously reported by De Wied and associates after injection of equally minute amounts of ACTH 4-10.[15] However, the addition of one amino acid to the alpha endorphin molecule (resulting in gamma endorphin, β-LPH 61-77) produces the opposite results on extinction and enhances its occurrence.[16] More recently, De Wied and associates have shown similar effects with the synthetic peptide des-Tyr-gamma endorphin and have suggested that these compounds may be related to a peptide that acts as an endogenous neuroleptic.[17]

We sought to determine the extent to which these results could be generalized across different types of behavioral situations. For example, if these peptides were acting on a substrate peculiar to aversive situations then tests in an unconditioned aversive task should produce similar results. However, if the mechanism of action of these peptides is more generally related to motivational mechanisms then the action of the peptides on extinction of both aversive and appetitive tasks should be similar. To test this hypothesis, rats were tested in three behavioral situations: extinction of an active avoidance task, a dark/light emergence test, and extinction of a water-reinforced appetitive task. For the active avoidance task, a pole jump shock avoidance test was used and the procedures of De Wied and co-workers were carefully followed.[14] Following acquisition of the task, all animals were given ten trial extinction sessions and then injected with peptide. The injections were made at a dose of 10 μg/kg dissolved in saline and injected subcutaneously. For the dark/light test hooded rats weighing 250g were injected with either Met-enkephalin (120 μg/kg), alpha endorphin (40 μg/kg) or gamma endorphin (40 μg/kg) one hour before testing. The testing consisted of placing the rat in a closed dark compartment 28.0 cm x 12.8 cm x 20.5 cm and after 5 seconds the door was raised. Latency was recorded for the rat to emerge into a light compartment of identical size. For the appetitive task, rats weighing 250g were water deprived for 24 hours before training. Each animal was then tested daily for several days in a runway with one arm (61 cm in length) at a 60° angle from the first arm (60 cm in length). At the end of the 60° arm a metal drinking tube protruded 3.0 cm into the arm. The rats were allowed to drink for a total duration of 30 seconds upon reaching the goal box. For extinction testing the rats were allowed to drink a dry tube for at least 2 separate bouts and then were removed 30 seconds after the last bout. Immediately after the first extinction the rats were injected with 40 μg/kg of alpha or gamma endorphin and retested at 2, 4, and 6 hours later.

As has been previously reported by De Wied et al,[14] alpha and gamma endorphin produced opposite effects on the extinction of the pole jump task (Figure 3). After 3 days of training, alpha endorphin significantly increased responding to the conditioning stimulus at 2 hours post-injection. In contrast, after 4 days of training gamma endorphin significantly decreased responding at 4 and 6 hours post-injection.

In the dark/light test, Met-enkephalin and alpha endorphin significantly delayed the emergence from the dark compartment to the light compartment one hour after the second (6 p.m.) injection. However,

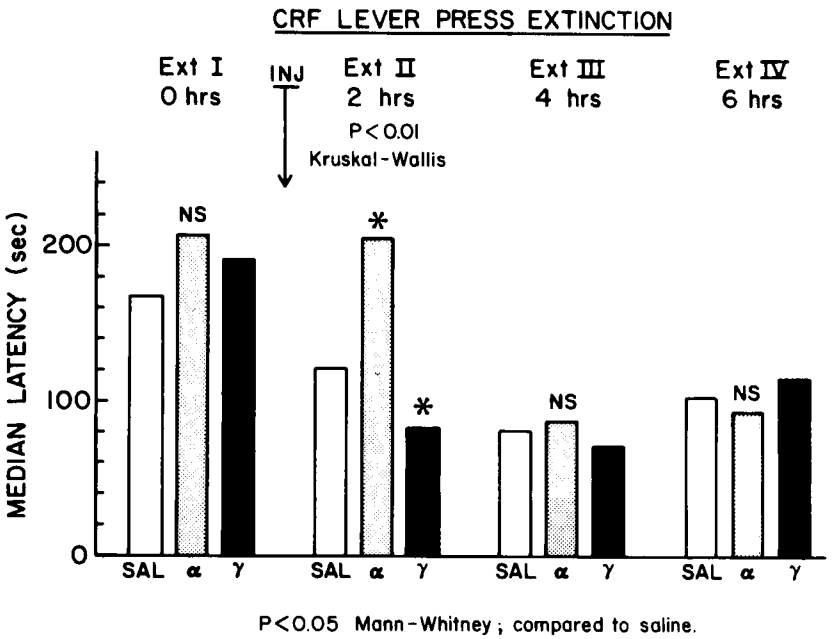

Fig. 3. Effects of subcutaneous injections of alpha or gamma-endorphin on extinction of lever press response for food pellet reward. Doses were 10 μg/rat injected subcutaneously in rats. Note that in contrast to effects of these same peptides on extinction of water-motivated drinking behaviors, in this appetitive task, as in the already reported active and passive avoidance tasks, alpha and gamma endorphin produce opposing effects.

at this time gamma endorphin significantly decreased the latency to emerge. These changes were evident in several repeated measures but the results of this test still vary considerably among rat strains (work in progress).

In the extinction of the water-reinforced runway task, rats given only saline injections showed a time dependent increase in the latency to approach the dry tube over the 3 extinction sessions. In contrast the rats receiving either alpha or gamma endorphin continued to run to the dry tube as quickly as during acquisition. In this test unlike the more aversive tasks, both peptides produced identical actions increasing resistance to extinction for at least 4 hours.

In the present study, as in those of De Wied et al,[14,16] alpha endorphin delayed extinction of an active avoidance task whereas gamma endorphin facilitated this extinction. However, in an appetitively motivated task for drinking, both alpha and gamma endorphin delayed extinction. These results and the results showing differential effects of

alpha and gamma endorphin on other "fear" motivated tasks, e.g. the light/dark test, might suggest that these peptides are acting on a substrate more related to aversive situations than to general motivational or learning mechanisms. How such a mechanism can differentially affect extinction in aversively-motivated versus appetitively-motivated tasks and where such a substrate is located remains to be determined.

In initial attempts to define this locus, we injected behaviorally effective doses of α-endorphin subcutaneously and then assayed both blood and brain for the presence of this peptide which is usually undetectable in either sample. We found some immunoreactive α-endorphin had reached the circulation in the first 20 minutes but was never detectable in brain (Figure 4). Given the amounts present in brain (see reference 6) and the amounts required to generate overt electrophysiologic effects after intraventricular injection (see below and 18), these brief and extremely low levels in blood are difficult to equate with the behavioral changes observed. Nevertheless, the present results confirm that systemic injection of small amounts of endorphin peptides can alter ongoing behavior. These behavioral effects require extremely small doses and they appear to last several hours after injection. The presence of systems responsive to peptide-like substances opens a new dimension in the biochemical analysis of behavior.

Pharmacological Characterization of Endorphin Receptors *In Vivo*

Previously[18] we have reported that nanomol doses of β-endorphin injected intracerebroventricularly will induce naloxone-reversible subcortical epileptiform activity in limbic regions without signs of behavioral convulsions and at doses below the threshold for analgesia.[18] More recently, we have evaluated several synthetic analogues of the endorphins and enkephalins for effectiveness in producing this EEG response, and have employed the pharmacological classification scheme of Martin[19] to characterize the opiate nature of this endorphin-elicited response. Martin has described three populations of opiate receptors in the spinalized dog preparation which he terms u, k and σ; for each receptor class there are specific key agonists, but opiate drugs which are agonists on one of these three receptors can also act as antagonists at the other receptors (see 19). In our studies on the EEG responses of the rat, we have employed several indices of response, including the sensory responses of the animal. The results of these studies (Tables II and III)

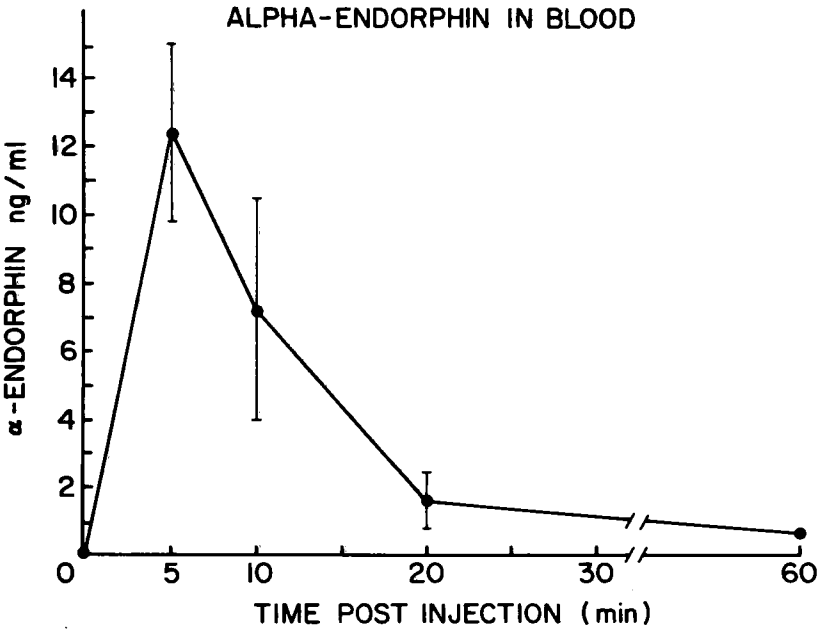

Fig. 4. Changes in alpha-endorphin-like immunoreactivity in the blood of rats sacrificed at various times after injection of 10 μg alpha endorphin subcutaneously as in behavioral experiments described in text. Note that levels are initially below detection, and that levels become detectable only during the 20 minutes following injection and are again negligible at 1 hour, even though behavioral effects last for several hours depending upon the task under evaluation (unpublished data).

indicate that the response to β-endorphin which elicits limbic epileptiform activity best fits the characteristics of the Martin u receptor. Pretreatment of rats with cyclazocine or with SKF 10,047 attenuates these effects of β-endorphin, as well as diminishing the EEG effects which are produced by morphine (but these effects of morphine on rat EEG are only seen with doses above the threshold for analgesia). When tested at

Table II. I.C.V. Administration of Opiate Agonist/Antagonists

SUBSTANCE	DOSE (I.C.V.)	BEHAVIOR	EEG	ANALGESIA
WIN-35, 197-2	100nM	SEDATED	↑ SYNCH	0→+
CYCLAZOCINE	150nM	SEDATED	NORMAL	0→+
SKF - 10047	100nM	HALLUCIN	NORMAL	0
MORPHINE SULFATE	100nM	SEDATED	SYNCH	+ +
β-ENDORPHIN	1-5nM	↑ THEN ↓	EPILEPT./SYNCH	0←+

Table III. Antagonism of Morphine and β-Endorphin Effects

SUBSTANCE	DOSE	EEG	ANALGESIA
MORPHINE	37μM/KG I.P.	CTX SYNCH	+ +
+ CYCLAZOCINE	37μM/KG I.P.	NORMALIZE	+
+ SKF 10047	37μM/KG I.P.	NORMALIZE	0
+ WIN-35, 197-2	37μM/KG I.F.	↑ SYNCH	↓
MORPHINE	100nM I.C.V.	CTX SYNCH	+ +
+ SKF 10047	37μM/KG I.P.	SYNCH	0
β-ENDORPHIN	3-5nM I.C.V.	EPILEPT.	0→+
+ CYCLAZOCINE	37μM/KG I.P.	NORMALIZE	+
+ SKF 10047	37μM/KG I.P.	NORMALIZE	0
+ WIN 35, 197-2	37μM/KG I.P.	EPILEPT.	+
+ NALOXONE	2-10MG/KG I.P.	NORMALIZE	0

doses two orders of magnitude above the EEG threshold for β-endorphin, neither k or σ agonists were able to elicit endorphin-like EEG changes. Thus it would appear that β-endorphin has greater activity at those u receptors leading to the limbic EEG response than does morphine.

Acknowledgements

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β-ENDORPHIN: ASPECTS OF STRUCTURE-ACTIVITY RELATIONSHIPS BY SYNTHETIC APPROACH

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Among various fragments of β-LPH[1] having opiate activity, only β-endorphin (β-EP) exhibits potent analgesia activity by intravenous administration.[2] It is also the most active peptide when injected directly into the brain.[3] In man, β-endorphin is a potent analgesic by intraventricular administration.[4]

In addition to its analgesia activity, β-endorphin causes catatonia,[5,6,7] hypothermia,[7] salivation,[8] and wet-dog shakes[8] in the rat. When injected centrally in the cat, β-EP induces visual "hallucinations".[9] Intravenous injections of low doses of β-EP in the cat produced high frequency licking and diminished locomotion;[10] however, at higher doses the responses included vomiting and relaxation of the nictitating membrane.[10] In man, β-EP suppresses acute withdrawal in drug addicts when injected intravenously.[11,12]

It has also been shown that β-EP stimulates prolactin release in human subjects[13] and inhibits urine flow in rats[14] by intravenous administration.

Figure 1 presents the amino acid sequence of various β-EP.[15] The only variations occur in residue positions 23, 27 and 31. These differences do not alter the biological activity.

```
                                    5                   10
Human:  H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-

                                   15                  20
        Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-

                                   25                      31
        Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH

Porcine:          Val             His                 Gln-OH

Camel,ovine,bovine:  Ile          His                 Gln-OH
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Fig. 1. Amino acid sequence of β-endorphin from pituitary glands of various species.

Both camel[16] and human[17,18] β-endorphins have been synthesized[19,20] by the improved procedures of the solid-phase method. Solution synthesis of human β-EP has also been reported.[21,22] For the last three years, over fifty analogs of camel and human β-EP have been synthesized from our laboratory. Structure-activity relationships of some of these analogs have recently been reviewed.[15,23,24,25]

Analogs were synthesized by the solid phase procedure as described previously[20] for β_h-EP. For preparative scale, the following changes were made: (a) diisopropylethylamine was used for the neutralization step, (b) trifluorethanol was omitted for the coupling step, (c) Tyr-1 was protected by the benzyloxycarbonyl group, (d) 2-mercaptopyridine plus anisole were used as scavengers in the HF step, (e) 20% acetic acid was used for Sephadex G-10 column, and (f) isocratic elution with 0.1 M ammonium acetate was employed in the CM-cellulose chromatography. These changes did not change the yield of the synthetic product. In fact, the yield may occasionally be increased to nearly 45% based on starting resin. The synthetic products behaved as homogenous peptides in partition chromatography, paper electrophoresis, thin-layer chromatography, disc electrophoresis, amino acid composition and tryptic map.

Methods to estimate the biological potency of synthetic analogs — Narcotic agonist activities were measured from the depression of electrically-stimulated contractions of the guinea pig ileum prepared as previously described.[26] Opioid activity was also assayed by the inhibition of tritiated β_h-EP binding to rat membrane preparations prepared essentially as described.[27] The radioligand-binding assay was only recently developed using [^{3}H-Tyr^{27}]-β_h-EP (50 Ci/mmol)[28] as the primary ligand.[29] The binding is time dependent and saturable with $K_o = 0.29 \times 10^{-9}$M and a maximal binding capacity of 0.15 pmole per mg of membrane protein. The assay detects β-EP concentration as low as 10^{-10}M.

The analgesic activity was assessed by the tail-flick method[30] in mice. Peptides were injected intracerebroventricularly in a volume of 5 μl per mouse. Immunoreactivity was estimated by a sensitive radioimmunoassay[31] using a specific antiserum to β_h-EP.[32]

Analogs with shortened peptide chains — In order to explore the contribution of the "nonenkephalin" segment of β-EP (residues 6-31) to its biological profile, β_c-EP-(1-5)-(28-31), β_c-EP-(6-31) and β_h-EP-(1-5)-(16-31) were synthesized and their biologic properties were investigated.[33] As summarized in Table I, only β_c-EP-(1-5)-(16-31) was found to exhibit

some analgesic activity (0.3%) and the analgesia was only partially, not completely blocked by naloxone. In addition, mice became extremely hyperactive to sound and touch, attempting to jump out of their cages and escape. The analgesic response and hyperactivity lasted 10 to 20 minutes. However, the hyperactive response was not antagonized by pretreatment with naloxone (3 ml/kg), indicating that the response is not mediated via opiate-like mechanisms.

Table I. Morphine-like Activity of Synthetic Analogs with Shortened Peptide Chains[a]

		In vivo analgesic activity	
Synthetic peptide	In vitro opiate activity	AD_{50}[b]	Relative Potency[c]
β_c-EP	100	0.11(0.07-0.17)	100
β_c-EP-(6-31)	4	>86	<0.1
β_h-EP-(1-5)-(16-31)	135	25.1(16.8-37.1)	0.3
β_c-EP-(1-5)-(28-31)	35	>86	<0.1

[a] Taken from reference 23.
[b] AD_{50} in μg/mouse (95% confidence limit); 7-10 mice for each dose tested.
[c] Molar basis.

β_c-EP-(6-31) of doses of 42.5 and 85 μg produced analgesia in one out of five and four out of eleven mice, respectively. It is important to note that β_c-EP-(6-31) does not contain the Met-enkephalin segment and yet it appears to possess measurable analgesic activity.

In radioligand-binding assay, β_c-EP-(6-31) exhibits significant activity inhibiting tritiated β_h-EP binding to rat brain membranes (see figure 2).[29] Together with above observations, it may be postulated that β-EP has two types of receptors in the rat brain: one is a naloxone-reversible binding site (enkephalin receptor) and the other is a naloxone-irreversible binding site ("nonenkephalin" segment of β-EP receptor).

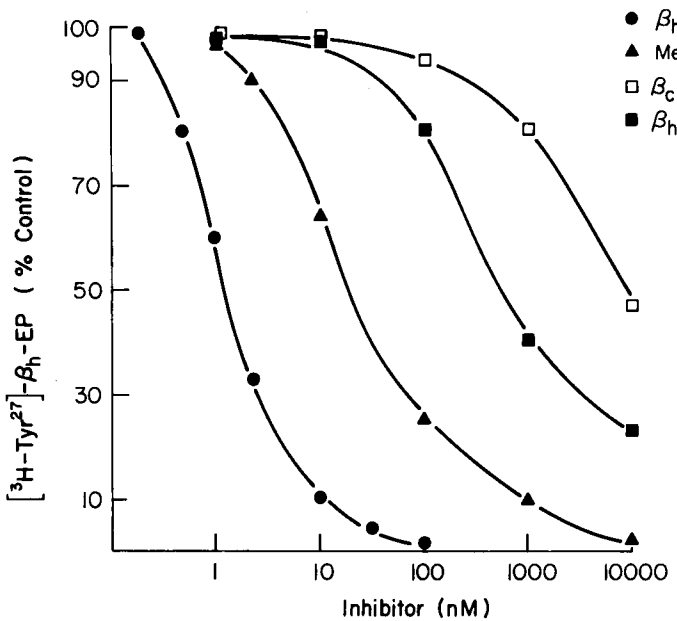

Fig. 2. Competition of the binding of [^{3}H-Tyr^{27}]-β_h-EP to rat membrane preparation by β_h-EP (•—•), Met-enkephalin (▲—▲), β_c-EP-(6-31) (□—□) and β_h-LPH (■—■).

Analogs with various chain lengths — Previous studies have shown that β-EP-(1-5) (Met-enkephalin), β-EP-(1-9), β-(1-16), β-(1-17) and β-EP-(1-19) are all less active than β-EP in the guinea pig ileum assay.[34,35,36] In opiate receptor assay, β-EP-(1-5), -(1-8), -(1-9), and -(1-27) exhibit less activity than β-EP, while β-EP-(1-29) appears to approach but not match the activity of the 31-residue peptide.[37,35] In order to explore further the influence of chain length on the biological activity of β-EP, the synthesis of β-EP-(1-15), -(1-21), -(1-26), -(1-28), -(1-29) and -(1-30) was accomplished and their biological activities were examined.[38,39,40]

Table II summarizes various biological activities of synthetic β_h-EP analogs with different chain lengths. In the guinea pig ileum assay, the extension of β-EP-(1-5) at its COOH-terminus to give β-EP-(1-15) leads to loss in potency. Further extension to give β-EP-(1-21) brings back activity exceeding that of the NH_2-terminal pentapeptide. No significant increment of activity is observed by further addition of five more residues to form β-EP-(1-26). The most dramatic gain in activity occurs as the chain is lengthened by addition of the next three residues. Thus, β_h-EP-(1-29) displays the full activity of β_h-EP in the guinea pig ileum assay.

Table II. Relative Potency of Synthetic Analogs with Various Chain Lengths

Synthetic Peptides	Guinea Pig Ileum Assay	Analgesic Activity in Mice	Immunoreactivity by RIA
β-Endorphin (β_h-EP)	100	100	100
β_h-EP-(1-30)	-	72	100
β_h-EP-(1-29)	104	20	95
β_h-EP-(1-28)	89	6	90
β_h-EP-(1-26)	48	8	65
β_h-EP-(1-21)	46	0.3	< 2
β_h-EP-(1-15)	17	< 0.1	< 1
β_h-EP-(1-5)	25	nil	0

The immunoreactivities of analogs of various chain lengths as measured by the β_h-EP radioimmunoassay system[40] are shown in Figure 3 and Table II. It is obvious that β_h-EP-(1-15) and -(1-21) are practically inactive. On the other hand, β_h-EP-(1-28 and -(1-29) are equally potent as the intact molecule on the weight basis, while β_h-EP-(1-26) is partially active.

The analgesic activity of synthetic peptides was assessed in mice by the tail-flick method[30] after being injected directly into the brain. As shown in Table II, the removal of one amino acid at a time starting with the COOH-terminus of β_h-EP reduced the potency of analgesic activity of β_h-EP in a stepwise fashion. Thus, removal of COOH-terminal Glu reduced analgesic activity by 28%, removal of -Gly-Glu reduced by 80% and removal of -Lys-Gly-Glu by 94%. These data indicate that all 31 amino acid residues are required for full analgesic activity.

It has been shown[41] that β-endorphin in water shows little, if any, secondary structure and methanol promotes the formation of helical structure to an extent as much as one half of the peptide molecule. It is therefore of interest to investigate the relationship of chain lengths to the

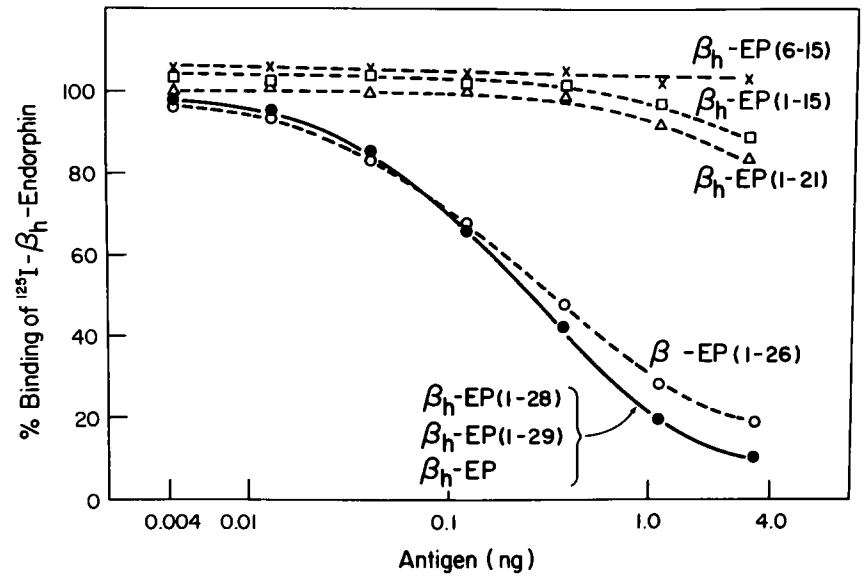

Fig. 3. Radioimmunoassay of β_h-EP and analogs with various chain lengths.

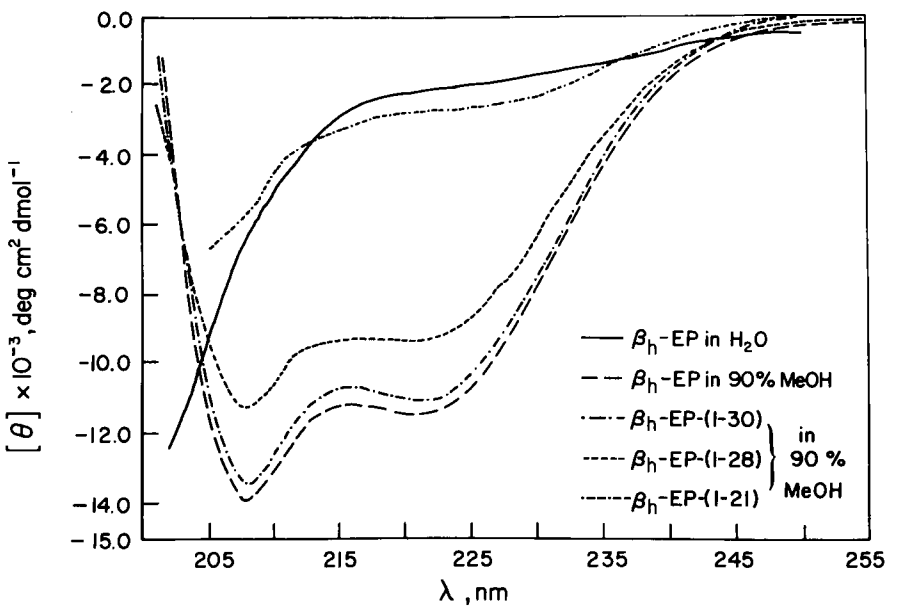

Fig. 4. CD spectra of β_h-EP with different chain lengths in 90% methanol at far UV region.

formation of α-helix in β_h-EP by circular dichroism (CD) spectra in 90% methanol. It may be seen in Figure 4 that β_h-EP-(1-30) achieves nearly full helical structure when compared with β_h-EP. It is reasonable to conclude that structural conformation is essential for the expression of full activity in β_h-EP.

Analogs with deletion of a single amino acid — It has recently been reported[42] that omission of Leu-14 or Asn-20 in the β_c-EP structure abolishes immunoactivity as evidence by radioimmunoassay but retains significant biological activities. It is now found that omission of Gly-2 abolishes biological activities but retains considerable immunoreactivity (see Table III). As shown in Table III, omission of Thr-6 or Ser-10 does not change significantly biological activities as well as immunoreactivity.

Table III. Biological Activity of Synthetic β_c-Endorphin Analogs (Single Amino Acid Deletion)

Synthetic peptides	Analgesic potency (ICV in mice)	Opiate activity (G.pig ileum)	Immuno-reactivity (RIA)
β_c-Endorphin	100	100	100
[Des-Gly^2]-β_c-EP	nil	1	82
[Des-Met^5]-β_c-EP	20	7	82
[Des-Thr^6]-β_c-EP	73	97	106
[Des-Ser^{10}]-β_c-EP	92	117	73
[Des-Gln^{11}]-β_c-EP	79	100	9
[Des-Thr^{12}]-β_c-EP	96	117	3
[Des-Pro^{13}]-β_c-EP	23	110	7
[Des-Leu^{14}]-β_c-EP	75	140	0
[Des-Val^{15}]-β_c-EP	24	123	0
[Des-Asn^{20}]-β_c-EP	46	110	0
[Des-Ile^{22}]-β_c-EP	57	197	29

As in the case of des-Leu[14] and des-Asn,[20] omission of Gln-11, Thr-12, Pro-13 or Val-15 destroys immunoreactivity but exhibits potent analgesic and opiate activities (see Table III). It is evident that there is lack of correlation between *in vivo, in vitro* and immunological activities.

Analogs modified at the carboxyl terminus with increased activities — The synthesis of [Gly[31]]-β_h-endorphin, [Gly[31]]-β_h-endorphinamide, and [Gly[31]]-β_h-endorphinylglycine was accomplished by the solid-phase method by use of procedures used for the synthesis of β_h-endorphin.[20] Purification of the analogs was effected by chromatography on carboxymethylcellulose and by partition chromatography on Sephadex G-50. The highly purified peptides were characterized by thin-layer chromatography, paper electrophoresis, and amino acid analysis.

The biological activities of the analogs were measured by *in vitro* and *in vivo* procedures as summarized in Table IV. The single replacement of Glu-31 in β_h-EP by Gly appears to substantially raise the *in vitro* activity (Table IV) but not significantly alter the analgesic potency.

Table IV. Biological Activities of Synthetic β_h-EP Analogs Substituted at Position 31 and Extended at the COOH-Terminal

Synthetic Peptides	Analgesic potency (ICV in mice)	Opiate Activity (G.pig ileum)	Radioligand-binding assay (rat brain membranes)
β_h-Endorphin (β_h-EP)	100	100	100
[Gly[31]]-β_h-EP	83	168	127
[Gly[31]]-β_h-EP-NH_2	225	200	153
[Gly[31]]-β_h-EP-Gly-OH	217	132	250

The opiate activities of the synthetic analogs as measured by the rat brain radioligand-binding assay are summarized in Figure 5 and Table IV. All the analogs exhibit greater potency than β_h-EP in the receptor and

guinea pig ileum assays. Interestingly, the analog with a COOH-terminal amide appears to be the most active. In the radioligand-binding assay (Table IV), [Gly^{31}]-β_h-endorphinamide is almost three times more potent in comparison with the parent peptide.

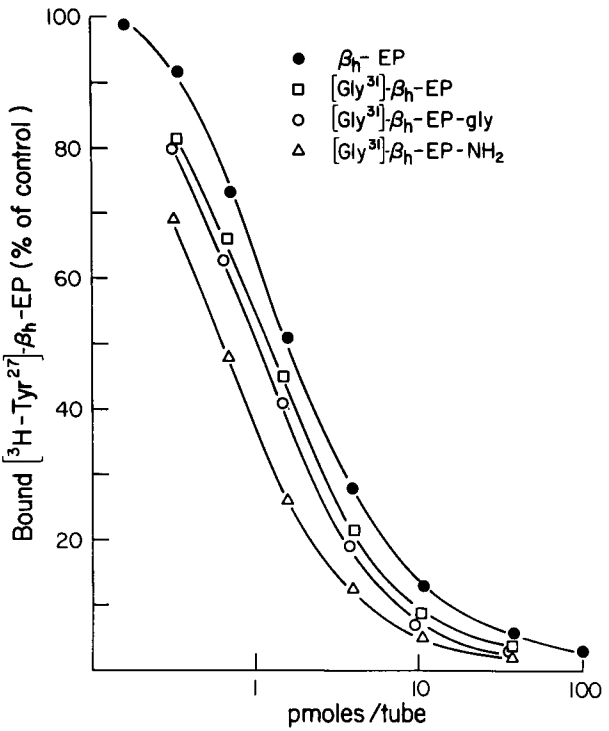

Fig. 5. The radioligand-binding assay of β_h-EP and analogs.

The analgesic potency of β_h-EP appears to be practically unchanged by the replacement of Glu-31 by Gly, indicating that the side-chain of Glu is not necessary for this activity. Conversion of the COOH-terminal carboxyl group of [Gly^{31}]-β_h-EP to an amide or extension by an additional Gly residue results in increases in analgesic potency. In view of the fact that the entire chain length of β_h-EP is required for full analgesic activity (see above), it is evident that even limited enzymatic attack at the COOH-terminus could rapidly destroy its activity. Thus, modification of position 31 and extension at the COOH-terminus may be one approach toward analogs with greater biological activity than β-endorphin.

Summary

Stereospecific radioligand-binding assay for opioid peptides using tritiated β_h-EP as the primary ligand is described. Complete primary structure is required for full analgesic activity. Omission of Met-enkephalin or a central segment does not abolish biological activities. Deletion of a single amino acid residue abolishes immunoreactivity but retains opiate potency. Modifications at position 31 and extension at the COOH-terminus enhances analgesic activity.

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ACTIVATION OF ENDORPHINS: SELECTIVE MECHANISMS FOR GENERATING SPECIFICITY WITH POTENCY

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One of the most intriguing aspects of the opiate field is the number and variety of naturally occurring peptides that appear to have opiate like properties. After the isolation and identification of the pentapeptides, methionine and leucine enkephalins, from brain[1] attention focussed on a 31-residue peptide and another of 27-residues, the C-Fragment and the C'-Fragment of lipotropin, which were known to possess the same N-terminal sequence as the enkephalins and occur in large quantity in pituitary;[2,3] and subsequently a number of other peptides with sequences corresponding to fractions of the C-terminal region of lipotropin were also shown to be present in pituitary and brain and possess a degree of opiate activity.[4–6] With the exception of leucine enkephalin, whose sequence does not occur in lipotropin, this series of peptides have appeared to share a common origin and they are frequently described as a 'family' of endogenous opiates (Figure 1). Recent evidence, however, suggests that the enkephalins may originate from precursors different from lipotropin: the five residues of leucine enkephalin have been found to constitute the N-terminal section of a 15 residue opiate peptide, α-neo-endorphin,[7] which is not related to lipotropin and it seems likely that methionine enkephalin may also originate from an independent biologically active precursor.[8,9]

Of the series of opiate peptides shown in Figure 1, only the C- and C'-Fragments are generated from lipotropin by cleavage at sequences of paired basic residues, with a specificity similar to that involved in the activation of intracellular prohormones. Thus the C- and C'-Fragments bear a special relationship to each other and to lipotropin and seem likely to have physiological importance.

The first steps towards elucidating the functions of the opiate peptides must involve identification of the potent 'endorphins' and study of their distribution in the various regions of the CNS where opiate receptors occur. Our present studies, conducted in the pituitary and brain of the rat, have concentrated on the peptides that are derived from

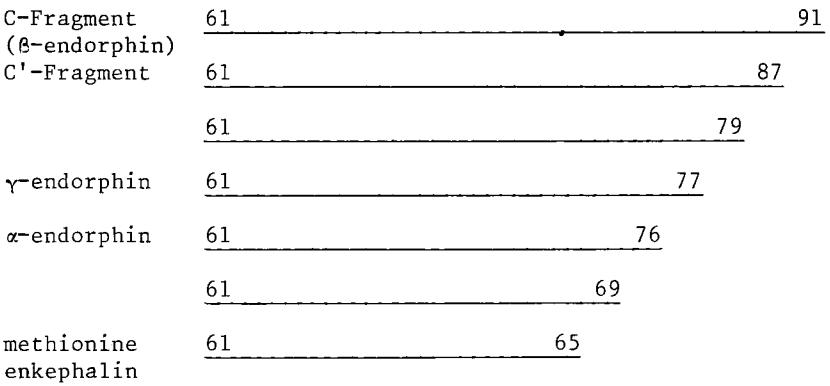

Fig. 1. Naturally occurring fragments of lipotropin with opiate activity

The numbers indicate residue positions in the sequence of lipotropin (itself a fragment of the 31K ACTH-endorphin prohormone). Current evidence indicates that methionine enkephalin, though having the same sequence as residues 61-65 of lipotropin, may originate biosynthetically from a different precursor.

lipotropin by cleavage at paired basic residues, the C- and C′-Fragments and their α, N-acetylated derivatives.[10] The results reveal that while the distribution of these lipotropin fragments differs in different tissues, it does so in a highly organized manner, which would indicate that the processing mechanisms may be under physiological control.

The distribution of the four peptides, together with their precursors (lipotropin and the 31K prohormone), was studied by the method of immunofluorescent staining using an antibody raised against the C-Fragment.[11] In agreement with other studies,[12] only isolated cells in rat anterior pituitary showed peptide staining whereas in the pars intermedia there was intense staining in all the cells: there was no reaction in the posterior pituitary. In rat brain, the regions shown by fluorescence to contain peptides with lipotropin C-terminal immunoreactivity were the hypothalamus, mid-brain, hippocampus, amygdala, and the periaqueductal gray. The majority of the fluorescence was in the hypothalamus. In order to identify the material visualized by this technique, the peptides were extracted from the tissues and resolved by gel filtration and ion exchange chromatography before estimation by immunoassay. This extensive purification was necessary since the antibody employed in the fluorescence study showed reactivity against a variety of peptides with overlapping sequences, including the C′-Fragment, the α, N-acetyl derivatives of the C- and C′-Fragments, the 31K ACTH-endorphin precursor and lipotropin, in addition to the C-

Fragment itself; and of these peptides only the C-Fragment (β-endorphin) shows potent analgesic activity.[13]

It was found by gel filtration that the pars intermedia contains a negligible amount of the 31K precursor or lipotropin, but it contains a large quantity of peptides the size of C-Fragment. Ion exchange chromatography of this fraction (Figure 2) revealed, suprisingly, that the

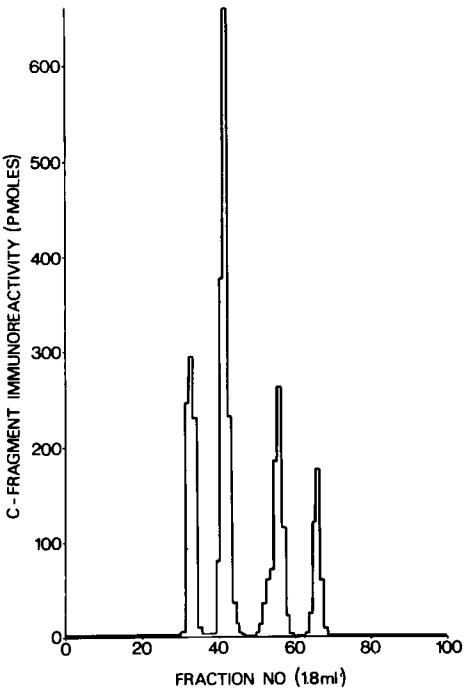

Fig. 2. Resolution of peptides with the approximate molecular size of lipotropin C-Fragment (31 residues) present in extracts of rat pars intermedia. The material obtained from 60 rats was fractionated by gel filtration, then chromatography (shown) was performed on a column (60 x 0.6cm) in 50% acetic acid using a gradient (100ml mixer volume) from 0 to 0.6M NaCl. Fractions (1.8ml) were collected and portions (1μl) were immunoassayed using an antiserum raised against the C-Fragment. The four peaks of immunoreactivity were in the positions of the porcine marker peptides, acetyl C'-Fragment, C'-Fragment, acetyl C-Fragment and C-Fragment, respectively. The antiserum was four times less reactive with the C'-Fragment than with the C-Fragment; the acetyl peptides were immunologically identical to the respective NH_2-peptides. The values have not been corrected for immunoreactivity.

C-Fragment accounted for less than 4% of the peptides with lipotropin C-terminal immunoreactivity. In the anterior pituitary, on the other hand, there was a substantial quantity of both the 31K prohormone and lipotropin in addition to a group of peptides the size of C-Fragment, and the C'-Fragment with its acetyl derivative were again present in

the largest quantity, the C-Fragment representing only 10% of the immunoactive peptides. Thus in the two regions of rat pituitary, the potent opiate peptide lipotropin C-Fragment appears to represent no more than a minor component compared with the other fragments of lipotropin. This indicates that in the rat the circulating endorphins derived from pituitary are essentially inert.

In the brain, as in the pars intermedia, there was negligible lipotropin or 31K prohormone. However, in the hypothalamus, in striking contrast to the pituitary, the principal immunoreactive peptide was found to be the C-Fragment (Figure 3). The processing of lipotropin in the hippocampus gave rise to the C'-Fragment as the principal 'opiate peptide' in that region (Figure 4) whereas in the mid-brain the four peptides were observed in more equal amounts (Figure 5). Thus, it is only in the hypothalamus that efficient processing of lipotropin takes place to form lipotropin C-Fragment.

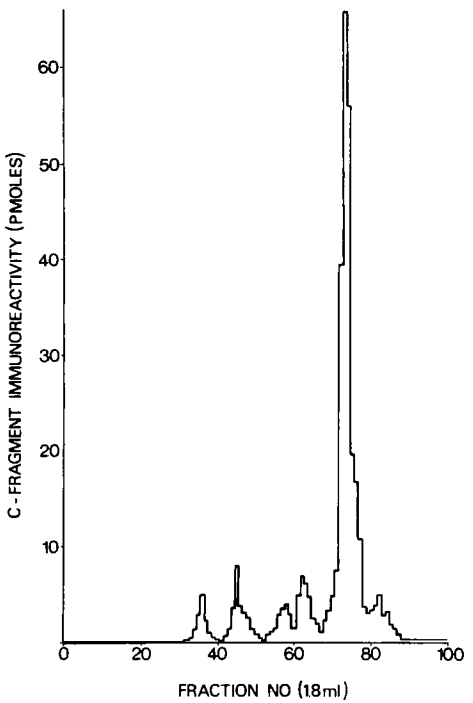

Fig. 3. Resolution of peptides the size of lipotropin C-Fragment present in extracts of rat hypothalamus. The conditions were as in Figure 2; 30μl portions were immunoassayed.

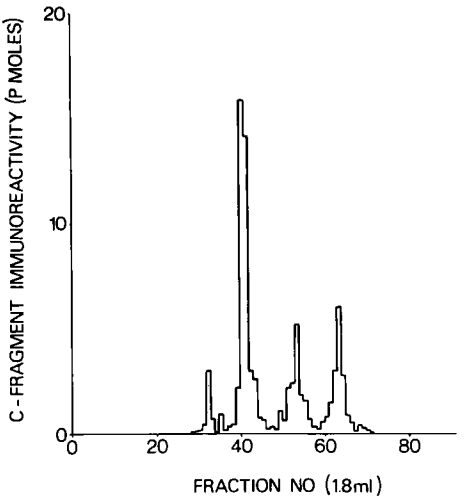

Fig. 4. Resolution of peptides the size of lipotropin C-Fragment present in extracts of rat hippocampus. The conditions were as in Figure 2; 100μl portions were immunoassayed.

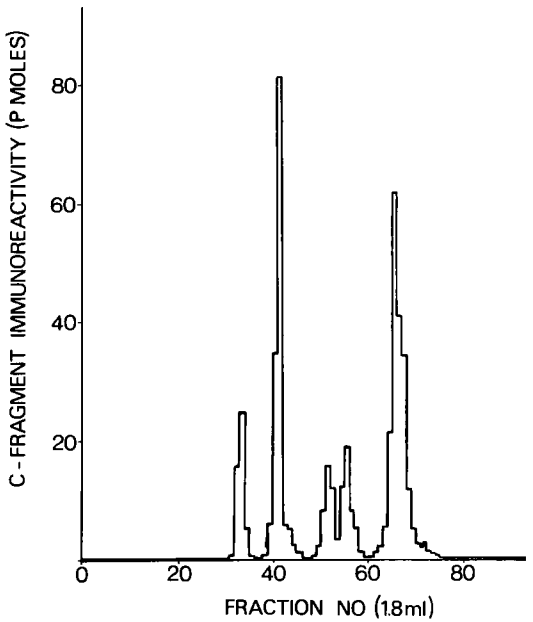

Fig. 5. Resolution of peptides the size of lipotropin C-Fragment present in the mid-brain region of the rat. The conditions were as in Figure 4.

The production and storage of inactive fragments, namely C'-Fragment and the N-acetyl derivatives of the C- and C'-Fragments which are essentially inert as opiates,[10] seems to indicate that certain peptides are formed during physiological mechanisms intended for negating the activity of the C-Fragment. The evidence suggests that in the pituitary the processing of the ACTH-endorphin prohormone is organized for the production of corticotropin and the potential for opiate activity which is associated with the C-terminal region of the prohormone is not realized, either as a consequence of N-acetylation or of proteolysis at the C-terminus. It appears that activation of the ACTH-endorphin prohormone to generate the C-Fragment is initiated by cleavage of the Arg-Tyr bond at positions 60 to 61 of lipotropin but further cleavage at the Lys-Gly bond at positions 89 to 90 leads to the formation of the relatively inert C'-Fragment, which may be regarded as a 'switch off' mechanism. The C- and C'-Fragments thus represent active and inactivated peptides, respectively.

Related pairs of peptides can also be discerned in α-neo-endorphin and its N-terminal fragment (leucine enkephalin) and in 'β-neo-endorphin' (the putative precursor of methionine enkephalin) and methionine enkephalin (Table I). It is notable that all these peptides appear to be generated by enzymic mechanisms involving cleavage at paired basic residues.

Table I. Proposed Biosynthetic Origins of Opiate Peptides. [a]The precursor of methionine enkephalin (β-neo-endorphin) has not so far been identified.

PRECURSOR	ACTH-lipotropin prohormone	pro-α-neo-endorphin	pro-β-neo-endorphin
	↓	↓	↓
ENDORPHIN	C-Fragment	α-neo-endorphin	β-neo-endorphin
	↓	↓	↓
ENDORPHIN FRAGMENT	C'-Fragment	leucine enkephalin	methionine enkephalin [a]

Each of the opiate peptides derived from lipotropin exhibits a similar potency in the guinea pig ileum assay[14] and on this basis each one could fulfil a role as a physiological opiate with a central or peripheral function. In certain assays, however, the C-Fragment of lipotropin exhibits a unique potency. As an analgesic agent, for example, it is at least 500 times more active than the C'-Fragment[13] and it is much more potent

in displacing ^{3}H naloxone from brain opiate receptors *in vitro*.[15] This would seem to imply that it is the C-Fragment and not the C′-Fragment which is functionally significant with respect to the opiate receptors involved in these assays. In contrast, in the ileum assay only α-neo-endorphin exhibits a unique potency; its activity is approximately 40 times that of leucine enkephalin.[7] This suggests that the receptors in the ileum complement the structure of α-neo-endorphin more fully than they do the structure of leucine enkephalin or the other opiate peptides (including β-endorphin). It thus appears that distinct populations of opiate receptors respond preferentially to different opiate ligands, the analgesic receptors to β-endorphin and the ileum receptors to α-neo-endorphin. By analogy it is anticipated that a third group of receptors may react preferentially to 'β-neo-endorphin', the putative precursor of methionine enkephalin.

These observations may be rationalized in molecular terms by the following hypothesis: that the common N-terminal tetrapeptide of β-endorphin and α-neo-endorphin (Tyr.Gly.Gly.Phe) be regarded as the 'activator core' of the endorphin peptides while the contiguous sequence constitutes an 'address' component. The activator peptide, when stabilized against degradation, is capable of eliciting a full opiate response[16] but the address components appear to fulfil an equally important role. In β-endorphin the address sequence contributes greatly to the analgesic potency but it does not have a significant effect on activity in the ileum. In contrast the address sequence which follows the activator tetrapeptide in α-neo-endorphin strongly potentiates its action on the ileum. These data indicate that the address sequences, which distinguish the endorphins, confer specificity and potency for their own opiate receptors.

A distinction between 'activator' and 'address' regions in the sequence of a biologically active peptide has previously been made in the study of corticotropin.[17] The address sequence was considered to be formed from the amino acids that add to the specific binding affinity between the activator (or 'message') region of ACTH and the ACTH receptor and as with the endorphins the address sequence alone is unable to trigger a biological response. This principle is now extended to the study of neuroactive peptides, where it appears that the address sequences confer the additional property of specificity for complementary receptors (Table II).

In the potent endorphins the address sequences serve also to stabilize the activator peptide against degradation. The structures of β-endorphin

Table II. Proposed Relationship between Endogenous Opiate Peptides and Their Specific Receptors.

Opiate ligand	Activator sequence	Address sequence	Complementary receptor
L_1 β-endorphin	Tyr Gly Gly Phe-	Met Address[1]	β-endorphin receptor (R_1)
L_2 α-neo-endorphin	Tyr Gly Gly Phe-	Leu Address[2]	α-neo-endorphin receptor (R_2)
L_3 β-neo-endorphin (predicted)	Tyr Gly Gly Phe-	Met Address[3]	β-neo-endorphin receptor (R_3)
L_4 methionine enkepthalin	Tyr Gly Gly Phe-	Met	$R_1R_2R_3$
L_5 leucine enkephalin	Tyr Gly Gly Phe-	Leu	$R_1R_2R_3$

and α-neo-endorphin are more resistant than those of the smaller peptides to attack by aminopeptidases in brain[18] and accordingly it is to be expected that they will have long lasting central actions. A long duration of action — the effects of β-endorphin persist for several hours — may necessitate a physiological mechanism to ensure that only one peptide can express its activity at a time, since diffusion could lead to overlap between different opiate activities. In this context it may be relevant that each of the potent endorphins contains within its sequence paired basic residues which invite the action of processing enzymes. It is an intriguing possibility that selective cleavage at a particular dibasic site could play a role in reducing the effectiveness of certain endorphins, by conversion to the less active C'-Fragment or enkephalin, while ensuring that another can express its specific activity.

It is of interest that in methionine and leucine enkephalins the activator tetrapeptide is linked to a single amino acid residue. These short peptides are relatively unstable in brain and the lack of a major address component may render them relatively non-specific in their actions at opiate receptors. It should be noted, however, that such properties are compatible with a neurotransmitter role.

The concept of activator and address regions in the structures of β-endorphin and α-neo-endorphin not only supports the prediction that a third potent endorphin will be found but it implies that each endorphin will have a high affinity for its complementary receptor and produce a different form of opiate activity. Furthermore the hypothesis of different

address regions in the endogenous opiate ligands is consistent with data demonstrating the existence of multiple opiate receptors.[19-21] It leads to the view that the three endorphins would act independently as potent neuromodulators in the central nervous system.

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BIOCHEMICAL EVIDENCE FOR TYPE 1 AND TYPE 2 OPIATE RECEPTORS

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Distribution of GTP "Sensitive" and "Insensitive" Opiate Receptors

Since Martin originally proposed that opiate receptors were heterogenous,[1] different subcategories of receptor heterogeneity have been invoked to explain various kinds of experimental data. Well-documented structure-activity relationships and naloxone reversibility differences strongly suggest that the guinea pig ileum and mouse *vas deferens* have different opiate receptors.[2] Several laboratories[2,3,4] have observed that some opiate alkaloids require higher concentrations to compete with opiate receptors labeled by opiate peptides, suggesting separate "peptide" and "alkaloid" opiate receptors. Opiate receptors labeled with [^{3}H]naloxone are readily accessible to agonists in the absence of sodium ion but selectively resist them in its presence.[5] Unfortunately, opiate receptors still lack a simple classification scheme which integrates the copious biochemical and pharmacological data available. Kebabian's recent elegant classification of dopamine receptors into adenylate cyclase coupled (Type 1) and uncoupled (Type 2) subtypes[6] has prompted us to explore the possibility that opiate receptors are analogously organized. Rodbell first reported that glucagon's requirement for guanyl nucleotides during activation of adenylate cyclase also manifests itself as a GTP-induced decrease in glucagon binding,[7] and analogous GTP effects on agonist binding have since been shown to occur with β-receptors,[8] α-receptors,[9] dopamine receptors,[10] and, very recently, opiate receptors.[11,12] We have used GTP's reduction of opiate agonist binding as an index of the extent of adenylate cyclase coupling. The more direct measure, opiate receptor-mediated alterations in adenylate cyclase activity, have been well studied only in neuroblastoma-glioma cells,[13]

while pioneering experiments with rat brain homogenates,[14] monkey amygdala,[15] and midbrain slices have proven difficult.

Beginning with micropunches of rat brain tissue on the assumption that small areas might contain more homogenous receptors, we observed GTP reduced met-enkephalin's inhibitory potency only in certain regions (Table I). In the guinea pig ileum, bovine adrenal medulla, and most brain areas, including three shown to mediate analgesia (periaqueductal gray, nucleus gigantocellularis, and reticular formation),[16] GTP (2 μM) elicited large and reproducible reversals of met-enkephalin inhibition. Membranes obtained from neuroblastoma-glioma cells, pituitary gland, Drosophila heads and the only four areas of the limbic system examined (amygdala, frontal cortex, nucleus accumbens, and hypothalamus) were completely resistant to this effect of GTP.

Different Ligand Selectivities of GTP "Sensitive" (Midbrain) and GTP Insensitive (Frontal Cortex) Opiate Receptors

We chose the rat midbrain and frontal cortex for further study since they are large, easy to dissect reproducibly, and enriched in the GTP-sensitive and GTP-insensitive sites, respectively. While the goal of molecular pharmacology is to understand the biochemical differences which are responsible for receptor differences, differences in receptor subtypes are defined classically by variations in their order of selectivity for a group of ligands. Our confidence in a classification scheme based on GTP effects was bolstered by our ability to demonstrate variations in the ligand selectivity of membranes prepared simultaneously from rat midbrain and frontal cortex. Moreover, the ligand selectivity pattern suggested by the binding experiments correlated well for all opiate receptors whose pharmacologies and GTP sensitivities had both been characterized.

Not only does the ratio of "specific opiate receptor binding" between the two prototype brain regions vary dramatically depending on which tritiated opiate ligand is used as a probe, but these ligands show the same pattern of preference in their [^{3}H]diprenorphine binding displacement potencies (Table II). Moreover, this ligand selectivity is most marked when incubations are performed in the presence of GTP (2 μM). Membranes prepared from Drosophila heads[17] support 60-80% specific [^{3}H]diprenorphine and [^{3}H]-D-Ala^2-met-enkephalinamide binding, but have no detectable [^{3}H]naloxone or [^{3}H]dihydromorphine specific binding, a ligand selectivity consistent with their "Type 2" classification

Table I. Opiate Receptors from Different Sourves Vary in Their GTP Sensitivity as Assessed by GTP's Ability to Reverse Met-Enkephalin (0.1 μM)[a].

Region	% Specific [^{3}H]Diprenorphine Binding		Region	% Specific [^{3}H]Diprenorphine Binding	
	-GTP	+GTP		-GTP	+GTP
Bovine Adrenal Medulla	71	100*	Rat Amygdala	53	53
Guinea Pig Ileum	67	85*	Rat Hypothalamus	61	61
Rat Periaqueductal Gray	48	78*	Rat Pituitary	79	79
Rat Caudate Nucleus	51	64*	Rat Frontal Cortex	70	67
Rat Thalamus	48	65*	Rat Nucleus Accumbens	60	60
Rat Reticular Formation	45	65*	NGH 109-15		
Rat Pontis Caudalis	45	65*	(Neuroblastoma x Glioma)	73	70
Rat Nucleus Gigantocellularis	57	76*	Drosophila Heads	75	74
Rat Whole Brain	58	77*			
Rat Locus Coeruleus	59	77*			
Rat Ventral Tegmentum	59	71*			

[a]Micropunches of frozen rat brain were pooled, weighed, homogenized in 100 volumes 50 mM Tris-HCl, pH 7.5 at 4°C, using a Brinkman polytron (setting 5, 15 sec), and centrifuged at 39,000 x g for 15 min. The pellets were resuspended in 10 volumes buffer. Specific binding was assayed in duplicate by incubating 150 μl membranes, bacitracin (100 μg/ml), and NaCl (100 mM) in a total volume of 2 ml with 0.98 nM [^{3}H]diprenorphine (6.6 Ci/mmol, Amersham), at 25°C for 1 hr in the presence and absence of 1 μM levallorphan (the "blank") and GTP (2 μM). Membrane-bound [^{3}H]diprenorphine was separated from free ligand by rapid filtration under vacuum through Whatman GF/B filters as described previously.[5] NGH-109-15 were assayed at 0.33 mg protein per tube and Drosophila heads were prepared as described by Schmidt-Nielsen *et al.*[17]

*$p<0.01$

Table II. Differences in Opiate Ligand Selectivity Between Midbrain and Frontal Cortex.

OPIATE LIGANDS	MIDBRAIN TO (Ratio of [^{3}H]Diprenorphine Inhibitory Potencies)	FRONTAL CORTEX (Ratio of [^{3}H] Specific Opiate Ligand Binding)
Cyclazocine	1	—
Ketocyclazocine	1	—
N-Allyl-Normatozocine	1	—
β-Endorphin	1	—
Met-Enkephalin	1:3	1:5
Leu-Enkephalin	1:4	1:10
D-Ala^2-Met-Enkephalinamide	1:2	1:2
Morphine	2:1	3:2
Naloxone	2:1	3:2
Diprenorphine	—	1:1.5

based on their GTP insensitivity. Presumably, the inability to demonstrate stereospecific [^{3}H]naloxone or dihydromorphine binding to invertebrate membranes[18] is due to the presence of Type 2 opiate receptors which lack selectivity for opiate alkaloids.

Regional Differences in Type 1 and Type 2 Receptors in Rat Brain

Since the GTP-sensitive and insensitive receptors have demonstrably different ligand selectivities, the regional distribution of the binding of [^{3}H]leu-enkephalin, the ligand with the most marked preference for Type 2 receptors, can be compared with [^{3}H]-naloxone binding, which markedly prefers Type 1 receptors to estimate the relative enrichment of these receptor types. Table III shows the results of such an analysis which was carried out on 25 micron serial sections of unfixed, frozen rat brain from which all but the neuroanatomically designated area had been scraped away. Specific binding was calculated by subtracting a "blank" (10^{-6} M levallorphan plus 10^{-6} M D-Ala^2-met-enkephalinamide) included for each region. Triplicate determinations resulted in ranges of less than 5% although the counts per minute obtained by placing the part of the glass containing the section of Aquasol^R and analyzing their radioactivity

Table III. Estimates of Relative Type 1 and Type 2 Opiate Receptors on Frozen Sections (25 Microns) of Rat Brain.

Brain Nucleus	Type 1	Type 2
	Specific [^{3}H]Naloxone Binding (cpm/unit area)	Specific [^{3}H]Diprenorphine Binding (+GTP) (cpm/unit area)
Caudate putamen	36	29
Globus pallidus	30	23
Hypothalamus (arcuate region	7	0
Habenula	50	10
Amygdala	26	14
Parafascicular n.	38	8
Substantia nigra	48	7
Interpeduncular nucleus	77	50
Periaqueductal gray matter	22	7
Mesencephalic reticular formation	30	35
Mesencephalic reticular formation (n. oralis)	19	43
Inferior colliculus	9	17
N. reticularis pontis caudalis	13	10
Raphe magnus	11	0
N. reticularis magnacellularis	13	10
N. reticularis gigantocellularis	35	7
Spinal cord	78	8

content by photospectrophotometry were very low (20-200 cpm).

Consistent with our GTP sensitivity data, regions of rat brain which have been shown to elicit analgesia[13] showed high ratios of [^{3}H]naloxone to [^{3}H]leu-enkephalin binding. These areas included the spinal cord,

periaqueductal gray and nucleus gigantocellularis. "Control" areas which are analgetically inert were obtained at several levels and found to contain equivalent or enriched [^{3}H]leu-enkephalin binding, suggesting that a high Type 1/Type 2 receptor ratio may reflect a peculiar synaptic organization typical of analgesic areas. The parafasicular nucleus, which supports morphine-induced analgesia in the monkey and has not been studied in the rat, shows the same pattern of relative Type 1 receptor enrichment. Indeed, these experiments are consistent with the suggestion that the guinea pig ileum has opiate receptors which have ligand selectivities similar to the receptors mediating analgesia in some rodent tests,[6] since analgesic areas and guinea pig ileum share the high GTP sensitivity and ligand selectivities[2] typical of Type 1 opiate receptors.

It must be pointed out that the early studies of opiate receptor distribution, of course, measured the contributions of Type 1 and Type 2 receptors to differing extents depending on the ligand and conditions used. For example, it is not surprising that the opiate receptor distribution pattern observed by Hillman *et al.*[19] for human brain showed a much more striking association with the limbic system than similar studies by Kuhar *et al.*[20] The use of [^{3}H]-etorphine,[19] which we now realize has the ability to bind to Type 2 opiate receptors, can be contrasted with the [^{3}H]naloxone and [^{3}H]-dihydromorphine or marked Type 1 preference used by Kuhar *et al.*[20]

Future Studies

Since Type 1 opiate receptors appeared for the first time with the advent of the vertebrates, we are fascinated by the possibility that the evolution of nervous systems of increasing complexity has featured the gradual addition of neural circuitry with Type 1 opiate receptors which now compose the bulk of mammalian brain, while the "primitive" Type 2 opiate receptors have been conserved at those anatomical sites which mark the phylogenetically oldest circuits, hence those with the greatest survival value. This notion is consistent with the enrichment of Type 2 opiate receptors in limbic areas which we have already observed. Thus careful autoradiographic studies of Type 2 opiate receptor distribution performed across many phyla as well as throughout development might reveal the "oldest" and perhaps most "hard-wired" synaptic areas. This last assumption seems plausible since Type 2 opiate receptors lack the potential for modulatory control which is bestowed on the Type 1 receptor by its ability to activate adenylate cyclase and, thus, initiate the cascade of events which produce alterations in synaptic membranes.

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ISOLATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF A NEURODEPRESSING HORMONE FROM THE EYESTALKS OF *PENAEUS VANNAMEI* (BOONE)

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The sinus gland of decapod crustaceans is a neurohemal organ located in the eyestalks and constituted of axon terminals which end at or close to a blood sinus. A large number of hormones stored by the sinus gland have been described but only a few have been isolated and purified.[1] The release of a hormonal agent from the eyestalk resulting in depression of locomotor activity was originally suspected on the basis of locomotor activity enhancement after eyestalk ablation and subsequent depression induced by eyestalk extract injection.[2,3] We have previously shown the presence in the sinus gland of the fresh-water Mexican crayfish *Procambarus bouvieri* (Ortmann) of a low molecular weight peptide which depresses the responsiveness of sensory and motor neurons, inducing the behavioral phase of low activity during daytime in crustaceans and which we have named the neurodepressing hormone (NDH). In other words, the NDH acts physiologically as a humoral modulator of circadian rhythms.[4–6] We have shown that this activity is present in all decapod crustaceans tested (to be published) and we have undertaken to purify and compare the hormone from two sources: a fresh-water crayfish and a marine organism, the white shrimp of the Pacific, *Penaeus vannamei* (Boone), which preceded the crayfish in evolution.

The activity of the NDH was assessed in vitro by its effectiveness in reducing the spontaneous firing rate of motoneurons. The abdominal chain of crayfish was isolated and the activity of the third root of the third ganglion was recorded with a suction electrode. The recorded motoneuron corresponds to f-5 of Wine *et al.*[7] For quantification, each reduction of 1% beyond 10% (spontaneous fluctuation) was taken as one unit of NDH activity.

Results and Discussion

A large number of eyestalks (6000 or 9000) were heated in water at 90° for 1 minute and then frozen rapidly. They were ground to a fine powder in the frozen state using powdered dry ice as grit. The powder was treated with acetone and chloroform in order to completely disrupt membranes and then dried. Next, it was extracted with water at 45° and centrifuged. This step was repeated until the extract was colorless. The combined extracts were then thoroughly dialysed against water and the diffusate, which contained all the NDH activity, was concentrated and fractionated successively on Sephadex G-25 Fine (twice) and G-15 columns. Active fractions were identified by bioassay, concentrated and submitted to the next fractionation step. The last Sephadex fraction was further purified by preparative paper electrophoresis at pH 1.8 and 10.0. An aliquot of this last preparation was hydrolyzed in 6N HCl for analysis in a Durrum D-500 amino acid analyzer by Dr. Paul Fletcher Jr. of Yale University and for dansylation and bidimensional TLC in polyamide plates. The results of the purification of NDH from 9000 eyestalks are shown in Table I.

Table I. Purification of the Neurodepressing Hormone from *Penaeus vannamei* (Boone)

FRACTION	TOTAL ACTIVITY [a] (ES eq.)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY [b] (ES eq/mg)	RECOV. %	PURIFICATION
Crude extract	9000	6200	1.45	-	1
Sephadex G-25 (1st)	8917	1246	7.16	99	4.9
Sephadex G-25 (2nd)	8835	393	22.46	98	15.5
Sephadex G-15	8775	91	96.43	97.5	66.5
Electrophoresis [c]	8000	0.116 [d]	68,850	88	47,450

[a] ES = eyestalk equivalent, determined by bioassay
[b] Eyestalk equivalents per mg of protein
[c] After two preparative electrophoretic purifications at pH 1.8 and 10.0
[d] Calculated from the amino acid analysis of 1000 ES eq. based on M.W. 1200

The neurodepressing hormone from *Penaeus vannamei* (Boone) has the same general characteristics as the one from *Procambarus bouvieri* (Ortmann). It is dialysable, thermostable and neutral. Its apparent molecular weight is 1200 as determined by gel filtration in Sephadex G-15, using Vitamin B12 and LH-RH (1355 and 1181 M.W. respectively) as markers. The activity of NDH is destroyed by the action of pronase and chymotrypsin. No N-terminal group could be detected with dansyl chloride and the peptide is ninhydrin negative. Besides, there is no electrophoretic mobility at 4 pH values tested. This means that both ends are blocked or that this is a cyclic peptide.

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CONFORMATIONAL STUDIES OF NEUROPEPTIDES USING THROUGH-SPACE AND THROUGH-BOND INTERACTIONS: OXYTOCIN

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We report PMR studies of three sequences in the peptide oxytocin using principally difference NOE methods.[1,2] The double Karplus ring-closure approach[3] was applied to the Pro^7 residue conformation. Despite many NMR studies several conformational features of oxytocin still remain to be rigorously demonstrated. For example, (a) no ψ angles have been experimentally determined, (b) Pro^7 ring conformation is undetermined, (c) unequivocal demonstration of both proposed β-turns still remains, and (d) the Cys^1-Cys^6-Pro^7 tripeptide contains several unsolved conformational problems.

Although the NOE has been successfully applied to several crystallographically-defined peptides[4] and to gramicidin S[2] and tyrocidin A[5], detailed applications to peptide hormones have yet to appear.

Results and Discussion

The Tyr^2-Ile^3-Gln^4-Asn^5 Moiety — Several conformational features of this sequence of oxytocin have been derived from scalar coupling constants, temperature dependence of chemical shifts, ^{13}C relaxation rates, theoretical calculations and Hα-Hβ spin-spin analysis.[6,7,8] Although it is by no means proven rigorously, all data favor the original hypothesis[9] of a β-II turn. Preliminary NOE data[10] supported this conclusion. Here we report NOE data at 270 and 360 MHz on a 2 mM sample of oxytocin in DMSO-d_6. At these low concentrations the spectral S/N ratio, and hence NOE errors, were barely acceptable for this size peptide, nevertheless, the data clearly rule out a β-I and favor a β-II turn. This was done using NOE ratios[2] alone but several rϕ, rψ, and r$\phi\psi$ distances[5] calculated for the larger NOE's also supported the proposed conformation. The NOE corresponding to the r$\phi\psi$ distance between the Asn^5 NH and the Gln^4 NH confirmed the β-turn conclusion but the NOE

ratios, ($Ile^3\phi^+/Ile^3\psi^-$) and ($Ile^3\psi^+/Gln^4\phi^-$), eliminate a β-I turn and confirmed a β-II turn.

The Cys^1-Cys^6-Pro^7-Leu^8-Gly^9NH_2 Moiety — Relatively little has been definitely established about the conformation of the sequence 6-7-8-9.[6] The Pro^7 residue possesses a trans conformation whilst Gly^9 has relatively high mobility. Although a 6-7-8-9 β-turn has been proposed to be consistent with NMR data, the diagnostic Gly^9 NH hydrogen bond has not been detected. Our NOE ratio ($Pro^7\psi^+/Leu^8\phi^-$) = 3.7 at 270 MHz firmly eliminates any β or γ-turn for the sequence 6-7-8-9 and the Pro^7 $\psi+$ NOE is too large for a β-I turn. The absence of an NOE > 0.5% between the Leu^8 NH and the Gly^9 NH also supports this. The lack of an amide proton on Pro^7 reduced the number of $\phi\pm$, $\psi\pm$ and $\phi\psi\pm$ NOE's for the proposed 6-7-8-9 β-turn but irradiation of Cys^6 Hα gave NOE's which helped delineate the 1-6-7 tripeptide conformation. Studies of residues 1 and 6 of the disulfide bridge using $^3J\alpha$-β values gave two alternative conformations — the classical rotamer and the rigid eclipsed.[8,11]

Table I.

	270 MHz NOE				360 MHz NOE			
Residue	ϕ^+	ϕ^-	ψ^+	ψ^-	ϕ^+	ϕ^-	ψ^+	ψ^-
Ile^3	3.6	2.5	4.9	5.3	10.6	1.3	6.1	12.4
Gln^4	4.0	2.9	3.2	2.8	8.7	5.5	3.6	9.2
Asn^5	5.1	2.6	6.5	8.3	8.1	5.9	10.6	22.5
Cys^6	2.1	2.9	---	---	5.2	---	---	---
Pro^7	---	---	11.2	5.1	---	---	13.0	18.4
Leu^8	1.2	3.0	0.0	0.0	9.1	---	0.0	0.0

Table II.

NOE ratio[a]	OBSERVED[b]		CALCULATED FOR β-TURN[b]	
	270 MHz	360 MHz	β-I	β-II
$Ile^3\phi^+$ / $Ile^3\psi^-$	0.6±0.3	0.8±0.4	3.8	0.35
$Ile^3\psi^+$ / $Gln^4\phi^-$	1.7±1.0	1.1±1.0	0.3	1.5

[a]For nomenclature see Ref. 5

[b]Calculated assuming rφ and rψ distances given in Ref. 6.

The 7.5% NOE between Hα^6 and Hα^1 was consistent with several, but not all, of the proposed disulfide bridge conformations.[8,11,12] The existence of a 2.5% NOE between Cys^6 Hα and Pro^7 Hδ was consistent with Cys^6 ψ being 180° but not −70°[12] and further restricted the tripeptide conformation. This also meant that the tail does not fold over the ring.

Pro^7 Ring Conformation — The double-Karplus and ring-closure criteria were used to completely establish the prolyl ring conformation. It is clear that only the Ramachandran B and not A conformation fits the data for Pro^7 and that the lower and upper field proton resonances belong to the S and R protons, respectively.

Conclusions

NOE data for oxytocin in DMSO-d_6 — (a) confirmed the β-II conformation for the sequence 2-3-4-5, (b) were not consistent with a β or a γ-turn for the sequence 6-7-8-9, (c) confirmed the trans conformation for Pro^7 and (d) delineated several conformational features of the Cys^1-Cys^6-Pro^7 tripeptide sequence. ^{3}J Values and a novel approach to prolyl residue conformational analysis established the R/S β proton configurations and a Ramachandran B conformation for Pro^7.

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OPIOID PEPTIDES IN BOVINE ADRENAL MEDULLARY GRANULES

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The identification of Met-enkephalin[1] as the 61-65 sequence of the pituitary peptide β-lipotropin (β-LPH) led to the supposition of a precursor-product relationship. However, studies have shown that in the striatal region of the brain, where the enkephalin levels are the highest, no β-lipotropin or β-endorphin could be detected.[2] Additionally, a possible Leu-enkephalin precursor termed α-neo-endorphin has been identified and, except for the enkephalin sequence, shown to be unrelated to β-endorphin.[3] Based on the immunocytochemical observations that large amounts of enkephalin immunoreactive material are present in the adrenal medulla[4,5] this organ was chosen as a possible source to study the biosynthetic pathway of the enkephalins. In this report our initial findings on the opioid peptides in chromaffin granules of the bovine adrenal medulla are presented. Further details have been submitted for publication.[6,7]

High performance liquid chromatography (HPLC) was used for peptide separations.[8] A fluorometric (fluorescamine) detection system was used to monitor peptides in column effluents.[9] Aliquots of collected fractions were tested for opioid activity by a radioreceptor binding assay employing neuroblastoma x glioma hybrid cells (NG 108-15).[10]

Chromaffin granules from bovine adrenal medulla were prepared according to a modified procedure of Smith and Winkler.[11] The granules were lysed at low pH in the presence of protease inhibitors. Membrane fragments were removed by centrifugation and proteins in the supernatant were precipitated with trichloroacetic acid (TCA). After removal of TCA and lipids by ether extraction, the sample was applied to a reverse-phase HPLC column (Figure 1). Opioid activity was detected not only in the calibrated Met- and Leu-enkephalin regions, but also in two regions before the elution position of Met-enkephalin (fractions 6

and 7) and one region after the elution position of Leu-enkephalin (fraction 16). These peptides eluted at their original positions when rechromatographed under the same conditions. This demonstrates that there are opioid peptides other than Met- and Leu-enkephalin in the chromaffin granules. The enkephalin concentration in chromaffin granules was found to be 8.5 nmol/mg protein after TCA precipitation with the Met-analog comprising about 65% of the total. As much as 1.2 nmol of enkephalin was recovered from 1 g of adrenal medulla. The active peptide eluting at the Leu-enkephalin position was purified to homogeneity and shown to have the amino acid composition of Leu-enkephalin. Fractions 6, 7 and 16 were present respectively in concentrations of 0.4, 0.5 and 0.5 pmol/μg protein. When protease inhibitors were deleted during the isolation procedure, the yields were significantly lower.

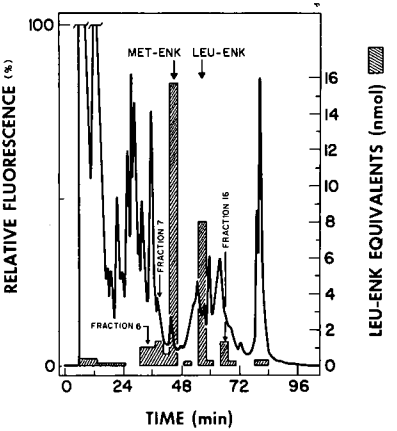

Fig. 1. Chromatography of peptides from chromaffin granules. Chromaffin granules from 20 g of adrenal medulla were lysed and the resulting supernatant was deproteinized with TCA. This peptide fraction was loaded on an RP-18 column (4.6 x 250 mm) which was eluted at 35 ml/hr with 0.5 M formic acid — 0.4 M pyridine (pH 4.0) using a step-wise gradient of n-propanol: 0% (5 min), 5% (5 min), 10% (25 min), 15% (30 min) and 40% (17 min). A portion (5% of the column effluent) was diverted to the fluorescamine monitoring system. Fractions (1.75 ml) were collected and aliquots (20 μl) were lyophilized and assayed for opioid activity with a radioreceptor assay.

In an alternative procedure, acid extracts of bovine adrenal medulla were applied to a Sephadex G-100 column (Figure 2). Five peaks of activity corresponding in molecular weights to approximately 20,000 (I), 10,000 (II), 5,000 (III), 2,000 (IV) and $<$ 1,000 (V) were eluted. Peaks I and II were detectable only when the material was digested with trypsin prior to assay. The activity of peak III was also greatly increased by trypsin digestion.

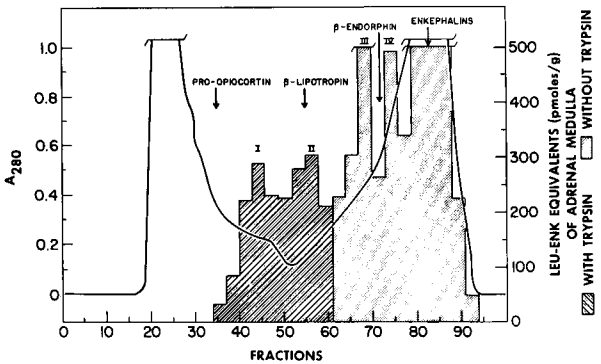

Fig. 2. Gel filtration of an acid extract of adrenal medulla on a Sephadex G-100 column. Aliquots (0.5 ml) of fractions (6 ml) 10-61 were treated with trypsin and assayed for opioid activity. The aliquots of fractions 61-99 were assayed directly for opioid activity.

Trypsin digests of peaks I and II were resolved by HPLC. Both digests showed the same pattern of active fragments, none of which corresponded to the elution position of β-LPH 61-69, the active tryptic fragment from β-endorphin and its precursors. These findings demonstrate that the opioid peptides in peaks I and II are structurally related to one another but differ from the precursor peptides found in the pituitary. Although the molecular weight of the peptide in Peak III was comparable to that of β-endorphin, the two peptides were found to be different by HPLC. Since peaks I, II and III all showed considerably greater activity in the radioreceptor assay when predigested with trypsin, the opioid sequence is contained within a larger peptide.

Lysed chromaffin granule supernatant was subjected to the same chromatography on Sephadex G-100 as described above. The same five peaks of activity were observed, but the relative amount of peak I was found to be more than 3-fold higher in the whole medulla than in granules. This may indicate uptake of the largest peptide by the granules followed by proteolytic processing within the granule. The relative amounts of the other four peaks were the same in both preparations.

As in the striatum,[2] no β-endorphin, β-lipotropin or pro-opiocortin could be detected in the adrenal medulla indicating that these are not the enkephalin precursors. The chromaffin granules may thus present a system in which to study the biosynthesis of the enkephalins for clarification of the route of the precursor molecules to opioid activity.

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BOMBESIN-LIKE PEPTIDES: REGIONAL DISTRIBUTION IN RAT BRAIN

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Introduction

Bombesin (BN), a tetradecapeptide isolated from frog skin, is active in the gastrointestinal tract and brain.[1] In the central nervous system BN induces hyperglycemia[2] and hypothermia[3] with a well defined structure-activity relationship. In particular, numerous amino acid residues near the C-terminus are required for BN to bind to the BN receptor and induce these responses.[4]

Endogenous BN-like peptides have been detected using an antibody which recognizes the C-terminus of BN.[5] This antiserum was used to map the regional distribution of BN-like peptides in rat brain.

Methods and Results

Using the indirect immunohistochemical procedure of Coons et al.[6] combined with the modifications described previously,[7] BN-like immunofluorescence was observed in discrete varicose nerve fibers such as those presented in Figure 1a. Perikarya, which contain BN-like peptides were not visible, however, until treatment with vinblastine. Figure 1b shows that after this treatment cell bodies were observed in the anterior hypothalamic nucleus. Also, perikarya were detected in the preoptic and mammilary nuclei as well as the mesencephalon.

The regional distribution of BN-like peptides was quantitated using a radioimmunoassay combined with microdissection techniques. Sections of 0.3 to 1 mm of frozen rat brain were microdissected and extracted with 2N acetic acid. The samples were prepared as described previously[5] and assayed for immunoreactivity using a 1:100,000 antiserum dilution.

Table I shows that the density of BN-like immunoreactivity is greatest in the nucleus tractus solitarius, substantia gelatinosa, arcuate nucleus and interpeduncular nucleus. The peptide density is intermediate

Table I. Regional Distribution of Bombesin-like Immunoreactivity

Rat Brain Region	Bombesin-Like Peptides (fmol/μg protein)
Cortex	
Cingulate cortex	0.29 ± 0.05
Hippocampus	0.22 ± 0.01
Striatum	
Caudate n.	0.22 ± 0.03
Globus pallidus	0.53 ± 0.05
Septal area	
N. accumbens	0.56 ± 0.07
Septum lateralis	0.89 ± 0.12
Interstitial n. of stria termnalis	1.16 ± 0.10
Hypothalamus and preoptic area	
Paraventricular	1.52 ± 0.15
Arcuate n.	2.6 ± 0.5
Lateral preoptic n.	0.84 ± 0.04
Anterior hypothalamic n.	1.10 ± 0.07
Amygdala	
Central amygdaloid n.	1.22 ± 0.10
Midbrain	
Central gray	1.45 ± 0.10
Substantia nigra, reticular part	1.04 ± 0.07
Interpeduncular n.	2.21 ± 0.20
Hindbrain	
Substantia gelatinosa	5.9 ± 0.7
N. tractus solitarius	4.7 ± 0.55

Each value represents the mean ± S.E. of 5 to 7 determinations

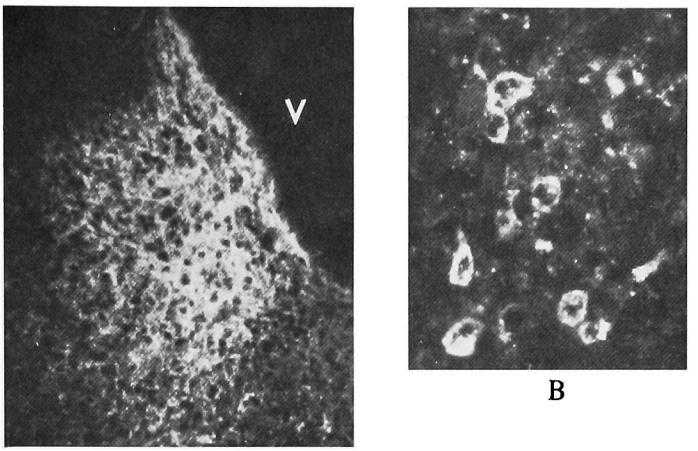

Fig. 1. BN-like immunofluorescence using 1:200 antiserum dilution. [a] Discrete varicose nerve terminals in the nucleus (n.) interstitialis stria terminalis (X 145). [b] Cell bodies in the anterior hypothalamic nucleus 4 days after intraventricular injection of vinblastine (20 μg) (X 390).

in the amygdala, certain hypothalmic as well as midbrain nuclei and the interstitial nucleus of the stria terminalis. Other septal areas as well as the striatum and cortex have a low density of BN-like immunoreactivity.

Conclusions

This work demonstrates that BN-like peptides are distributed in discrete loci of the rat brain. The density of immunoreactive BN is greatest in certain hindbrain (n. tractus solitarius and substantia gelatinosa), hypothalamic (arcuate n.) and midbrain (interpeduncular n.) nuclei. The density of immunoreactive BN is 10-30 fold lower in certain cortical (cingulate cortex and hippocampus) and striatal (caudate nucleus) areas.

Using immunocytochemical techniques, this BN-like immunoreactivity was localized to discrete varicose nerve fibers and cell bodies. Also, the density of immunofluorescence correlated well with the data shown in Table I.

Recently, the immunoreactive component present in rat midbrain and hypothalamus extracts was characterized using HPLC (in preparation). One major peak of immunoreactivity was observed which eluted before BN. One minor peak of immunoreactivity was present

which coeluted with Substance P. The structure of this major component remains to be determined. Because of the presence in neurons it is suggested that this endogenous BN-like component may function as a peptidergic neuroregulator.

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β-ENDORPHIN AND ANALOGS: 360 MHZ PROTON NMR SPECTROSCOPY

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The conformational properties of the naturally occurring opioid peptides, methionine enkephalin and β-endorphin, have been investigated by circular dichroism spectroscopy in the presence and absence of added non-aqueous solvents and in the presence of detergents.[1,2,3] Enkephalin and a variety of analogs have also been examined by proton and ^{13}C NMR spectroscopy.[4,5,6] The above studies have indicated a probable β-turn in enkephalin and considerable secondary structure in β-endorphin in the presence of nonaqueous solvents or detergents. However, no reports have heretofore been presented on the investigation of β-endorphin (Figure 1) by proton NMR spectroscopy.

```
                    5                   10
H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-

                   15                   20
Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-

                   25                   31
Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
```

Fig. 1. Amino acid sequence of human β-endorphin. Camel β-endorphin is identical except it has histidine-27 and glutamine-31.

As shortened or modified sequences of β-endorphin are known to have altered biological activities,[7,8] and since these changes may be related to conformational variations, proton NMR spectra were run of β_c-EP, β_h-EP and various analogs. Those analogs were: β_h-(1-5), -(1-9), -(1-15), -(1-21), -(1-29), -(1-30), [I_2-Tyr^1]-β_h-EP, [I_2-Tyr^{27}]-β_h-EP and [I_2-Tyr^1,I_2-Tyr^{27}]-β_h-EP. In addition, omission analogs of β_c-EP (lacking a single amino acid in position 1,4,5,6, or 7 were investigated. The spectra of the above peptides, along with decoupling information, enable us to

unequivocably assign virtually all the signals in the proton NMR for β-endorphin.

Figure 2 shows the spectra of β_h-EP-(1-5), β_h-EP-(1-9), β_h-EP-(1-15), β_h-EP-(1-21), and β_h-EP. These spectra were performed on the Stanford University Magnetic Resonance Laboratory's HXS-360 MHZ NMR. The spectra were carried out at pH 6.7 in Aldrich 100.0% D_2O at 26°C and approximately 1.4 nmolar concentration. Representative values obtained from the above spectra include: Thr-16 (d) 1.12, Thr-12 (d) 1.21, Thr-12 (d) 1.24, Ala-26 (d) 1.28, Ala-21 (d) 1.37, Met-5 (s) 2.07, Tyr-27 (d,d) 6.83 and 7.12, Tyr-1 (d,d) 6.88 and 7.17 P.P.M.

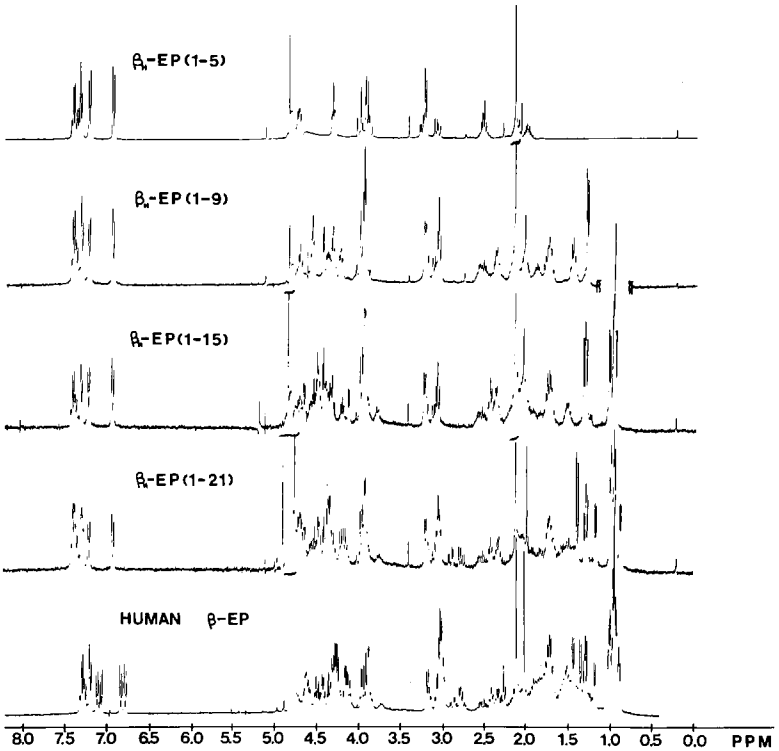

Fig. 2. 360 MHZ Proton NMR (pH 6.7) for human β-endorphin (1-5), -(1-9), -(1-15), -(1-21), and β_h-EP.

The spectra of β_h-EP were run in the above solvent and the temperature was varied between 25° and 75°C. Relatively minor changes occurred with this temperature variation. This was also true for pH changes between 4 and 8, except that upon deprotonation at

approximately pH 7.5 the aromatic protons of Tyr-1 were shifted upfield to a position very near those of Tyr-27. Addition of large molar quantities of Ca^{++}, Mg^{++}, K^{+}, or Na^{+} caused no discernible change in the spectra of β_h-EP. Under identical conditions β_h-EP-(1-29), β_h-EP-(1-30), and β_h-EP showed no significant differences in spectral properties. For the omission analogs examined, in each case marked and distinctive changes were found for the omission of a single amino acid from β_c-EP, but nothing which was attributable to significant conformational changes.

While there was no change in the NMR spectra found upon addition of methanol or SDS to β_h-EP-(1-9), there were significant changes in the spectra of β_h-EP in the presence of 50% methanol (Figure 3) or 0.1% SDS.

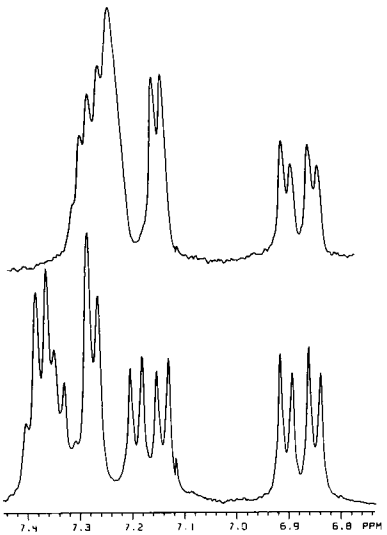

Fig. 3. Upper — Human β-endorphin in 50% CD_3OD + 50% D_2O; Lower — Human β-endorphin in 100% D_2O; 26°C, pH 6.7.

From the above work, we conclude that the altered biological activity of shortened sequences of β-EP is not due to significant conformational changes in non-lipophilic, aqueous solution, but may arise from preferential changes for these peptides in lipophilic medium.

Acknowledgement

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α-NEO-ENDORPHIN: A "BIG" LEU-ENKEPHALIN FROM PORCINE HYPOTHALAMI

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Introduction

The critical structure for endogenous opiate activity is Tyr-Gly-Gly-Phe-X, where X is Met or Leu in Met- and Leu-enkephalin.[1] The homology between the β-lipotropin (β-LPH) sequence 61-65 and Met-enkephalin and the relationship between the β-LPH C-terminal subsequences and the known "big" Met-enkephalin such as α-, β-, γ-, δ-endorphins lead to the elucidation of the biosynthetic pathway of Met-enkephalin relatives from the common precursor to ACTH and MSH's.[7,8]

The existence of a precursor to Leu-enkephalin in brain has been suggested[9] but even a "big" Leu-enkephalin had not been discovered until the first isolation from porcine hypothalami of α-neo-endorphin which has the Leu-enkephalin moiety at its N-terminus.[10]

α-Neo-endorphin proved to be a "big" Leu-enkephalin. The occurrence of this peptide in the brain was strong evidence for the existence of a separate precursor to Leu-enkephalin and supported by the finding that the nucleotide sequence of cloned cDNA for ACTH-β-LPH precursor does not contain the coding for Leu-enkephalin.[11]

N-terminal sequence (1-8) of α-neo-endorphin was determined by dansyl-Edman method to be: Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg-[10]. Another dansyl-Edman degradation using 5 μg of α-neo-endorphin revealed that a proline residue is located at position 9. The sequence (1-9) of the peptide was established to be: Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg-Pro-(Gly, Tyr_2, Lys_2, Arg). Syntheses of the peptides corresponding to

the N-terminal sequences (1-6) and (1-9) of α-neo-endorphin were carried out and their opiate activities were compared with natural α-neo-endorphin and the known opioid peptides.

Experimental

α-Neo-endorphin was purified from the acid extracts of 30,000 pig hypothalami in ca. 50 μg yield.[10]

Sequence Analysis: α-Neo-endorphin (5μg) was subjected to dansyl-Edman degradation.[10]

Syntheses of α-Neo-endorphin-(1-6), -(1-9) and -(1-9)-amide were carried out by the solution method. Analytical data are as follows:

α-Neo-endorphin-(1-6): $[\alpha]_D$ +7.5° (c=1, H_2O); Anal. Calcd. for $C_{34}H_{49}N_9O_8 \cdot AcOH \cdot 3/2H_2O$: C, 54.12; H, 7.07; N, 15.78. Found: C, 54.13; H, 7.06; N, 15.78. Composition: $Gly_{2.06}$, $Leu_{1.00}$, $Phe_{0.98}$, $Tyr_{0.89}$, $Arg_{0.99}$. α-Neo-endorphin-(1-9): $[\alpha]_D$ −39.0° (c=1, H_2O); Anal. Calcd. for $C_{51}H_{80}N_{16}O_{11} \cdot 3AcOH \cdot 3H_2O$: C, 51.22; H, 7.47; N, 16.77. Found: C, 51.35; H, 7.36; N, 16.63. Composition: $Gly_{2.04}$, $Pro_{0.99}$, $Leu_{1.00}$, $Phe_{1.03}$, $Tyr_{0.95}$, $Arg_{2.02}$, $Lys_{1.01}$. α-Neo-endorphin-(1-9)-amide: $[\alpha]_D$ −37.3° (c=1, H_2O); Anal. Calcd. for $C_{51}H_{81}N_{17}O_{10} \cdot 4AcOH \cdot 4H_2O$: C, 50.45; H, 7.54; N, 16.95. Found: C, 50.56; H, 7.47; N, 16.67. Composition: $Gly_{2.06}$, $Pro_{0.96}$, $Leu_{1.00}$, $Phe_{0.99}$, $Tyr_{0.93}$, $Arg_{2.04}$, $Lys_{1.02}$, $NH_{3\ 0.93}$.

Opiate Activity — Assay of the opiate activities of the synthetic peptides were performed by the guinea-pig ileum method.[12] Naloxone was obtained from Sankyo Co., Japan. Synthetic Met- and Leu-enkephalin and β-endorphin were obtained from Protein Research Foundation, Minoh, Japan. IC_{50}(50% inhibition concentration) values were expressed in molar concentration and determined from dose-response curves shown.

Results and Discussion

Sequence (1-9) of α-Neo-endorphin — The N-terminal sequence (1-8) has been established by dansyl-Edman degradation at a nanomolar scale but further information could not be obtained in the previous work.[10] Because of limited material, conventional methods were not applicable. The strategy that succeeded in the case of LH-RH,[13] was attempted but in vain. After digestion with chymotrypsin or trypsin, the mixture, containing peptide fragments along with the enzyme used, was submitted to the dansyl-Edman method. α-Neo-endorphin underwent

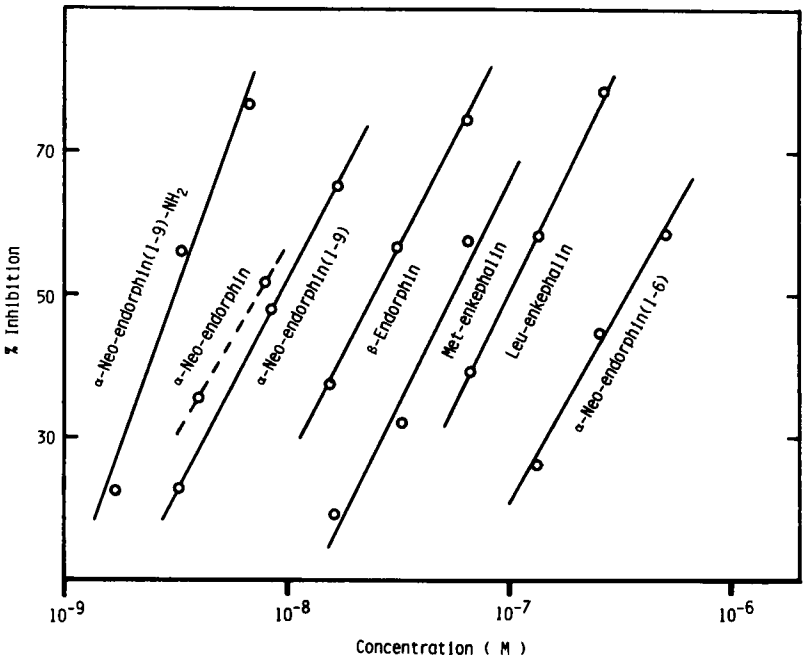

Fig. 1. Dose-response curves of opioid peptides (guinea-pig ileum)

Table I. IC_{50} and Relative Potency of Opioid Peptides (Guinea-Pig Ileum)

Opioid Peptide	IC_{50}(nM)	Relative Potency
Met-enkephalin	33	100
Leu-enkephalin	130	24
β-Endorphin	27	120
α-Neo-endorphin	(5)	(670)
α-Neo-endorphin(1-6)	430	8
α-Neo-endorphin(1-9)	10	330
α-Neo-endorphin(1-9)-NH_2	4	800

too much fragmentation. Sacrificing 5 μg of the peptide, another dansyl-Edman degradation was carried out. The experiment described herein indicated a proline residue at position 9.

Synthesis and Opiate Activity of N-Terminal Peptides — On the basis of structural data three kinds of N-terminal peptide sequences (1-6) and (1-9) were synthesized and their opiate activities determined. Dose-response curves are shown in Figure 1. The curve for natural α-neo-endorphin (dotted line) has not yet been completed, because of shortage of the natural peptide. Opiate activities of all peptides were completely antagonized with naloxone. From Figure 1 and Table I, synthetic peptide (1-6) with Arg-extension at the C-terminal of Leu-enkephalin showed only 1/3 of the activity of Leu-enkephalin. However, α-neo-endorphin (1-9), with the further extension of -Arg-Lys-Arg-Pro, showed remarkably increased activity, almost comparable to that of the natural α-neo-endorphin. The C-terminal amidation of peptide (1-9) enhanced its activity beyond that of the natural α-neo-endorphin. The potent morphino-mimetic activity of natural α-neo-endorphin appears mostly due to the established sequence (1-9).

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ARG°-β_h-ENDORPHIN: A SYNTHETIC β-LIPOTROPIN FRAGMENT (60-91) WITH POTENT BIOLOGICAL ACTIVITY *IN VIVO*

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Introduction

β_h-Endorphin or lipotropin C fragment, a 31-peptide with potent analgesic activity, has been isolated and characterized.[1] The amino acid sequence of β_h-endorphin corresponds to the C-terminal region of β-lipotropin (61-91).[2] By analogy to proinsulin and other prohormones, it is reasonable to assume that β_h-endorphin is a degradation product of β-lipotropin. *In vitro* bioassays of β-lipotropin showed no morphine-like activity when compared with β_h-endorphin[3]. However, endogenous morphine-like peptides can be generated by incubating β-lipotropin with rat brain enzymes[3]. One of the cleavage points of β-lipotropin by enzymes is located between positions 60 and 61 (an Arg-Tyr bond). Based on these observations, we synthesized Arg°-β_h-endorphin (β-lipotropin (60-91) (Table I), by solid-phase methods. Comparisons of the biological activity of this polypeptide with β_h-endorphin, both *in vitro* and *in vivo,* have been made, and the results of these studies are reported here.

Table I. Amino Acid Sequence of ARG°-β_h-Endorphin

```
    1                   5                  10                  15
Arg-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-
                   20                  25                  30
Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
```

Materials and Methods

Boc-Glu (OBzl) was attached to chloromethyl resin (3 mmoles Cl in 4 g of 1% cross-linked resin) by the $CsHCO_3$ method[4]. Analysis showed 0.4 mmole Glu per gram of resin[5]. A Schwarz/Mann Synthesizer was used and one 1.5 minute wash unless otherwise specified in the following system: Prewash and 20 min deprotection with 40% TFA in CH_2Cl_2

containing 0.5% indole, $CHCl_3$, 33% dioxane in CH_2Cl_2, CH_2Cl_2, pre-wash and 10 min neutralization by 10% Et_3N in CH_2Cl_2, three washes CH_2Cl_2, 5-fold excess of Boc-AA in 10% DMF/90% CH_2Cl_2, 5-fold excess of DCC, couple 2 hours, wash twice CH_2Cl_2. Asn and Gln were coupled as Onp derivatives for 15 hours. Each coupling was monitored for completion by the ninhydrin test[6]. Hydroxyls of Thr and Ser and the γ-carboxyl group of Glu were protected with benzyl groups. The ϵ-amino group of Lys was protected by 2-Cl-Z and the phenolic group of Tyr by *o*-Br-Z.

The peptide was deblocked by TFA, washed with CH_2Cl_2 and methanol, and dried *in vacuo*. The peptide was cleaved from the resin by HF (10 ml/gm, 0°C for 60 minutes with 5 ml/gm anisole). HF was removed by vacuum and the resin was washed with ether and extracted with 10% HOAc. The extract was lyophilized to give 205 mg of crude endorphin. This was passed through a P-6 column (Figure 1). The absorptions at 280 nm were determined and tubes containing peptide were examined by TLC (silica gel Polygram, n-BuOH : pyridine : H_2O 15 : 10 : 3 ; 12). Peptides were located with ninhydrin and Pauly sprays. The desired peptide (135 mg) was purified on CMC, eluting with 0.025, 0.05, 0.1, and 0.2 M NH_4OAc (Figure 2). The 0.1 M NH_4OAc fractions gave 63.7 mg of Arg°-β_h-endorphin, which was further purified by partition chromatography to give 37 mg of pure peptide (Figure 3).

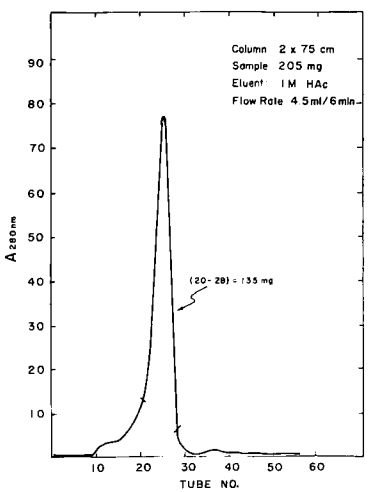

Fig. 1. P-6 Filtration of Arg°-β_h-Endorphin

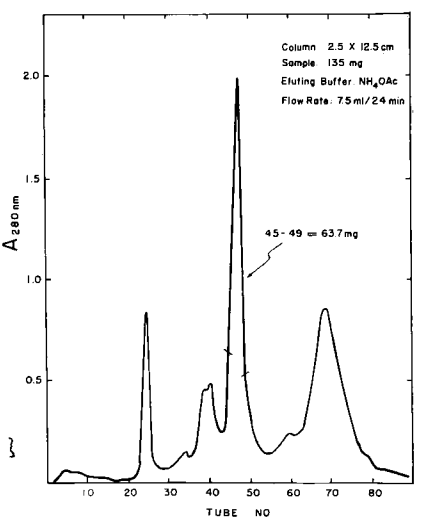

Fig. 2. Ion-Exchange of Arg°-β_h-Endorphin

TLC showed R_f^1 0.44 (cellulose glass plate, n-BuOH : pyridine : HOAc : H_2O 42 : 24 : 4 : 30); R_f^2 0.72 (cellulose Polygram, n-BuOH : pyridine : HOAc : H_2O 15 : 10 : 3 : 12); and R_f^3 0.45 (silica gel Polygram, n-BuOH : pyridine : HOAc : H_2O 15 : 10 : 3 : 12). Movement on electrophoresis was 10.7 cm to cathode (Whatman No. 3 paper 11.5 x 57 cm, pH 3.8 pyridine-acetate buffer, 500 V for 4 hours). The amino acid analysis showed the correct composition, within experimental error.

Results and Discussion

The ability to inhibit stereospecific ^{3}H-naloxone binding in the presence of sodium chloride has been shown to be closely correlated with the analgesic potency of a large number of opiates examined, including the enzyme-resistant synthetic enkephalin analogs such as D-Ala^2-Met-enkephalinamide.[7] Arg°-β_h-endorphin was found to have 2% and 0.2% of the binding affinity of β_h-endorphin in the absence and presence, respectively, of sodium chloride (Table II). This weak binding activity of Arg°-β_h-endorphin was identical to the binding activity of the large molecule, β-lipotropin[3]. However, Arg°-β_h-endorphin showed 10% of the analgesic activity of β_h-endorphin when microinjected into rat brain (Figure 4). When 30 μg of this analog was injected intravenously into adult male rats pretreated with estradiol and progesterone, or a 750 μg intraventricular injection was given to adult rhesus monkeys, Arg°-β_h-endorphin was almost as active as β_h-endorphin in releasing prolactin. It is reasonable to assume that the high potency of this polypeptide is due to enzymatic cleavage of the Arg-Tyr bond and formation of β_h-endorphin during the *in vivo* bioassay. Interestingly, Arg-Met-enkephalin, a fragment of β-lipotropin 60-65, was found to be inactive *in vivo*[8]. The discrepancy between Arg°-β_h-endorphin and Arg-Met enkephalin can be explained by the fact that Arg-Met-enkephalin was cleaved by enzyme *in vivo,* not only at the Arg-Tyr bond but also at the Tyr-Gly bond to form the inactive Gly-Gly-Phe-Met fragment.

Table II. Opiate Receptor Affinities of β_h-and Arg°-β_h-Endorphin

Compound	ID_{50} (Na^+ = 0)	ID_{50} (Na^+ = 100 µM)	Sodium Response Ratio
β_h-endorphin	4 x 10^{-8}	4 x 10^{-7}	10
Arg°-β_h-endorphin	2 x 10^{-6}	2 x 10^{-4}	100

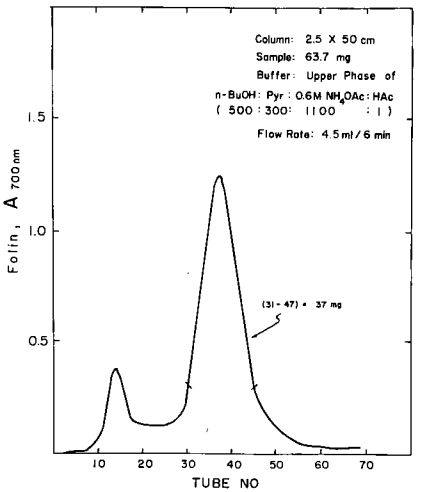

Fig. 3. G-50 Partition of Arg°-β_h-Endorphin

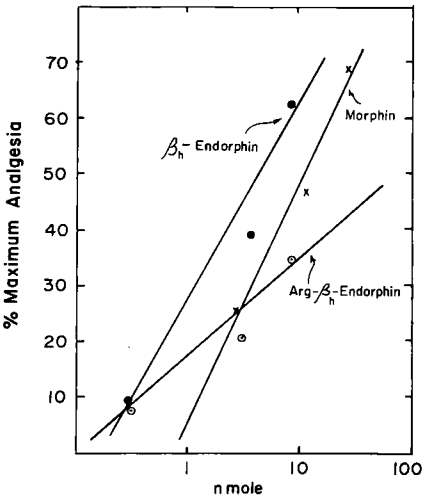

Fig. 4. *In Vivo* Bioassay of Arg°-β-Endorphin

Acknowledgments

We would like to thank Dr. Candace Pert of the National Institutes of Health for providing the opiate-receptor binding assay, Dr. Agu Pert, also of the National Institutes of Health, for the *in vivo* tail-flick task and flinch-jump test, and Dr. C. Y. Bowers, School of Medicine, Tulane University, New Orleans, Louisiana, for the prolactin-releasing hormone assay.

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INSULIN-STIMULATED SODIUM TRANSPORT IN TOAD URINARY BLADDER

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Because many of its ion transport properties bear a close resemblance to those of the distal nephron of the mammalian kidney, the urinary bladder of the toad is often used as a model for the study of transport processes, in particular those regulated by hormones. In 1965 Herrera observed that insulin stimulated sodium transport in the toad bladder[1]; however, the levels of hormone required to achieve the effect were so high that the physiological relevance of insulin-stimulated sodium transport was uncertain. Since it is now recognized that physiological levels of insulin increase sodium reabsorption by the human kidney through a direct effect on the distal nephron[2], we have reinvestigated insulin-stimulated sodium transport in toad bladder as a first step toward elucidating the molecular mechanisms by which insulin regulates sodium transport in the kidney and other epithelial tissues.

An increase in short circuit current (SCC), a measure of transepithelial sodium transport, can be discerned within 15 minutes after the addition of porcine insulin to the toad bladder and the increase persists for 20 hours or more. Using inhibitors of RNA or protein synthesis, the response of the tissue SCC to insulin can be dissected into one component which begins rapidly, but is transient in nature and a second component which is slow in onset and of long duration[3].

The results of such an experiment with actinomycin D, an inhibitor of RNA synthesis, are presented in Figure 1. To insure that all mRNA synthesis was effectively blocked, one group of bladders was preincubated with the inhibitor for 160 minutes prior to hormone addition. Porcine insulin at a concentration of 7.8×10^{-9}M was added to both the actinomycin D-treated hemibladders and the paired, control tissues. The SCC of the tissues to which insulin alone was added increased by 35% within 30 minutes after exposure and remained elevated for at least 5½ hours thereafter. The SCC of the group preincubated with actinomycin D also increased by 35% within the first 30 minutes of exposure to

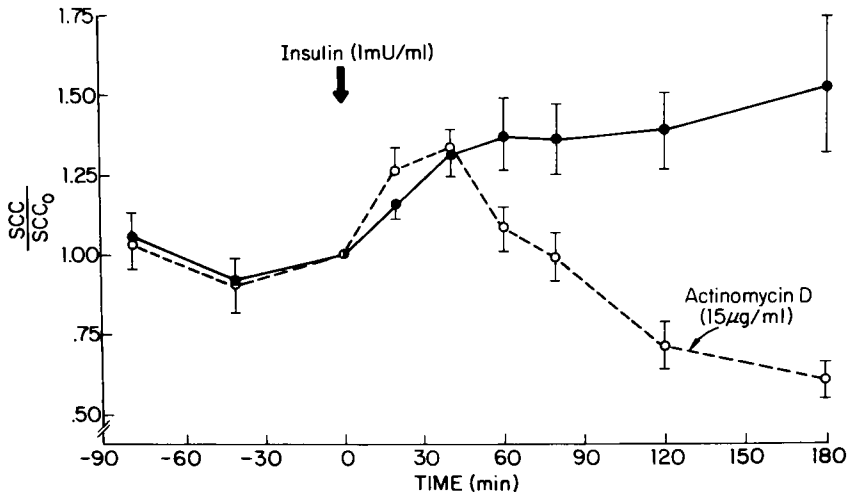

Fig. 1. The effect of actinomycin D on insulin-stimulated SCC.

insulin; however, after 90 minutes of hormone treatment the average SCC of this group of hemibladders was nearly identical to the average SCC before insulin was added. The duration of the response to insulin and the effects of inhibitors are independent of insulin concentration; high and low doses of insulin elicit both the rapid and the sustained components of transport and in all cases the long-term response is sensitive to inhibitors of protein or RNA biosynthesis. Since we find that cycloheximide and puromycin have the same effect as actinomycin D on insulin-stimulated SCC, the sustained response to insulin apparently requires the synthesis of specific proteins.

We examined the biological activities of several insulins with regard to SCC and found that porcine insulin from a commercial source (Sigma Chemical Co.) had only approximately 1% the activity of porcine insulin provided by Eli Lilly & Co. The low activity of the commercial preparation probably accounts for reports by several investigators that concentrations of insulin above 10^{-8}M are required to stimulate SCC in this tissue. As shown in Table I, porcine insulin from Lilly stimulates the SCC at concentrations as low as 1.6×10^{-10}M, and gives a half-maximal response at $\sim 10^{-8}$M. Bovine insulin is slightly more potent than porcine insulin, while porcine proinsulin causes a half-maximal increase in SCC just below 10^{-7}M. A cross-linked insulin analog A1-suberoyl-B30 porcine insulin (kindly provided by Dr. D. Brandenburg), which had from 5-11% of the activity of insulin in a fat cell assay[4], was very similar in activity to proinsulin. Neither of these two peptides at concentrations as

Table I. Stimulation of Sodium Transport by Insulin Analogs

Analog	% Increase in SCC Concentration (M)				
	7.8×10^{-10}	7.8×10^{-9}	3.9×10^{-8}	1.1×10^{-7}	3.9×10^{-7}
Porcine Insulin	21	38	72	77	100
Bovine Insulin	43	55	--	98	--
Dsu-Insulin	29	45	48	--	51
Porcine Proinsulin	10	21	33	66	53

high as 10^{-6}M (5 μg/ml) elevated SCC by more than 70% of the maximum increase caused by porcine insulin.

Studies in amphibia and fishes suggest that insulin native to these species is 10 to 100 times more potent in stimulating ion transport in these animals than is mammalian insulin[5]. Because purified toad insulin is not available to us, we are examining the effects of angler-fish insulin on sodium transport in the toad bladder to see if this insulin may be more potent than mammalian insulin. Angler-fish insulin increases SCC at concentrations as low as 7×10^{-11}M (Table II), well below the lowest

Table II. Effect of Angler-Fish Insulin on Short Circuit Current in Toad Bladder Epithelium

Time (min)	SCC/SCCo Insulin Concentration (M) Angler Fish					Porcine
	7×10^{-11}	7.8×10^{-10}	7.8×10^{-9}	7×10^{-8}	7.8×10^{-7}	7.8×10^{-7}
30	1.00	1.14	1.19	1.24	1.62	1.26
60	1.20	1.10	1.29	1.42	1.62	1.42
90	1.00	1.27	1.38	1.68	1.97	1.58
120	--	1.37	1.36	--	2.29	1.58
690	1.15	1.54	--	--	1.51	1.35

concentration of porcine insulin giving a discernible effect. Furthermore, the maximum elevation of the SCC caused by angler-fish insulin is about twice as great at each time interval as that caused by the mammalian

insulins. Increasing the porcine insulin concentration by 5 fold does not stimulate sodium transport further.

We conclude that insulin at physiological concentrations is a potent stimulator of sodium transport across the toad urinary bladder. Detailed analysis of this effect, which appears to include two separate components, may be useful in determining the mechanisms by which these effects on ion movement are achieved.

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PURIFICATION OF THE ENKEPHALIN GENERATING ENDOPEPTIDASE OF RAT BRAIN MEMBRANES

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Brain proteases, responsible for the synthesis and degradation of neuropeptides may provide important neurochemical control mechanisms. To study these enzymes, we developed a generally useful, radiochemical assay based upon the availability of amphiphilic substrates. To search for enzymes which produce enkephalin from larger precursors, we synthesized β-Lipotropin-(61-69) and labeled the C-terminal Lys by guanidination with ^{14}C O-methylisourea. The labeled product of proteolysis by rat brain enzymes does not absorb to dextran coated charcoal tablets (I.E.M. Screening Systems Inc. North Hollywood, CA 91601) and is thereby separated from the substrate (as well as from enkephalin). In previous experiments[1] we found that enkephalin aminopeptidase can be inhibited by 10^{-5}M puromycin, and

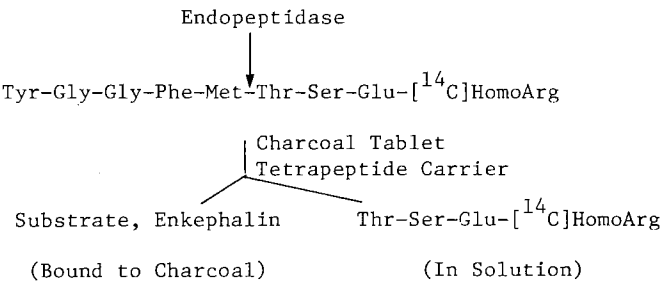

Fig. 1. Endopeptidase assay.

this substance was routinely included in the endopeptidase assays. The aminopeptidase is assayed with ^{3}H Tyr-Met enkephalin[1] as previously described except that dextran coated charcoal tablets are used instead of polystyrene columns. The assay procedures used are described

diagrammatically in Figure 1 for the case of the endopeptidase substrate.

Approximately half of the β-LPH-(61-69) endopeptidase activity of brain homogenate is particulate. The K_m values for the soluble and membrane enzymes are 2.1 x 10^{-6}M and 7.7 x 10^{-6}M, respectively, and both activities are inhibited by mercuribenzoate with an IC_{50} near 2 x 10^{-6}M. Thus they are thiol proteases. The soluble activity is inhibited strongly by other peptides such as neurotensin, 88%, Tuftsin 74% and angiotensin II 71% at 10^{-5}M whereas the membrane enzyme is only weakly inhibited by these peptides. The pH optimum for both enzymes is near 7, indicating that the enzymes are not lysosomal in origin. When reactions were assayed both by the charcoal method and by chromatography, equimolar amounts of radioactive product and enkephalin were found. Therefore the rate limiting step in the liberation of radioactivity is cleavage at the Met-Thr bond. After electrophoresis the

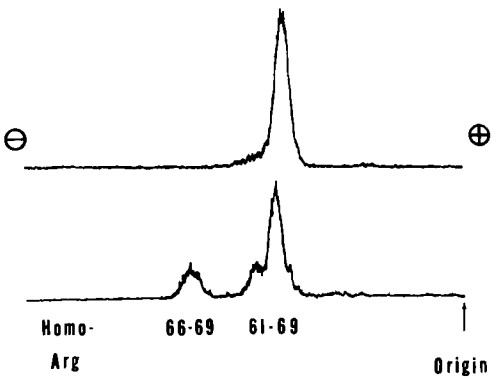

Fig. 2. Radioactivity after electrophroesis of an endopeptidase reaction at 0 time (upper) and 30 min (lower).

only radioactive product seen is the carboxyl terminal tetrapeptide fragment β-LPH 66-69 (Figure 2). Endopeptidase activity was determined in eight regions of the rat brain. The soluble activity was fairly evenly distributed throughout the brain but the membrane enzyme was relatively concentrated in the striatum, hippocampus and midbrain regions, which contain large amounts of neuropeptides. The membrane enzyme was purified since it may be more relevant to enkephalin biosynthesis than is the soluble activity.

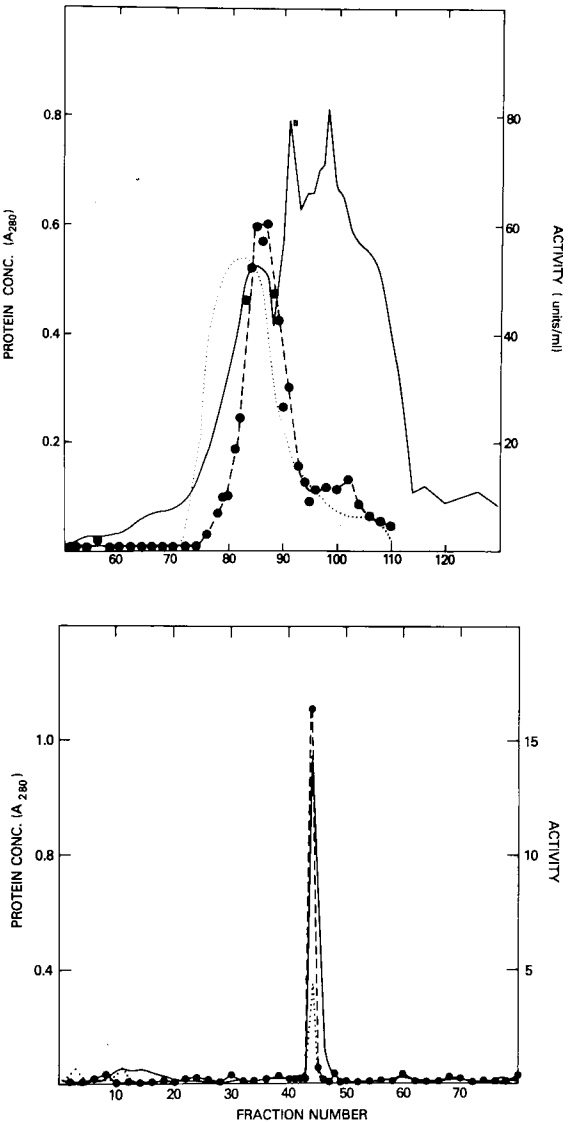

Fig. 3. Top: Chromatography of the 40-70% ammonium sulfate fraction on phenyl sepharose 4B with a gradient of decreasing NaCl concentration. Bottom: Chromatography of fractions 83-90 from the upper chromatogram on hydroxylapaptite with a KH_2PO_4 gradient. In both parts of the figure the solid line represents absorbance at 280 nm, endopeptidase activity is shown by the points and dashed line, and aminopeptidase activity is indicated by the dotted line.

Table I. Endopeptidase Purification Summary

Fraction	Total A_{280}	Total Activity pmol/min	Specific Activity pmol/min/A_{280}
Washed Membranes	1600	153000	96
Triton Extract	1060	256000	242
40-70% $(NH_4)_2SO_4$	180	102000	568
Phenyl Sepharose	13.5	40800	3020
Hydroxylapatite	2.5	37300	14900

The 1000 x g supernatant solution of a homogenate of 24 rat brains in 0.32 M sucrose, 0.01 M Tris pH 7.5 was sedimented at 100,000 x g for 30 minutes and washed once. The resuspended pellet was treated with 0.1% Triton X-100 for 30 minutes and recentrifuged. The solubilized proteins were fractionated by ammonium sulfate followed by chromatography on phenyl sepharose and subsequently on hydroxylapaptite. The activity emerged essentially in one fraction (Figure 3). As summarized in Table I, the activity has so far been purified 150-fold over the washed membranes, with 24% recovery. The purified endopetidase was highly active in generating enkephalin from β-LPH (61-69) as well as from γ-endorphin but much less so from β-endorphin. Apparently, β-endorphin is resistant to the action of a number of peptidases, perhaps for conformational reasons.[3]

Aminopeptidase activity copurifies with the endopeptidase in many steps. The enrichment of aminopeptidase in the most purified endopeptidase fraction is 67-fold. The purified aminopeptidase is inhibited by puromycin and therefore is not dipeptidyl peptidase of Swerts *et al.*[4]

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SYNTHETIC AND PHARMACOLOGICAL STUDIES WITH ENKEPHALIN ANALOGS IN RELATION TO STRUCTURAL FEATURES OF MORPHINE

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In sharp contrast to their counterparts in the morphinan or benzomorphan family whose receptor-bound conformation is defined by their rigid molecular framework, the pharmacological response to enkephalin may not reside in the native molecular shape but is realized only after adapting to the geometrical requirements of the binding site. A clearer insight regarding the final *bound* conformation may be gained by chemically restricting the conformational freedom of the essential pharmacophores. Based on the above consideration, the bridged enkephalin analogs shown in Figure 1 were synthesized and evaluated for analgesic activity *in vivo* and *in vitro*.

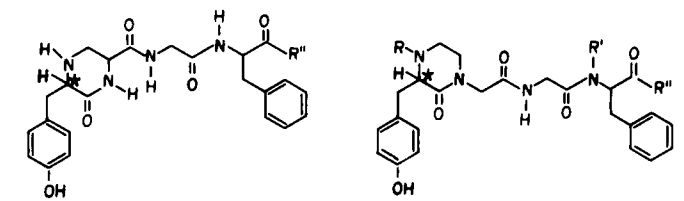

Fig. 1. Conformationally restricted analogs of enkephalin.

The orientation of the N-lone pair has been shown to be a determinant parameter for analgesic activity in morphinans[1,2]. Invariably, when it is oriented axial and into the plane of the aromatic ring the compounds lose their antinociceptive property. In contrast to the morphinans the most active of which are tertiary alkyl bases[3], enkephalin bears a primary

amine function with significant rotational freedom. Alkylation of the basic nitrogen may introduce some rotational barriers owing to unfavorable steric passing interactions. It follows that inversion, and the number of rotational conformers would be reduced such that the lone pair would either be optimized or misaligned. To ascertain whether the N-lone pair orientation effect manifests itself in opioid peptides, the *N*-methyl, *N,N*-dimethyl, and the trimethyl quaternary iodide salt of [Leu-OCH_3^5]-enkephalin were synthesized and evaluated for morphinomimetic activity *in vivo* and *in vitro* relative to the primary parent compound.

Results and Discussion

With the exception of 2-b (R″ = Nle R = R′ = H) none of the cyclic piperazinone analogs displayed any morphinomimetic activity (Mouse hot plate test ICV). Furthermore, 2-b was inactive in the rat tail-flick test and none of the compounds, including 2-b, displayed any affinity for opiate receptors in the binding assay based on [^{3}H]-naloxone displacement from rat brain nor were they active in the guinea pig ileum assay. Compound 2-b was about 3 times more potent than [Met^5]-enkephalin (MHP) and its action could only be reversed by very high concentration of naloxone suggesting that it is not mediated by the interaction with the μ receptor[4] for which morphine has high affinity.

The lack of analgesic activity and receptor affinity of *cis*-amido (1-a, 1-b) and *trans*-amido (2-a) piperazine peptides and the unique properties of 2-b have several implications regarding the binding mode of enkephalin to μ receptors. In 1-a and 2-a the mobility of the tyramine moiety is restricted in space and is perfectly superimposable upon that same fragment in morphine. Therefore, the results obtained suggest that the tyramine portion of tyrosine does not necessarily adopt an analogous receptor-bound conformation as that in morphine[5]. Furthermore, when the configuration about the designated carbon is inverted to D as in 1-b and 2-b the entire piperazinone ring mimics the corresponding piperidine counterpart of rigid opiates. Although the tyramine moiety appears to be an indispensable feature[6], our results do not endorse a common binding mode between the tyrosyl region of enkephalin and morphine and suggest that the basic nitrogens do not share a common binding locus.

That the N-lone pair orientation is an essential component of analgesic activity may be inferred from Table I. The receptor binding data reveal that whereas the desmethyl and *N*-methyl analogs are equipotent, the *N,N*-dimethyl and quaternary compounds show only 4% and .6%

affinity respectively, and this trend is closely paralleled in the guinea pig ileum assay. The low affinity of (6) is in agreement with values observed for *N*-methyl quaternary salts of other potent agonists[7]. If simple electrostatic forces were governing the interaction of the nitrogen atom with a complementary anionic receptor site then the receptor affinity would not be expected to be reduced drastically in passing from a monomethyl to a dimethyl group.

Table I. Relative Potencies of Methylated [LeuCH$_3$[5]]-Enkephalin Analogs.

R-Tyr-Gly-Gly-Phe-Leu-OCH$_3$[a]

	R	# MICE SHOWING ANALGESIA[b] # TESTED 2	4	6	8	10 min	GPI	OPIATE RECEPTOR
3	NH_2	0/6	1/6	0/6	0/6	0/6	100	100
4	H-N-CH_3[c]	1/6	3/6	0/6	0/6	0/6	218 ± 86	107 ± 23
5	$N(CH_3)_2$[c]	1/6	4/6	3/6	2/6	1/6	8.1 ± 3.2	4.0 ± 0.7
6	$^+N(CH_3)_3\,^-I$[c]	Inactive					0.2 ± 0.1	0.6 ± 0.3

[a] HCl salts, (3 = 100%) [b] 50 μg/mouse ICV [c] convulsions

This situation may very likely be simulated by a new class of narcotic analgesics in the tricyclic bridged aminotetralin series[8,9]. Similar to enkephalin these compounds are characterized by a primary amine function and optimum activity resides in the β unsubstituted nitrogen epimer. If a solvent-exposed orientation of the lone pair is critical for activity, then disubstitution forces it into an inverted position because the *N*-methyl substituents cannot be accommodated into the polyethylene bridge.

In contrast to the results obtained in the *in vitro* assays, the *N,N*-dimethyl analog displays the highest activity in the *in vivo* analgesic test. This finding could be due to the existence of multiple classes of opiate receptors[4] and demonstrates the necessity of testing enkephalin analogs in several different assay systems.

Acknowledgements

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A COMPARISON OF LOW ENERGY STRUCTURES OF ENKEPHALIN ANALOGS

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A study on the conformational preferences of a series of active enkephalin (ENK) analogs is made to elucidate a common structural feature which could help to explain their physiological activity. The molecules studied are: [Leu]ENK(I), [Met]ENK(II), [D-Ala^2, Met^5]-ENK amide(III), [D-Ala^2, Met^5]ENK(IV), [D-Met^2,L-Pro^5]ENK amide (V), [D-Ala^2, D-Phe^5]ENK(VI), [D-Ala^2, D-Leu^5]ENK(VII), [D-Ala^2, (N-Me)Phe^4, Met^5]ENK amide (VIII), and [D-Ala^2, (N-Me)Met^5]ENK amide(IX). Computational results are obtained from empirical energy calculations using ECEPP.[1,2] The nomenclature and conventions are those given by IUPAC-IUB.[3] Starting conformations were taken from studies on dipeptides,[4] N-methylated residues,[5] and enkephalins.[5-7]

Energy minimization on the above analogs resulted in three standard classifications of conformers, denoted E1, E2 and E3. It was found that the ϕ, ψ values of the first four residues of all the analogs belong to the following ϕ, ψ regions: ($\pm$,+; +,–; –,–; and –,+). The values of all the dihedral angles averaged over all the equivalent low-energy structures for all the analogs are given in Table I. The differences between E1, E2 and E3 appear in the backbone conformation around the fifth residue, that is, E1 has (ϕ_5,ψ_5) = (+,+), E2 (–,+) and E3 (–,–). In each case it was found that only certain values of the side-chains of the first and fourth residues were allowed, for a given set of ϕ_5, ψ_5 values.

In Table II the minimum energy values for E1, E2 and E3 and the global energy minimum are given. Figure 1 shows that for E1 the side-chains of residues 4 and 5 point in the same direction, while in both E2 and E3 the side-chains of the Tyr^1 and residue 5 point nearly parallel to one another, and Phe^4 points away from the backbone structure.

Those analogs with D-residues in position 5 (VI and VII) are expected to strongly prefer conformer type E1 since ϕ_5 and ψ_5 are both positive, and that preference is shown energetically in Table II. Note that some E1 conformers are of low energy even when the 5th position contains an L-residue. In the case of analog IX, E1 is the minimum energy

Table I. Average Conformers for Active Enkephalin Analogs[a]

Residue[b]		Dihedral Angles, deg E1	E2	E3	Residue[b]	Dihedral Angles, deg E1	E2	E3
Tyr[1]	ϕ_1	175	167	-172	R4 ϕ_4	-139	-134	-98
	ψ_1	151	158	159	ψ_4	142	73	160
	χ_1^1	180	60	-60	χ_4^1	180	-60	-60
R2	ϕ_2	80	81	86	R5 ϕ_5	55	-142	-63
	ψ_2	-84	-76	-91	ψ_5	63	138	-50
	χ_2^1	-60	-60	-60	χ_5^1	-60	180	180
Gly[3]	ϕ_3	-76	-163	-77				
	ψ_3	-53	-68	-64				

[a]All ω's are trans (for I to VII Δ is $\pm 10°$ and for VIII and IX, $\pm 20°$). For V: $\phi_5 = -75°$ and $\chi_2^1 = 60°$ in E1, E2 and E3. For VI and VII: (ϕ_5, $\psi_5 \simeq 154°$, 32°) in E2; $\chi_2^1 = 60°$ in E1 and E2. For IX: $\psi_4 = 100°$ in E1, $\psi_5 = 78°$ in E2, 71° in E3.

[b]R2 is Gly[2], D-Ala[2], D-Met[2]; R4 is Phe[4], (N-Me)Phe[4]; R5 is Leu[5], Met[5], Pro[5], D-Phe[5], D-Leu[5], (N-Me)Met[5].

Table II. Energies of Enkephalin Analogs in the Three Conformers

Analog	Conformer (Energy in Kcal/mol) E1	E2	E3	E_{min}[b]	Activity[a] %	Ref.
I	-6.5	-3.5	-4.7	-9.5[c]	119	8
II	-6.4	-3.9	-5.3	-9.7[c]	100	8
III	3.6	4.4	4.0	-1.2	528	9
IV	-5.4	-2.4	-4.3	-6.1	716	8
V	-6.5	-3.8	-8.0	-10.1	2,490	10
VI	-9.7	-3.0	-3.4	-9.7	522	9
VII	-7.8	1.7	-0.3	-7.8	684	9
VIII	18.5	13.7	17.8	12.0	$\sim 10^6$[d]	11
IX	13.1	20.2	17.4	13.1	711[e]	9

[a]Activities are taken relative to [Met]ENK as 100%.

[b]E_{min} corresponds to the global minimum energy for each analog.

[c]Recalculated from an earlier work.[7]

[d]The activity corresponds to [D-Ala[2], (N-Me)Phe[4], Met[5](O)]ENK-ol[11].

[e]Activity given for [D-Ala[2], (N-Me)Leu[5]]ENK-amide.

conformer. The case of analog VIII is unique, in that it is the only one in which type E2 is clearly preferred. In the super active form of VIII[11], [D-Ala[2], (N-Me)Phe[4],-Met[5](O)]ENK-ol, the C-terminal alcohol group (not calculated here) would be expected to decrease the energy of types E1 or E3. It is also interesting that the side-chain orientations of [Leu]ENK in the crystal structure[12] resemble the orientations found in E3, but the structures differ considerably in the backbone dihedral angles.

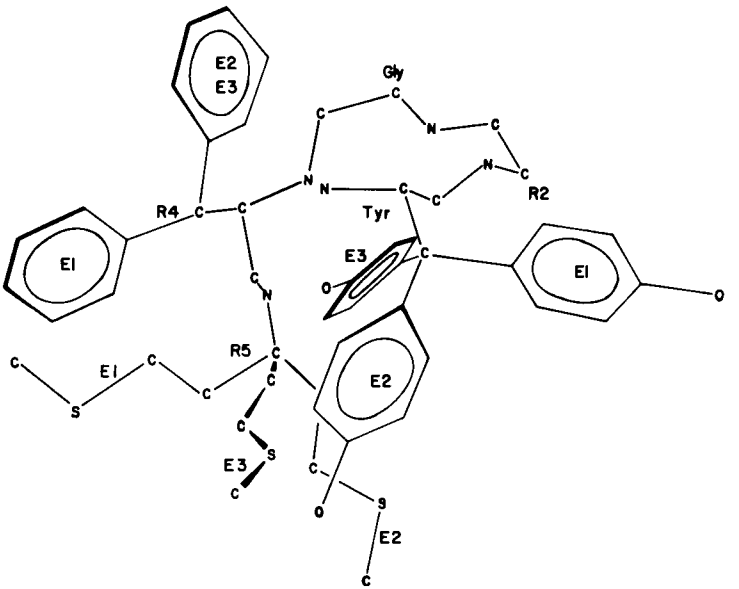

Fig. 1. Side-chain configurations in conformers E1, E2 and E3.

From this work we have shown that all the active analogs studied here can achieve equivalent configurations and still be of low energy. The precise structure recognized by the receptor will most probably be one of the three configurations found here. Further studies will attempt to delineate this structure.[5]

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IDENTIFICATION OF α-MELANOCTYE STIMULATING HORMONE IMMUNOREACTIVITY IN RAT AND HUMAN BRAIN AND CEREBROSPINAL FLUID

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Introduction

An extensive extrahypophyseal system of nerves containing α-melanocyte stimulating hormone (α-MSH), an acetyl tridecapeptide amide, has been described in the rat brain.[1,3] The presence of a regional distribution of this peptide combined with the fact that α-MSH administration produces potent central nervous system effects[4] suggests a neuromodulator or neurotransmitter role for α-MSH in the central nervous system. The present report describes the distribution of α-MSH-like immunoreactivity in the rat and human brain and identification of α-MSH immunoreactivity in cerebrospinal fluid.

Methods and Results

Identification and Characterization of α-MSH-like Immunoreactivity in Brain and Cerebrospinal Fluid (CSF). — The α-MSH-like immunoreactivity in the rat and human brain and rat CSF was characterized by combining high pressure liquid chromatography (HPLC) with radioimmunoassay. Immunoreactive material was extracted from brain tissue by boiling in 2N acetic acid. Lyophylates were resuspended in 40% acetonitrile in water and then chromatographed on a 4 mm x 30 cm column of μBondapak/C_{18} (Waters Associates, Milford, MA).

Radioimmunoassay of HPLC fractions demonstrates a non-homogeneity of the immunoreactive substance from extracts of whole brain and hypothalamus. Four peaks of immunoreactive material were

noted in each sample. Furthermore, as determined in retention times, the same four immunoreactive compounds were present in rat and human brain and rat CSF. The similarity of elution patterns of synthetic α-MSH with one immunoreactive peak of brain extracts is suggestive of chemical identity between this immunoreactive peak and synthetic α-MSH since this system is capable of resolving peptides from closely related analogues. The close temporal relationship of the retention times of the other peaks to the α-MSH peak combined with the fact that they are both recognized by the α-MSH antibody suggests that they are peptides of similar size and structure to α-MSH.

Distribution of α-MSH Immunoreactive Compounds in Rat and Human Brain. — The distribution of α-MSH immunoreactivity in rat and human brain was determined quantitatively by combining radioimmunoassay and microdissection techniques. The antibodies were raised against α-MSH conjugated to bovine serum albumin in our laboratory or generously provided by Drs. M.C. Tonon and H. Vaudry, Laboratorie d'Endocrinologie, Mont-Saint-Aignan, France. The post-mortem human specimens were obtained from: Human I-25 year old female, cause of death — cystic fibrosis, specimens obtained 15 hours post mortem; Human II — 33 year old male, melanoma, specimens obtained 14 hours post mortem. As Table I shows, there are significant and many similar concentrations of α-MSH immunoreactivity in the human and rat brain. The greatest concentrations of α-MSH appear to be in the periventricular nucleus of the thalamus and in the hypothalamic nuclei with particularly high concentrations in the median eminence and the arcuate nucleus, the site of α-MSH containing perikarya. The mesencephalic central gray has moderate α-MSH levels. Cortical and striatal regions have low α-MSH concentrations.

Quantitation of Immunoreactive α-MSH in Human and Rat CSF — In lumbar tap human CSF of a random group of patients at the National Institutes of Health, α-MSH immunoreactivity has been detected in concentrations ranging from 4.0 to 73.3 pg/ml with a mean of 22.9 pg/ml. Rat ventricular CSF was measured in an attempt to measure the *in vivo* release of neural α-MSH into the CSF. The lateral ventricle of chloralose-urethane anesthetized rats was cannulated and perfused with artificial CSF. The perfusate was collected from an indwelling cannula in the cisterna magna at 20 minute intervals. Approximately 180 pg/20 minute interval was detected in the perfusate by radioimmunoassay. Rats hypophysectomized for 2-3 weeks had CSF α-MSH concentrations similar to controls.

Table I. α-MSH Concentrations in Selected Regions of the Rat and Human Brain

REGION	α-MSH CONCENTRATION (pg/μg of PROTEIN ± SEM)		
	RAT	HUMAN	
		I	II
Cerebral Cortex	0.20 ± 0.09	0	0
Striatum			
Caudate N.	0.13 ± 0.03	-	-
Globus Pallidus	0.39 ± 0.15	-	.03
Septum			
N. accumbens	0.33 ± 0.05	1.46	-
L. Septal n.	1.29 ± 0.16	15.28	-
Hypothalamus + Preoptic area			
Medial Preoptic n.	3.75 ± 0.32	5.51	-
Lateral Preoptic n.	0.97 ± 0.06	0	-
Anterior hypothalamic n.	3.49 ± 0.55	3.75	1.61
Periventricular n.	7.19 ± 0.73	-	-
Paraventricular n.	5.75 ± 0.35	15.72	2.5
Supraoptic n.	1.95 ± 0.41	9.34	0.36
Arcuate n.	8.72 ± 1.33	6.97	1.63
Median eminence	11.02 ± 1.60	16.10	2.03
Ventromedial n.	3.23 ± 0.63	5.71	2.39
Dorsomedial n.	8.95 ± 0.65	5.95	1.7
Posterior hypothalamic	4.32 ± 0.56	3.92	2.76
Periventricular n. of thalamus	7.19 ± 0.73	20.24	0.42
Mesencephalic Central Gray	3.44 ± 0.67	0.42	0.21

Conclusion

These results describe the presence and distribution of α-MSH in human and rat brain and extend immunocytochemical findings.[1-3,5] Recent results indicate that α-MSH immunoreactivity in rat brain is derived primarily from the arcuate nucleus and not the pituitary gland.[3,6] As hypophysectomy was without effect on rat α-MSH CSF concentrations, much CSF α-MSH may be derived from neural release. Consistent with this suggestion is the finding of a potassium stimulated, calcium dependent α-MSH release from hypothalamic slices[7] or synaptosomes.[8] These findings support an α-MSH neuroregulatory role in rodent as well as human brain.

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SYNTHESIS, SPECTROSCOPIC AND BIOLOGICAL PROPERTIES OF DELTA-SLEEP INDUCING PEPTIDE AND ITS ANALOGS

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In recent years several attempts have been described to isolate and identify brain constituents that could act as humoral factors for the induction and maintenance of sleep.[1-3] Recently an endogenous linear nonapeptide (the delta sleep inducing peptide — DSIP) was found capable of enhancing the delta EEG slow wave sleep in rabbits after intracerebroventricular or intravenous injection.[4,5]

With the aim of launching a structure-function study of this peptide (1) we have carried out its synthesis and that of a number of its analogs (2-6) with the aid of solution methods. The present report gives the initial results of the study which is still in active progress.

The synthetic routes to these peptides are represented in Figure 1. Protected 1-4 and 5-9 segments were prepared by stepwise chain elongation and these were then coupled. The N-terminal amino group was protected by Boc or Z, and the serine and tyrosine side chains by formation of the benzyl ether, whereas aspartic and glutamic acid residues were protected in the form of benzyl esters. The amino acid derivatives were coupled by means of the activated ester or DCC/HOBT procedures shown in Figure 1. Coupling of the Tyr(Bzl) in the form of its p-nitrophenyl ester was catalyzed by HOBT. The protected 1-4 and 5-9 segments were joined using the mixed anhydride method. Coupling yields were 70% or higher.

The identity of the peptides was assessed by tlc on Merck silica gel plates, by elementary microanalysis, and by amino acid analysis. The protected nonapeptides were purified by HPLC on fractionated Sephadex LH-20 in dimethylformamide. After catalytic hydrogenation on Pd-black the resultant nonapeptides were run through a DEAE-Sephadex A-25 ion exchanger column in NH_4OAc buffers 0.1-0.3 M, pH

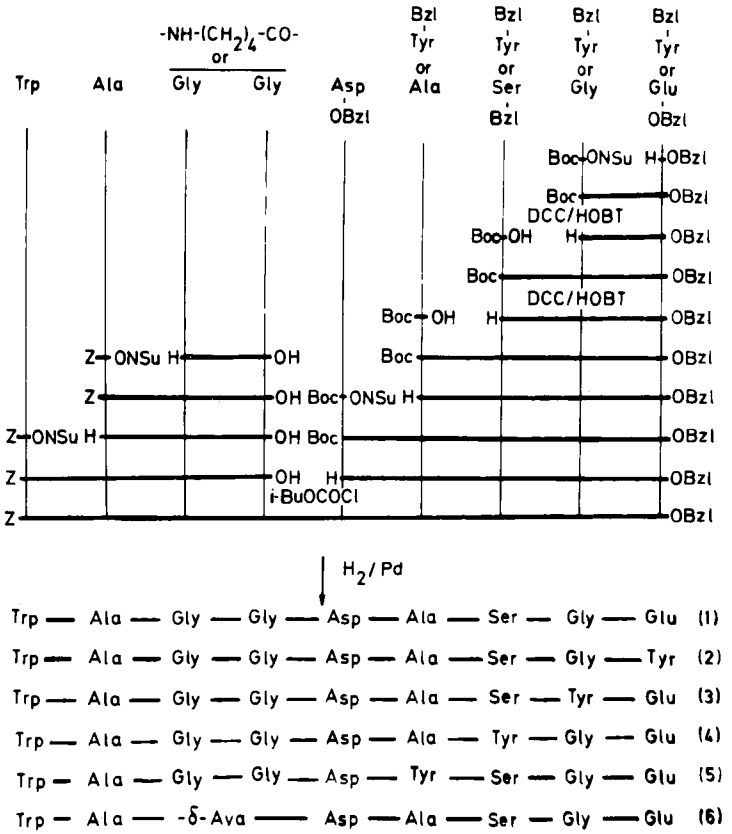


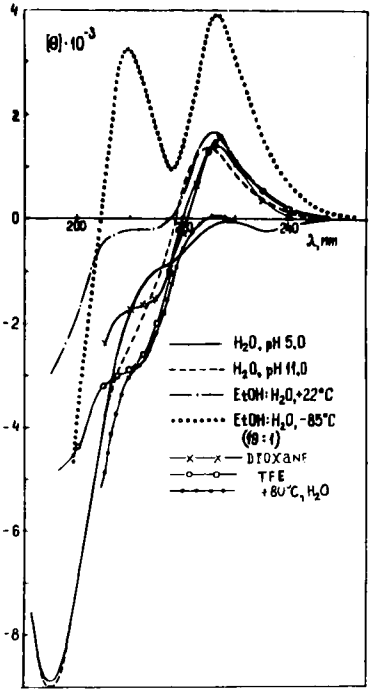
Fig. 1. Scheme for the synthesis of DSIP and its analogs.

6.7-7.6. Homogeneity was checked by tlc, electrophoresis, HPLC on a DC-6A resin, and amino acid analysis. Digestion of DSIP with leucine aminopeptidase yielded all the amino acids in their theoretical ratio, indicating their optical purity and the absence of contaminating products with β-Asp bond.

Biological testing of DSIP on rabbits was in full accord with the data of Monnier.[5] We also investigated the effect of DSIP in rats (after intraventricular administration). The peptide caused a 60% increase in overall sleep during the first hour after injection of a dose of 6 nmoles per kg weight.

The conformational properties of DSIP and its analogs have been investigated by the combined use of NMR, CD, fluorescence, and Laser-Raman techniques. Here we shall describe the results obtained mainly on DSIP and its Tyr^7-analog using NMR, CD, and fluorescence spectroscopic techniques.

The CD spectrum of DSIP in H_2O (Figure 2) displays a 250-310 nm aromatic $A_{1g} \rightarrow B_{2u}$ transition and two weak bands at 220-240 nm due to $A_{1g} \rightarrow B_{1u}$ and n-π^* transitions. A strong negative band at 195 nm is due to a π-π^* Cotton effect. The CD spectra of DSIP do not show any interpretable shifts with change in solution pH or solvent polarity. Cooling causes a drastic increase in the aromatic and n-π^* band intensities presumably due to diminished flexibility of the peptide backbone and of the indole side chain.



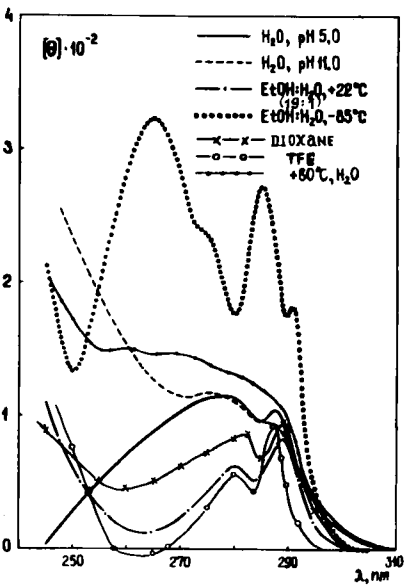


Fig. 2. CD spectra of DSIP

The CD spectra of Tyr^7-DSIP (not shown) are similar to those of DSIP, differences being attributable to the additional aromatic chromophore. The NH chemical shifts of DSIP in water at pH 4.66 show a strong temperature dependence ($\Delta\delta/\Delta T = (5\text{-}6)\cdot 10^{-3}$ ppm/°C), evidence of hydrated amide protons. The $^3J_{NH-C\alpha H}$ couplings range from 6.5 to 8.0 Hz which is typical of an equilibrium mixture of interconverting conformers. A striking feature of pH-δ plots from the PMR spectra of DSIP and the Tyr^7-DSIP in water recorded at various pH values (Figures 3 and 4) is the high sensitivity of the Ala-2 and Ser(Tyr)-7 NH chemical shifts to the ionic state of the carboxyls. The Ala-2 NH protons in both

cases are (Figures 3 and 4) sensitive to titration of the aspartic acid side chains (pK 3.9 for DSIP and 4.3 for Tyr^7-DSIP) and of the C-terminal carboxyls (pK 3.6 and 3.3, respectively). The Ser-7 and Tyr-7 NH protons are sensitive to titration of the aspartic acid β-COOH while the Ser and Tyr side chain signals do not shift in the same pH range. These data could be accounted for by assuming the presence of a turn at the 5-7 site. At the same time the titration data showed no indication of the proximity of the N and C termini: the indole CH signals do not shift with change in pH while the N_1H indole proton is only slightly sensitive to titration of the glutamic acid γ-COOH.

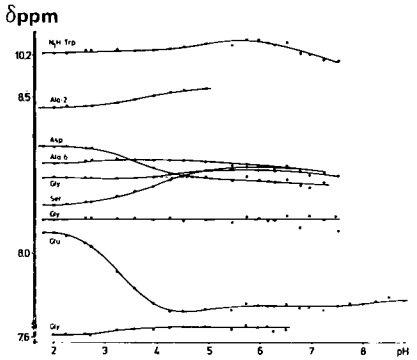


Fig. 3. pH Dependence of the chemical shifts of DSIP NH proton signals at 32°C, 300 MHz.

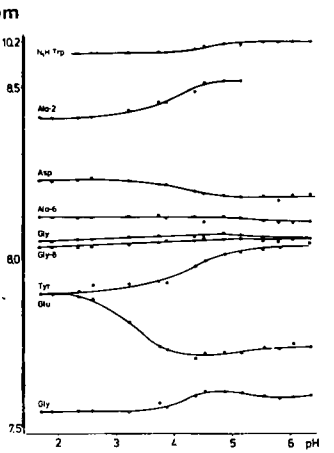


Fig. 4. pH Dependence of the chemical shifts of Tyr^7-DSIP NH proton signals at 32°C, 300 MHz.

For the Tyr-containing DSIP analogs the distances R_x between the fluorescent donor Tyr and the fluorescent acceptor Trp were estimated from the respective energy transfer measurements. In the case of Tyr^6-DSIP and Tyr^8-DSIP, R_x proved to be less than 12 A, while in Tyr^7-DSIP, it is 14±1 A. These results suggest a folded structure of DSIP and its analogs.

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DEGRADATION AND BINDING STUDIES OF SUBSTANCE P PEPTIDES USING RAT BRAIN FRACTIONS AND HUMAN PLASMA

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Introduction

Substance P (SP), a putative transmitter or modulator in CNS of mammals, found in brain, in various nerve fibers, in the intestinal tract, and in serum exhibits sialogogic, hypotensive, smooth muscle activity and depolarizing action in certain neurons. The physiological function has as yet not been clarified. It seems to be connected with discrimination processes in sensory (peptidergic) neurons. Degradation studies with organ fractions did not prove conclusive with regard to the physiological role of SP.

With high probability degradation by membrane bound proteases is involved in receptor interactions in the synaptic cleft. This may be the main reason for the difficulties encountered in receptor binding studies. Recently Nakata et al.[1] were able to separate binding to a synaptic fraction with K_D = 2.7 nM from nonspecific binding using ^{3}H-SP with 187 Ci/mmol. We directed our studies to assess the biological relevance of SP degradation.

SP-Peptides and 11-Norleucine-SP (full biological activity), Arg-Pro-Lys-Pro-Gln-Gln-(4-^{3}H-Phe)$_2$-Gly-Leu-Nle-NH_2, 27 Ci/mmol, were synthesized by Bienert et al.[2] Rat brain subfractions were prepared after Whittaker. P_2 is a crude synaptosomal fraction. Breakdown products from ll-Nle-SP were separated by polyacrylamide gel electrophoresis and detected by LSC of gel sections. For details see Berger et al.[3]

Results

a) Degradation of 11-Nle-SP by P_2 and human plasma correlated directly with the loss of activity on guinea pig ileum.[3]

b) Degradation velocity increased linearly with protein content of P_2 but not with increasing plasma concentration.

c) From 10^{-10}M to 10^{-5}M 11-Nle-SP only one K_M was detected: for P_2: 3.6×10^{-5}, for diluted plasma (20%): 8×10^{-6}. Below 10^{-7}M the half life time at 37°C was 9.3 min for P_2 (1 mg protein/ml) and 24 min for undiluted plasma in vitro (Figure 1). Degradation velocity at 25°C was 40% and at 0°C 4% of the 37°-value, pH optimum was 7-8 for P_2, and 7.5 for plasma.

d) Both enzyme systems were inhibited by o-phenanthroline (K_I = 0.1mM), bacitracin (K_I = 0.11 mg/ml), EDTA: 1mM ca. 50%, dithiothreitol: 5mM ca. 50%, and only weakly by aprotinin. Degradation was inhibited also by SP and 11-Nle-SP (to the same degree), bradykinin and angiotensin II, by N-terminal SP-tetrapeptide, and depended on the length of C-terminal SP-sequences (Figure 2). X-Pro dipeptidyl aminopeptidase substrates gave no inhibition.

e) The proteolytic activity of P_2 could not be removed or decreased by washing and is an intrinsic one.

f) ^{3}H-Phenylalanine was formed very quickly as the main degradation product to be displaced by Phe and other hydrophobic amino acids. Proteolytic and radiolytic degradation products proved to be disturbing factors in SP binding studies.

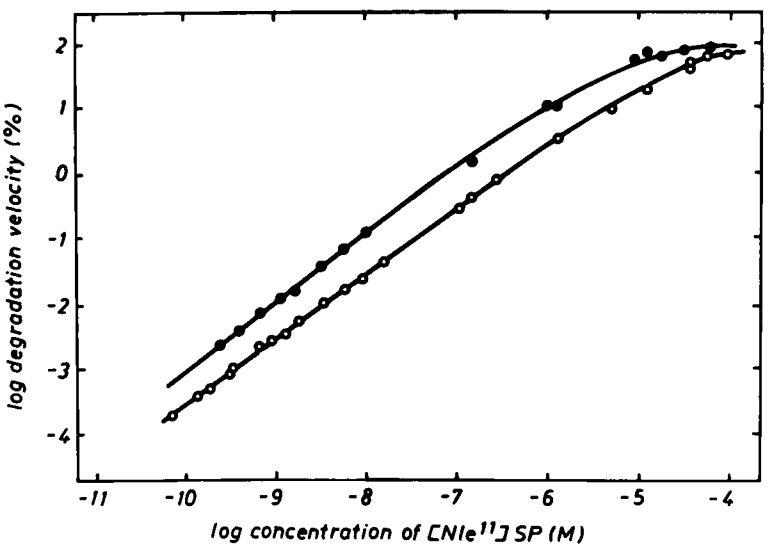

Fig. 1. Dependence of degradation velocity of 11-Nle-SP on peptide concentration, 37°C, in percent of maximum activity. P_2-Fraction, 0.2-0.5 mg protein/ml. Human plasma, 20% (v/v).

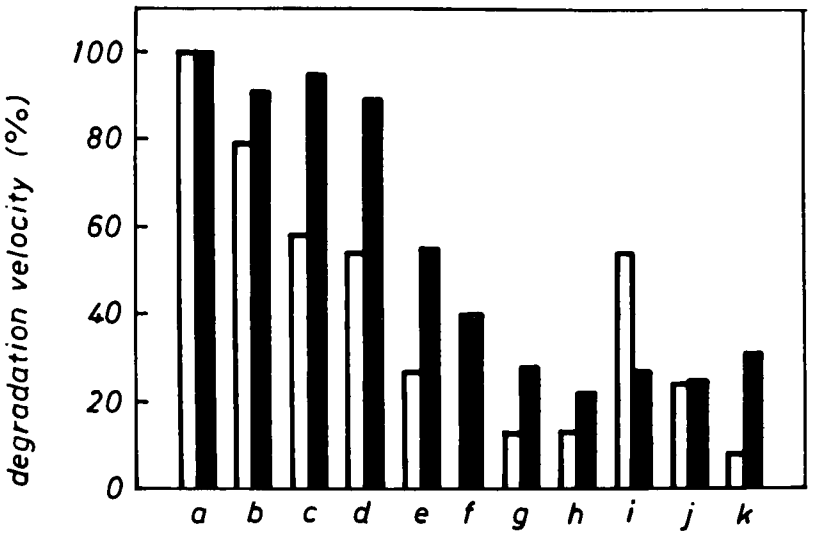

Fig. 2. Influence of peptides, 8.9×10^{-5}M, on ^{3}H-Nle-SP degradation, 2.3×10^{-8}, 37°C. P_2, 0.5 mg protein/ml, pH 7.25. Human plasma, 20%, pH 7.4, a) control, b) SP 10-11, c) SP 8-11, d) SP 6-11, e) SP 3-11, f) SP 2-11, g) SP, h) 11-Nle-SP, i) SP 1-4, j) bradykinin, k) angiotensin II.

g) Uptake of 11-Nle-SP (2×10^{-9}M) by P_2 in the presence of o-phenanthroline and serum albumin at 0°C was diminished only to one half of the 37°-value and increased in the absence of Na^+ ions.

h) With an 11-Nle-SP concentration of 10^{-9}M a specific displacement of binding to P_2 by a SP excess was visible (Figure 3). Plasma did not reveal any binding properties.

Conclusions

It is possible to get biologically relevant information from in vitro peptide degradation systems, also at higher concentrations, when a constant rate has been proved down to the physiological concentration range and when biologically estimated inactivation is in good correlation (Figures 1 and 2).

Enzymatic degradation is likely to be responsible for terminating biological action of SP in the CNS (synaptic cleft) as a part of regulation. No specific uptake of the peptide into synaptosomal brain fractions was observed (due to low temperature dependence and increase of binding without Na^+ ions).

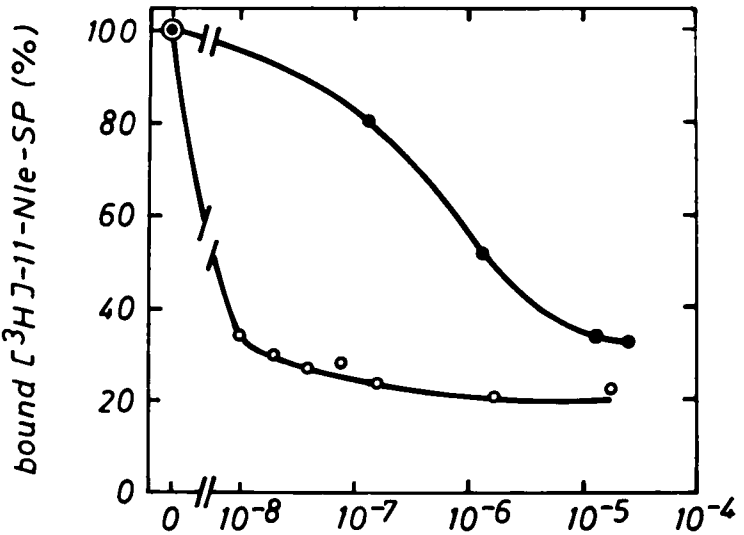

Fig. 3. Displacement by 11-Nle-SP of ^{3}H-Nle-SP binding to P_2(●): 1.4×10^{-8} (O): 1.5×10^{-9}, 1.6 mg protein/ml, 20 min., 0°C. 100% means saturation. 6.8% (●) and 10.9% (O) were bound from peptide in supernatant. Absisse: conc. of unlabelled 11-Nle-SP.

A similarity to peptidase systems described by Benuck and Marks[4] and Lee et al.[5] is obvious.

The essential component of the SP-degrading system in plasma and P_2 is a neutral metallopeptidase. The basic N-terminal sequence of SP was found to be important for inactivation, probably by enzyme binding, whereas the C-terminus is responsible for action.

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NOVEL TRH ANALOGS WITH INCREASED NEUROPHARMACOLOGICAL ACTIVITY

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Introduction

The neuropharmacological (CNS) effects[1] of TRH and the observation that it may have antidepressant activity in man[2] has attracted much interest. However, commonly used modifications for investigating structure-function relationships in peptides (incorporation of D-amino acids or N-methylation of amide linkages) have been shown to be of limited applicability in the case of TRH. In this communication we describe the synthesis and biological evaluation of a novel series of analogs in which the C-terminal region of the tripeptide is stabilized by the introduction of methyl substituents adjacent to the proline carboxamide group.

Chemistry

Analogs were synthesized by the route exemplified in Scheme 1. The use of N-hydroxy-5-norbornene-2,3-dicarboximide (HONBI), introduced by Hatanaka *et al*[3], gave improved yields at steps (i) and (iv). After deprotection, the analogs were purified by ion-exchange chromatography on S.P. Sephadex and gave satisfactory elemental and amino-acid analysis and nmr spectra. L-*trans*-3-Methylproline (Mep), was synthesized from L-isoleucine.[4] L-3,3-Dimethylproline (Dmp) was synthesized by an adaptation of the proline synthesis of Cox *et al.*[5]; the amino acid was resolved as its amide by crystallization of the D(+) tartrate salt.

Pharmacological Methods

(a) Reversal of reserpine-induced hypothermia: the assay was carried out in mice as described in Askew.[8] The mean area under the 4 hour temperature-rise/time curve was calculated for each dose level and

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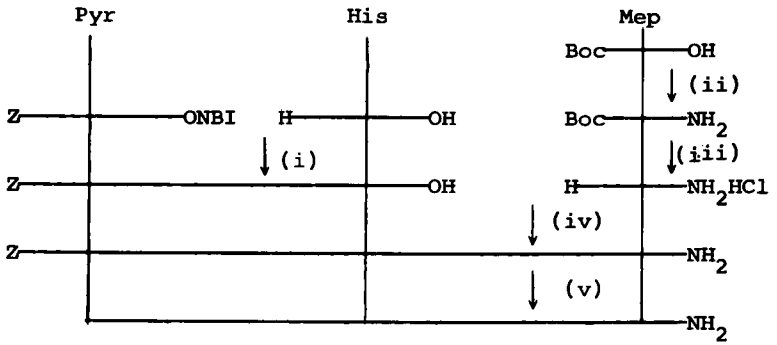

Reagents: (i) Salt coupling, $NaHCO_3$; (ii) isobutyl chloroformate ammonia; (iii) HCl in ethyl acetate; (iv) DCCI/HONBI; (v) H_2/10% Pd on C.

Scheme 1. Synthesis of TRH-Analogs.

the potency relative to TRH calculated using a parallel line assay. The minimum effective dose (med) was defined as that dose giving a statistically significant temperature rise relative to control.

(b) Rabbit hyperthermia test: the assay was based on the method described by Horita and Carino.[9] Potency ratios were calculated as in (a) using the area under the two hour temperature rise/time curve. The med was defined as the minimum dose to cause a 1° rise in temperature within 60 minutes.

(c) Endocrinology: Male Sprague-Dawley rats (160-180 g) were used. Test compounds were administered intravenously five minutes prior to blood collection in rats which had been treated subcutaneously for four days with β-estradiol-3-benzoate in corn oil vehicle.[7] Blood samples were obtained by cardiac puncture and plasma separated by centrifugation (750 g x 20 min. at 4°C). Blood samples were stored at -20°C until assayed using NIAMDD rat prolactin and TSH radioimmunoassay kits.

Discussion

We have found that incorporation of a methyl group adjacent to the C-terminal carboxamide function of TRH yields an analog with increased CNS potency and greater stability to plasma enzymes.[6] The CNS potency of this "stabilized" TRH analog (Table I, 2) could be further increased by methylation of the histidine τ-nitrogen (Table I, 3) or by alkylation of the C-terminal carboxamide (Table I, 4). The most dramatic increase was affected by further substitution on the proline ring;

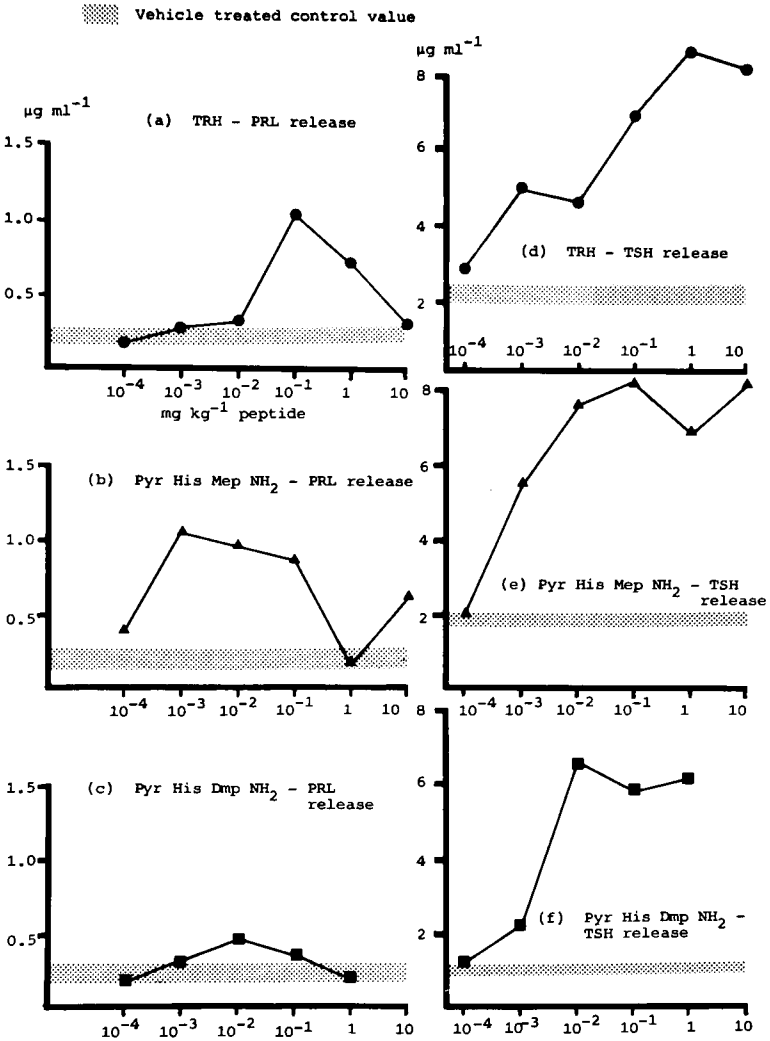

Fig. 1. Endocrinological evaluation of TRH analogues

thus, the dimethyl analog (Table I, 5) was up to 200 times more potent than TRH in neuropharmacological assays. In contrast to their CNS effects, TRH and analogs (2) and (5) appeared to evoke release of TSH at similar dose levels (Figure 1d,e,f). The effect on PRL release was more complex with all three peptides giving rise to bell-shaped dose-response relationships. Whilst the Mep analog (2) evoked maximal release of PRL at doses 10-30 times less than TRH (Figure 1a, b) the Dmp analog (5) evoked a small but significant PRL release only at 10 $\mu g\ kg^{-1}$ (Figure 1c).

Table I. CNS Pharmacology of TRH Analogs.

Structure	Reserpine reversal test (mouse)		Rabbit hyperthermia test	
	m.e.d. $^{-1}$ mg kg i.v.	p.r. vs TRH	m.e.d. µg i.c.v.	p.r. vs TRH
1. TRH	1	-	10	-
2. Pyr-His-MepNH$_2$	0.3	4	0.1	12
3. Pyr-MeHis-MepNH$_2$	0.1	32	<0.1	N.C.
4. Pyr-His-MepNHcyclohexyl	0.1	22	0.1	71
5. Pyr-His-Dmp-NH$_2$	0.03	50	0.003	199

MeHis = τ-methyl-L-histidine; Mep = L-*trans*-3-methylproline; Dmp = L-3,3-dimethylproline.

Thus the incorporation of methyl groups adjacent to the C-terminal carboxamide group of TRH yields a series of analogs with differing biological profiles. In particular, the Dmp analog (5) has greatly increased neuropharmacological potency and a reduced ability to evoke release of prolactin.

Acknowledgements

It is a pleasure to acknowledge the contribution of Mr. K.P. Guest, Mr. A. Lynn, Mr. C.R. Moon, and Mr. T. Watson to this work.

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SYNTHESIS OF <AAD-HIS-(4R,5R)-5-METHYL-TZL-NH_2, A TRH ANALOG WITH HIGH CNS ACTIVITY

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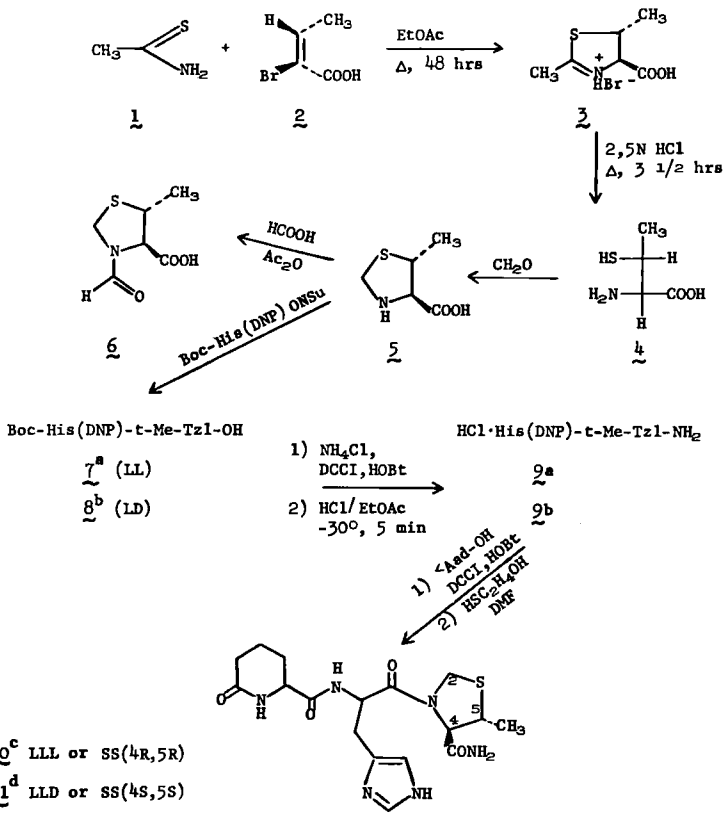
Selectively enhanced CNS activities have been reported for the TRH analogs <Aad-His-Tzl-NH_2(I) and <Aad-His-Pro-NH_2,[1] as well as for other TRH analogs containing β-alkylated prolines.[2] For example, analog I was 10-100 times more potent than TRH in reversing pentobarbital induced hypothermia.[3] Relative to TRH, all of these analogs have small changes of steric, lipophilic, and electronic nature. The β-alkyl substitution of proline[2] might also reduce susceptibility of a peptide to the action of peptidases.[4] These observations and our interest in TRH analogs with novel biological properties resulted in the synthesis of L-<Aad-L-His-L-trans-5-methylthiazolidine-4-carboxamide (10), a β-methylated analog of I. Analog 10 shows high CNS activity. We describe herein the synthesis of this TRH analog and the diastereomeric tripeptide 11 having the D configuration at the carboxyterminal amino acid.

The required carboxy terminal amino acid, trans-5-methylthiazolidine-4-carboxylic acid (t-Me-Tzl) (5), was synthesized stereoselectively as shown in Scheme I utilizing a procedure for 4 previously reported for the synthesis of a series of N-alkylated cysteine derivatives.[5] Condensation of cis α-bromocrotonic acid[6] (2) with thioacetamide (1), acid hydrolysis of the resultant thiazolinium salt 3 to β-methylcysteine (4) and subsequent reaction with formaldehyde resulted in the formation of racemic 5. The trans relationship of C-4 and C-5 hydrogens was confirmed by ^1H and ^{13}C NMR comparisons of the N-formylated derivative 6 with known literature data.[7] The merit of this method for the synthesis of β-alkylcysteines compared to other methods[8] lies in its shortness, stereoselectivity, and potential versatility with respect to alkyl substituents in the β-position.

Conversion of DL-5 into the diasteriomeric tripeptides <Aad-His-trans-β-methyl-Tzl-NH_2 (10 and 11) was accomplished by stepwise synthesis in solution as outlined in Scheme I. Reaction of 5 with Boc-

His(DNP)-hydroxysuccinimide ester gave the diasteriomeric dipeptide acids 7 and 8 which were separated by silica gel chromatography (silica gel 60) using 80-20-2 ($CHCl_3$-MeOH-conc. NH_4OH) as eluant. Conversion of 7 and 8 to the blocked dipeptide amides with NH_4Cl using DCCI/HOBt activation,[1] and removal of the t-butyloxycarbonyl group with saturated HCl in EtOAc at -30° gave 9 in high purity. Low temperature deblocking conditions and short reaction time (5 min.) were required to minimize side reactions of the acylated thiazolidine derivative. Coupling of 9a and 9b with <Aad-OH in CH_2Cl_2 using DCCI/HOBt activation and removal of the histidine blocking group gave the diasteriomeric tripeptide products 10 (LLL) and 11 (LLD), respectively. The yield of isolated product for the 4 steps of converting dipeptide acid 7 to 10 was 64%.



Scheme 1.

[a] $[\alpha]_D^{25}$ -21.3° (c, 0.3, MeOH); R_f 0.29[e]. [b] $[\alpha]_D^{25}$ +11.9°, (c, 0.3, MeOH); R_f 0.35[e]. [c] $[\alpha]_D^{25}$ -93.2 (c, 0.08, 50% HOAc); R_f 0.64[f], 0.34[g]. [d] $[\alpha]_D^{25}$ +17.4 (c, 0.07, 50% HOAc); R_f 0.68[f], 0.34[g]. [e] Silica gel, 80-20-2 ($CHCl_3$-MeOH-NH_4OH). [f] Silica gel, 70-30-3 ($CHCl_3$-MeOH-NH_4OH). [g] Silica gel, 70-30-3 ($CHCl_3$-MeOH-H_2O).

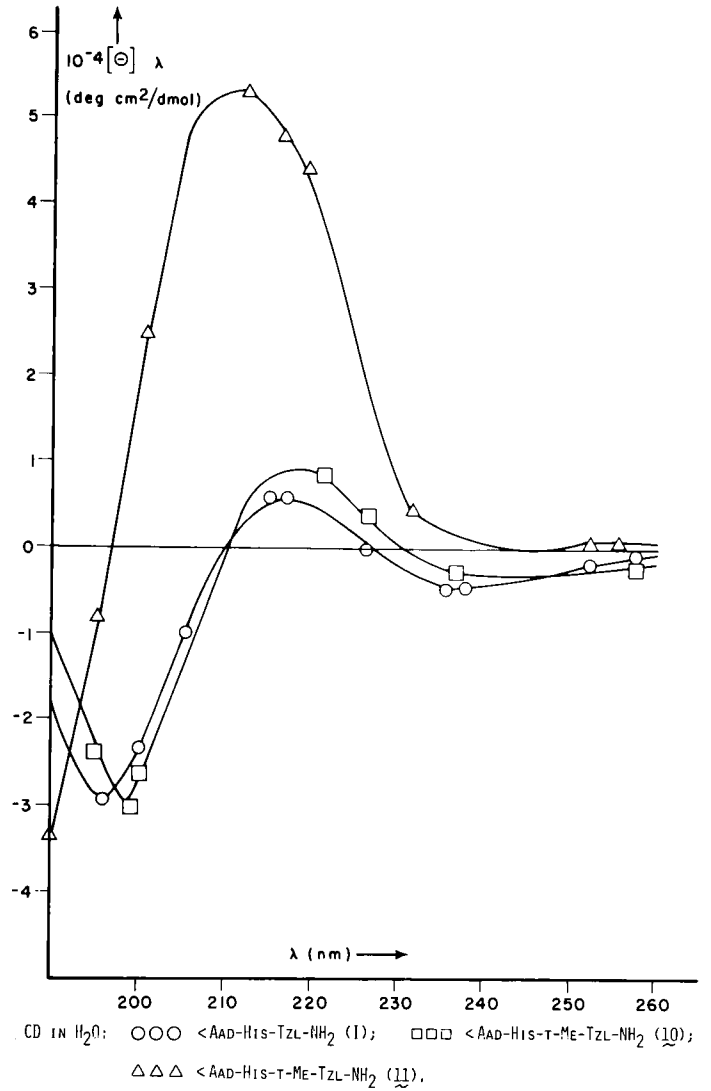


Fig. 1. CD-Spectra of TRH-Analogs.

Configurational assignments with respect to the carboxy terminal amino acid were made by CD (Figure 1) and NMR measurements through comparison with the previously prepared L-<Aad-L-His-L-Tzl-NH_2(I). The CD spectrum of 10 in H_2O resembled that of I more closely than did the spectrum of 11, and analog 10 was therefore assigned the all

"L" configuration. ^{1}H NMR spectra of 10 in DMSO-d_6 showed characteristic structural similarities to TRH and I, such as the nonequivalence of the His-β-carbon protons, nonequivalence of the t-Me-Tzl C-2 protons, and nonequivalence of the primary amide protons.[9] Chemical shifts were at 2.85 and 3.15 (His β-CH_2), 4.3 and 5.1 (t-Me-Tzl δ-CH_2), and 7.2 and 8.25 (CO-NH_2) ppm downfield from TMS. On the contrary, 11 showed more equivalent geminal proton chemical shifts at 2.86 and 2.94 (His β-CH_2), 4.64 and 4.69 (t-Me-Tzl δ-CH_2), and 7.0 and 7.37 (CO-NH_2) ppm downfield from TMS.

Analog 10 reverses the hypothermic action of chlorpromazine demonstrating a potency nearly as great as that observed[10] for I. The diasteriomeric peptide 11 did not show significant activity at the highest doses tested.

These studies demonstrate a practical method for the stereoselective synthesis of β-alkylthiazolidine carboxylic acids and establish their application in peptide synthesis. CD and NMR are useful methods for configurational assignments of the new alkylated products through reference to the corresponding unsubstituted peptide.

Acknowledgements

We are indebted to Dr. Byron Arison for help in obtaining and interpreting NMR spectra, to Dr. William Randall for help with the CD spectra and to Dr. George Yarbrough for biological evaluation of the analogs.

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LOCALIZATION OF SPECIFIC HIGH-AFFINITY BINDING OF VASOACTIVE INTESTINAL POLYPEPTIDE (VIP) IN RAT BRAIN

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Introduction

Vasoactive intestinal polypeptide (VIP) has been localized immunohistochemically and by radioimmunoassay in the mammalian central nervous system.[1-4] Recently, the existence of specific VIP receptors in rat brain was demonstrated in our laboratory.[5] In this we paper report our investigation of the subcellular localization of binding and preliminary results of lesion studies on the subregional distribution of binding. These findings further support a possible role of VIP in neurotransmission.

Materials and Methods

Natural porcine VIP was obtained from V. Mutt (Stockholm, Sweden) and the synthetic peptide from J.-K. Chang (Peninsula Labs, San Carlos, CA). Peptides were iodinated with chloramine-T.[5] Unilateral brain lesions were made electrolytically in the rostral portion of the thalamus. The loci and size of all lesions were comparable. Cortical layers were removed from sagittal sections by hand under a light microscope (Herkenham *et al.,* in preparation). Washed tissue homogenates and subcellular fractions were prepared as previously described.[5] The binding assay involving separation of bound from free ^{125}I-VIP by centrifugation has been described.[5]

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Results and Discussion

Subcellular Distribution of VIP Receptor. — Table I shows that the greatest amount of binding activity was found in the crude mitochondrial fraction and that slightly less was found in the nuclear pellet. Subfractionation of the P_2 pellet revealed over two-thirds of the activity in the synaptosomal fraction.

The subcellular localization of ^{125}I-VIP binding to synaptosomes within the crude mitochondrial fraction is consistent with the possible association of receptors with membranes derived from nerve terminals. High levels of VIP-stimulable adenylate cyclase are found in synaptosomal fractions of guinea-pig brain.[6]

Table I. Subcellular Localization of Specific ^{125}I-VIP Binding in Rat Brain.

Subcellular Fraction	Percent of Total Recovered Binding Activity
Nuclear (P_1)	43.3 ± 4.2
Mitochondrial (P_2)	53.4 ± 3.3
Myelin (A)	5.5 ± 1.9
Synaptosomes (B)	69.7 ± 12.3
Mitochondria (C)	24.8 ± 9.9
Soluble (S_2)	3.3 ± 2.0

Results are means ± SEM from eight different preparations.

Subregional Distribution of VIP Binding and Effects of Thalamic Lesion. — In view of reports relating VIP with arousal[8] and the high levels of both VIP receptors[5] and peptide in the cortex,[1-4,7] making possible a role for VIP in higher nervous processes, we investigated the binding of VIP in cellular layers from the cerebral cortex. Layers I and VI possessed more VIP binding sites than layers II-IV or V (Table II) while there was no difference at benzodiazepine or bombesin binding sites (Herkenham *et al.*, in preparation). Compared to whole brain, all cortical layers were enriched in binding for both ^{125}I-VIP and ^{3}H-flunitrazepam. White matter showed the least binding for both ligands.

Table II. Specific ^{125}I-VIP Binding in Cell Layers of Rat Cerebral Cortex.

Layer	Specific ^{125}I-VIP Binding	Specific ^{3}H-Flunitrazepam Binding*
I	82.8 ± 27.2 (5)	228 ± 23 (3)
II-IV	47.5 ± 19.0 (5)	244 ± 14 (3)
V	41.3 ± 13.6 (5)	226 ± 11 (3)
VI	57.8 ± 21.3 (5)	198 ± 4 (3)
White matter	11.7 ± 1.7 (4)	49 ± 3 (3)
Whole brain	39.3 ± 6.4 (3)	143 (1)

Values are mean fmol/mg protein ± SEM (number of preparations) with 1 nM ^{125}I-VIP or with 0.3 nM ^{3}H-flunitrazepam.
*Data from Herkenham *et al.* (in preparation).

Fuxe *et al.*[2] have shown a concentration of VIP-immunoreactive cell bodies in layers II-IV of the rat neocortex. This finding is not necessarily in contradiction to ours concerning the localization of VIP receptors, considering that layer I is devoid of cell bodies and consists of dendrites, axons, and terminals.[9] Thus, the high concentration of receptors in layers I and VI may represent terminal fields for intrinsic cortical neurons in layers II-IV, which are known to ascend and descend to other layers.[9] The alternative possibility, that the receptors in layers I and VI were postsynaptic to thalamo-cortical projections demonstrated to terminate there[10] was investigated by making lesions in the source nuclei within the thalamus on one side of the brain.

Table III. Specific ^{125}I-VIP Binding in Cortical Layers Following Unilateral Thalamic Lesions.

Layer*	Unlesioned Side	Lesioned Side
I + VI	6.3 ± 1.0	8.3 ± 1.6^{+}
II - V	5.9 ± 0.6	6.3 ± 0.8

Values are mean fmol/mg protein ± SEM for five animals.
*Layers in each animal were pooled.
$^{+}P<0.05$ vs unlesioned side (paired Student's t-test, D = 2.0 ± 0.7).

By two weeks after thalamic lesion, pools of cortical layers I and VI from the lesioned sides of the brain showed a significantly greater amount of VIP binding than similar pools from the unlesioned sides (Table III). No such effect was observed in layers II-V nor in any layer at two days post-lesion (not shown). This apparent VIP receptor supersensitivity may be due to direct denervation supersensitivity. A second explanation is that deprivation of thalamo-cortical inputs to intrinsic VIP neurons was responsible for the observed supersensitivity. The behavioral significance of these observations is currently unknown.

Acknowledgement

D.P.T. was supported by an ADAMHA National Research Service Award (DA-05101).

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SPECIFIC CLEAVAGE OF β-LPH, ACTH AND PRO-OPIOMELANOCORTIN BY TONIN

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Introduction

Recently, tonin, an enzyme capable of cleaving at pH optimum of 6.8 angiotensinogen and angiotensin I to angiotensin II, has been purified from rat submaxillary glands.[1] The sequence of the first 40 NH_2-terminal residues was determined.[2] This serine protease is capable of cleaving β-LPH at pH 6.8 into β-LPH 1-50, 1-51, 51-60, 52-60, 79-91 and the opioid fragment 61-78.[3] The aim of the present study is to investigate in greater detail its cleavage pattern on substrates such as β-endorphin, Met-enkephalin, β-LPH, ACTH, and the 31,000 dalton pro-opiomelanocortin.[4]

Results

Effect of pH and Time of Incubation — The release of the opiate-like peptide β-LPH (61-78) can be monitored by a specific opiate radioreceptor assay, involving the displacement of ^{3}H-naloxone from rat brain homogenate opiate receptor preparations.[3] From Figure 1, it is apparent that 24 hour digestions at pH 5 was optimal for the release of opiate-like peptides.

Effect of Tonin pH 5 on Met-Enkephalin, β-Endorphin and β-LPH — Both ovine β-endorphin and Met-enkephalin were shown by high performance liquid chromatography (HPLC) and by opiate radioreceptor assay[3] to be resistant to tonin attack, whereas ovine-β-LPH was cleaved into peptides binding to opiate receptors (Figure 2). The resistance of both β-endorphin and Met-enkephalin to tonin cleavage was found to be true for pH varying between 4 and 8.5 (data not shown). Using a combination of Sephadex G-50 and HPLC on a Waters μ-CN column (0.38 x 30 cm) eluted with a linear gradient of CH_3CN with 0.02 M triethylamine phosphate pH 3 aqueous buffer, it was possible to show

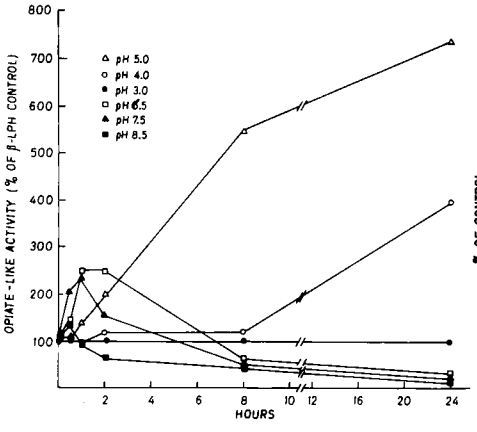

Fig. 1. Effect of pH and time of incubation of tonin release of opiate-like peptides from β-LPH using 1:100 tonin: β-LPH ratio.

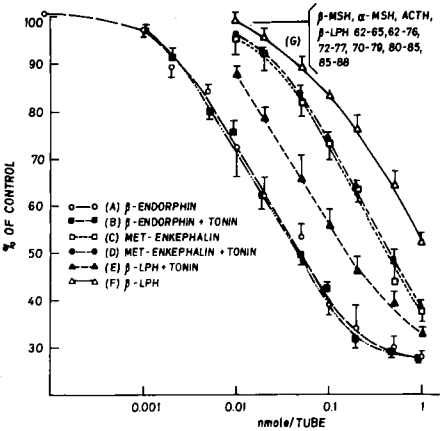

Fig. 2. Opiate radioreceptor assay of tonin effect at pH 5 on Met-enkephalin, β-endorphin and β-LPH.

after amino acid analysis that at pH 5, tonin cleaves β-LPH into β-LPH 1-23, 24-51, 52-78, and a major opioid 60-91 which has an Arg residue NH_2-terminal to the β-endorphin segment. These results clearly point out the importance of the three dimensional structure of the substrate in directing the site of cleavage by tonin.

Comparison of Tonin Cleavage of ACTH at pH 5 and 6.8: — Figure 3 depicts the HPLC separation of the resulting peptides from tonin digestion at pH 5 and 6.8. From Figure 3A, it is seen that ACTH is

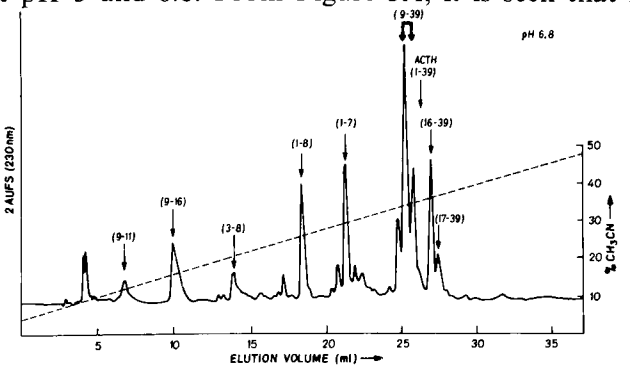

Fig. 3A. Tonin cleavage of ACTH at pH 6.8

cleaved at pH 6.8 at specific Arg 8, Lys 15,16, Phe 7, and Tyr 2 residues. The peptides ACTH 17-39 and 16-39 represent the peptide 18-39 known as CLIP[5] with one or two Lys residues at its amino terminus. Such CLIP peptide was found to be abundant in the intermediary lobe of the

pituitary[5] and quantitative changes in its relative amounts seem to occur during fetal development.[6] In contrast at pH 5 (Figure 3B) tonin cleaves ACTH only between Arg 8 - Trp 9.

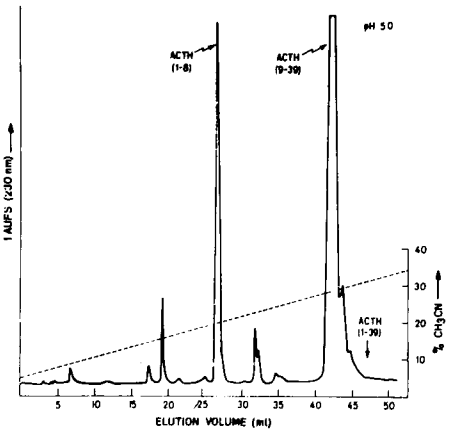

Fig. 3B. Tonin cleavage of ACTH at pH 5

Tonin cleavage of pro-Opiomelanocortin at pH 5 — When rat pituitary intermediary lobe cells are incubated with ^{35}S-Met for 20 minutes, and the cells extracted with 5M acetic acid, a 31,000 dalton molecule containing both ACTH and β-LPH, known as pro-opiomelanocortin can be isolated in pure form.[4,7] This molecule is known to contain two Met residues, one at position 4 of ACTH and the other at position 65 of β-LPH.[7,8] The cleavage of such a model molecule, by tonin, is shown in Figure 4. Control experiments showed that the precursor is stable under the conditions of the incubation, and that trypsin at pH 5, only releases the expected β-LPH 61-69 and ACTH 1-8 peptides.

Preliminary information on the nature of the peptides released by tonin was obtained both by tryptic peptide mapping and micro-sequencing.[7-9] In Figure 4, fraction 18-29 contained ACTH 1-8 and fraction 57-63, 73-77, 79-85, and 89-91 yielded (i) C-terminal fragments of β-LPH starting at residues 52 and 53, based on microsequencing (Met 13 and 14) and tryptic digestion (β-LPH 61-69). (ii) N-terminal ACTH related peptides (Met 4 by sequence and ACTH 1-8 by tryptic digestion). One of them coelutes with ACTH 1-39 in HPLC (Figure 4). The power of such a partial microsequencing approach for protein identification has recently been recognized.[8-10]

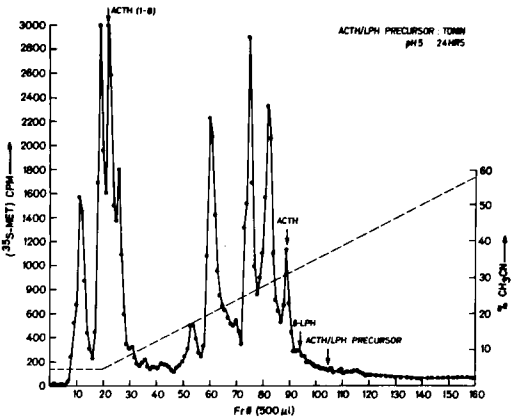

Fig. 4. HPLC separation of the products of 24 h digestion of pro-opiomelanocortin by 5μg tonin pH 5. The position of the peptide markers ACTH 1-8, ovine ACTH and β-LPH and rat pro-opiomelanocortin or ACTH/LPH precursor are shown.

Conclusions

Although β-endorphin and Met-enkephalin are resistant to tonin, this enzyme is capable of cleaving ACTH, β-LPH, and their precursor at specific Arg, Lys and Phe residues. The possible release of ACTH-like and endorphin-like peptides by tonin has been demonstrated.

Acknowledgements

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ISOLATION AND SPECIFICITY OF A CATION-SENSITIVE NEUTRAL ENDOPEPTIDASE FROM BOVINE PITUITARIES

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Introduction

Progress in studies on the identification, localization, and function of biologically active peptides has stimulated interest in the mechanisms of their formation and degradation. Accumulated evidence suggests that most active peptides are derived from larger precursor molecules by specific proteolytic cleavage. Peptide bond cleavage is also involved in peptide inactivation. The enzymes, however, involved in these processes are not known. We have previously reported partial purification and characterization of a new endopeptidase from bovine pituitaries,[1] which was strongly inhibited by monovalent cations. In the present work we describe the isolation of an apparently homogeneous preparation of the enzyme and studies on its specificity with synthetic and natural peptides.

Results

The first four purification steps were the same as those described previously.[1] Further purification was achieved by DEAE-cellulose chromatography at pH 8.3. Elution was carried out with a linear gradient established between 200ml of 0.01M Tris-EDTA and 200ml of 0.5M Tris-EDTA. The enzyme emerged from the column as a peak of activity close to the end of the gradient. Active fractions were pooled, concentrated by ultrafiltration and applied to the top of an AcA-34 ultrogel column (2.5 x 45cm; LKB Instruments Inc.) equilibrated with a 0.01M Tris-EDTA buffer (pH 7.5). The enzyme was eluted from the column as a sharp peak of activity coinciding with a protein peak. Enzyme activity was determined with Cbz-Gly-Gly-Leu-pNA as substrate and expressed in units as the number of μmoles of p-nitroaniline released per hour.[1] Specific activity is in units/mg protein. The isolated enzyme had a specific

activity of 10 to 11. Polyacrylamide gel electrophoresis was performed under non-dissociating conditions[2] in 4% and 6% gels, using a Tris-HCl buffer (0.05M; pH 8.3 or pH 8.7). A single, sharp protein band was observed. An Ouchterlony double gel diffusion test with several immune-serum dilutions from immunized rabbits revealed a single precipitation line. A preliminary estimate of the molecular weight was obtained by gel filtration on a Sephrose-6B column[3] (2.6 x 60cm) equilibrated with 0.5M Tris-HCl buffer (pH 7.5). The void volume of the column was determined using a suspension of an attenuated strain of influenza virus. The column was calibrated with thyroglobulin, ferritin, catalase, and aldolase. The enzyme was eluted slightly ahead of thyroglobulin suggesting a molecular weight of about 700,000. A broad pH optimum between 7.5 and 9.0 was found with Cbz-Gly-Gly-Leu-pNA as substrate in 0.05M Tris-HCl buffer.

The substrate specificity of the enzyme was studied with several synthetic substrates (Table I) and natural peptides (Table II). Reaction products were identified as described previously[1] and also by amino acid analysis of the incubation mixtures before and after separation of the products by HPLC[4] followed by acid hydrolysis of the isolated peptides.

Table I. Specificity of the Neutral Endopeptidase with Synthetic Substrates.

	Substrate	Relative Activity	
1.	Cbz-Gly-Gly-Leu-pNA	100	(100)
2.	Cbz-Gly-Gly-Tyr-Leu-pNA	53	(54)
3.	Cbz-Gly-Gly-Tyr-Ala-pNA	6.5	(41)
4.	Cbz-Leu-Leu-Glu-2NA	120	(124)
5.	Cbz-D-Ala-Leu-Arg-2NA	52	(52)
6.	Bz-Phe-Val-Arg-pNA	16	(25)
7.	Cbz-Gly-Gly-Arg-2NA	6	(6)

The concentration of each substrate was 0.4 mM. Incubation mixtures (0.25ml) contained substrate (10μl of 10mM solution in DMSO), Tris-HCl buffer (0.05M; pH 7.8) and enzyme (0.4 units). Incubations were at 37° for 15 to 90 min. Release of pNA or 2NA was determined after diazotization.[1] Values in parenthesis show the rate in the presence of excess aminopeptidase M; an increased rate indicates cleavage between two adjacent amino acids in addition to that between the C-terminal amino acid and chromogen. Heavy arrows show major cleavage sites; light arrows show slow cleavage. Substrates were synthesized in this laboratory by Dr. S. Pearce.
Cbz-, α-*N*-benzyloxycarbonyl-; pNA, *p*-nitroanilide; Bz, α-*N*-benzoyl; 2NA, 2-naphthylamide

Although interpretation of the reaction rates given in Table I requires caution, because all substrates were studied at the same concentration, and Km values were not determined; a rather characteristic property of the enzyme is its broad specificity. This is noticeable both with synthetic substrates and natural peptides. The enzyme preferentially cleaves bonds in which the carboxyl group of a hydrophobic amino acid is linked to pNA or to a small neutral amino acid. In addition to this chymotrypsin-like activity the enzyme exhibits a trypsin-like activity by cleaving bonds in which the carboxyl group of arginine is involved (Table I). Of interest is also cleavage of the bond on the carboxyl side of glutamate (substrate 4) and on the amino side of hydrophobic amino acids as seen in angiotensin and neurotensin, creating the impression of a carboxypeptidase-like activity. However, as reported previously,[1] the enzyme does not attack simple model chymotrypsin, trypsin, carboxypeptidase or amino-peptidase substrates. That a hydrophobic center before the cleaved bond greatly accelerates the rate of reaction was seen both with synthetic and natural peptides. As reported previously the enzyme was inhibited by sodium and potassium ions and sulfhydryl blocking agents.[1]

Table II. Cleavage of Natural Peptides by Neutral Endopeptidase.

Structure of peptide and site of cleavage
Angiotensin II: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Neurotensin: pGlu-Leu-Tyr-Gly-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
Bradkykinin: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
LHRH: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
α-MSH: Ac-Ser-Tyr-Ser-Met-Gly-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

Rapid cleavage indicated by heavy arrows. Simple arrows indicate slow cleavage. Incubation mixtures (0.1ml) contained peptide (68nmol), buffer (0.025M Tris-HCl; pH 8.0) and enzyme (0.3 units) in the absence and presence of excess aminopeptidase M.

The high molecular weight of the enzyme and its broad specificity posed the question whether the different activities are due to a single catalytical unit or to a multienzyme complex. Some preliminary data favor the second possibility. Thus, although the activity with all substrates was inhibited by NaCl, the activity with the arginine-containing substrates (substrate 5, 6 - Table I) was preferentially inhibited by leupeptin and N-ethylmaleimide. Furthermore, the activity with the glutamate-containing substrate (substrate 4, Table I) was inhibited at

increasing enzyme concentrations, while that with other substrates increased linearly with enzyme concentration. It is of interest that progressive enzyme inactivation was observed at 37° in very dilute enzyme solutions and in the presence of high buffer concentration (0.4M Tris-HCl; pH 8.0) suggesting the presence of a dissociating system. Further studies of the various activities and their regulation should provide insight into their significance in the conversion of pituitary hormones.

Ackowledgement

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INACTIVATION OF NEUROPEPTIDES BY BRAIN ARYLAMIDASES AND endo-OLIGO-PEPTIDASES

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Introduction

Several biologically active peptides present in the CNS act as neurohormones, or neurotransmitters, or both.[1] The modulation of their activity requires the existence of proteolytic pathways to release, convert, and inactivate neuropeptides. In previous studies, we demonstrated kinin converting activity for brain peptidases[2] and isolated and partially characterized two kinin inactivating endopeptidases.[3,4] Endopeptidases A and B are thiol-activated peptidases active at physiological pH which inactivate bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) at the Phe^5-Ser^6 and Pro^7-Phe^8 peptide bond, respectively. In this paper we present a chromatographic system for the separation of brain endo- and exopeptidases, report their activity for the inactivation of angiotensin I and II, substance P, LH-RH and neurotensin, identify the sites of cleavage responsible for inactivation of the angiotensins and LH-RH and show that the activity of endopeptidases A and B is limited by the size of the substrate.

Results

Figure 1 shows the elution diagram obtained when the pH 5 supernatant fraction of rabbit brain is chromatographed on DEAE cellulose. The six pools of the effluent containing bradykinin inactivating activity were characterized by determining peptide fragments and amino acids released from bradykinin and Lys-bradykinin with an amino acid analyzer.[3,4] Pools 1 through 3, containing essentially all of the endopeptidase A (Pools 1 and 2) and B (Pool 3), accounted for 80% of the

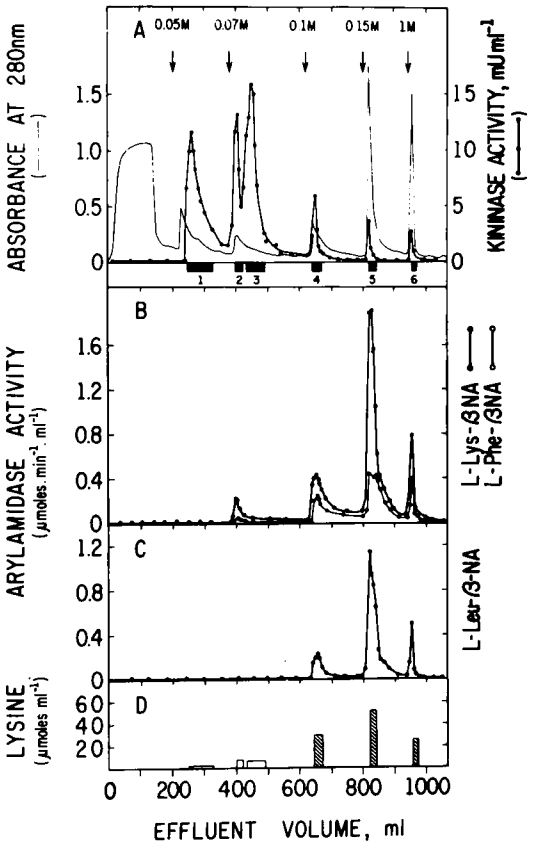

Fig. 1

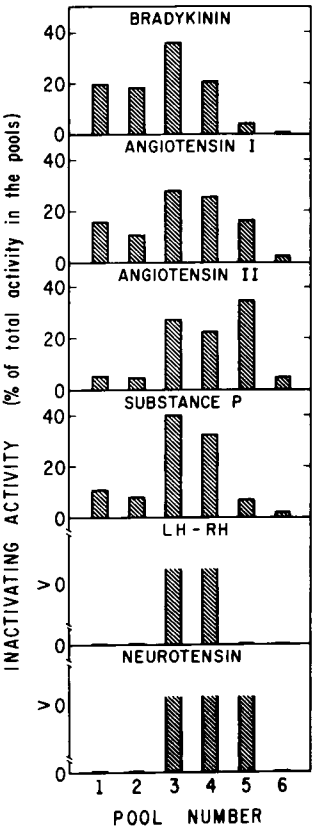

Fig. 2

Fig. 1. Chromatography of the pH 5.0 supernatant fraction from rabbit brain on DEAE cellulose. Panel D: Lysine released from Lys-bradykinin. The filled bars indicate pools which convert Met-Lys-bradykinin and Lys-bradykinin to bradykinin.

Fig. 2. Inactivation of neuropeptides by Pools 1 through 6 (Figure 1) derived from rabbit brain. Data for LH-RH and neurotensin are reported as 0 to 100% inactivation.

recovered kininase activity, whereas Pools 4 through 6 accounted for 98% of the recovered arylamidase activity. Figure 1 also indicates a multiplicity of arylamidase activities in Pools 4-6, demonstrable by their different relative rates of hydrolysis of Leu-, Lys-, and Phe-β-NA. Bradykinin, angiotensin I and II and substance P were inactivated by all of the pools, whereas LH-RH and neurotensin were inactivated by Pools 3 and 4 and 3, 4 and 5, respectively (Figure 2). These results suggest that brain peptidases have some specificity for the inactivation of neuropeptides.

The sites of hydrolysis of angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) and angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (Greene, *et al.*, ms. in preparation) and LH-RH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) (unpublished results) by endopeptidase B were determined on the basis of the stoichiometric and quantitative recovery of both products from each peptide. The Pro^7-Phe^8 peptide bond of angiotensin I and II and the Pro^9-Gly^{10}-NH_2 bond of LH-RH were identified as the only sites of hydrolysis. In contrast, neutral brain proteinase hydrolyzes bradykinin and LH-RH each at more than one peptide bond.[5,6]

The ability of brain endopeptidases to hydrolyze small peptides but not native or denatured proteins[3,4] led us to compare them with pancreatic proteolytic enzymes with respect to hydrolysis of a synthetic peptide related to bradykinin, $(Gly)_3$-Arg-bradykinin, either free or bound to succinylated polylysine (3,000 daltons). Table I shows that brain endopeptidase A and B do not hydrolyze $(Gly)_3$-Arg-bradykinin when this peptide is coupled to succinylated polylysine through the amino terminal group, whereas chymotrypsin and carboxypeptidase B hydrolyze the bradykinin moiety both free and when covalently bound to polylysine. Similar results were obtained when $(Gly)_3$-Arg-bradykinin was bound to Affi-gel 10 or to succinylated polylysine (180,000 daltons).[7]

Table I. Hydrolysis of the Bradykinin Moiety of $(Gly)_3$-Arg-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg Bound to Succinylated Polylysine.

Enzyme	10 min	20 min	60 min	120 min
		% of total		
Chymotrypsin	35	25	100	100
Carboxypeptidase B	55	95	100	100
Brain Endopeptidase A	0	0	0	0
Brain Endopeptidase B	0	0	0	0

The bradykinin moiety of the molecule not hydrolyzed was released by trypsin and measured by bioassay.[7]

Conclusions

The multiplicity of endo- and exopeptidases present in nervous tissue that hydrolyze biologically active polypeptides irreversibly modify

their biological properties. Although there are differences in specificity for amino acid sequences near or at the scissile bond, brain endopeptidases A and B share a common property: selectivity for certain low molecular weight substrates. The same dependence on substrate size has also been suggested for brain arylamidases.[8] This view of the specificity of intracellular peptidases depending on both amino acid sequence and size of substrate reconciles the early concepts of Fischer and Bergmann and those of Walschmidt-Leitz[9] and strongly suggests the existence of two distinct proteolytic systems, i.e. proteolysis of high[10] and low molecular weight polypeptides. Thus the peptidases which we have described may be part of the intracellular chain of low molecular weight proteolysis that modulates the conversion and inactivation of biologically active peptides.

Acknowledgement

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SYNTHESIS OF L-GLUTAMIC ACID γ-DIHYDROXYANILIDES AS POTENTIAL SPECIFIC AGENTS AGAINST MELANOMA

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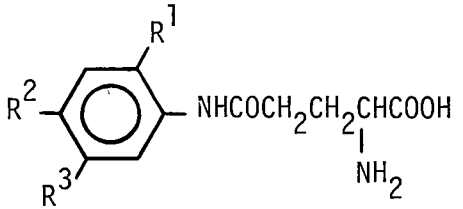
L-Glutamic acid γ-(*p*-hydroxyanilide) *(1)* is a natural growth-regulatory substance which occurs in the gills of the common mushroom *Agaricus bisporus* and is an efficient substrate for the enzyme tyrosinase[1]. In the presence of the enzyme, the colorless phenol is converted rapidly to a red product with an absorbance maximum at 490 nm. Although it was originally proposed that this is an *ortho*-quinone[2,3], recent evidence points to other possible structures[4]. The natural role of the quinone metabolite in the lifecycle of the mushroom is believed to be the induction and maintenance of spore dormancy, a "cryptobiotic" state in which energy utilization and macromolecular synthesis virtually cease[5]. The quinone is a potent thiol reagent and inhibits a number of SH-containing enzymes of bacterial and mammalian origin[4,6-9]. The phenol has also been reported to inhibit the growth of the B16 melanoma tumor in the mouse, presumably because this tumor secretes large amounts of tyrosinase and can therefore bring about quinone formation *in situ*[9,10].

Metabolic conversion of the phenol to the 490 nm quinone in *Agaricus bisporus* was postulated to occur via L-glutamic acid γ-(3,4-dihydroxyanilide) *(2)* as an intermediate[4]. The latter compound was subsequently isolated from the related mushroom species *Agaricus campestris* and named agaridoxin, and its catechol structure was confirmed by chemical synthesis[11]. The published route to agaridoxin is unsuited to the synthesis of *meta* and *para* analogs since it makes use of an isopropylidene protecting group, which is specific for *ortho* diols. We therefore developed a more general approach utilizing readily obtainable dimethoxyaniline derivatives as starting materials. By this two-step route, we prepared both agaridoxin and L-glutamic acid γ-(2,5-dihydroxyanilide) *(3)*, a hitherto inaccessible positional isomer which may be viewed as being a potential alternative quinone precursor under biological oxida-



	R^1	R^2	R^3
1	H	OH	H
2	H	OH	OH
3	OH	H	OH

tion conditions. Compounds *2* and *3* produced >95% inhibition of calf thymus DNA polymerase α at $1x10^{-5}$ M, and were cytotoxic to cultured B16 mouse melanoma cells with ID_{50} values of $5x10^{-5}$ M and $2x10^{-4}$ M respectively. However, the single-dose LD_{50} value for *2* and *3* in BDF_1 mice was 125 mg/kg, and neither compound extended the lifespan of BDF_1 mice with ip implanted B16 melanoma at doses of up to 75 mg/kg (qd 1-21).

Experimental Section

L-Glutamic Acid γ-(3,4-Dihydroxyanilide) (Agaridoxin, 2). To an ice-cold solution of 4-aminoveratrole (950 mg, 6.2 mmol), L-glutamic acid α-benzyl ester (1.85 g, 5.0 mmol), and 1-hydroxybenzotriazole (1.62 g, 6.2 mmol) in dry THF (20 ml) was added dropwise a cold solution of DCC (1.2 g, 5.8 mmol) in THF (4 ml). After 1 hr at 0° C and overnight storage at room temperature, a small amount of glacial acetic acid was added and the mixture was stirred for 15 min, filtered, and evaporated under reduced pressure. The residue was triturated with 10% citric acid, washed with water, then dilute $NaHCO_3$ and again water, and finally air-dried. The resultant solid was taken up in EtOAc (100 ml) and the solution cooled until crystals of *N,N'*-dicyclohexylurea were deposited, which were filtered and discarded. Solvent evaporation and purification of the residue by dry-column chromatography (Woelm activated silica gel, grade III/30 mm, ICN Pharmaceuticals, Inc., Cleveland, Ohio) with $CHCl_3$ as the eluent yielded L-glutamic acid γ-(3,4-dimethoxyanilide) α-benzyl ester as a pale-pink solid (1.49 g, 50%): mp 155-158° C; $\delta(CDCl_3)$ 1.8-2.6 (m, 5H, CH_2CH_2CH), 3.83 (s, 6H, OCH_3), 5.08 and 5.15 (two s, 4H, OCH_2), 5.6-8.0 (m, 15H, aromatic protons and NHCO). *Anal.* Calcd for $C_{28}H_{30}N_2O_7$:

C, 66.38; H, 5.96; N, 5.53. Found: C, 66.59; H, 6.09; N, 5.37. To a solution of the foregoing compound (250 mg, 0.49 mmol) in dry CH_2Cl_2 (5 ml) cooled to -78° C (Dry Ice/acetone) was added dropwise under N_2 a solution of boron tribromide (1.3 g) in the same solvent (5 ml), likewise precooled to -78° C. The reaction mixture was allowed to come to room temperature overnight, with careful protection from moisture throughout. After a total of 45 hr, the suspension was cooled and treated carefully with degassed MeOH (3ml). Vacuum evaporation yielded a pale-brown residue which was triturated with deionized water, extracted with ether (5 x 10 ml), and filtered. The pale-pink aqueous layer was lyophilized and the residue dissolved in a small volume of deionized water. The solution was added to a column of Dowex-50WX8(H^+) resin which had previously been equilibrated with 0.4 M ammonium formate (pH 4). Elution with the same buffer and lyophilization of pooled TLC-homogeneous fractions gave a pale-yellow powder (80 mg). This was redissolved in a small volume of water, the pH adjusted to 5.0 with dilute ammonia, and the solution lyophilized again to obtain the final product (65 mg, 52%): mp 205° C (softening above 180° C); R_f 0.34 (Eastman 13254 cellulose sheet with fluorescent indicator, 3:1:1 *n*-BuOH-AcOH-H_2O); positive ninhydrin test; green color with 3% ethanolic $FeCl_3$; $[\alpha]_D^{25}$ + 25.77° (*c* 0.26, 1 N HCl); δ (D_2O) 1.93-2.76 (m, 4H, CH_2CH_2), 4.06 (t, 1H, CH), 6.76 (broad m, 3H, aromatic protons). *Anal.* Calcd for $C_{11}H_{14}N_2O_5 \cdot 0.5H_2O$: C, 50.18; H, 5.74; N, 10.64. Found: C, 50.51; H, 5.86; N, 10.36.

L-Glutamic Acid γ-(2,5-Dihydroxyanilide) (3). This compound was prepared as described in the preceding experiment (coupling step, 51% yield; deblocking step, 41% yield): mp 180° C dec; R_f 0.40 (cellulose, 3:1:1 *n*-BuOH-AcOH-H_2O); positive ninhydrin test; orange-brown color with 3% ethanolic $FeCl_3$; $[\alpha]_D^{25}$ + 26.15° (c, 0.26, 1 N HCl); δ (D_2O) 1.93-2.86 (m, 4H, CH_2CH_2), 3.66 (broad t, 1H, CH), 6.60-6.96 (m, 3H, aromatic protons). *Anal.* Calcd for $C_{11}H_{14}N_2O_5 \cdot 0.33H_2O$: C, 50.63; H, 5.66; N, 10.73. Found: C, 50.70; H, 5.70; N, 10.94.

Acknowledgment

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DNA BINDING PROPERTY OF 7-BULKY ARALKYLAMINO SUBSTITUTED ACTINOMYCIN

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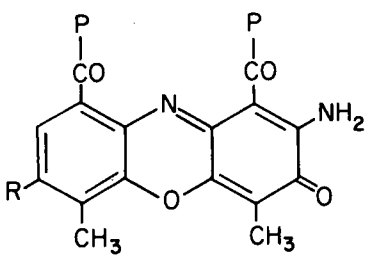
Introduction

Actinomycin D (AMD) is an antitumor antibiotic having a 2-aminophenoxazin-3-one chromophore and two cyclic pentapeptide lactones (Figure 1). The biological activity of AMD is considered to be due to its ability to bind to double-stranded DNA, thereby inhibiting DNA-dependent RNA polymerase[1]. We have studied C-7-chromophore substituted bulky aralkylamino AMD analogs (Figure 1) primarily by circular dichroism (CD) to determine the nature of their interaction with native DNA.



AMD, R=H; P=Thr-D-Val-Pro-Sar-MeVal—O—

ANALOG I, R=-NH-CH_2-(pyrrolyl); P= "

ANALOG II, R=-NH-CH_2-(3,4-dichlorophenyl); P= "

ACT, R=H; P=-NH-$(CH_2)_2$-$N(C_2H_5)_2$

Fig. 1. Structures of AMD and analogs.

To enhance the therapeutic efficacy we synthesized C-7 substituted aralkylamino AMD analogs and established that they bind to DNA, inhibit RNA synthesis, and exhibit improved *in vivo* antitumor activity (Table I)[2,3]. The CD spectra of two of these AMD analogs, *N*-7-(2′-pyrrolyl)methylamino AMD (I) and *N*-7-(3′,4′-dichloro)benzylamino AMD (II), with DNA, are shown in Figure 2. Comparison of these spectra with those of the parent AMD and actinomine (ACT, Figure 1), the chromophore compound lacking the peptide moieties, may give some insight into the mode of interaction of analogs I and II with DNA.

Table I. Properties of Actinomycin D and 7 Position Analogs

COMPOUND	ΔT^{a}_{M}	$K_{AP}{}^{b}(M^{-1})$	ED^{c}_{50}, RNA(NG/ML)	ED^{d}_{50}, P388(NG/ML)
AMD	7°	23×10^{6}	810	50
7-AMINO AMD	8°	23×10^{6}	830	300
AMD ANALOG I	16°	92×10^{6}	900	250
AMD ANALOG II	7°	26×10^{6}	950	100

[a],[b],[c] DNA: calf thymus; buffer: [a]sodium cacodylate (0.01M, pH. 7.0); [b]phosphate (0.01M, pH 7.0, 20°), K_{AP}—apparent binding constants (Scatchard plot); [c]ED_{50}, RNA-using E. coli polymerase; [d]ED_{50}, P388—using leukemic cells in culture; [c],[d]units show concentration of inhibitor in ng/ml (37°C, pH 7.4) for 50% reduction of total RNA synthesis.

Results and Discussion

The CD spectrum of DNA shows characteristic ellipticity at 240(-) and 275(+) nm and none from 300 to 600 nm (Figure 2). The two AMD analogs I and II have similar CD spectra with a small negative ellipticity at 350-360 nm and also ellipticities at 260(-) and 240(+) nm (see Figure 2). When DNA and analog I associate, large changes in the CD spectrum occur. The chromophore band (-) at 350 nm is enhanced and an additional broad negative band develops from 480 to 600 nm. The DNA ellipticities at 240(-) and 275(+) nm are intensified and shifted to slightly longer wavelengths. For AMD the chromophore bands at 380 and 460 nm are intensified and shifted to slightly higher wavelengths. These changes in spectra upon DNA binding are accentuated in the difference CD spectra (Figure 3). The CD spectral changes for AMD + DNA and

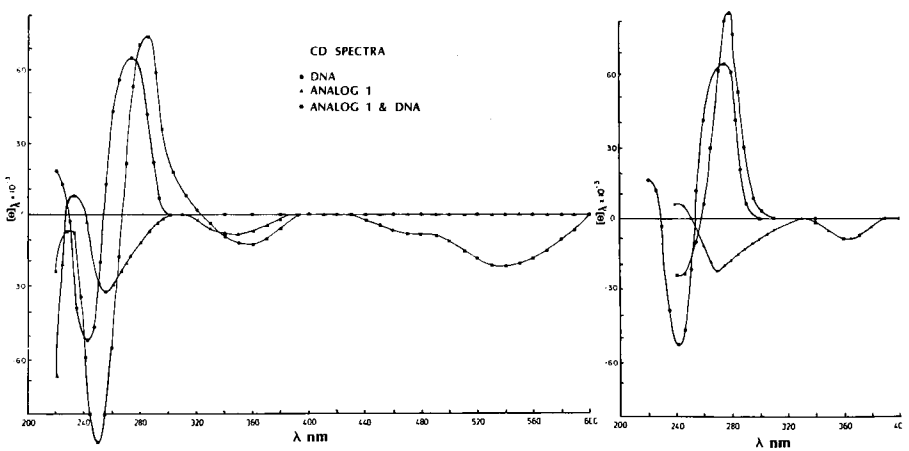


Fig. 2. CD Spectra: calf thymus DNA, 0.01M phosphate buffer, pH 7.0, 20°C, [Drug]/[DNA-nucleotide] = 1:10. Values for ●, Δ and ■ are 0 beyond 375 nm.

7-amino AMD + DNA have been interpreted in terms of the intercalation of the chromophores between dyad related G-C base pairs and cooperative binding of the peptide moieties with the DNA minor groove via van der Waals and hydrophobic forces and hydrogen bonds[4].

Both AMD and analog I show similar CD spectral changes in their chromophore regions when complexed with DNA (Figure 3). The negative Cotton effects are enhanced and shifted in those wavelength regions between 360 and 600 nm corresponding to the maxima of their respective absorption spectra. In the DNA region AMD and analogs exhibit enhancement of the 260 nm trough and the 290 nm peak. However, the effect at 260 nm is greater for analog I. This may be due to a difference in sugar phosphate conformation as a result of additional binding interactions between the pyrrolyl group and the bridging phosphate group at the site of intercalation (Figure 4).

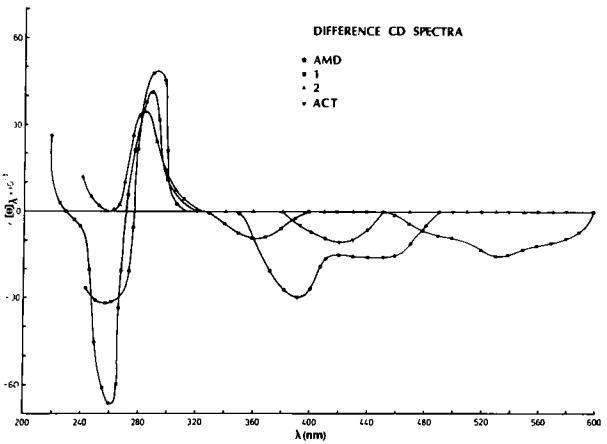


Fig. 3. Difference CD-Spectra of AMD and analogs.

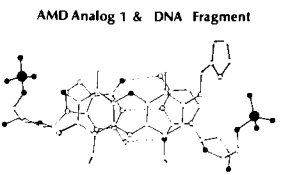


Fig. 4. Possible conformation of AMD and its binding product to DNA fragment.

C N O P

For AMD analog II there is no enhancement of the small negative chromophore peak at 380 nm upon addition of DNA. The only significant change in the CD spectrum involves an intensification of the positive DNA peak at 275 nm indicating that AMD analog II may not interact with DNA in the same fashion as AMD or analog I. The lack of change in the chromophore region of the CD spectrum implies that intercalation may be minimal. The intensification and shift (to 280 nm) of the 275 nm peak indicates a change in DNA conformation upon complexation with analog II.

The intensification of the ellipticities of DNA at 240(-) and 275(+) nm upon complexation with AMD and analogs I and II are similar to the

CD spectra of free RNA[5]. The difference in CD spectra between RNA and DNA is due not only to the difference in sugar moieties but also to the ensuing conformational differences in orientation of bases with respect to the helical axis, base stacking pattern, and helical parameters. Perhaps the CD spectra of DNA complexed with AMD and its analogs reflect conformational changes in the DNA that mimic the A form rather than the B form of DNA. This may explain inhibition of DNA-dependent RNA polymerase, which presumably binds only to the B form of DNA in the domain of the binding site. The presence of the cyclopentapeptides in the minor groove of DNA appears to be an important factor stabilizing this A-like conformation as supported by the CD spectrum of DNA complexed with actinomine which lacks the peptide moieties. Upon ACT-DNA complexation, a negative band occurs in the chromophore region at 380 nm, but there is no intensification or shift in ellipticity in the DNA region (Figure 3). The A-conformation of DNA has a larger minor groove than does the B form, enabling it to better accommodate the peptide moieties of AMD and its analogs.

Conclusion

The presence of bulky substituents at C-7 of AMD does not preclude complexation with DNA. The CD studies suggest that the mode of binding of substituted AMD analogs to the DNA duplex may be different from the parent AMD and each other as indicated by characteristic changes in ellipticity in the chromophore and DNA regions of the CD spectra. Despite these differences, DNA binding affinity and biological activity do not significantly change relative to AMD.

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MONOHAPTENIC MODEL PEPTIDES THAT ELICIT PASSIVE CUTANEOUS ANAPHYLAXIS

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The immune-specific elicitation of anaphylactic reactions requires compounds with two or more haptenic groups that are able to bridge antibodies fixed on histamine releasing cells whereas monovalent haptens generally inhibit such reactions.[1] However, a variety of monohaptenic compounds were found to be elicitors rather than inhibitors of anaphylaxis mainly in studies on passive cutaneous anaphylaxis (PCA) in the guinea pig. Some of these elicitors require hydrocarbon moieties of discrete size in addition to the single haptenic group in order to be effective. Thus, *N*ε-Dnp-L-lysine when carrying benzoyl-, benzyloxycarbonyl-, octanoyl- or decanoyl- substituents on the α-amino group is an efficient elicitor of Dnp-specific PCA.[2,3] Similarly, 1,6-diaminohexane carrying a dinitrocarboxyphenyl- (Dncp) substituent on one terminus and a benzylpenicilloyl- (Bpo) on the other one (N^1-Dncp-N^6-Bpo-diaminohexane) is an elicitor of Dncp-specific reactions with the benzyl side chain of the Bpo group providing the necessary hydrocarbon moiety.[4]

This paper deals with model peptides carrying an N-terminal Dncp-substituent as a single haptenic group and C-terminal Phe providing with its phenyl residue the hydrocarbon moiety for PCA elicitation. The capacity of the derivatives to evoke Dncp-specific PCA reactions was studied in guinea pigs according to standard procedures. The peptide derivatives listed in Table I were synthesized in solution starting at the benzyl ester protected C-terminal amino acid and using a liquid-liquid extraction strategy for intermediate purification (two-phase method[5]). Nα-Boc-amino acid *N*-hydroxysuccinimide esters were used for stepwise chain elongation. The final N-deblocked peptides were hydrogenated with Pd on C and reacted with 2-chloro-3, 5-dinitrobenzoic acid at pH 9.6-10.2 in aqueous solution. The final purification involved chromatography on dry silica gel columns and preparative silica gel thin layers. Particular emphasis was on removal of traces of 2-chloro-3,5-dinitrobenzoic acid. The final preparations were homogeneous when run

Table I. Dinitrocarboxyphenyl (Dncp) Derivatives Synthesized for the Study of PCA

	Compound	R_f (TLC) [a]	R_f (HPTLC) [a]
I	N^1-Dncp-N^6-benzoyl-diaminohexane	0.64 A	
II	Dncp-Phe	0.49 A	
III	Dncp-Ala-Phe	0.66 A	
IV	Dncp-Gly-Gly-Phe	0.89 A	0.45 B
V	Dncp-Ala-Ala-Gly-Phe	0.47 A	0.45 B
VI	Dncp-Ala-Ile-Gly-Phe	0.61 A	0.60 B
VII	Dncp-Gly-Ala-Ala-Gly-Phe	0.52 A	0.40 B
VIII	Dncp-Gly-Gly-Ala-Ala-Gly-Phe	0.42 A	
IX	Dncp-Leu-Ala_2-Leu-Glu-Gly_3-Phe	0.56 B	
X	Dncp-Ala-Ala-Gly-Ala	0.33 B	

[a] Retardation factor of product upon thin layer chromatography (TLC) or high performance thin layer chromatography (HPTLC) on silica gel in solvent A: dioxan/H_2O, 5:1; B: dioxan/H_2O, 4:1.

on analytical thin layer plates and in some instances on high performance thin layer plates. They showed correct amino acid ratios. N^1-Dncp-N^6-benzoyl-diaminohexane and Dncp-Phe were prepared according to published methods and purified by recrystallization.[4] They gave correct elementary analyses.

PCA was performed on out-bred guinea pigs (300 g) by intradermally injecting 0.1 ml doses of anti-Dncp antiserum in the dilutions 1/40, 1/80, 1/160, 1/320, 1/640. Elicitation was performed 15-20 hours later by intravenous injection of Dncp-peptide together with Evans blue. Blue spots at the site of intradermal injections indicate positive reactions.[6] The antiserum was raised in rabbits by administering Dncp-substituted bovine gamma globulin in complete Freund's adjuvant. It had an affinity of approximately 10^6 M^{-1}.

The eliciting capacities of the peptide derivatives are summarized in Table II. The lower members of the series up to the tripeptide (IV) were inactive even at fairly high doses. The diaminohexane hapten (I) was

Table II. Elicitation of Cutaneous Anaphylactic or Anaphylactoid Reactions

Compound	μmoles injected per animal	number of [a] positive / all animals	full development of reaction	
			time	color intensity
I	1.0-2.0	0 / 6	-	-
	4.0	4 / 4	30 min.	moderate
II	0.1-1.0	0 / 9	-	-
III	0.3-0.6	0 / 6	-	-
IV	1.0-2.0	0 / 6	-	-
V	0.5-1.0	7 / 7	40 min.	full
VI	0.1-1.3	2 / 5	20 min.	pale
VII	0.5-1.0	6 / 6	40 min.	full
VIII	0.3-0.7	3 / 6	70 min.	pale
IX	ca: 0.05	7 / 8	40 min.	moderate
X	0.6-1.2	0 / 6	-	-
Dncp-Cl [b]	0.01-0.025	0 / 6	-	-

[a] In all animals one test site was injected with normal rabbit serum 1:5. This was negative in all instances.

[b] Dncp-Cl: 2-chloro-3,5-dinitrobenzoic acid.

however active at 4 μmoles per animal, a dose which could not be reached with the peptides for solubility reasons. It may be noted that the related hapten N^1-Dncp-N^6-BPO-diaminohexane studied previously gave strong standard PCA reactions at 0.1 μmole per animal under strictly comparable conditions (identical antiserum).[4] Somewhat retarded reactions of full intensity were elicited by a tetra- and a pentapeptide (V, VII). The tetrapeptide VI with Ile replacing an Ala of V exhibited only weak activity. Also active were a hexapeptide and a nonapeptide (VIII, IX). Dncp-Ala_2-Gly-Ala-OH (X) corresponding to the active derivative V except that C-terminal Phe has been replaced by Ala was inactive. This result corroborates the notion that the aromatic side chain of Phe acts as a hydrocarbon helper group in elicitation of anaphylaxis.

The studies of this series of model peptides suggest that on exposed protein chains a single antigenic determinant adjacent to a Phe position

may be sufficient for elicitation of anaphylactic or anaphylactoid reactions in sensitized individuals.

Acknowledgement

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THE IMMUNE RESPONSE TO BI- AND TRI-FUNCTIONAL ANTIGENS

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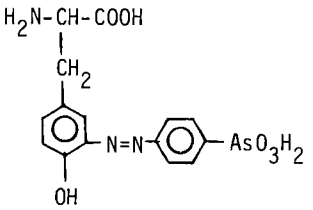
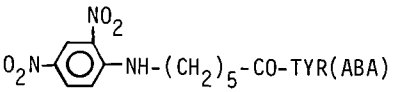
Investigation of antigen structural requirements for lymphocyte activation in the immune response showed that L-tyrosine-azobenzene-*p*-arsonate [Tyr(ABA)] activates T lymphocytes and serves as an effective carrier for haptens, such as the dinitrophenyl (DNP) group in the induction of anti-hapten antibody responses[1]. The murine anti-hapten antibody response to bifunctional antigens composed of one Tyr(ABA) carrier epitope and one DNP haptenic epitope (BI-1) is extremely weak and consists exclusively of IgM antibody in both primary and secondary responses. Conventional hapten-protein conjugates normally elicit IgM followed by IgG antibody responses. Since different helper T cells are synergistic in the induction of optimal antibody responses[2], antigens with more than one carrier epitope were tested for their quantitative and qualitative effect on the response.

Strain A/J mice were primed and boosted with 100 μg of the antigens listed below. Plaque forming cells (PFC) producing anti-TNP (trinitrophenyl) antibody of the IgM class were detected by the method of Jerne, and those producing IgG were detected by first inhibiting IgM PFC with goat anti-mouse IgM (heavy chain-specific) serum and then developing the plaques with rabbit anti-mouse IgG serum (heavy and light chain-specific).

Only IgM producing PFC were detected in primary responses. TRI induced about twice as many PFC as BI-1 (Table I, Figure 1a). However, whereas no IgG appeared in secondary responses to BI-1, both IgM and IgG PFC were seen in secondary responses to TRI (Figure 1b).

Structural differences between TRI and BI-1 are: two Tyr(ABA) carrier epitopes in TRI vs. one in BI-1; nine proline residues as spacer in TRI vs. none in BI-1; longer distance between DNP haptenic epitope and one carrier epitope separated by 6-aminocaproyl-Tyr(Pro)$_9$ in TRI vs. shorter distance of spacer (6-aminocaproyl) in BI-1. To define the critical factors which activate IgG antibody, additional antigens with selected

Table I. Antigens

Monofunctional		TYR(ABA)
Bifunctionals		BI-1
	$DNP-(PRO)_{10}-TYR(ABA)$	BI-2
	$Ac-TYR(ABA)-(PRO)_9-TYR(ABA)$	BI-3
	$DNP-NH-(CH_2)_5-CO-(PRO)_9-TYR(ABA)$	BI-4
Trifunctional	$DNP-NH-(CH_2)_5-CO-TYR(ABA)-(PRO)_9-TYR(ABA)$	TRI

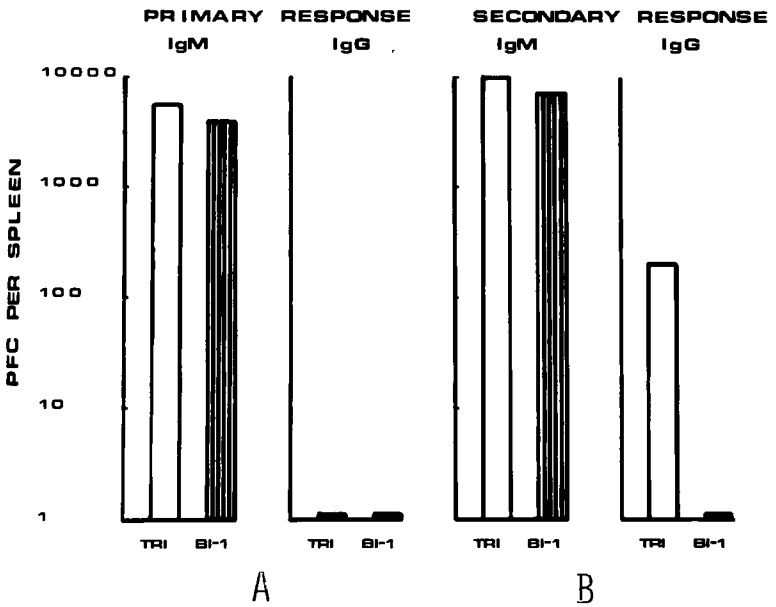


Fig. 1. Anti-TNP antibody reponse in mice to the synthetic antigens TRI and BI-1. (a) Primary response assayed on the tenth day after priming; (b) Secondary response assayed on seventh day after boosting with the same antigen.

properties were prepared (BI-2, BI-3, BI-4). Analysis of the antibody response to these model antigens is to pinpoint the properties of TRI responsible for activating IgG antibody production.

Synthesis

The antigens (Table I) except BI-1 were synthesized by the solid phase method, starting with Boc-Tyr(OBz)-polymer and using Boc-$(Pro)_3$-OH symmetrical anhydride[3] for fragment coupling. The tripeptide was synthesized in the following way:

$$\text{Boc-Pro-OH} + \text{H-Pro-OBzl} \rightarrow \text{Boc-}(\text{Pro})_2\text{-OBzl} \xrightarrow{H_2\text{-Pd}}$$

$$\rightarrow \text{Boc-}(\text{Pro})_2\text{-OH} + \text{H-Pro-OBzl} \rightarrow \text{Boc-}(\text{Pro})_3\text{-OBzl} \xrightarrow{H_2\text{-Pd}} \text{Boc-}(\text{Pro})_3\text{-OH}$$

Symmetrical anhydrides were chosen to avoid chain termination by the trifluoroacetylation reaction observed in the antigen BI-2 synthesis[4]. However, appreciable chain termination occurred also with this method. The by-product was not a mixture of trifluoroacetylated peptides. More byproduct was seen with increasing chain length. It was particularly pronounced in the stages of $(Pro)_{18}$-Tyr → $(Pro)_{39}$→Tyr (Figure 2) in preparation of a series of antigens DNP-$(Pro)_{10\text{-}40}$-Tyr(ABA) in a related study[5]. The byproduct was investigated in some detail at the stage of $(Pro)_{21}$-Tyr. The desired peptide H-$(Pro)_{21}$-Tyr-OH was separated from byproduct by preparative high voltage electrophoresis. Amino acid analysis gave Pro/Tyr ratio of 21.4, that of the byproduct was 12.2. The byproduct amounted to 30% by weight; it contained no free amino groups.

The peptides were terminated as DNP-derivatives or acetylated and cleaved from polymer by HF. All peptides were subjected to preliminary purification on Sephadex G-15 or LH-20 in water or dilute ammonia. Amino acid analyses gave the expected Pro/Tyr ratios. The products were subject to diazonium coupling with arsanilic acid. To remove unavoidable byproducts[6], the diazonium coupling products were successively chromatographed on Sephadex G-15 in 0.1*N* ammonia, silica gel 60 in $CHCl_3$-methanol-conc. NH_4OH, 3:3:1, and Sephadex LH-20 in 0.1*N* ammonia.

The antigen BI-1 was synthesized by reaction of DNP-6-aminocaproic acid with tyrosine methyl ester; the ester was hydrolyzed and the peptide coupled with diazonium arsenylate. The product was purified by repeated chromatography on Sephadex G-15 and LH-20 in 0.1*N* ammonia.

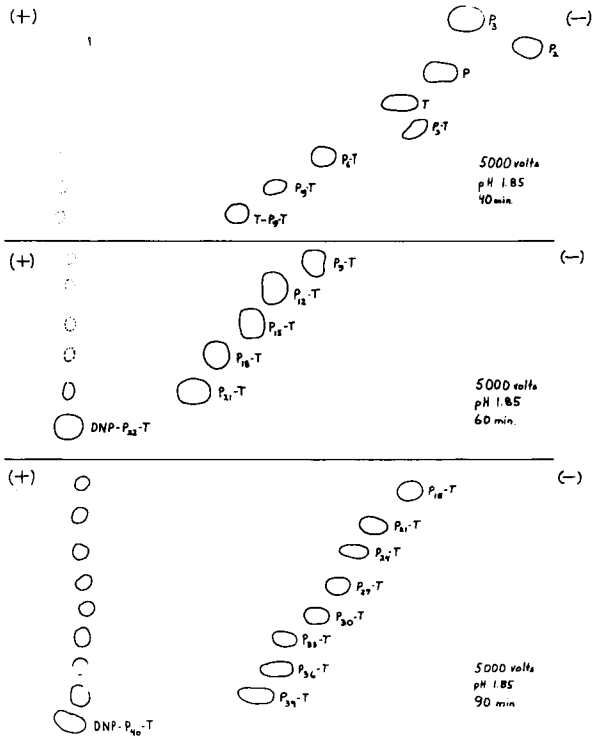


Fig. 2. Electrophoretic migration of the peptides of proline (P) and tyrosine (T).

High voltage electrophoresis (Figure 2) and thin layer chromatography ($CHCl_3$:methanol:conc. NH_4OH = 2:2:1) were used to monitor purification steps.

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SYNTHESIS OF BRANCHED PEPTIDE DERIVATIVES FOR USE AS LARGE HAPTENIC REAGENTS

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In order to test the multispecificity hypothesis[1] quantitatively, it will be necessary to assess the monovalent binding capabilities of individual molecular species of immunoglobulins with many, diverse, chemical structures (haptens). Selected haptens should be large enough to fill most potential binding sites within Fab combining regions; otherwise, their structures need bear no resemblance to that of the eliciting antigen. Since the expected frequency of multispecific binding is relatively low, this study requires a large number of compounds of molecular size comparable to a tetra- or pentapeptide which possess a uniquely reactive functional group allowing covalent attachment to affinity supports or immunizing carriers. The low number and/or high cost of such compounds commercially available made it necessary for us to consider a systematic synthesis of specificity probes as large haptenic reagents. The main goal of our synthesis program is to incorporate a substantial variety of structures into such reagents by following a plan that minimizes the overall labor.

Binding phenomena will be evaluated by quantitative affinity chromatography[2] where radiolabeled antibodies are passed through many columns each containing gel particles bearing a different, covalently bound hapten. Retention will be measured with buffer and free hapten elution.

Figure 1 shows five parts of an idealized haptenic reagent. The desired characteristics of such a reagent are the following: (1) a chemically stable determinant portion having a molecular mass of approximately 450-700 daltons, (2) a unique functional group for covalent attachment placed at the terminus of a relatively featureless spacer arm of 8 or more linear atoms, (3) reasonable water solubility ($\geqslant 10^{-4}$M), (4) purity >99%, (5) modular units which can be altered in various ways.

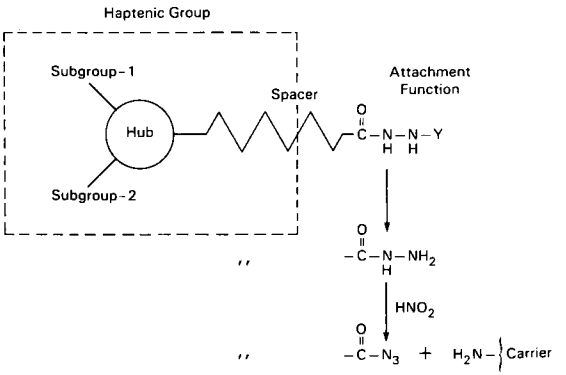

Fig. 1. General features of a large haptenic reagent. The latter is a free or protected carbonylic acid hydrazide where Y = H, Boc or Msc (see Figure 2, bottom). The hub is a small trifunctional moiety to which two subgroups of varied structure are attached. The remaining position bears a spacer arm terminating in an attachment function. Hydrazides are deprotected and converted to reactive acyl azides just prior to forming an amide linkage to an adsorbent matrix or carrier.

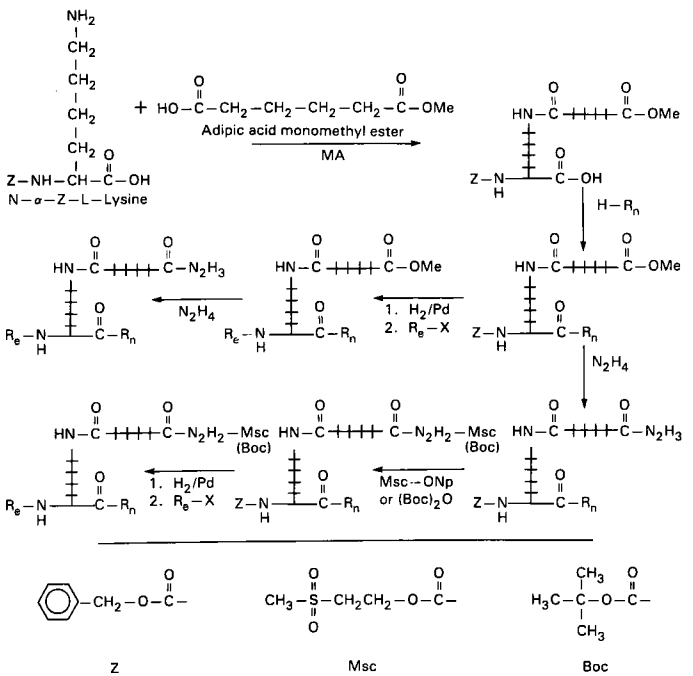

Fig. 2. Synthetic pathways to lysine-based haptenic reagents. MA = *via* mixed anhydride; R_n-H = a nucleophilic subgroup; R_e-X = an activated electrophilic subgroup with leaving group, X; -ONp = p-nitrophenyl group.

Haptenic reagents based on L-lysine or L-ornithine as trifunctional hub structures are currently being synthesized. Various subgroups having molecular weights of around 150-200 are directly available or easily prepared. Figure 2 shows some pathways we are using in synthesis. Different haptens can be prepared by coupling subgroups in various combinations using minimal functional group protection, in order to reduce the number of steps per final reagent. Also, steps can be saved by introducing the hydrazide and spacer function at an early stage of synthesis, preferably in the initial step; the hydrazide then would be protected. We have been experimenting with the 2-(methylsulfonyl) ethoxycarbonyl (Msc) group[3] for this purpose because it holds promise of increasing crystallizability and water solubility. Furthermore, the Msc group is stable during removal of obligatory Boc or Z groups and can be removed under mild alkaline conditions in the presence of a scavenger such as methanol. Boc hydrazides may be prepared in the lysine series. This attachment function was found to be quite satisfactory in earlier work on large haptens.[4,5]

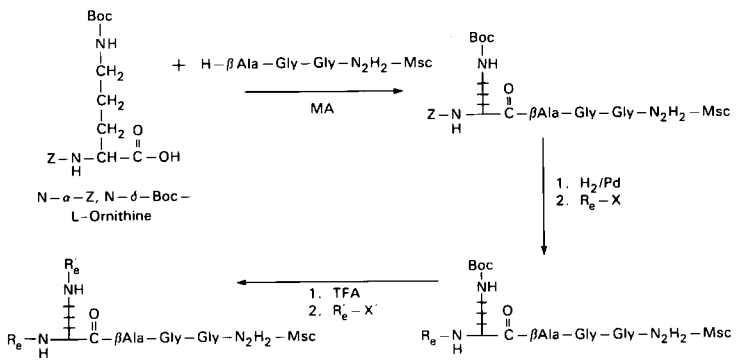

Fig. 3. Synthetic pathway to ornithine-based haptenic reagents.

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DELINEATION OF AN ANTIGENIC SITE OF LIPOPROTEIN A-II

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Introduction

There has been considerable interest in the precise structure of the antigenic determinants of proteins, and the ultimate objective has been to understand the immunological responses and reactions in molecular terms. By 1975 Atassi[1] reported that the antigenic reactive regions of proteins are small and possess short boundaries. The size, surface location, and the shape of these reactive regions make them quite accessible for binding with the antibody combining sites. It has been difficult to study small antigens by conventional immunoassay. Askenase[2] developed a solid phase radioimmunoassay in which antiserum was coated on the wall of the test tubes which were incubated with the ^{125}I labeled antigens for 16 hours. Tubes were then washed and counted. A better approach for peptide antigens was adopted by Smith et al.[4] The fragments of Sperm Whale Myoglobin were synthesized on a support. The side chain protecting groups were selected so that they could be removed easily under mild conditions while peptides remained attached to the resin. This approach posed a problem of selection of protecting groups and conditions for their deprotection. To circumvent these problems and to make this method of general use, we report the synthesis of an improved resin, on which peptides can be synthesized using conventional solid phase techniques and commercially available protected amino acids.

Experimental

A high porosity chloromethyl polystyrene resin was modified to obtain Boc-11-aminohendecanamido-11-hendecanamido methyl resin.[4] p-Bromomethyl benzoic acid was coupled to the resin thus obtained to yield *p*-Bromomethylbenzamido-11-hendecanamido-11-hendecanamido

methyl polystyrene resin. (Br=0.3 meq/g). The first amino acid was coupled to this resin as its cesium salt.[5] The ester linkage of this amino acyl resin was found to be resistant to hydrogen fluoride. This resin was used to delineate one of the antigenic sites of apolipoprotein A-II.

Several fragments of apoA-II were synthesized on the above resin and the protecting groups were removed by treating the resin with hdyrogen fluoride at 0°C for 1/2 hour. The deprotected peptidyl resins were analyzed for the amino acid composition and sequenced on a LKB 4020 solid phase peptide sequencer. No deleted sequences were observed. The anti-apoA-II was purified using affinity column chromatography and was labeled with ^{125}I. The immunoassay was performed in a buffer containing 1% BSA, 0·5% Tween 20 and 0.1 M sodium borate, pH 8.5. A typical incubation mixture contained 5 mg of peptidyl resin, 20μl p-dioxane, 20μl of ^{125}I anti-apoA-II (50,000 cpm) and sufficient buffer to bring the volume to 1 ml. The samples were incubated at 37° for 24 hours. The buffer was removed and the resin beads were washed and counted.

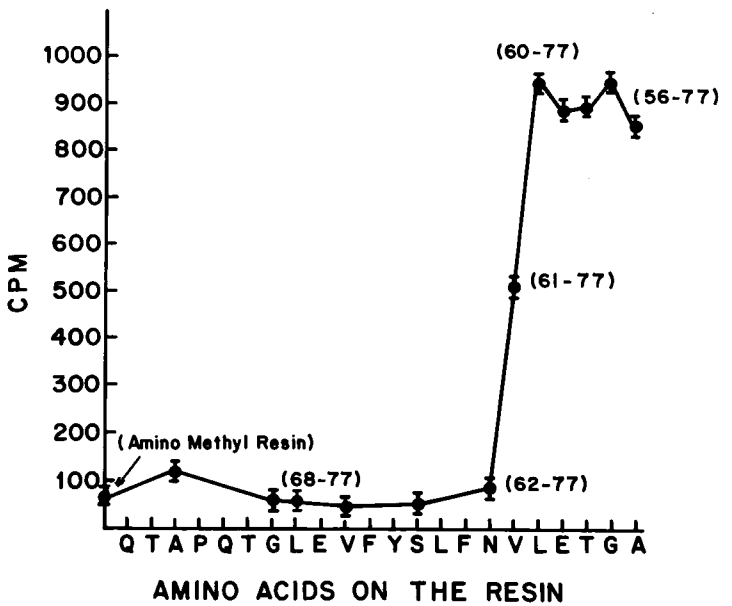

Fig. 1. Binding of ^{125}I anti-apoA-II to resin bound apoA-II sequences

Results

Figure 1 shows that the coupling of the first sixteen residues to the resin did not produce any appreciable binding of anti-apoA-II but after two more residues the immunoreactivity of the peptidyl resin was increased remarkably above the nonspecific binding. The specificity of the immunoabsorption of anti-apoA-II was determined by competitive inhibition of the binding by synthetic (60-77) fragment of apoA-II (Figure 2) and by the dose response curves of different resins (Figure 3).

Discussion

During our search for an acid resistant benzyl ester anchoring linkage, we found that benzyl ester linkage of Boc-amino acyl-4-oxymethyl benzamido-11-hendecanamido-11-hendecanamido methyl resin was completely resistant to anhydrous liquid hydrogen fluoride. This linkage on a macropore resin has proven to be useful for the location of antigenic sites of proteins.

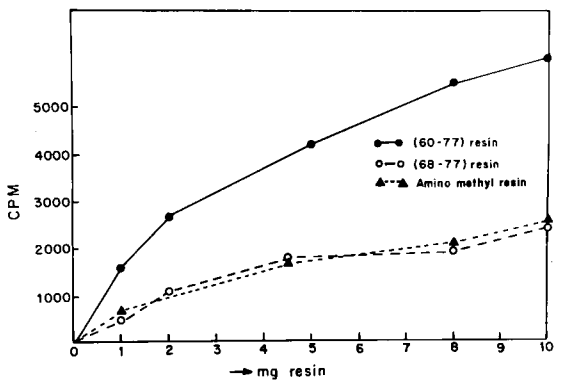

Fig. 2. Dose response curves of binding of ^{125}I anti-apoA-II to resin bound (60-70) apoA-II, resin bound (68-77) apoA-II, and amino methyl resin (control).

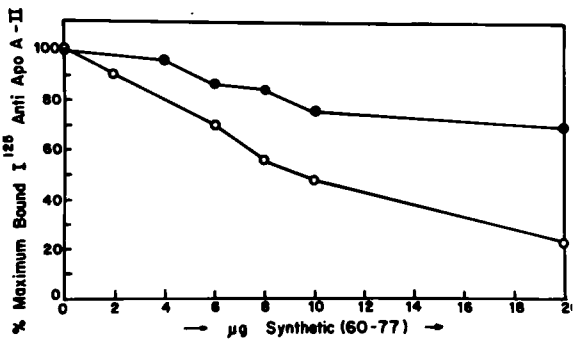

Fig. 3. The test for the specificity of immunoabsorption of anti-apoA-II to resin bound (60-77) apoA-II by inhibition assay (O—O). Resin bound (68-77) apoA-II was used as a control (●—●).

ApoA-II is an appropriate model for this study. It sequence is known and Mao et al.[6] have delineated several antigenic fragments using several enzymatic cleavage and chemical modification reactions. These data show that peptidyl resin (60-77) binds to antibody specifically indicating that one of the antigenic sites of apoA-II lies between the residues (60-77). The sharp increase and plateau in the binding of anti-apoA-II with the peptidyl resin containing an appropriate number of amino acids indicate that the antigenic determinant has sharp boundaries. The results in Figure 1 also confirm that the fragment (60-77) has the same antigenicity as does the fragment (56-77). This observation was not possible using conventional radioimmunoassay techniques. This technique provides a quick screening procedure for the primary antigenic determinants of protein and also provides a means of studying the spatial and structural requirements of antigenic determinants.

Acknowledgements

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STRUCTURE OF CLONED cDNA FOR THE BOVINE CORTICOTROPIN-β-LIPOTROPIN PRECURSOR PROTEIN

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The pituitary hormones corticotropin (ACTH) and β-lipotropin (β-LPH) are formed from a large common precursor protein[1-7]. The two hormones in turn are known to contain small component peptides having biological activity: α-melanotropin (α-MSH) and corticotropin-like intermediate lobe peptide (CLIP) are derived from ACTH[8]; β-melanotropin (β-MSH), endorphins, and methionine enkephalin are liberated from β-LPH[9,11]. The locations of these various peptide components of the precursor protein are shown in Figure 1. The intracellular level of the messenger RNA (mRNA) encoding the common precursor protein is depressed by glucocorticoids, which appear to act at the transcriptional level by means of the glucocorticoid receptor[12,13]. Although the general positions of ACTH and β-LPH on the common precursor molecule have been known for several years, earlier studies had provided no information about the exact structural relationships of these peptides. Moreover, ACTH and β-LPH account for only one-third to one-half the molecular weight of the precursor protein and thus, there has been considerable interest in and speculation about primary structure and possible biological functions of the peptides in the remaining ("cryptic") portion.

The techniques of DNA cloning[14] and nucleotide sequence analysis[15,16] together provide a powerful tool for understanding the organization of eukaryotic genes and the regulation of their expression. Using bacterial plasmids or phage vectors, it has been possible to propagate DNA sequences from a wide variety of plant and animal cell sources within bacterial cells (for reviews, see 17-19). Procedures have been described for the introduction of randomly generated fragments of

mammalian chromosomal DNA into *Escherichia coli* K12[20], for the cloning of complementary DNA (cDNA) copies of RNA species that predominate in certain tissues[21-23], and for the propagation in bacteria of chemically synthesized nucleotide chains that correspond to the amino acid sequence of small peptide hormones[24,25].

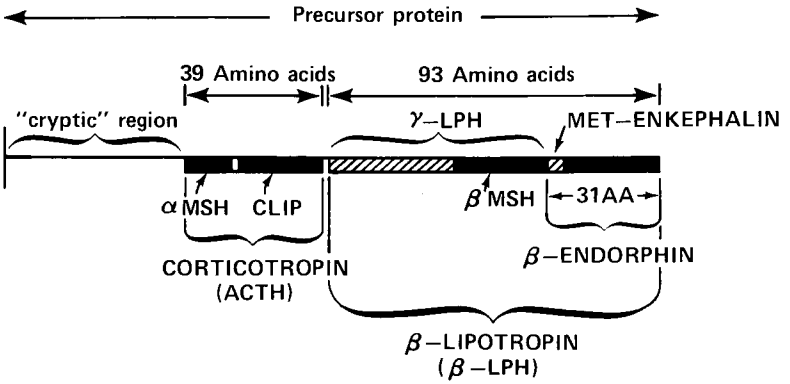

Fig. 1. Map of corticotropin-β-LPH precursor protein showing previously known peptide components. The precise relationship of corticotropin (ACTH) and β-lipotropin (β-LPH) on the precursor molecule has been identified by analysis of cloned cDNA derived from mRNA encoding the precursor[26]. The locations of the peptide components of ACTH and β-LPH were determined earlier by amino acid analysis. The length shown for β-LPH (93 amino acids) has been assigned from the nucleotide sequence of the cDNA insert of the pSNAC20 plasmid (see text), and differs from the 91 amino acid along commonly accepted sequence for β-LPH[33]. Lengths of the various segments of the precursor protein are not drawn to scale. Abbreviations used are; MSH (melanotropin), CLIP (corticotropin-like intermediate-lobe peptide), ACTH (corticotropin), and LPH (lipotropin).

Recently, we reported the cloning and analysis of a double stranded cDNA species complementary to the mRNA that encodes the corticotropin β-LPH precursor peptide[26,27]. Our analysis of this cDNA has shown that the precursor protein is comprised of several repetitive units that are separated by pairs of the basic amino acid residues lysine and arginine. One of these repetitive units is contained within the "cryptic" portion of the molecule and includes a previously unknown third melanotropin sequence.

Cloning and Characterization of Corticotropin-β-Lipotropin cDNA

Our experiments have utilized ACTH-β-LPH mRNA that was purified from the neurointermediate lobe of bovine pituitaries[3]. Avian myeloblastosis virus reverse transcriptase was used for the sequential synthe-

sis of the two strands of duplex cDNA. Homopolymeric poly-dC extensions were added to the longest species of fractionated double-stranded cDNA and complementary poly-dG extensions were added to the *PstI* endonuclease-cleaved DNA of the tetracycline-resistance plasmid pBR322. The two DNA species were joined by annealing and the chimeric molecules were introduced by transformation into *E. coli* strain χ1776. Tetracycline-resistant transformants were isolated, and those bacterial clones that contained ACTH-β-LPH cDNA were identified by an *in situ*-colony hybridization procedure using ^{32}P-labeled mRNA as a probe. The various steps of the procedure followed for the synthesis and cloning of ACTH-β-LPH cDNA have been described elsewhere[26,27] and are shown in Figure 2.

Plasmid DNA isolated from twelve bacterial clones that showed a positive reaction with the ACTH-β-LPH mRNA probe was isolated and analyzed by electrophoresis of restriction endonuclease-cleaved DNA in agarose gels. The cDNA inserts synthesized varied in length from 190 to 1220 base pairs. All of the inserts longer than 535 base pairs in length contained an *Eco*RI site at the same location as the single *Eco*RI cleavage site observed to be present in the molecules of the synthetic cDNA preparation prior to cloning, suggesting that they were derived from transcripts of a homogeneous species of the mRNA. Computer analysis of the amino acid sequence of ACTH and β-LPH indicated that the nucleotide sequence encoding the amino acids glutamic acid and phenylalanine, which are situated near the carboxyl terminus of ACTH, is the possible locus within these two proteins for an *Eco*RI cleavage site. Since the position of the *Eco*RI cleavage site we had identified in the cDNA insert suggested that the site was not contained within the DNA segment encoding the cryptic portion of the precursor protein, it seemed likely that this *Eco*RI cleavage site would be strategically located near the carboxyl terminus of ACTH.

Nucleotide Sequence Analysis of Cloned cDNA

DNA sequence analysis[16] of the region surrounding the solitary *Eco*RI cleavage site of one of the hybrid plasmids (pSNAC20) confirmed the prediction that this site is near the gene location that corresponds to the carboxyl terminus of ACTH. Furthermore, our analysis revealed that nucleotides encoding corticotropin and β-lipotropin are separated on the cDNA by only a six base pair sequence that specifies lysine and arginine, indicating that the carboxyl terminus of corticotropin is connected on the

precursor peptide with the amino terminus of β-lipotropin by these two amino acids.

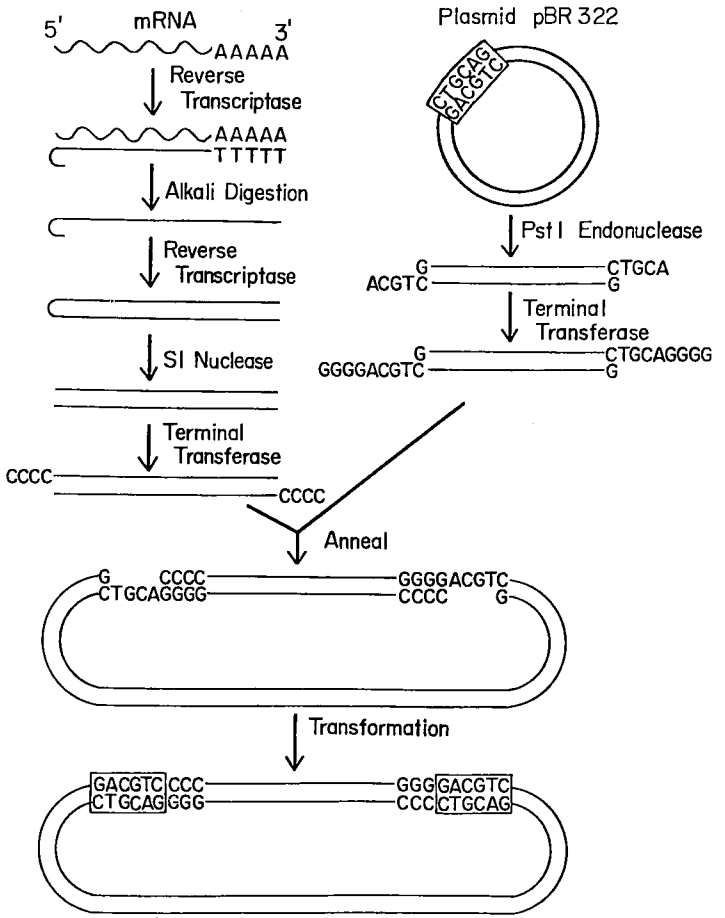

Fig. 2. Outline of protocol used for cloning of corticotropin-β-lipotropin mRNA. Precursor mRNA purified from the neurointermediate lobe of bovine pituitaries served as template for the synthesis of a complementary DNA strand using avian myeloblastosis virus reverse transcriptase (AMV-RT). The RNA-DNA hybrid was treated with alkali to remove the mRNA strand, and a second strand of DNA was synthesized using AMV-RT. The duplex cDNA was treated with S1 nuclease to digest the single-stranded connecting loop as shown. The double-stranded cDNA was fractionated by electrophoresis in acrylamide gel and homopolymeric dC "tails" were added to the largest species. Complementary homopolymeric poly dG extensions were added to the 3' termini of pBR322 plasmid DNA that had been cleaved in the β-lactamase (Ap-resistance) gene by the *PstI* endonuclease. Following annealing of the dG-dC extensions, the chimeric DNA molecules were introduced into *E. coli* strain χ1776 by a transformation procedure, and the Tc-resistance gene carried by the plasmid vector was used in the selection of transformants. The experimental details are described elsewhere.[26]

In subsequent experiments, we determined the sequence of the entire pSNAC20 cDNA insert, which was found to contain 1091 nucleotides (excluding the poly dG-dC tails used for linking the cDNA to the vector plasmid). The details of this analysis, which involved determination of the primary DNA sequence of a series of overlapping endonuclease generated DNA fragments, have been described elsewhere[27]. The nucleotide sequence of the mRNA molecule that was the template for the cDNA insert cloned in the pSNAC20 plasmid is shown in Figure 3. Certain important features of the precursor protein could be inferred from the nucleotide sequence of its mRNA, and some of these are indicated in the figure.

In assigning the amino acid sequence of the cryptic portion of the precursor protein, the translational reading frame was inferred from codons corresponding to the known amino acids of ACTH and β-LPH. The location that we proposed as the most probable translational initiation site for this mRNA[27] has been verified by recent analysis of peptide fragments derived from the previously cryptic portion of the molecule ([27a] E. Herbert, personal communication; R. E. Mains, personal communication). On the basis of this assignment, the molecular weight of the ACTH-β-LPH precursor protein is calculated to be 29,259.

The first 26 amino acid residues following the initiative methionine (at residue minus 131) include a large proportion of hydrophobic amino acids (16 non-polar residues, including 7 leucine residues), consistent with the putative role for this segment as a signal peptide. A high content of hydrophobic amino acids is characteristic of the signal peptide segment of proteins destined for secretion[28-31].

As shown in Figure 3, the nucleotide sequence of the β-LPH segment of the molecule reveals the presence of two additional amino acids, Ala-Glu between positions 35 and 36 of the generally accepted structure of bovine β-LPH. This finding is consistent with a structure that contains 93 amino acid residues, as reported by Pankov[32]; however, the residue at position 16 is Gln, which is in agreement with the assignment at this position by Li *et al*[33].

Computer analysis of the amino acids of the cryptic portion of the precursor protein, as inferred from our nucleotide analysis of the cDNA insert of the pSNAC20 plasmid, showed that the sequence of amino acids in the positions minus 55 to minus 44 is strikingly similar to the amino acid sequences of α-MSH and β-MSH, which are known to have structural and biological features in common (Figure 4). This segment of the precursor protein contains the characteristic tetrapeptide sequence His-

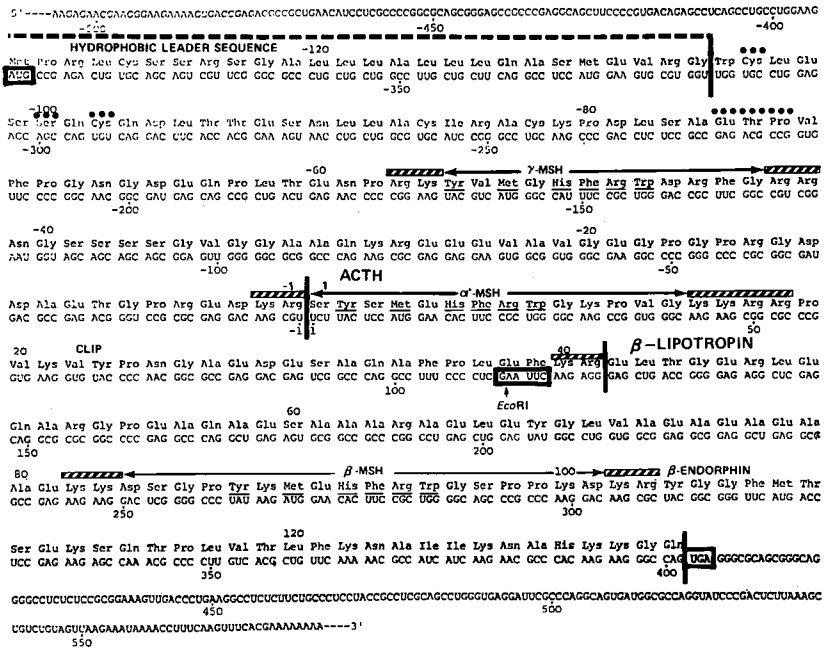

Fig. 3. Nucleotide sequence of corticotropin-β-LPH precursor mRNA as determined by analysis of the 1091 base pair cDNA insert of pSNAC20 plasmid. The cDNA was synthesized on an ACTH-β-LPH precursor mRNA template and cloned in *E. coli* χ1776 as described in Figure 2. Nucleotide sequence of the cDNA insert was carried out by the procedure of Maxam and Gilbert[16] as described[27]. The amino acid sequence was inferred from the nucleotide sequence, using the known amino acid sequence of ACTH (beginning at nucleotide position 1 and amino acid position 1) to establish the translational reading frame. Nucleotide and amino acid positions are numbered at the bottom and top of each panel, respectively, using plus and minus designations with respect to position 1 of ACTH. The untranslated regions at the 5′ and 3′ ends of the mRNA are shown without corresponding amino acids. The hydrophobic putative leader segment is indicated, as are the positions of α, β, and γ MSH.

Amino acids that are at corresponding positions in the "cryptic" segment of the precursor protein and in calcitonin are shown by ●●●●●. Pairs of basic amino acid residues that punctuate the component peptides of the precursor protein are indicated by ▨. The single *Eco*RI site within the cDNA insert, the translational start codon (AUG) for Met, and the UGA translational termination codon are indicted by boxes.

Phe-Arg-Trp, that is required for melanotropic activity, plus tyrosine and methionine residues in the same positions as they are found in known MSHs. Moreover, the MSH-like peptide we identified in the cryptic part of the precursor molecule is flanked on both sides by a pair of basic amino acid residues, suggesting that the new MSH-like peptide (like α-MSH and β-MSH) can be formed by proteolytic processing of the precursor. Because of its remarkable structural similarity to α-MSH and

β-MSH, we have named the newly found peptide, γ-melanotropin (γ-MSH)[27]. It is interesting to note that still another peptide fragment within the putative signal peptide segment of the precursor molecule (amino acid residues minus 111 to minus 105) exhibits some structural similarity to the MSHs; this fragment contains methionine and tryptophan residues at positions characteristic of the MSH structure and shares with α-MSH serine and glutamic acid residues at equivalent positions.

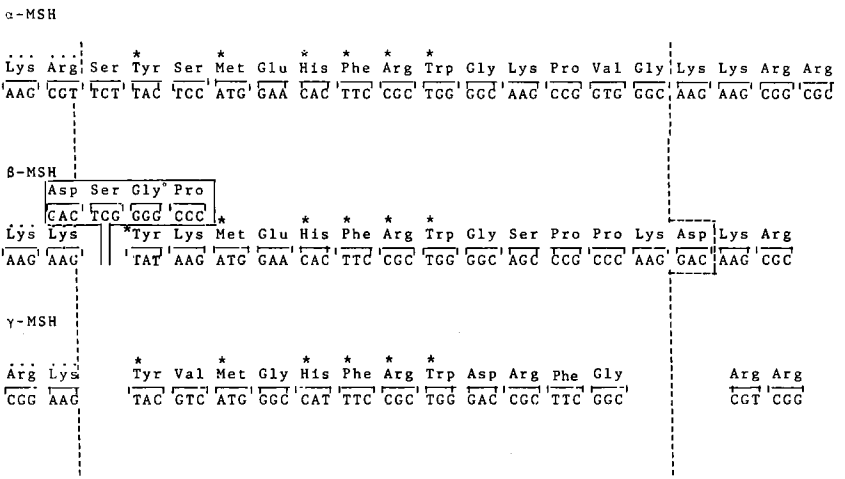

Fig. 4. Comparison of nucleotide and amino acid sequences in regions of corticotropin-β-lipotropin precursor protein that contain α, β, and γ MSH. The amino acids common to all three peptides in corresponding positions are indicated by asterisks. The basic amino acid residues that separate all three MSH peptides from adjacent regions of the precursor protein are set-off by dashed lines. Corresponding amino acids in the three MSH peptides are encoded by identical nucleotides, except for His, which is encoded by CAC in α-MSH and β-MSH and by CAT in γ-MSH and for Tyr, which is encoded by TAC in α-MSH and γ-MSH and by TAT in β-MSH.

It is also worth noting that the cryptic part of the ACTH-β-LPH precursor molecule contains several amino acid residues in positions equivalent to those found in the hormone calcitonin[34]. The Cys at position 2 of the peptide that is produced by excision of the hydrophobic leader sequence is followed by a Ser and Cys at positions 6 and 8 and by a Glu-Thr-Pro at positions 31, 32, and 33. These amino acids are found in analogous positions in bovine calcitonin[34].

Repetitive Nature of the DNA Sequence Encoding the Structure of the Precursor Protein

As shown in Figure 3, the structure of the bovine ACTH-β-LPH precursor peptide, as inferred from the nucleotide sequence of the cDNA insert, indicates that the precursor protein consists of a series of repetitive units, and that combinations of the basic amino acid residues lysine and arginine are located at the junctions of these units. Each of the repetitive structures of the precursor protein contains a peptide core segment having a MSH sequence, and this sequence is followed by or lies within an individually different sequence. The repetitive units, and the MSH sequences themselves are flanked on both sides by pairs of basic amino acid residues or by a terminus of the precursor molecule. The occurrence of a translational termination codon (UGA) following the final amino acid (Gln) of β-endorphin identifies the beginning of the 3′ untranslated region of the mRNA molecule. The structure of the precursor protein suggests that each of its units is destined to be separated from its neighbor by proteolytic processing and that further cleavage occurs within the units to yield smaller peptides.

In addition to the MSH-associated repetitive sequences, other shorter segments of the cDNA sequence appear to be duplicated within the ACTH-β-LPH precursor peptide[26,27]. In certain instances, mismatch between two largely homologous DNA segments results in a shift of the translational reading frame. Thus, despite extensive sequence homology observed at the nucleotide level, such segments encode different amino acid sequences. The potential role of such DNA sequence duplications in the evolution of the gene encoding the precursor protein is of considerable interest.

It is also worth noting the high G + C content (65%) of the structural gene sequence for the translated segment of the ACTH-β-LPH precursor. This contrasts with the 39% G + C content for total bovine DNA. As we have reported[27], the codon choices used for translation of the message for the precursor protein indicate that certain codons are strongly preferred for some amino acids and the choices reflect the high G + C composition of the DNA.

Biological Significance of Experimental Findings

The repetitive nature of the nucleotide and amino acid sequence of the ACTH-β-LPH precursor molecule raises other questions concerning

the biological characteristics and possible functions of peptide fragments that may be derived from it. The presence of several largely homologous units within the same protein molecule suggest that the functions of the component peptides may be related to or coordinated with each other. It is well established that ACTH is essential for the induction and maintenance of glucocorticoid production in adrenocortical cells. However, additional effects of the pituitary on adrenocortical cells, such as stimulation of mitogenic activity and mineralocorticoid production cannot be accounted for solely by the action of ACTH. It is reasonable to speculate that other peptides that may be elaborated from the precursor molecule are involved in these actions.

Another possibility is that these structurally-related peptides are involved in neural functions. Several studies have demonstrated that not only endorphins and methionine-enkephalin, but also MSHs and related peptides are present in various regions of the central nervous system. In addition to the potent opiate activity of endorphins and methionine-enkephalin, MSH-related peptides have been reported to exert certain effects, such as learning and retention of new behavior, on brain functions. It is thus intriguing to speculate that various peptides derived from the ACTH-β-LPH precursor may act together or antagonistically as modulators of neural functions.

In the experiments discussed here, DNA cloning techniques have defined the structural relationship of polypeptide components of the ACTH-β-LPH precursor protein, have led to the discovery of a new peptide (γ-MSH) within the precursor protein, and have provided other valuable information about the structure of the previously cryptic part of the precursor molecule. The repetitive DNA sequences observed in the cDNA we have cloned have intriguing implications for an understanding of the evolution of the gene encoding this peptide, as does the occurrence within this DNA of sequences encoding amino acids in positions equivalent to the positions of the same amino acids in the calcitonin molecule. However, it is worth noting that the conclusions summarized here result from a study of the cDNA copy of a single mRNA molecule. In any case, the DNA cloning procedures used in our investigations have proved to be a valuable tool for elucidating the structure and function of these peptide hormones and for study of the genes that encode them.

Acknowledgements

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STRUCTURE AND EXPRESSION OF GROWTH HORMONE GENES BY RECOMBINANT DNA TECHNOLOGY

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Introduction

There are many polypeptides of biologic and medical interest which have not been fully studied because they cannot be obtained in adequate quantity. Recent advances in biochemistry and recombinant DNA technology have made possible the controlled synthesis of specific proteins in organisms or under conditions in which they are not normally synthesized. Such methods employ enzymes and subcellular components of the protein synthesizing machinery of living cells, either *in vitro* in cell-free systems, or *in vivo* in microorganisms. In either case, the key element is the provision of a nucleic acid template specifying the desired amino acid sequence. Two basic facts underlie the application of these technologies: first, DNA and RNA from all living organisms are chemically similar and second, the genetic code is universal.

DNA- and RNA-directed protein synthesizing systems allow the preparation and detection of radioactively labelled polypeptides[1,2]. These techniques have been very useful for studying the regulation of transcription and translation, but the small amount of material made constitutes a serious limitation to synthesis of preparative quantities.

Recombinant DNA technology has made it possible to isolate specific genes from higher organisms and to transfer them into the genomes of microorganisms, such as bacteria or phages. The transferred gene is replicated and propagated when the host microorganism replicates. As a result, large quantities of the original gene can be obtained in a very short time (amplification process) and its structure can be readily investigated. In addition, if care has been taken to insert the foreign gene in the proper orientation and reading frame and adequately located in the bacterial genome, the microorganism may transcribe and translate it as one of its own, making important amounts of the encoded protein available

(expression process). This is especially desirable when dealing with medically needed products such as growth hormone, insulin, etc.

We have isolated and amplified in bacteria the DNA coding for rat growth hormone (rGH)[3], human growth hormone (hGH)[4] and human chorionic somatomammotropin (hCS)[5]. The comparison of the primary structure of these DNAs shows a high degree of homology which supports the hypothesis derived earlier from the amino acid sequences[6,7], that the GH and CS genes arose by duplication from a common ancestral precursor[4]. From the DNA sequences, we have deduced the amino acid sequences of both rat and human pregrowth hormone and also confirmed most of the amino acid sequence of hCS. Finally, the cloned GH DNAs have been transferred to specially designed "expression plasmids" as a means to have bacteria synthesize GH[4,8]. This is of particular importance since there is not enough hGH available to meet all the medical needs, nor to explore others of its possible therapeutic uses.

This report summarizes our use of recombinant DNA technology to study the structure, evolution, and expression bacteria of these peptide hormone genes.

Molecular Cloning of Complementary DNA

When a double stranded DNA fragment is available in purified form, its molecular cloning is a well established procedure and is described elsewhere[9]. Briefly, the DNA fragment is first covalently linked to the DNA of a suitable vehicle, such as a bacterial plasmid. Plasmids are extrachromosomal, double stranded, circular DNAs which may exist in some bacteria. They generally carry genes coding for antibiotic resistance and enter and establish themselves in the bacteria by a process known as "transformation". The plasmid pBR322 has been specially designed for cloning[10]. It contains the genes coding for ampicillin and tetracycline resistance and many sites specifically recognized by a variety of hydrolytic enzymes termed restriction endonucleases. These enzymes recognize and cleave mirror image DNA segments ("palindromes") 4 to 6 nucleotides long. The sequence $\overset{5'\text{...}AAGCTT\text{...}3'}{3'\text{...}TTCGAA\text{...}5'}$, specifically cleaved by the restriction enzyme Hind III into $\overset{5'\text{...}A}{3'\text{...}TTCGA} + \overset{AGCTT\text{...}3'}{A\text{...}5'}$, was chosen in our studies as the insertion site for the hormonal DNAs. This sequence occurs only once in pBR322 and is located in the gene coding for tetracycline resistance. The plasmid with the foreign DNA insert (recombinant plasmid) is used to transform the bacterium host (*Escherichia coli* in our case) which, when grown, will replicate faithfully the infecting plasmid and yield unlimited amounts of the starting DNA.

Obviously, if this starting DNA is heterogeneous, so will be the population of transformed bacteria. In this case an additional screening step will be necessary to select those bacterial clones containing the recombinant DNA of interest. The difficulty of this step depends, of course, on the relative abundance of the desired DNA species in the original mixture.

In our present studies, the critical step was to obtain a sufficiently enriched preparation of the DNA sequences coding for GH and CS. At least two copies of the genes coding for these proteins are present in any human or rat somatic cell. Therefore, in principle, these genes could be isolated from the chromosomes of any tissue. However, this was not selected as a first approach for several reasons. First, intervening sequences (or introns) have been found in most eukaryotic genes[11]. These segments of DNA, often of considerable length, interrupt the coding regions of genes; they are transcribed into the initial nuclear RNA but their RNA transcripts are excised when nuclear RNA is processed into mRNA. Since bacteria do not have introns and do not process their RNA in the same manner, they are not able to express eukaryotic genes containing intervening sequences into meaningful protein. The second limitation of this procedure is in the high complexity of the eukaryotic genome (about 5×10^6 different genes) requiring a high sensitive assay to detect the desired sequences.

The strategy for obtaining the starting DNA material to be cloned was to obtain messenger RNA preparations enriched in either the GH or CS sequences and to reverse transcribe them into complementary DNA (cDNA)[12,13]. The advantage of this approach is obvious. First, the cDNA does not have any intervening sequences since it is an exact copy of the mRNA; second, the cDNA can be radioactively labelled during synthesis, greatly facilitating its handling and analysis; third, the complexity of the mRNA population (and therefore, the cDNA population) is relatively low (about $1\text{-}5 \times 10^4$ different mRNAs/cell); finally, adequate selection of the tissue and of its physiologic treatment may increase the concentration of the mRNA of interest. We have used human placentas obtained from term pregnancies as source of mRNA for the cloning of hCS cDNA[5], cultured rat pituitary cells treated with thyroid and glucocorticoid hormones for the cloning of rGH cDNA[3] and human pituitary tumors removed by surgery from acromegalic patients for the cloning of hGH cDNA[4].

Total RNA was isolated by homogenizing the tissue or cells in guanidine thiocyanate solution and centrifuging the RNA through a

CsCl solution[14]. Polyadenylated RNA (containing most of the mRNA) was obtained by affinity chromatography on oligo-dT cellulose[15]. The integrity of the mRNA preparations and their degree of enrichment in the mRNA of interest was verified by cell-free translation[2]. Figure 1 shows an example of such an analysis, which strongly suggests that the rat and human GH mRNAs and the hCS mRNA were the most abundant species in our mRNA preparations.

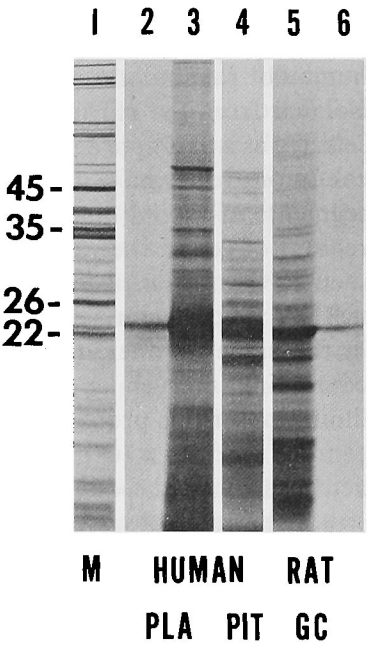

Fig. 1. Cell-free translation products of the mRNA preparations. An aliquot of the mRNA preparations was translated in the wheat-germ protein synthesizing system. The ^{35}S-labelled protein products were analyzed by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel and autoradiographed. Lane 1: molecular weight markers[4]; lane 3: products coded by term human placenta mRNA; lane 4: products coded by human pituitary tumor mRNA; lane 5; products coded by mRNA extracted from rat cultured pituitary cells (GC) induced by thyroid and glucocorticoid hormones. Lane 2 is an immunoprecipitate of the products shown in lane 3 with antiserum to hCS; lane 6 is an immunoprecipitate of the products shown in lane 5 with antiserum to rGH.

Double stranded cDNA was synthesized from the mRNA preparations by reverse transcription. At this stage, analysis of the double stranded cDNA by polyacrylamide gel electrophoresis showed a pronounced size heterogeneity. This is the result of several factors including priming with short poly=dT pieces at different sites of the much longer poly-A tails, the difficulty in obtaining full-length transcripts from long RNA molecules with reverse transcriptase, and the sequence complexity of the RNA template. Cleavage of cDNA with restriction enzymes eliminates some heterogeneity and, on gel electrophoresis, bands corresponding to distinct DNA fragments appear clearly[16]. This is illustrated in Figure 2 using hGH cDNA as an example. The assumption that the detectable fragments originated from the most abundant DNA species

present (GH or CS DNA based on mRNA translation) has been directly verified by extracting these fragments from the gel and determining their DNA sequences. The fragments obtained in this way were valuable, either to clone as such (as in the case with hCS and rGH) or as markers for the identification of cloned full-length cDNA (as in the case with rGH and hGH).

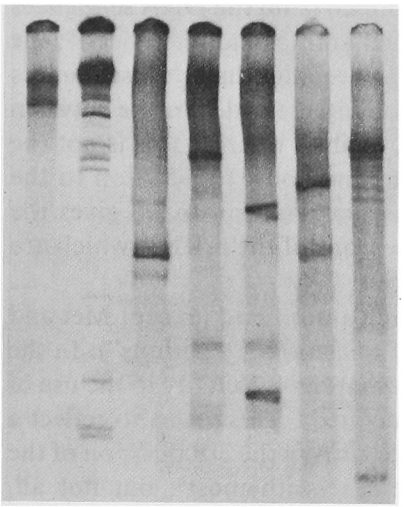

Fig. 2. Analysis of cDNA synthesized from mRNA extracted from a human pituitary tumor producing growth hormone. Aliquots of the cDNA preparation were cleaved with various restriction endonucleases and electrophoresed on a polyacrylamide gel. The figure shows an autoradiogram of the gel: a: uncleaved cDNA; b: molecular weight markers; c: cDNA cut by Pst I and Bgl II; d: cDNA cut by Pvu II; e: cDNA cut by Pvu II and Bgl II; f: cDNA cut by Hinf I and Sma I; g: cDNA cut by Hae III (taken from ref. 4).

For inserting cDNA in the Hind III site of the plasmid pBR322, synthetic tetranucleotides (pApGpCpT) complementary to the Hind III sticky ends were (see above) ligated to the 5′ extremities of the cDNA fragments[17]. Then, Hind III-opened plasmid and cDNA were mixed so as to allow their complementary ends to hybridize. The chains were ligated with DNA ligase and the resulting material used to transform *E. coli*. Bacteria containing a recombinant DNA plasmid were selected by their antibiotic resistance characteristics (Amp^R Tet^S vs. Amp^R Tet^R for bacteria transformed with intact pBR322 and Amp^S Tet^S for nontransformed bacteria). Selected clones were grown in quantity, and their plasmid DNA isolated. This DNA was digested with Hind III and various other restriction nucleases and analyzed by gel electrophoresis. In every case, an appreciable number of clones harbored an inserted DNA whose restriction map matched the one obtained with double stranded cDNA prior to cloning; these were tentatively identified as GH or CS clones.

Sequence Analysis of the Cloned DNA

The definitive identification of the cloned DNA was performed by sequencing the inserted DNA. This was done either by the chemical cleavage technique of Maxam and Gilbert[18] or by the chain termination technique of Sanger *et al*[19]. From the DNA sequences, the corresponding mRNA and protein sequences were deduced; they are shown in Figure 3, 4 and 5. The amino acid sequences determined from the DNA sequences are generally consistent with the published amino acid sequences[20]; the only exceptions concern amide groups. We feel that the DNA sequences are correct in this respect, since by this technique, the difference between Asp and Asn or Glu and Gln are readily visible, whereas this is not the case with the classical protein sequencing methods. In addition to the sequences coding for the proteins themselves, our approach gives the sequences of the signal peptides and of regions of the mRNA which are not translated into protein.

As the genetic code is redundant, each amino acid (except Met and Trp) may be coded by several triplets of nucleotides ("codons"). In the three genes we have sequenced, there is a marked selectivity in the use of one triplet over another for certain amino acids. This seems to reflect a strong preference for G or C rather than U or A in the 3rd position of the codon. This has been found to be the case with most[4], but not all, eukaryotic[21] mRNAs sequenced to date.

Comparison of the Primary Structure of the Genes

Table I shows homologies between the nucleotide and the amino acid sequences of hCS, hGH, and rGH. Two points should be noted. First, there is more homology between the nucleic acid sequences than between the amino acid sequences; this strengthens the hypothesis that GH and CS genes evolved from a common ancestral gene by a duplication mechanism[4,6,7]. Second, hGH has more homology with hCS than with rGH; this suggests that the duplication event occurred after the human and rat species had already separated. Since both genes exist in both species, if we assume a comparable evolution rate for both genes, this implies that the same duplication mechanism may have occurred independently more than once[4].

In contrast to the 5′ untranslated and coding regions, the 3′ untranslated regions of hGH and rGH mRNAs seem relatively unrelated (only 38% homology). If appropriate gaps are introduced, a homology of more

```
                        -26                     -20
                        met ala ala asp ser gln thr pro trp leu
GUGGACAGAUCACUGAGUGGCG  AUG GCU GCA GAC UCU CAG ACU CCC UGG CUC

                        -10
leu thr phe ser leu leu cys leu leu trp pro gln glu ala gly ala
CUG ACC UUC AGC CUG CUC UGC CUG CUG UGG CCU CAA GAG GCU GGU GCU

  1                                 10
leu pro ala met pro leu ser ser leu phe ala asn ala val leu arg
UUA CCU GCC AUG CCC UUG UCC AGU CUG UUU GCC AAU GCU GUG CUC CGA

            20                                      30
ala gln his leu his gln leu ala ala asp thr tyr lys glu phe glu
GCC CAG CAC CUG CAC CAG CUG GCU GCU GAC ACC UAC AAA GAG UUC GAG

                              40
arg ala tyr ile pro glu gly gln arg tyr ser ile gln asn ala gln
CGU GCC UAC AUU CCC GAG GGA CAG CGC UAU UCC AUU CAG AAU GCC CAG

     50                                 60
ala ala phe cys phe ser glu thr ile pro ala pro thr gly lys glu
GCU GCG UUC UGC UUC UCA GAG ACC AUC CCA GCC CCC ACC GGC AAG GAG

                      70                                      80
glu ala gln gln arg thr asp met glu leu leu arg phe ser leu leu
GAG GCC CAG CAG AGA ACU GAC AUG GAA UUG CUU CGC UUC UCG CUG CUG

                                  90
leu ile gln ser trp leu gly pro val gln phe leu ser arg ile phe
CUC AUC CAG UCA UGG CUG GGG CCC GUG CAG UUU CUC AGC AGG AUC UUU

            100                                     110
thr asn ser leu met phe gly thr ser asp arg val tyr glu lys leu
ACC AAC AGC CUG AUG UUU GGU ACC UCG GAC CGC GUC UAU GAG AAA CUG

                            120
lys asp leu glu glu gly ile gln ala leu met gln glu leu glu asp
AAG GAC CUG GAA GAG GGC AUC CAG GCU CUG AUG CAG GAG CUG GAA GAC

    130                                 140
gly ser pro arg ile gly gln ile leu lys gln thr tyr asp lys phe
GGC AGC CCC CGU AUU GGG CAG AUC CUC AAG CAA ACC UAU GAC AAG UUU

                    150                                     160
asp ala asn met arg ser asp asp ala leu leu lys asn tyr gly leu
GAC GCC AAC AUG CGC AGC GAU GAC GCU CUG CUC AAA AAC UAU GGG CUG

                                170
leu ser cys phe lys lys asp leu his lys ala glu thr tyr leu arg
CUC UCC UGC UUC AAG AAG GAC CUG CAC AAG GCA GAG ACC UAC CUG CGG

            180                                     190
val met lys cys arg arg phe ala glu ser ser cys ala phe AM
GUC AUG AAG UGU CGC CGC UUU GCG GAA AGC AGC UGU GCU UUC UAG GCA

CACACUGGUGUCUCUGCGGCACUCCCCCGUUACCCCCCUGUACUCUGGCAACUGCCACCCCUA

CACUUUGUCCUAAUAAAAUUAAUGAUGCAUCAUAUCAAAAAAAAAAAAAAAAAA
```

Fig. 3. Nucleotide sequence of rGH mRNA and its inferred amino acid sequence (taken from ref. 3).

```
                                    -26                     -20
                                    met ala thr gly ser arg thr ser
GGAUCCUGUGGACAGCUCACCUAGCUGCA       AUG GCU ACA GGC UCC CGA ACG UCC

                                    -10
leu leu leu ala phe gly leu leu cys leu pro trp leu gln tyr ser
CUG CUC CUG GCU UUU GGC CUG CUC UGC CUG CCU UGG CUU CAA UAU UCA

         1                                  10
gly pro phe pro thr ile pro leu ser arg leu phe asp asn ala met
GGA CCU UUC CCA ACC AUU CCA UUA UCC AGA CUU UUU GAC AAC GCU AUG

                    20                                      30
leu arg ala his arg leu his gln leu ala phe asp thr tyr gln glu
CUC CGC GCC CAU CGU CUG CAU CAG CUG GCC UUU GAC ACC UAC CAG GAG

                                   40
phe glu glu ala tyr ile pro lys glu gln lys tyr ser phe leu gln
UUU GAA GAA GCC UAU AUC CCA AAG GAA CAG AAG UAU UCA UUC CUG CAG

              50                                    60
asn pro gln thr ser leu cys phe ser glu ser ile pro thr pro ser
AAC CCC CAG ACC UCC CUC UGU UUC UCA GAG UCU AUU CCG ACA CCC UCC

                                70
asn arg glu glu thr gln gln lys ser asn leu glu leu leu arg ile
AAC AGG GAG GAA ACA CAA CAG AAA UCC AAC CUA GAG CUG CUC CGC AUC

     80                                     90
ser leu leu leu ile gln ser trp leu glu pro val gln phe leu arg
UCC CUG CUG CUC AUC CAG UCG UGG CUG GAG CCC GUG CAG UUC CUC AGG

                    100                                     110
ser val phe ala asn ser leu val tyr gly ala ser asp ser asn val
AGU GUC UUC GCC AAC AGC CUG GUG UAC GGC GCC UCU GAC AGC AAC GUC

                                   120
tyr asp leu leu lys asp leu glu glu gly ile gln thr leu met gly
UAU GAC CUC CUA AAG GAC CUA GAG GAA GGC AUC CAA ACG CUG AUG GGG

            130                                     140
arg leu glu asp gly ser pro arg thr gly gln ile phe lys gln thr
AGG CUG GAA GAU GGC AGC CCC CGG ACU GGG CAG AUC UUC AAG CAG ACC

                               150
tyr ser lys phe asp thr asn ser his asn asp asp ala leu leu lys
UAC AGC AAG UUC GAC ACA AAC UCA CAC AAC GAU GAC GCA CUA CUC AAG

    160                                    170
asn tyr gly leu leu tyr cys phe arg lys asp met asp lys val asp
AAC UAC GGG CUG CUC UAC UGC UUC AGG AAG GAC AUG GAC AAG GUC GAC

                    180                                     190
thr phe leu arg ile val gln cys arg ser val glu gly ser cys gly
ACA UUC CUG CGC AUC GUG CAG UGC CGC UCU GUG GAG GGC AGC UGU GGC

191
phe AM
UUC UAG CUGCCCGGGUGGCAUCCUGUGACCCCUCCCCAGUGCCUCUCCUGGCCCUGGAAGUUG

CCACUCCAGUGCCCACCAGCCUUGUCCUAAUAAAAUUAAGUUGCAUCAAAAAAAAAAAA
```

Fig. 4. Nucleotide sequence of hGH mRNA and its inferred amino acid sequence (taken from ref. 4).

```
     24                        30
    ala ile asp thr tyr gln glu phe glu glu thr tyr ile pro lys
G   CCC AUU GAC ACC UAC CAG GAG UUU GAA GAA ACC UAU AUC CCA AAG

     40                                      50
asp gln lys tyr ser phe leu his asp ser gln thr ser phe cys phe
GAC CAG AAG UAU UCG UUC CUG CAU GAC UCC CAG ACC UCC UUC UGC UUC

                    60                                      70
ser asp ser ile pro thr pro ser asn met glu glu thr gln gln lys
UCA GAC UCU AUU CCG ACA CCC UCC AAC AUG GAG GAA ACG CAA CAG AAA

                                       80
ser asn leu glu leu leu arg ile ser leu leu leu ile glu ser trp
UCC AAU CUA GAG CUG CUC CGC AUC UCC CUG CUG CUC AUC GAG UCG UGG

             90                                        100
leu glu pro val arg phe leu arg ser met phe ala asn asn leu val
CUG GAG CCC GUG CGG UUC CUC AGG AGU AUG UUC GCC AAC AAC CUG GUG

                               110
tyr asp thr ser asp ser asp asp tyr his leu leu lys asp leu glu
UAU GAC ACC UCG GAC AGC GAU GAC UAU CAC CUC CUA AAG GAC CUA GAG

    120                                     130
glu gly ile gln thr leu met gly arg leu glu asp gly ser arg arg
GAA GGC AUC CAA ACG CUG AUG GGG AGG CUG GAA GAC GGC AGC CGC CGG

                    140                                     150
thr gly gln ile leu lys gln thr tyr ser lys phe asp thr asn ser
ACU GGG CAG AUC CUC AAG CAG ACC UAC AGC AAG UUU GAC ACA AAC UCG

                                   160
his asn his asp ala leu leu lys asn tyr gly leu leu tyr cys phe
CAC AAC CAU GAC GCA CUG CUC AAG AAC UAC GGG CUG CUC UAC UGC UUC

            170                                       180
arg lys asp met asp lys val glu thr phe leu arg met val gln cys
AGG AAG GAC AUG GAC AAG GUC GAG ACA UUC CUG CGC AUG GUG CAG UGC

                               190 191
arg ser val glu gly thr cys gly phe AM
CGC UCU GUC GAG GGC ACG UGU GGC UUC UAG GUGCCCGAGUAGCAUCCUGUGACC

CCUCCCCAGUGCCUCUCCUGGCC
```

Fig. 5. Nucleotide sequence of a fragment of hCS mRNA and its inferred amino acid sequence (taken from ref. 5).

than 90% appears in a short region at the end of the molecules. This region is located just before the poly-A tail and includes the AAUAAA sequence found in almost all eukaryotic mRNAs. Sequence conservation may indicate a specific function for this region.

Table I. Amino Acid and Nucleic Acid Sequence Homology Between rGH, hGH and hCS.

	Homology (%)	
	hGH vs. rGH	hGH vs. hCS
Nucleic Acid:		
5'-noncoding	73	90*
Presequence	76	90*
Coding	76	92
3'-noncoding	38	94
Amino Acid:		
Presequence	58	84
Coding	67	86

Data are taken from ref. 4 except for the ones labelled with an asterisk which are from unpublished results[22].

Expression of Growth Hormone in Bacteria

To see whether bacteria can use the cloned DNA to synthesize GH protein sequences, we tried two types of "expression plasmids", first with rGH and then with hGH. An organism seldom uses all of its genetically endowed capabilities at any given time. For example, *E. coli* seems to express only those operons coding for the proteins it needs in a given environment[23]. An operon is a group of genes, clustered together in continuous sequence and regulated as a whole, which code for proteins carrying out related functions in the bacterium. Each operon contains, proximal to its N-terminal amino acid codon, a DNA region called the "control region", which includes a variety of controlling elements such as the operator, promoter, and ribosome binding site. The function of this "control region" is to direct the initiation of transcription and translation of the operon when the cell needs it. Therefore, to be expressed in *E. coli*, the GH gene must be properly and precisely located with respect to a given *E. coli* control region. Since in both prokaryotes and eukaryotes translation is initiated by the incorporation of methionine coded by ATG, the GH gene should ideally be inserted behind a bacterial control region such that its first ATG codon substitutes for the ATG codon of the bacterial protein it replaces. Genes synthesized *in vitro* by organic chemistry techniques may fulfill such precise construction. However, when dealing with natural genes one must rely on the naturally occurring restriction sites for cleaving and splicing the DNAs; as a consequence,

our experiments lead, in a first step, to the expression of rat and human GH linked at their N-terminal to bacterial protein sequences[4,8].

rGH sequences were expressed in bacteria by inserting the previously cloned rGH cDNA in the gene coding for beta-lactamase, on a plasmid derived from pBR322, named "rGH-expression plasmid"[8]. This was performed by taking advantage of two Pst I restriction sites, one between the nucleotides coding for amino acids 182 and 183 of the beta-lactamase gene and the second between the nucleotides coding for amino acids -24 and -23 of the presequence of rGH cDNA. Figure 6 shows the junction between the two genes and how the correct reading frame is maintained. The resulting hybrid gene codes for a protein of 395 amino acids containing the N-terminal 81 amino acids of beta-lactamase connected by an ordinary peptide linkage to the 214 residues of rat pregrowth hormone. The synthesis of the hybrid beta-lactamase-rGH product was demonstrated in two ways. First, the proteins encoded by the expression plasmid were compared on sodium dodecyl sulfate-polyacrylamide gel to those encoded by pBR322 (Figure 7). Pre beta-lactamase and beta-lactamase synthesized by pBR322 were missing in the expression plasmid products; they were replaced by a new polypeptide of about 47,000 daltons which is within the experimental error of the MW expected for the rGH hybrid protein. Second, colonies of *E. coli* containing either the expression plasmid or pBR322 were compared for their content of an rGH immunoreactive product. The solid phase immunological screening test[24] showed that only the colonies containing the rGH-expression plasmid reacted with purified ^{125}I-anti-rGH IgG. We estimated that about 25,000 molecules of beta-lactamase-rGH were synthesized per *E. coli* cell.

hGH sequences were expressed in bacteria by using the tryptophan expression plasmid designed by Hallewell and Emtage[4,25]. Derived from pBR322, this plasmid contains a portion of the *E. coli* tryptophan operon[26], namely the entire *trp* control region, the entire first *trp* gene (*trp*E) and 275 base pairs of the second *trp* gene (trpD) located ahead of the Hind III site. Any DNA inserted at the Hind III site of this plasmid should therefore be transcribed and translated as a continuation of the *trp*D gene, under the regulation of the *trp* control region. The cloned hGH cDNA was inserted at this site, in the same reading frame as the *trp*D sequence (Figure 8). The anticipated product was a hybrid protein containing the N-terminal region of the *trp*D protein, 13 amino acids coded by the junction and the 5′ (normally untranslated) region of hGH mRNA, the 26 amino acids of the signal peptide of prehGH and the 191

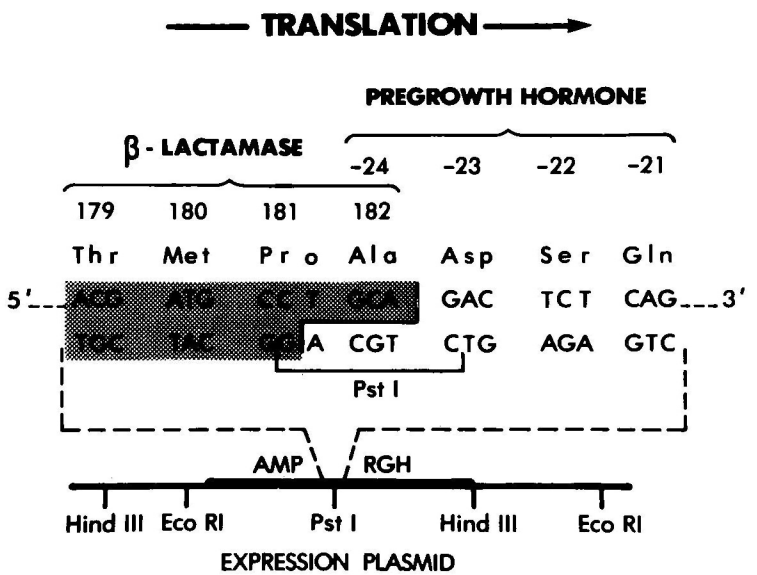

Fig. 6 Postulated nucleotide sequence at the junction between the beta-lactamase and rGH genes in the rGH-expression plasmid (taken from ref. 8).

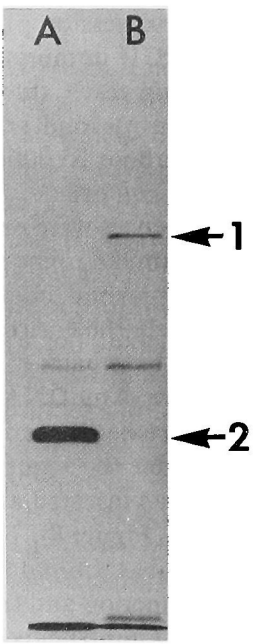

Fig. 7. Autoradiogram of a sodium dodecyl sulfate-polyacrylamide gel displaying the proteins synthesized from pBR322 (lane A) and from the rGH-expression plasmid (lane B). Arrow 1: fusion protein; Arrow 2: beta-lactamase (taken from ref. 8).

amino acids of hGH itself. When *E. coli* containing this hGH-*trp* expression plasmid were treated with 3β-indolylacrylic acid (which specifically interacts with the *trp* control region, promoting transcription of the *trp* operon[17]) large amounts of the expected hybrid product were induced. This was demonstrated by pulse labelling the bacterial proteins at various times after the derepression (Figure 9). The hybrid *trp*D-hGH protein was immunoprecipitated with antiserum to hGH and quantitated by its incorporated radioactivity. The hybrid protein represented about 3% of the proteins synthesized in derepressed *E. coli*, or about 10^6 molecules per cell.

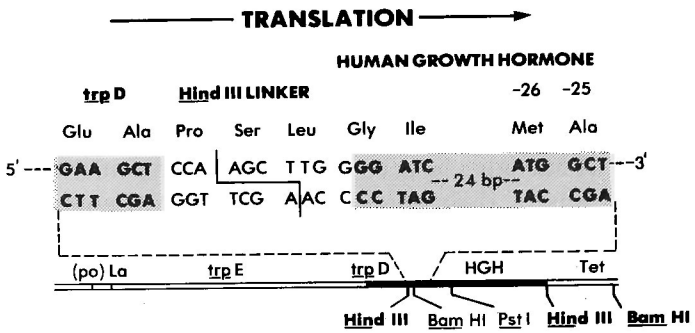

Fig. 8. Postulated nucleotide sequence at the junction between the *trp*D and hGH genes in the hGH-*trp* expression plasmid (taken from ref. 4).

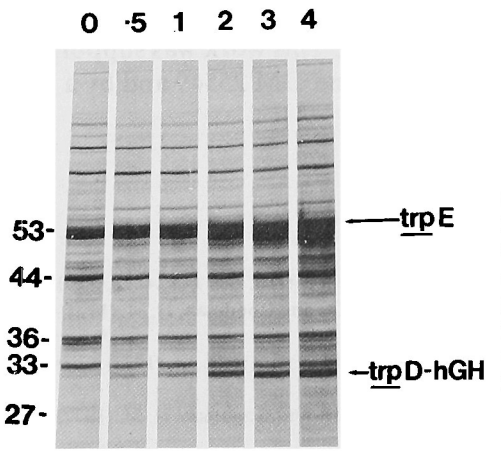

Fig. 9. Autoradiogram of sodium dodecyl sulfate-polyacrylamide gel displaying the pulse labelled proteins from *E. coli* harboring the hGH-*trp* expression plasmid. The cultures were induced by 3β-indolylacrylic acid for the number of hours indicated above each lane. The molecular weight x 10^3 are shown on the left (taken from ref. 4).

Conclusion

This report shows how developments in recombinant DNA technology provide new methods to study the primary structure of proteins. In fact, by looking directly at the gene level, appreciably more information is gained: structure of the signal peptide of the hormones, structure of the portions of the mRNAs which are not translated into proteins, but could play a role in the regulation of their synthesis, etc. These additional data provide new tools to approach more directly many problems including those concerning the evolution of genes and the regulation of their expression. The cDNAs obtained by cloning serve many other purposes not discussed here, e.g., they constitute the "probes" used to isolate their chromosomal counterparts[11]: genomic DNA generally contains intervening sequences whose locations, structure and processing may be important for understanding the molecular steps involved in gene expression. Cloned cDNAs may also be used to titrate, by molecular hybridization, the *in vivo* levels of their complementary mRNA and nuclear RNA precursors[28]. Finally, our studies with the GH expression plasmids represent an important step toward the production of peptide hormones in bacteria.

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CHEMICAL DNA SYNTHESIS AS AN APPROACH TO PEPTIDE SYNTHESIS: THE HUMAN INSULIN PROJECT

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Introduction

In the last two years, several bacterial strains have been constructed that produce mammalian peptide hormones and other eukaryotic proteins (Table I). It is now clear that genetically engineered bacteria will be a valuable source of polypeptides. In this paper, we will focus on our human insulin project and try to give the reader an overview of the recombinant DNA and chemical DNA synthesis techniques that made this research feasible.

Two approaches have been used for the molecular cloning and expression of genes for mammalian polypeptides. One successful approach starts with messenger RNA and uses reverse transcriptase to make a DNA copy, which is then cloned. Our work has used an entirely different approach that has great generality and flexibility[1,2]. Knowing the desired amino acid sequence, we use the genetic code and write a DNA sequence that should command the production of the desired peptide. Chemical DNA synthesis is then used to make the gene. Thus, the gene is a completely artificial or man-made one with a DNA sequence that probably is quite unlike the natural gene.

The Insulin Project

A. Overview. Figure 1 illustrates the overall scheme that we used to produce human insulin[2]. The human insulin chains (21 amino acid A chain and 30 amino acid B chain) were made in separate bacterial strains

Table I. Production of Eukaryotic Hormones and Proteins in *E. coli.*

Peptide or Protein	Form	Cleavable Free Active Form	Reference
A. HORMONES			
Somatostatin	Fused to β-Galactosidase	Yes	Itakura, *et al.*[a]
Rat Insulin	Fused to β-Lactamase	No	Villa-Komaroff, *et al.*[b]
Rat Growth Hormone	Fused to β-Lactamase	No	Seeburg, *et al.*[c]
Human Insulin A and B-chain	Fused to β-Galactosidase	Yes	Goeddel, *et al.*[d]
Human Growth Hormone	Fused to trpD peptide	No	Martial, *et al.*[e]
Human Growth Hormone	Free	—	Goeddel, *et al.*[f]
B. PROTEINS			
Ovalbumin	8 Amino acid β-Galactosidase Precursor	No	Fraser and Bruce[g]
Ovalbumin	8-Amino acid β-Galactosidase Precursor	No	Mercereau-Puijalon *et al.*[h]
Dihydrofolate Reductase	Free?	—	Chang, *et al.*[i]
Hepatitis Core Antigen	Probably fused to β-Lactamase	—	Burrell, *et al.*[j]

[a]Itakura, K., Hirose, T., Crea, R., Riggs, A. D., Heyneker, H. L., Bolivar, F., and Boyer, H. W. (1977) *Science* **198,** 1056-1063. [b]Villa-Komaroff, L., Efstratiadis, A., Broome, S., Lomedico, P., Tizard, R., Naber, S. P., Chick, W. L., and Gilbert, W. (1978) *Proc. Nat. Acad. Sci. USA* **75,** 3727-3731. [c]Seeburg, P. H., Shine, J., Martial, J. A., Ivarie, R. D., Morris, J. A., Ullrich, A., Baxter, J. D., and Goodman, H. M. (1978) *Nature* **276,** 795-798. [d]Goeddel, D. V., Kleid, D. G., Bolivar, F., Heyneker, H. L., Yansura, D. G., Crea, R., Hirose, T., Kraszewski, A., Itakura, K., and Riggs, A. D. (1979) *Proc. Nat. Acad. Sci. USA* **76,** 106-110. [e]Martial, J. A., Hallewell, R. A., Baxter, J. D., and Goodman, H. M. (1979) *Science* **205,** 602-607. [f]Goeddel, D. V., Heyneker, H. L., Hozumi, T., Arentzen, R., Itakura, K., Yansura, D. G., Ross, M. J., Miozzari, G., Crea, R., and Seeburg, P. H. (1979) *Nature* in press. [g]Fraser, T. H. and Bruce, B. J. (1979) *Proc. Nat. Acad. Sci. USA* **75,** 5936-5940. [h]Mercereau-Puijalon, O., Royal, A., Cami, B., Garapin, A., Krust, A., Gannon, F., and Kourilsky, P. (1978) *Nature* **275,** 505-510. [i]Chang, A. C. Y., Nunberg, J. H., Kaufman, R. J., Erlich, H. A., Schimke, R. T., and Cohen, S. N. (1978) *Nature* **275,** 617-624. [j]Burrell, C. J., Mackay, P., Greenaway, P. J., Hofschneider, P. H., and Murray, K. (1979) *Nature* **279,** 43-47.

as tails on a rather large precursor protein, the enzyme beta-galactosidase. The insulin chains were efficiently clipped from the precursor protein by treatment with cyanogen bromide, a methionine specific cleavage reagent. Because synthetic DNA was used to make the insulin genes, we were able to arrange that the insulin tails are attached to beta-galactosidase by a methionine linkage. After purification of the separate insulin chains, they were joined by air oxidation[3] to give active insulin.

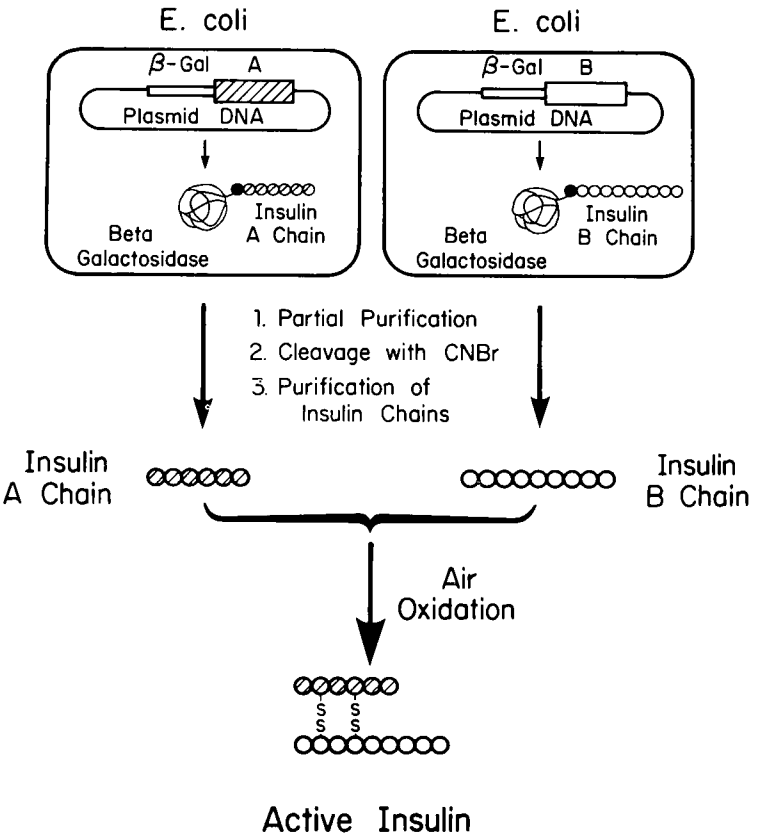

Fig. 1. Schematic overview of the strains and procedures for the production of human insulin by bacteria. Two *E. coli* strains were constructed having chemically synthesized insulin A or B chain genes inserted into the beta-galactosidase gene of a plasmid cloning vector. *In vivo*, a fused protein is made, mostly beta-galactosidase, but with an insulin tail joined by a methionine. *In vitro*, the insulin peptide chain is clipped off by treatment with cyanogen bromide. After separate purification, the insulin A and B chains are joined by air oxidation. For details cf. Ref. 2

B. Chemical DNA synthesis. Although there are alternative methods[4] the fastest way to make DNA is the phosphotriester method[5,6] illustrated in Figure 2. Some details of the method are given in the legend of Figure 2, but here we will just state that recent improvements in the method, such as the rapid synthesis of trimers, together with the extensive use of high performance liquid chromatography for analysis and purification of the oligodeoxyribonucleotides, have dramatically reduced the time

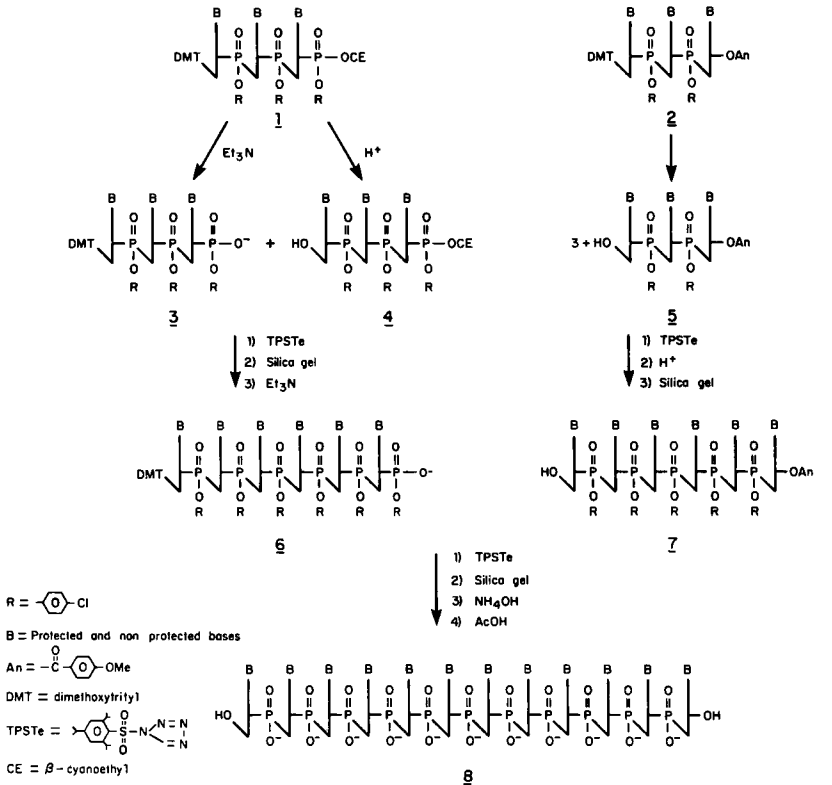

Fig. 2. The chemical synthesis of oligodeoxyribonucleotides by the improved triester method[5,6]. Starting with nucleosides, a library of fully protected triester trimers, such as *1* and *2* are made. The type *2* trimer, with the anisol 3′-protecting group will become the 3′ end of the oligonucleotide. The type *1* trimer is bifunctional, and depending on the treatment (either mild acid or base) will be either the 5′-end component or an internal sequence component. Because of the chlorophenyl protecting groups attached by ester linkage to the phosphate groups (forming phosphotriesters), the trimers and intermediate oligonucleotides (e.g., *3, 4, 5, 6, 7*) are not soluble in water. Therefore all condensations and purifications are done in nonaqueous solvents such as chloroform. Trimers can be condensed to yield hexamers (e.g., *3 + 4* yields *6*) and hexamers can be condensed to yield dodecamers (e.g., *6 + 7* yields *8*, still in fully protected triester form). The next to last step in a typical synthesis is the removal of all protecting groups by treatment with acetic acid and NH_4OH, generating the desired water soluble single stranded DNA fragment. The last step is a careful purification of the DNA fragment by high performance liquid chromatography[5].

necesary for the construction of DNA fragments[5]. A library of trimers has been established and longer oligonucleotides can be assembled quickly from the trimer units (which correspond to amino acid codons).

To make the insulin A and B chain genes, it was necessary to make 29 oligonucleotides that were assembled and joined by ligation to make a total of 181 base pairs of duplex DNA. Starting from the trimer library, the DNA fragments were made by four people in about three months. It is apparent that techniques have developed to the point where the genes necessary for altering the bacteria can be made with relatively modest expenditures of time and money.

C. Molecular cloning. Figure 3 illustrates how the insulin A chain was assembled, cloned, and positioned at the end of beta-galactosidase. Step 1 (shown in Figure 3) was joining the small (13 base average) oligonucleotides. Because they were designed to have complementary overlaps, they assemble themselves, and were joined to give duplex DNA by the action of the T4 DNA ligase. The gene was designed to have restriction enzyme sites at each end (Eco R1 on the left and Bam H1 on the right). Step 2 was preparation of the plasmid DNA cloning vector pBR322. Preparation included treatment with Eco R1 and Bam H1 restriction enzymes, which cuts out a small piece of the plasmid and provides a site for insertion of the synthetic A gene. In step 3, the prepared plasmid and synthetic DNA were mixed and joined by T4 ligase, followed by transformation of *E. coli* and molecular cloning. A clone was obtained that contained a correct insulin A gene, as verified by direct DNA sequencing. Next, a DNA fragment containing most of the *E. coli* lac operon, including the lac promoter, operator, and the first 1006 amino acid codons of beta-galactosidase, was inserted (steps 4, 5, and 6). This led to a clone making insulin-beta-galactosidase fused protein.

D. Expression. — Purification and Characterization. Approximately 20 percent of the bacterial protein produced by the insulin strains was found to be beta-galactosidase-insulin fused protein[2]. The fused protein is insoluble and was enriched to more than 50% purity by low speed centrifugation. The insulin chains then were cleaved from the fused protein by treatment with cyanogen bromide in 70 percent formic acid at room temperature overnight. The free insulin chains were converted to S-sulfonated derivatives and purified by ion exchange chromatography, gel filtration, and reverse phase high performance chromatography. In our published work[2], the B chain was not purified to homogeneity.

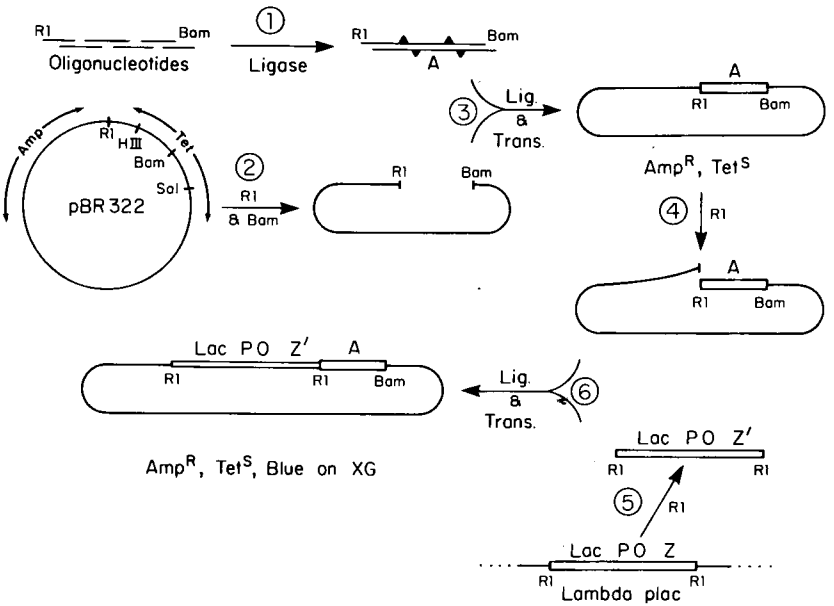

Fig. 3. The construction of a plasmid DNA containing a synthetic insulin A chain gene inserted at the end of a β-galactosidase gene. The procedures are described in the text and details are given in Ref. 2..

Only an explanation of the symbols is given here. The symbol A represents the synthetic A chain gene. pBR322 is a well-characterized plasmid cloning vector containing two antibiotic resistance genes, ampicillin (Amp) and tetracycline (Tet), and several convenient restriction endonuclease sites including Eco R I (R I) and Bam H I (Bam). Lambda plac is a lambda transducing phage carrying the entire *E. coli* operon, which includes the lac promoter (P), the lac operator (O), and the entire β-galactosidase structural gene (Z). There is an Eco R I endonuclease site to the left of the operon and also one near the end of the β-galactosidase gene; thus, the lac operon DNA fragment can be readily obtained. The phenotype of the bacterial strains successfully infected with the desired plasmid are shown. For example, the A chain producing strains would be ampicillin resistant (Amp^R), tetracycline sensitive (Tet^S), and the colonies would be blue on a special indicator agar called Xg. For further details cf. Ref. 2

However, its complete purification now has been accomplished (M. Ross and R. Wetzel, personal communication). The size, chromatograhic properties, and amino acid composition of the insulin chains are correct, and recently the complete amino acid sequences of the A and B chains have been determined to be correct (J. Shively, personal communication).

Chain joining, assay, and yields. S-sulfonated derivatives of the insulin chains were made prior to purification to insure stability by preventing the premature formation of disulfide bonds. Fortunately, the best method for joining chains[3] starts with the S-sulfonated derivatives. With a fivefold excess of A chain, up to 80 percent correct joining of the B

chain to the A chain can be obtained. To detect insulin chain production by the bacteria, we adapted the Katsoyannis reconstitution procedure to the microgram scale. Using this procedure, we had no difficulty producing radioimmune active insulin in control experiments, and with partially purified bacterial insulin chains[2]. The microreconstitution assay was used to follow the individual insulin chains during their purification.

We obtain approximately 1 mg of insulin chain per liter of culture and milligram amounts of the separate chains have been purified. Insulin reconstituted from the purified chains has been characterized by HPLC reverse phase chromatography and does stimulate glucose utilization and glycogen synthetase activity in fat cells (K. Kikuchi and J. Larner, unpublished). A comparison of radioimmune activity and biological activity indicates that all correctly joined A and B chains form fully active insulin.

Conclusion

There no longer is any doubt that bacteria can be used to produce active mammalian hormones. The only question remaining with respect to insulin is whether our approach will be commercially practical. In this regard, our present results are very encouraging and two firms, Genentech, Inc., and Eli Lilly, Inc., are developing procedures for large-scale production.

The techniques we used are quite general; thus, we are confident that bacteria can be engineered to produce any unmodified peptide hormone or hormone analogue that does not contain methionine. By using other cleavage methods, or accepting lower yields, even peptides that contain methionine probably can be made. In the long run, it almost certainly will be preferable to produce the peptide hormones without a precursor peptide. Growth hormone already has been produced as free hormone[7], but for most small peptides, research must be done to obtain mutant strains that do not degrade short, abnormal (to *E. coli*) peptides. For the present, a precursor peptide approach probably is best for small peptides.

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STUDIES ON PEPTIDE HORMONE/RECEPTOR INTERACTIONS

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This report shall concentrate on three aspects of the work going on in my laboratory: (i) the discovery that adrenocorticotropin-(1-24)-tetracosapeptide ($ACTH_{1-24}$) is automatically incorporated into planar bilayer lipid membranes without the intermediacy of receptors or proteins, (ii) the discovery that hormone packages such as tobacco mosaic virus particles carrying covalently bound hormone molecules develop properties of superactive hormones, and (iii) the use of 'fat' or 'super' amino acids as tools in structure-activity studies.

$ACTH_{1-24}$ Spans Lipid Bilayer Membranes (with P. Schoch and D. F. Sargent) — Polypeptide hormones are believed to react with specific receptors on the outside surface of their target cells. These receptors (or the discriminator parts of the holoreceptors[1] are sometimes described as being integral membrane proteins[2] although gangliosides may — by virtue of their glycosidic structures — also serve as discriminators for certain protein hormones and toxins[3]. Allegedly, simple polypeptide hormones do not penetrate the lipid bilayer membrane structures of their target cells.

In our laboratory, it was observed by fluorometric methods (fluorescence anisotropy, energy changes) that dansyl derivatives of adrenocorticotropic hormone (ACTH) and alpha-melanotropin (α-MSH) are bound to lipid micelles and vesicles from aqueous solutions. The nature of the binding remained unknown: it might have been aided by the presence of the dansyl group used as a fluorescent marker[4].

We have recently developed a new method that allows the observation of asymmetric binding of charges to artificial lipid bilayer membranes of the Mueller-Rudin and the Montal type. The method is called *Capacitance Minimization*[5]. Its application to the study of the interaction of adrenocorticotropin-(1-24)-tetracosapeptide ($ACTH_{1-24}$) with planar lipid bilayers has revealed an entirely unexpected feature. Following the addition of $ACTH_{1-24}$ to one side of a pure lipid membrane, part

of the ACTH molecule, presumably the N-terminus, is found to be exposed to the aqueous solution on the opposite side[6]. The chemical details of the reaction are still unknown, however, we are studying the incorporation of ACTH and other hormones into lipid bilayer liposomes by various labelling techniques[7].

Clearly, the reaction of $ACTH_{1-24}$ with egg lecithin and pure dioleoyl-L-α-phosphatidyl choline (a neutral lipid) follows a two-state mechanism:

$$ACTH_{free} \overset{A}{\rightleftharpoons} ACTH_{adsorbed} \overset{B}{\rightleftharpoons} ACTH_{incorporated}$$

Reaction A is a rapid step with ΔG_o = -3.35 kcal/mol (in 9 mM KCl + 1 mM 3-morpholino propanesulfonic acid, pH 7.2); the equilibrium B is slow, with τ (1/2) about 2-4 minutes and ΔG_o = 0.40 kcal/mol. The most probable transmembrane charge distribution of the incorporated species is 4 charges *cis* (remaining on the side of the addition) and 2 charges *trans* (appearing on the opposite side). The capacitance change of the membrane introduced by the binding process (A + B) is half-saturated at $[ACTH_{1-24}] = 2.5 \cdot 10^{-5}$M. The membrane then contains 35 adsorbed and 19 incorporated molecules of $ACTH_{1-24}$ per 10^6 A^2. However, membranes doped with 10% or less of a negatively charged lipid (e.g. a mixture of dioleoyl-lecithin and dipalmitoyl-phosphatidyl serine) react already at $[ACTH_{1-24}] = 10^{-9}$ M and physiological ionic strength.

The induced capacitance change is clearly a saturable process and, in this respect, resembles the biological effect of a hormone on its target cell. Saturability might result from the characteristics of membrane compressibility, from the appearance of positive charges on the *'trans'* surface, and from a saturation of binding (the number of $ACTH_{1-24}$ molecules bound to a membrane is eventually restricted by electrostatic repulsion between the molecules).

There are a number of similarities between $ACTH_{1-24}$/pure lipid planar bilayer interactions and $ACTH_{1-24}$/target cell membrane interactions. Saturability is one, the concentration range of interaction with negatively charged bilayers another, and a third is the rate constant of the off reaction. It ($k_{-1} \simeq 3\text{-}6 \cdot 10^{-3} \cdot s^{-1}$) has the same order of magnitude as that observed for the target cell interaction of other hormones, e.g. $7 \cdot 10^{-3} \cdot s^{-1}$ for vasopressin and $1 \cdot 10^{-3}$ - $3 \cdot 10^{-4} \cdot s^{-1}$ for insulin[8]. The fourth similarity is that between its two-stage mechanism and a two-stage

mechanism recently postulated and found to explain the partial agonism of ACTH-(5-24)-icosapeptide[9].

Despite these similarities, it remains to be proved whether the insertion mechanism occurs in natural membranes and has anything to do with the physiological process. We are actively studying the chemical mechanisms, other hormones, and other lipid systems. Should it be true that (even without the intervention of a receptor protein) the address portion[10] of the ACTH molecule remains outside the membrane and the message sequence[10] penetrates it, then a number of new possibilities could be envisaged for the triggering action of the hormone, e.g. (i) perturbation of lipid structure, (ii) reaction with integral membrane proteins (adenylate cyclase?) that are *not* exposed to the outer surface, (iii) reaction with inside peripheral or even cytoplasmatic proteins including microfilaments and microtubules, and (iv) abscision of the message and its transport or diffusion to other parts of the cell interior.

The phenomenon of adsorption and insertion would furthermore be interesting with respect to the ligand-receptor homing theories of Adams and Delbruck[11] and of Berg and Purcell[12] based on the reduction of dimensionality and on three-dimensional diffusion theory, respectively.

'Superhormones' (with Marly Kriwaczek and Alex Eberle) — Peptide hormones have been covalently attached to tobacco mosaic virus (TMV) *via* the side-chain amino group of the lysine[68] residues of individual capsomers[13,14]. The conjugates were prepared from mercaptosuccinyl TMV (TMV-SH) and hormone derivatives containing groups that react specifically with -SH (e.g. bromoacetyl, 6-maleimidohexanoyl).

The binding of TMV particles containing about 600 molecules of angiotensin II amide each, TMV-S-ANG′(600), to target cell membrane vesicles was demonstrated by density gradient centrifugation[14] and studied quantitatively with dynamic light scattering and transient electric birefringence (the Kerr effect)[15]. The binding was found to be exceptionally strong and practically irreversible with angiotensin II amide.

The biological activity of an alpha melanotropin conjugate, TMV-S-MSH′ (500), in frog skin was unexpectedly high and of very long duration (about 20% of the activity could not be removed by repeated washings).

We have studied the matter of high biological activity in more detail using frog skin *(Rana pipiens)* and *Cloudman* S91 mouse melanoma cell cultures. The virus conjugate was prepared from desacetyl-N^{α}-6-maleimidohexanoyl-α-MSH ($C_4H_2O_2$ N- CH_2-CH_2-CH_2-CH_2-CH_2-CO-Ser-Tyr-

Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val·NH_2; MH-MSH) and contained about 300 hormone residues. The following results were obtained with frog skin: MH-MSH had $8 \cdot 10^9$ U/mmol, Capsomer-S-MH-MSH (1) $6 \cdot 10^9$ U/mmol, and TMV-S-MH-MSH (300) $5 \cdot 10^{10}$ U/mmol. Every attached hormone molecule of the TMV conjugate was 6 times more active than a free MH-MSH molecule or 8 times more active than the capsomer-supported hormone.

Tyrosinase stimulation (24 h) in the melanoma cell cultures indicated that TMV-S-MH-MSH (300) is a full agonist compared to α-MSH but about 10 times more active on a molar basis (EC_{50} about $2 \cdot 10^{-10}$M; α-MSH had EC_{50} about $2 \cdot 10^{-9}$ M). Again each individual bound hormone molecule is more active than a free hormone molecule (it must be borne in mind that α-MSH is about 10 times more active than MH-MSH, so that the effect of conjugation on activity is about 100 fold in this case).

Preliminary results of Prof. Hans Kosterlitz, M.D., Aberdeen (personal communication), indicate that in the guinea pig ileum and mouse vas deferens assays, TMV-S-Enkephalin' (200) is also superactive (Enkephalin' in this case was Tyr-D-Ala-Gly-Phe-Leu-Lys ($COCH_2$-)·NH_2[16]).

What are the reasons for this superactivity? Our results indicate that it is dependent on the integrity of the TMV particle (capsomer derivatives are less active). From steric and dynamic considerations, the ability of each one of the many hundred bound hormone molecules to react with an individual cell surface receptor is strongly impaired (over 100 receptors, or 1/10 to 1/100 of the receptor population of a cell would have to become located in an area of 0.01 μm^2 or 1/10,000 to 1/100,000 of the total cell surface). If only one or two bound hormone molecules per virus particle were to find a receptor, then these molecules would have to be enormously superactive (over 100 times more active than the parent molecule). Intuitively, such an activity could result either from a strongly enhanced affinity or from a strongly enhanced probability of one and the same receptor being occupied again and again immediately after release of the preceding hormone ligand.

Strongly enhanced affinity could be caused by *Cooperative Affinity* phenomena[13,14] (one bound hormone ligand would cause, say $K_{ass} = 10^9$ l/mol, two simultaneously bound ligands $K_{ass} = 10^{18}$ l/mol, etc.) or by an affinity increase through *insertion of the hormones into the lipid bilayer portion of the plasma membrane.*

Enhancement of the probability of reoccupation of an occupied receptor could be explained by the probability of a neighboring hormone

molecule on the TMV particle to become attached to a receptor upon release of the original hormone molecule. In the surface diffusion situation[11,12], this probability is very great. For short, we could call this activity enhancement as being due to *'Reynold's Affinity'* phenomena.

We are actively pursuing the causes of the observed superactivity of TMV-agonist conjugates because they might provide additional insight into the nature of receptors. Particularly we are studying interactions with pure lipid bilayers.

'Fat' Amino Acids as Tools for Structure-Activity Studies (with Kim Quang Do and Jean-Luc Fauchere) — Carboranylalanine (Car)[17,18] has proved to be an interesting analogue of phenylalanine for probing structure-activity and structure-binding relationships in biologically active polypeptides[19,20]. Its main features are pseudoaromaticity and space-filling properties. In the latter respect, it closely resembles a molecule of phenylalanine in which the phenyl ring is rotating about its 1,4-axis. We expect carboranylalanine and similar 'fat' amino acids to be especially useful for experimentally distinguishing between the 'address' and 'message' quality[10] of the parent amino acid residue in a particular peptide hormone, for enhancing lipophilicity and receptor-binding properties[19,20,21], and for producing strong antagonists.

According to space-filling models, *(S)*-2-amino-3-(1-adamantyl)-propionic acid (adamantylalanine, Ada) has about the same overall size and shape as carboranylalanine, but lacks its pseudoaromaticity. The characteristics of its side-chain as a saturated hydrocarbon should make it a suitable analogue for probing into the structure significance of both phenylalanine and leucine. Instead of the two branches of leucine at the γ-carbon atom, it possesses three and, in addition, a cyclohexyl ring in chain conformation joining the three branches (Figure 1).

Adamantylalanine has recently been prepared[22], and an improved synthesis of carboranylalanine reported[18]. Other 'fat' amino acids have also been prepared, like β-methylvaline (*t*-butyl-glycine, Bug), or are in the course of being synthesized (γ-methyl-leucine or neopentylglycine, Neo) in our laboratory (J.L. Fauchere).

Interestingly, Car and Ada are the most lipophilic amino acids known, with Hansch π-values of + 1.20 and + 0.63, respectively (determined by J. L. Fauchère in the laboratory of C. Hansch). This means that they are at least 100 times more lipophilic than Phe or Leu. The enhanced binding of [Car^4, Leu^5]enkephalin to rat brain opiate receptors could be at least partially due to this property[20].

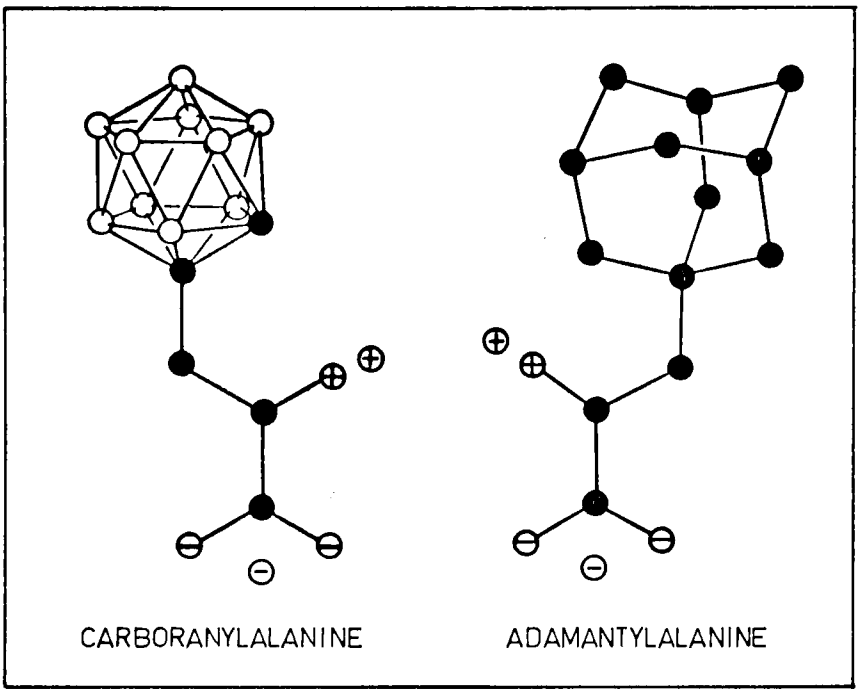

Fig. 1. A diagrammatic representation of carboranylalanine and adamantylalanine. The hydrogen atoms are omitted; ● represents carbon, O boron, ⊕ nitrogen, and ⊖ oxygen.

Together with Prof. P. Schiller, Montreal, and Prof. H. Kosterlitz, M.D., Aberdeen, we are studying the influence of these amino acids on the properties of enkephalins. First results are the following (P. Schiller, personal communications; the figures are relative potencies in the guinea pig ileum assay; ala stands for D-Ala):

1	H·Tyr-Gly-Gly-Phe-Met·OH	100 %
2	H·Tyr-Gly-Gly-Phe-Leu·OH	24 ± 8 %
3	H·Tyr-Gly-Gly-Car-Leu·OH	12 ± 4 %
4	H·Tyr-Gly-Gly-Car-Leu·NH_2	7 ± 0.5 %
5	H·Tyr-ala-Gly-Car-Leu·OH	121 ± 39 %
6	H·Tyr-ala-Gly-Car-Met·NH_2	411 ± 172 %
7	H·Tyr-ala-Gly-Ada-Leu·OH	5 ± 0.3 %
8	H·Tyr-ala-Gly-Ada-Leu·NH_2	8 ± 1 %

One observation is very striking: the [Ada[4]]enkephalins(7, 8) are very weakly active as compared to the corresponding [Car[4]]enkephalins (5, 6). This very probably reflects the difference between pseudoaromaticity of Car (replacing the aromatic Phe) and the purely aliphatic properties of Ada.

Because peptide bonds involving Car and Ada are not easily cleaved by proteolytic enzymes *(cf.*[19]*)*, these new amino acids might also prove to be interesting for the protection of hormones and other peptides against enzymatic degradation.

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GASTROINTESTINAL PEPTIDES: RECEPTORS AND MECHANISM OF ACTION IN PANCREATIC ACINAR CELLS

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The term "gastrointestinal peptides" is usually used to refer to those peptides that possess the ability to modify the functions of various gastrointestinal tissues. Each gastrointestinal peptide appears to possess the ability to alter the function of each gastrointestinal tissue in some way. For example, cholecystokinin (CCK) stimulates pancreatic enzyme secretion, augments the action of secretin on pancreatic fluid and electrolyte secretion, causes gallbladder contraction, increases gastric acid secretion and alters the contractility of gastrointestinal smooth muscle. Although a given gastrointestinal peptide may have different effects on different tissues, the biochemical basis of action of a given peptide appears to be the same in all target tissues. The present paper reviews the actions of various secretagogues on pancreatic acinar cells and in particular, the initial steps in the sequence of events that culminate in stimulation of pancreatic enzyme secretion. These initial events in acinar cells also appear to be the initial steps in the actions of gastrointestinal peptides in other gastrointestinal tissues.

All of the agents that have been found to be capable of stimulating pancreatic enzyme secretion appear to act by initiating one of two distinct sequences of biochemical changes[1]. Some secretagogues cause release of cellular calcium and, after a series of undefined steps, stimulation of pancreatic enzyme secretion. Other secretagogues cause activation of adenylate cyclase, increased cellular cyclic AMP, activation of cyclic AMP-dependent protein kinase and, after a series of unknown steps, stimulation of enzyme secretion. Although these two biochemical pathways are initially distinct, they interact at some presently unknown step to cause "potentiation" of the stimulation of pancreatic enzyme secretion[1-7]. That is, the increase in enzyme secretion caused by a secretagogue that increases cyclic AMP plus a secretagogue that increases calcium release is substantially greater than the sum of the effect of each secretagogue acting alone.

Receptors for Gastrointestinal Peptides

Table I summarizes the different classes of receptors that have been identified on pancreatic acinar cells, the agents that can interact with each class of receptors and the radiolabeled ligands that can be used to examine receptor function directly.

Receptors Causing Activation of Adenylate Cyclase — Pancreatic acinar cells possess 3 classes of receptors that mediate activation of adenylate cyclase and increased cyclic AMP. Two of these classes of receptors interact with both secretin and VIP. Studies of binding of ^{125}I-VIP to acinar cells have shown that one class of receptors has a high affinity for VIP and a low affinity for secretin (VIP-preferring receptors), whereas the other has a low affinity for VIP and a high affinity for secretin (secretin-preferring receptors)[8-12]. These two classes of receptors can also be distinguished by their abilities to interact with analogues of secretin and by their relative affinities for C-terminal fragments of secretin and VIP. These receptors do not interact with other known pancreatic secretagogues and do not interact with glucagon which has an amino acid sequence similar to that of VIP and secretin. In both secretin and VIP the intrinsic biologic activity resides in the N-terminal portion of the molecule. For example, secretin$_{1-14}$ has an efficacy equal to that of native secretin, whereas secretin$_{5-27}$ or secretin$_{14-27}$ have efficacies of less than 1% of that of secretin. Although C-terminal partial sequences of VIP and secretin have little or no intrinsic biological activity, these peptides are able to interact with secretin-preferring receptors as well as with VIP-preferring receptors. Thus, C-terminal fragments of secretin (e.g. secretin$_{5-27}$ or secretin$_{14-27}$) or VIP (VIP$_{14-28}$) are competitive antagonists of the action of VIP and secretin. both the N-terminal and the C-terminal regions influence the affinity of the peptide for its receptor. For example, the affinities of secretin$_{1-14}$ and secretin$_{14-27}$ for the secretin-preferring receptors are approximately 1000-fold lower than the affinity of secretin for this same class of receptors. One important feature of secretin and VIP that distinguishes them from the other peptides which stimulate pancreatic enzyme secretion is that, to date, no fragment of either peptide has been found to be as potent as the native molecule.

A third class of receptors that cause activation of adenylate cyclase are those that interact with cholera toxin. The receptors can be identified by measuring binding of ^{125}I-cholera toxin and there is a good correlation between the ability of native cholera toxin to inhibit binding of ^{125}I-cholera toxin and the ability of the native toxin to activate adenylate cyclase and

Table I. Classes of Receptors for Pancreatic Secretagogues

RECEPTOR INTERACTS WITH	ANTAGONIST	USED TO EXAMINE BINDING
SECRETAGOGUES THAT INCREASE CYCLIC AMP		
CHOLERA TOXIN	CHOLERAGENOID	^{125}I-TOXIN
SECRETIN (high affinity) VIP (low affinity)	SECRETIN$_{5-27}$ SECRETIN$_{14-27}$ VIP$_{14-28}$	^{125}I-VIP
VIP (high affinity) SECRETIN (low affinity)	SECRETIN$_{5-27}$ SECRETIN$_{14-27}$ VIP$_{14-28}$	^{125}I-VIP
SECRETAGOGUES THAT INCREASE CALCIUM OUTFLUX		
CCK GASTRIN CAERULEIN	BUTYRYL CYCLIC GMP	[^{3}H]CAERULEIN ?
BOMBESIN LITORIN RANATENSIN ALYTESIN	NONE	^{125}I-[Tyr^{4}]BOMBESIN
PHYSALAEMIN SUBSTANCE P ELEDOISIN	NONE	^{125}I-PHYSALAEMIN
ACETYLCHOLINE	CHOLINERGIC ANTAGONISTS	[^{3}H]QNB ?

increase cellular cyclic AMP[13]. Other peptides that increase pancreatic enzyme secretion do not interact with the cholera toxin receptor. A partial sequence of cholera toxin referred to as "choleragenoid" or "aggregated B subunits" possesses the ability to interact with the cholera toxin binding sites, has no intrinsic biologic activity and, therefore, functions as a competitive antagonist of the action of native cholera toxin. Recent studies[14,15] of the mechanism of action of cholera toxin have indicted that in contrast to other peptides that activate adenylate

cyclase, binding of the toxin molecule *per se* is not sufficient to elicit activation of adenylate cyclase. It appears that following binding of cholera toxin to the outer surface of the plasma membrane, a portion of the toxin molecule crosses to the cytoplasmic surface of the membrane and causes ADP ribosylation of a regulatory component of adenylate cyclase.

Receptors Causing Release of Cellular Calcium – Pancreatic acinar cells possess 4 classes of receptors for secretagogues that increase enzyme secretion by causing release of cellular calcium. One class interacts with CCK and gastrin as well as caerulein, a decapeptide originally isolated from amphibian skin and in which seven of its eight C-terminal amino acids are identical to those in the C-terminal octapeptide of CCK. These receptors can be identified by the abilities of butyryl derivatives of cyclic GMP to competitively inhibit the actions of CCK, gastrin and caerulein[7]. Studies of binding of [^{3}H] caerulein have identified binding sites that interact with CCK, gastrin, and caerulein as well as fragments and analogues of these peptides[16-18]. The *relative* potencies with which various secretaogues inhibit binding of [^{3}H] caerulein are similar to those with which they increase calcium outflux and enzyme secretion; however, there are still some important discrepancies that must be resolved before binding of [^{3}H] caerulein can be considered to reflect occupation of the CCK receptor. In contrast to secretin and VIP, the intrinsic biologic activity of CCK, gastrin and caerulein is a property of the C-terminal portion of the molecule[19]. In fact, C-terminal fragments of CCK are substantially more potent than the native peptide. The shortest C-terminal fragment which has a potency equal to or greater than that of native CCK is the C-terminal heptapeptide. Even shorter C-terminal fragments, such as the tetrapeptide, still possess intrinsic biologic activity but their potencies are less than that of native CCK. CCK also differs from VIP and secretin in that the region of the molecule which possesses intrinsic biologic activity also determines the affinity of the peptide for the receptor. Of the many fragments and analogues of CCK tested, none has been found to occupy the receptor and not cause a full biologic response.

A second class of membrane receptors that cause calcium outflux are those which interact with bombesin, litorin, ranatensin, and alytesin. These peptides were initially isolated from amphibian skin and are named after the frog from which they were derived. For example, bomesin was isolated from the skin of *Bombina bombina*. These peptides are not unique to amphibians because bombesin-like immunoreactivity

has been found in mammalian tissues. Receptors for bombesin and related peptides can be identified by measuring binding of ^{125}I-[Tyr^4]bombesin[20]. The intrinsic biologic activity of bombesin and structurally related peptides is a property of the C-terminal portion of the molecule, and the C-terminal nonapeptide of bombesin has the same potency and efficacy as does the native tetradecapeptide[21]. Shorter C-terminal fragments of bombesin still possess full biologic activity, but their potencies are less than that of native bombesin. The region of the bombesin molecule which possesses intrinsic biologic activity also determines the affinity of the peptide for its receptor. Of the fragments and analogues of bombesin which have been tested, none has been found to occupy the receptor and not cause a full biologic response. Thus, there are no known competitive antagonists of the action of bombesin and related peptides.

A third class of receptors that mediate calcium release from pancreatic acinar cells are those that interact with physalaemin, eledoisin and substance P. Physalaemin was originally isolated from amphibian skin and eledoisin was isolated from the posterior salivary gland of a mediterranean octopod. Substance P was originally isolated from brain and from gastrointestinal mucosa and has been found subsequently in spinal cord, dorsal ganglia, and plasma as well as enterochromaffin cells in intestinal mucosa. This class of receptors can be identified by measuring binding of ^{125}I-physalaemin[22]. Like the CCK-related peptides and the bombesin-related peptides, the intrinsic biologic activity of physalaemin, eledoisin and substance P is a property of the C-terminal region of the peptide. Eledoisin is approximately 50% more effective than physalaemin or substance P in stimulating amylase secretion. This finding raises the possibility that a peptide exists or can be synthesized which will be able to occupy the receptor but which will be devoid of intrinsic biologic activity. This finding also suggests that with physalaemin and structurally related peptides, the portion of the molecule that possesses intrinsic biologic activity is not congruent with the region of the molecule that influences the affinity of the peptide for its receptor.

In addition to receptors for gastrointestinal peptides, pancreatic acinar cells also possess receptors that interact with muscarinic cholinergic agents. This class of receptors can be identified by the abilities of atropine and other muscarinic antagonists to competitively inhibit the actions of acetylcholine and other cholinergic agonists[7,19]. Radiolabeled quinuclidinyl benzilate, [^{3}H]QNB, has been used to examine the interaction of various agents with muscarinic cholinergic receptors in several different tissues; however, no such studies using the pancreas have been reported in detail.

Mechanisms of Action

Cyclic AMP — In pancreatic acinar cells both the secretin-preferring receptors and the VIP-preferring receptors cause an increase in cyclic AMP; however, it is the increase in cyclic AMP caused by the VIP-preferring receptors that stimulates pancreatic enzyme secretion[12]. What function, if any, is altered by the increase in cyclic AMP caused by the secretin-preferring receptors is not known. Secretin and VIP cause endogenous activation of cyclic AMP-dependent protein kinase in acinar cells[23] and increase adenylate cyclase in plasma membranes from acinar cells[24-28]. Inhibitors of cyclic nucleotide phosphodiesterase augment the action of secretin and VIP on cellular cyclic AMP as well as on enzyme secretion[2,3]. Exogenous derivatives of cyclic AMP can reproduce the action of VIP and secretin on enzyme secretion and the increase in enzyme secretion caused by VIP or secretin plus exogenous cyclic AMP is the same as that obtained with VIP or secretin alone[3-7]. Gastrointestinal peptides that cause calcium release from pancreatic acinar cells do not increase cyclic AMP and do not alter the increase in cyclic AMP caused by VIP or secretin.

Although CCK does not increase cyclic AMP in acinar cells[1], it can activate adenylate cyclase in plasma membranes from acinar cells[24,26-29]. This action of CCK does not appear to be related to its ability to stimulate enzyme secretion because CCK does not increase cyclic AMP in acinar cells and the concentrations of CCK required to activate adenylate cyclase are substantially greater than those required to stimulate enzyme secretion. Moreover, the structural requirements for CCK to activate adenylate cyclase are different from the structural requirements for CCK to stimulate enzyme secretion.

Calcium — The increase in pancreatic enzyme secretion caused by various secretagogues is reduced or abolished when calcium is removed from the incubation medium[1,30-34]. Furthermore, divalent cation ionophores such as A23187 can increase pancreatic enzyme secretion and their actions are also reduced or abolished by removing extracellular calcium[35-35]. These observations were initially interpreted to indicate that secretagogues cause stimulation of pancreatic enzyme secretion by virtue of their ability to increase the inward movement of calcium from the extracellular medium into pancreatic acinar cells[35]. Subsequently, direct measurements of the effects of secretagogues on transport of ^{45}Ca by pancreatic acinar cells showed that secretagogues such as CCK, bom-

besin, physalaemin and cholinergic agents increase calcium outflux but do not alter the inward movement of calcium into the tissue[1,33]. Later, several different studies showed that those secretagogues which increase calcium outflux can, under certain conditions, also increase the inward movement of calcium from the extracellular medium into the tissue[38-41]. The secretagogue concentrations required to increase calcium influx have generally been at least 100-fold greater than those required to cause maximal stimulation of calcium outflux and enzyme secretion. Moreover, these effects of secretagogues on calcium influx have not been seen with concentrations of extracellular calcium below 1 mM. Finally, in some studies the secretagogue-induced increase in influx of ^{45}Ca probably resulted from uptake of radioactivity from the solution used to wash the cells, not from the incubation medium. Thus, virtually all investigators agree that secretagogues such as CCK, cholinergic agents and peptides derived from amphibian skin increase pancreatic enzyme secretion by increasing calcium outflux. The significance of the increased calcium influx seen with high concentrations of secretagogues remains to be determined.

The calcium released from pancreatic acinar cells by secretagogues can be accounted for completely by release of calcium from intracellular bound stores[42-44]. The mechanism by which this release of bound cellular calcium mediates the action of secretagogues in enzyme secretion is not known. That is, release of bound calcium is thought to cause a rise in the cytoplasmic calcium concentration;[45] however, it is not known whether the critical event in the action of the secretagogue is the loss of bound calcium *per se* or the resulting increase in cytoplasmic calcium. Secretagogues that increase cellular cyclic AMP (e.g. VIP, secretin and cholera toxin) do not alter calcium transport in pancreatic acinar cells and do not alter the effects of other secretagogues on calcium transport[1].

Secretagogues that increase calcium outflux also cause other effects such as increased cellular cyclic GMP[1], and depolarization and decreased resistance of the acinar cell-surface membrane[45]. These effects appear to be caused by secretagogue-induced mobilization of cellular calcium; however, it is the change in calcium, not the change in cyclic GMP or electrical properties, that appears to play the casual role in increasing enzyme secretion.

Thus, there are at least two roles for cellular calcium in the actions of secretagogues on pancreatic enzyme secretion[34]. 1) Release of cellular calcium mediates the action of secretagogues such as CCK, bombesin, physalaemin, and cholinergic agents on enzyme secretion. 2) Calcium

also plays a role in one of the more distal steps in the secretory process where it appears to function to support the stimulation of enzyme secretion caused by all secretagogues.

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X-RAY ANALYSIS AND THE CONFORMATION OF PANCREATIC POLYPEPTIDE, PP

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Introduction

Four polypeptide hormones — insulin, somatostatin, glucagon, and pancreatic polypeptide (PP) — are found in the human pancreas. Although PP is found only in the gut mucosa of cartilaginous fish, it is found in the endocrine pancreas of elasmobranchian cartilaginous fish and higher vertebrates[1]. It is thus phylogenetically the most recent islet hormone. Avian (aPP), porcine (pPP), bovine (bPP), ovine (oPP) and human (hPP) pancreatic polypeptides have been sequenced[2,3]; whereas there is close homology amongst the mammalian primary structures, there are considerable differences between these and the avian sequence. This is also reflected in the species specificity of immunological and some biological activities (see Ref. 3 for a review). Generally PP has gastrointestinal functions; aPP is a potent gastric secretagogue in chickens. It may also induce hepatic glycogenolysis and decrease plasma glycerol levels, but it does not induce changes in plasma glucose. Its physiological role is not properly understood but the rise of levels in circulation after feeding and the effect of aPP on genetically obese mice, indicates it may be a satiety factor[4].

We have studied aPP from turkey pancreas. This was first characterized and sequenced by Kimmel *et al*[2] and may be conveniently extracted as a by-product of insulin and glucagon extraction procedures[3]. The material has been crystallized by Wood *et al*[5] in a form suitable for X-ray analysis. In an earlier paper we outlined our preliminary crystallographic studies which indicted a symmetrical dimer of partly helical conformers[5]. More recently we have calculated a 3.0 A electron density map which confirms these observations and gives details of the conformation. In this paper we describe the conformation found in the crystals, we discuss its

relevance to solution studies and we compare the conformation to that of the other pancreatic polypeptide hormones, insulin and glucagon, for which the high resolution x-ray structure has been derived[6,7].

X-Ray Analysis

PP was isolated from turkey pancreas using the methods described by Wood *et al*[5]. Purified PP was dissolved at pH 6.9 in 0.1M Tris/HCl buffer to give a concentration of 5 mg/ml, and the solubility of the polypeptide was decreased by adding equimolar concentrations of zinc acetate. Crystallization was performed by leaving the filtered solution, after warming to 55° C, in a hot box for ten to fourteen days. Zinc ions have now been shown to be essential for crystallization.

Monoclinic crystals grow to 1.3 mm in their longest dimension. The crystals, which diffract to ~ 1 A resolution, are of the monoclinic space group C2 with cell dimensions $a = 34.18$A, $b = 32.91$A, $c = 28.45$A and $\beta = 105.26°$.

The crystal structure was solved using the method of isomorphous replacement combined with anomalous scattering from a single heavy atom derivative ($HgCl_2$). The calculated phases have an average figure of merit of 0.88 to 3.0 A resolution. The best electron density map using these phases indicates both open solvent regions and the path of the polypeptide chain. The most prominent feature in the electron density appears to be a metal ion and analysis of crystals using the energy dispersive analysis of X-rays (EDAX) on a scanning electron microscope indicates only the presence of zinc in sufficient quantities to explain the observed electron density. The asymmetric unit comprises one molecule; this is closely associated with an equivalent molecule to form a dimer about a crystallographic two-fold axis. Dimers are linked in the crystals through coordination to zinc ions.

The Secondary and Tertiary Structure

The main feature of the molecule is a stretch of well-defined α-helix involving residues 14 to 32. Residues 1 to 9 including Pro 2, Pro 5 and Pro 8 have an extended, polyproline-like structure while residues 10 to 14 form a loose bend which enables the α-helix and polyproline-like helix to close-pack in an antiparallel fashion (see Figure 1). The interactions between the chains are essentially hydrophobic with the three proline side-chains of the polyproline-like helix interdigitating with Leu 17, Phe

20, Tyr 27 and Val 30. These groups form part of the hydrophobic side of the helix as predicted correctly by Wood *et al*[5], using the helix-wheel method of Edmundsen and Schiffer. The carboxy terminal residues Arg 33, His 34, Arg 35 and Tyr 36 extend loosely from the helix.

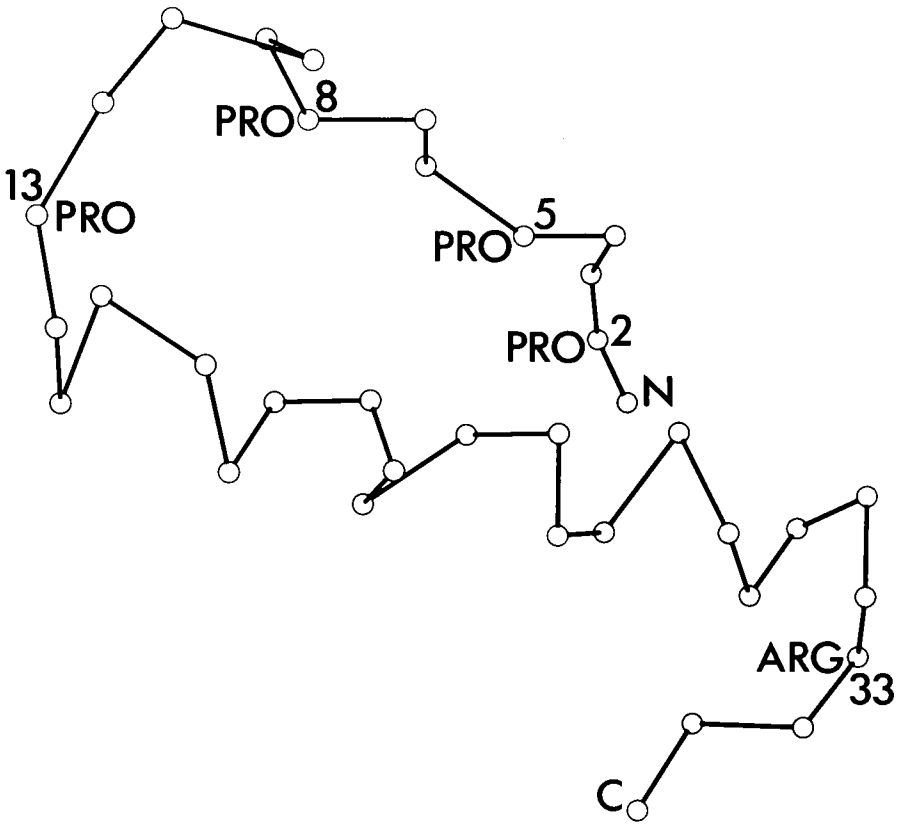

Fig. 1. The conformation of the main chain of avian pancreatic polypeptide (aPP). Each amino acid residue is indicated by a circle at the position of its α-carbon.

The overall impression is that the main fold of the molecule would be retained in solution but the carboxy terminal residues may be rather flexible. Residues Asp 10, Asp 11, Glu 15, Asp 16, Arg 19, Asp 22, Asn 23, Gln 25, Glu 26, Asn 29, Arg 33 and Arg 35 form a hydrophilic surface including one side of the helix. In the crystals most of these residues are extended into the solvent region.

Quaternary Interactions

Two protomers are arranged with their helices approximately anti-parallel (≅ 30° offset) and related by a crystallographic two-fold axis as shown in Figure 2 and suggested by our earlier studies. Contacts involve mainly non-polar residues which are not completely buried in the tertiary interaction. The hydrophobic nature of these interactions indicates that dimers would probably form in rather dilute neutral aqueous solutions.

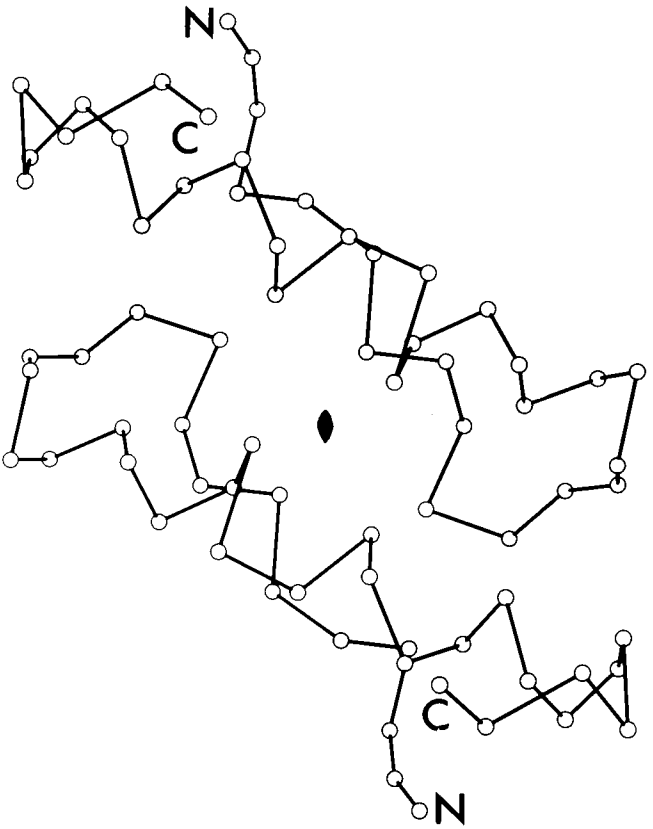

Fig. 2. The arrangement of two aPP molecules in the symmetrical dimer.

The zinc ions in the structure are each coordinated to three molecules and thus crosslink the lattice and stabilize the crystal structure. Each zinc is bound to the imidazole of His 34 (and possibly the C-terminal amide) of one molecule, to the α-nitrogen of Gly 1 of a second molecule, and to the amide of Asn 23 of a third molecule.

General Conclusions

Pancreatic polypeptide (PP) has a well-defined conformation which is stabilized by hydrophobic tertiary interactions. The crystallographic results are consistent with the far UV circular dichroism which indicates a substantial amount of helical secondary structure and which is stable in dilute aqueous solutions[8]. This is a surprising observation for such a small polypeptide (36 amino acids) with no disulphide bridges. Glucagon, which is only slightly smaller at 29 amino acids, has a helical structure under some conditions but in dilute aqueous solutions conformers with little secondary structure predominate.

Like glucagon and insulin, the PP monomers have extensive hydrophobic regions on their surfaces which give rise to self-association. The quaternary structures — glucagon trimers and zinc insulin hexamers —appear to play a role in storage of the hormones, although the monomers must predominate at equilibrium in the low concentrations characteristic of circulation. Models for receptor binding of insulin and glucagon through specific, partly hydrophobic interactions, which depend very much on the conformation of the polypeptides, have been proposed and it remains to be seen whether a similar model is also relevant to PP.

Acknowledgements

We are grateful to our many colleagues in the laboratory — especially John Jenkins, Maggie Crowe, Trevor Sewell, Sudhir Bedarkar —who have generously given their time to assist in the collection of turkey pancreas over the past four years. We are also grateful to Professor Joe Kimmel, Professor Sture Falkmer, Dr. Norman Lazarus and Mr. Ray Neville for helpful suggestions, and to the Science Research Council UK for support.

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STRUCTURE-FUNCTION STUDIES OF MELANOPHORE STIMULATING HORMONES (α-MSH AND β-MSH) AND THEIR ANALOGS ON MELANOMA PLASMA MEMBRANE ADENYLATE CYCLASE: COMPARISON WITH FROG SKIN MELANOPHORES

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Introduction

Melanophore stimulating hormone (MSH) has a profound effect on melanoma cell metabolism causing an induction of tyrosinase activity, changes in cellular morphology, and growth arrest of cultured melanoma cells[1]. Our laboratories are concerned with the properties of the MSH receptor-adenylate cyclase system in melanoma cells and in determining the structural requirements for hormone activity in these cells. Since it is difficult to isolate large amounts of the natural hormones, we have developed solid phase synthetic methods for preparing pure α-MSH and β-MSH in high yield[2]. These methods were also used for the synthesis and purification of the analogs reported here. The properties of these peptides were studied on a hormone sensitive melanoma membrane adenylate cyclase system we have prepared, and the results compared with the activities on the frog skin, which represents the normal melanophore and responds to MSH by darkening.

Results

Mouse melanoma membrane adenylate cyclase was isolated by a slight modification of standard procedures[3]. However, the specific activity of membrane fractions obtained by standard sucrose gradient techniques was not significantly greater than that of washed particulate (Table I). Hence, in all studies reported here, washed particulate was the

Table I. Adenylate Cyclase Activities of Membrane Protein Fractions Obtained from S-91 Melanoma Cells.

Membrane Fraction	Specific Activities[a]		
	Basal	NaF	α-MSH
Crude Homogenate	19.7 ± 4.8	240.8 ± 1.7	17.1 ± 1.9
Washed Particulate	24.7 ± 2.7	416 ± 8.9	40.5 ± 3.7
Sucrose Interface[b]	6.5 ± 1.1	137.6 ± 5.1	4.2 ± 1.3
Sucrose Top Float[c]	35.6 ± 3.1	482.1 ± 45.3	55.4 ± 3.2

[a]Adenylate cyclase activities obtained on the basis of 50 μg protein per assay under standard assay conditions for basal, 15 mM NaF, or 5.6×10^{-8} M α-MSH. Results represent mean value for four determinations ± S.E. from duplicate experiments.

[b]32.0/42.3% (wt/wt) sucrose interface.

[c]32% (wt/wt) sucrose top float.

source of membrane material. The sucrose top float was also examined in several cases and gave the same results.

The effects of various hormones on melanoma adenylate cyclase were determined over the entire dose-response range. As shown in Figure 1, MSH and prostaglandin E_2 (PGE_2) were potent activators of the system. β-Adrenergic agents such as ephinephrine and isoproterenol also are mild stimulators (causing an activity equivalent to 10% of the maximum MSH stimulation). The specificity of peptide hormones was illustrated by the inability of Leu[5]-enkephalin, glucagon, or secretin to activate this adenylate cyclase.

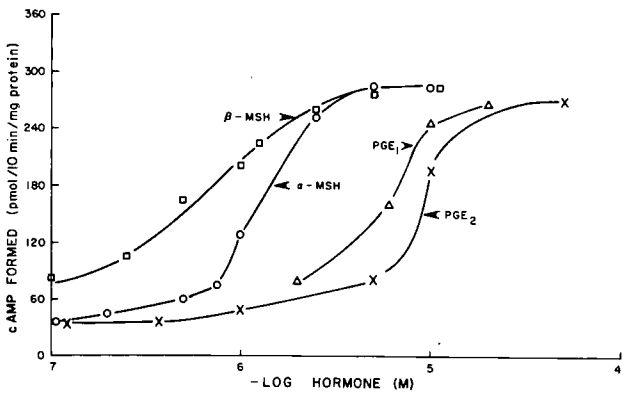

Fig. 1. Dose-response for activation of melanoma adenylate cyclase by hormones.

One objective of our research is to develop a binding assay for MSH, but we have been unable to radio-iodinate β-MSH[4] or α-MSH without concurrent loss of biological activity. Two likely positions of reaction are

at Tyr-2 and Met-4 in α-MSH (Tyr-5 and Met-7 in β-MSH), and we have examined several analogs modified in these positions (Figure 2 and Table II). The substitution of norleucine for methionine in either α- (Figure 2) or β-MSH (Table II) did not alter the biological activity of the hormones, but did enhance their potency. Indeed, [Nle^7]-β-MSH appears to be the most potent analog of β-MSH reported thus far. Substitution of the Tyr-2 by phenylalanine in [Nle^4]-α-MSH led to a slight decrease in potency, and substitution of the same residue by 3′,5′-diiodotyrosine or D-tyrosine in α-MSH led to further reductions in potency. However, all analogs were still full agonists. Interestingly, even N-Ac-α-MSH$_{7-13}$ possessed considerable potency (as measured by frog skin assay).

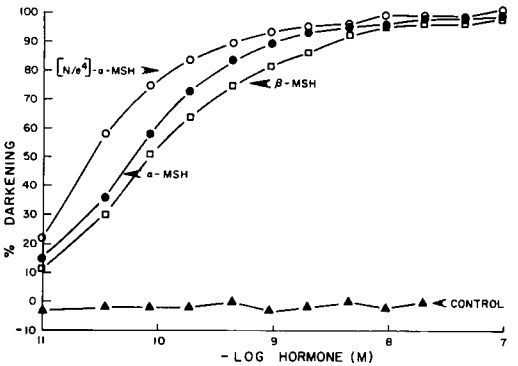

Fig. 2. Dose-response for darkening in the frog skin bioassay system by hormones.

Discussion

A simple high yield preparation of a hormone sensitive plasma membrane adenylate cyclase system from mouse melanoma cells has been developed. A highly reproducible 8-fold stimulation of adenylate cyclase is produced by α-MSH. Other ligands such as PGE_2, F^-, GTP, and isoproterenol also stimulate this adenylate cyclase, but other peptide hormones have no affect. The frog skin bioassay system can also be used to examine MSH activity in a dose-response manner[2], and we have used this standard assay for comparison with the melanoma cyclase system.

Both β-[4] and α-MSH are inactivated by chloramine-T in the absence or presence of iodide. [Nle^7]-β-MSH and [Nle^4]-α-MSH were prepared and found to be much more potent than the native hormones, and also were more resistant to oxidative inactivation. These results suggest that in α- and β-MSH the Met sulfur has a slight repulsive interaction with the receptors. [I_2-Tyr^2]-α-MSH has considerable activity, but it appears that either ionization of the hydroxyl group and/or

Table II. Relative Potency of MSH Analogs in Frog Skin and Melanoma Adenylate Cyclase Assays.

Hormone	Frog Skin Assay[a]	Melanoma Adenylate Cyclase Activity
α-MSH	1.00	1.00
[Nle^4]-α-MSH	2.33	2.00
[Phe^2, Nle^4]-α-MSH	0.74	1.00
[I_2-Tyr^2]-α-MSH	0.10	0.40
[D-Tyr^2]-α-MSH	0.025	-
α-MSH_{7-13}	0.010	-
β-MSH	0.75	1.50
[Nle^7]-β-MSH	5.2	4.17

[a]Relative potency = $\frac{\text{conc. of } \alpha\text{-MSH at 50\% response}}{\text{conc. of hormone at 50\% response}}$

steric bulk of the iodides interfere with binding. The reduced potency of [Phe^2, Nle^4]-α-MSH also suggests that the Tyr hydroxyl group is important for binding. The significant drop in potency for [D-Tyr^2]-α-MSH illustrates the importance of the correct enantiomeric relationship in the 2-position. These results, the significant potency of Ac-α-MSH_{7-13}, and the full agonist activity of all analogs, indicate that the N-terminal region of MSH is important for affinity but not agonism, and supports previous suggestions[5] that the N-terminal region of α-MSH is important for potentiating its biological activity. Finally, it should be noted that though the general trends in potency in both assay systems are similar, there are significant discrepancies, suggesting that the receptor systems of the normal and transformed cells may have somewhat different structural requirements.

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VASCULAR RECEPTORS FOR KININS

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Bradykinin[1] has been considered one of the most potent endogenous vasodilators. Recent studies have focused on the possible protective role of kinins against high blood pressure[2,3]. Research is currently directed at the development of inhibitors of the converting enzyme (kininase II) because these compounds are expected to reduce the activation of the vasoconstrictor renin-angiotensin system and prevent the inactivation of the vasodilator kallikrein-kinin system. For these reasons inhibitors of the angiotensin-converting enzyme are among the promising new antihypertensive agents[4,5].

Vascular receptors for kinins were studied to a) demonstrate their presence in arterial and venous smooth muscle; b) consider the possible existence of different receptor types for kinins, and c) promote the use of isolated vessels in pharmacological studies with kinins, earlier carried out on intestinal or uterine smooth muscles[6]. Our results are from pharmacological studies with kinins and their analogs on isolated rabbit arteries and veins.

Figure 1 shows the contractile actions of Lys-bradykinin (Lys-BK), bradykinin (BK), and des-Arg^9-bradykinin (des-Arg^9-BK) on isolated rabbit aorta, the anterior mesenteric, and the jugular veins.

In the first two preparations Lys-BK and des-Arg^9-BK are more active than BK. A different pattern is observed in the jugular vein (Figure 1). The results indicate that: a) kinins are potent stimulants of isolated vascular smooth muscles; b) the order of potency of kinins is not the same in all isolated vessels, suggesting the existence of at least two different receptor types.

Table I summarizes the results obtained with analogs of BK and des-Arg^9-BK. Activities are expressed in terms of α^E (intrinsic activity), pD_2 (apparent affinity of agonists), and pA_2 (apparent affinity of antagonists)[10,11,12]. Because of the role of aromatic residues in des-Arg^9-BK for binding to receptors and in the activation of them[9], we chose analogs in which Phe^5 or Phe^8 was replaced by Tyr(Me), Cha or Leu, to evaluate the

contribution made by the phenyl rings present in positions 5 and 8. The results obtained in the aorta and in the jugular vein are presented. The anterior mesenteric vein behaves similarly to the aorta.

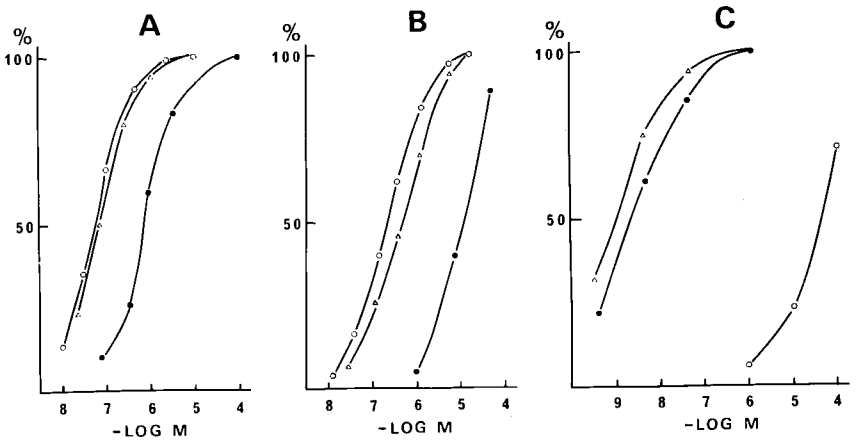

Fig. 1. Concentration-response curves measured with Lys-BK, (△—△) BK (●—●) and des-Arg^9-BK (o—o) on the aorta (A), the anterior mesenteric (B) and the jugular veins (C) of the rabbit. Tissues were suspended in oxygenated Krebs at 37° C and incubated for 6 hours (A and B) or 4 hours (C) before measuring the effects of the kinins. Technical details are described in ref. 7, 8 and 9. Points indicate the means of 6 to 8 determinations. Ordinate: Myotropic effect in per cent (%) of the maximal contraction. Abscissa: Concentration of kinins.

The results support the existence of two different receptor types, showing that octapeptide analogs are active in the aorta and practically inactive in the jugular vein, while nonapeptides are weak stimulants in the aorta. Certain nonapeptides (e.g. [Tyr(Me)^8]-BK) are more potent in the jugular vein than BK, others are generally more potent on venous than on arterial smooth muscle. Some of the octapeptides block the action of kinins on the aorta[13], but none of the octa or nonapeptides exerts an inhibitory action on the jugular vein. The inhibition of the aortic receptor by [Cha^8], des-Arg^9-BK and [Leu^8], des-Arg^9-BK is competitive and specific for kinins[9], providing strong evidence that the receptors for kinins in the aorta are different from those in the jugular vein. The functional role of Phe^5 and Phe^8 is definitely different in octapeptides. Replacement of Phe^5 with other residues modifies the affinity while that of Phe^8 influences primarily the intrinsic activity, suggesting that Phe^5 is primarily involved in binding while Phe^8 is the stimulating group. Replacement of Phe^8 with non-aromatic residues changes agonism to antagonism. Aromaticity in position 8 is therefore essential for activation of aortic receptors, while hydrophobicity and the ring size appear to be

Table I. Biological Activity of Three Natural Kinins (Lys-BK, BK and des-Arg^9-BK) and Analogs on the Aorta and Jugular Vein of the Rabbit.

COMPOUND	AORTA				JUGULAR VEIN			
	α^E	pD_2	R.A.	pA_2	α^E	pD_2	R.A.	Ant.*
BK	1.0	6.22	8.5	-	1.0	8.77	100.	-
des-Arg^9-BK	1.0	7.29	100.	-	-	4.34	<0.01	-
Lys-BK (Kallidin)	1.0	7.27	95.0	-	1.0	9.08	190.0	-
[Tyr(Me)^5],des-Arg^9-BK	1.0	5.27	0.95	0	-	-	<0.01	0
[Cha^5],des-Arg^9-BK	1.0	7.21	83.0	0	1.0	4.92	0.01	0
[Leu^5],des-Arg^9-BK	1.0	5.81	4.0	0	-	-	<0.01	0
[Tyr(Me)^8],des-Arg^9-BK	-	4.28	0.1	-	-	4.13	<0.01	0
[Cha^8],des-Arg^9-BK	0	-	-	6.42	1.0	5.42	0.04	0
[Leu^8],des-Arg^9-BK	0	-	-	7.27	-	-	0	0
[Tyr(Me)^5]-BK	0.9	5.91	4.1	-	1.0	8.37	43.0	-
[Cha^5]-BK	1.0	5.45	1.4	-	1.0	7.38	4.0	-
[Leu^5]-BK	0.7	4.61	0.2	-	1.0	7.49	5.2	-
[Tyr(Me)^8]-BK	-	4.70	0.25		1.0	9.00	160.0	-
[Cha^8]-BK	-	-	0	0	1.0	6.95	1.6	-
[Leu^8]-BK	-	-	<0.01	0	1.0	6.08	0.21	-

* The compounds have not been tested (-) for antagonism (Ant.) or they have been tested and found inactive (0) as inhibitors.

determinants for the binding contributed by Phe^5. In nonapeptides the two aromatic residues appear to be involved in binding only, since all analogs maintain full intrinsic activity. Phe^5 cannot be replaced with other aromatic or aliphatic residues without loss of affinity. Substitutions with aromatic residues in position 8 are well tolerated, even improving affinity (see [Tyr(Me)^8]-BK). Aliphatic residues in position 8 reduce affinity markedly.

Lys-Bk is as active as des-Arg^9-BK and more potent than BK in the aorta, suggesting that increased affinity for the B_1 receptor (aortic) can be achieved either by removing a positive charge at the carboxyl-end (des-Arg^9-BK) or by adding a similar charge at the amino-end (Lys-BK). To

obtain more potent agonists and antagonists for the B_1 receptor, two analogs of des-Arg^9-BK, prolonged at the N-terminus by the addition of Lys, were synthesized and tested. Table II shows that des-Arg^{10} kallidin is a full agonist with increased affinity, mostly due to prolongation of binding to the aortic receptor[14]. Similar changes are observed with the antagonist, the affinity of which increases 10 times (compare pA_2 values 7.27[14] of [Leu^8]des-Arg^9-BK with 8.37 for [Leu^9], des-Arg^{10}-kallidin). The addition of Lys at the N-terminus does not improve the affinity for the B_2 receptors of the jugular vein.

Table II. Biological Activities of Kinins on Vascular Receptors.

COMPOUND	AORTA				JUGULAR VEIN			
	α^E	pD_2	R.A.	pA_2	α^E	pD_2	R.A.	Ant.
des-Arg^9-BK	1.0	7.29	100	-	-	4.34	<0.01	-
des-Arg^{10}-Kallidin	1.0	8.61	1200	-	-	4.82	0.01	-
Leu^9,des-Arg^{10}-Kallidin	-	-	-	8.37	-	4.00	<0.01	0
BK	1.0	6.22	8.5	-	1.0	8.77	100.0	-

In conclusion, receptors of two different types for kinins have been found in arterial and venous smooth muscles. Isolated rabbit vessels responding to kinins with sustained contractions, are useful for structure-activity studies of these peptides.

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BINDING TO THE PARATHYROID HORMONE RECEPTOR: DELINEATION OF STRUCTURAL DETERMINANTS IN THE AMINO-TERMINAL REGION OF THE MOLECULE NECESSARY FOR BINDING

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Introduction

Analysis of structure-activity relations for parathyroid hormone (PTH) have revealed that full bioactivity resides within the NH_2-terminal 34 amino acids[1]. Within this fragment is a region essential for activation (positions 1 and 2) and a sequence (positions 3-34) containing structures necessary for binding to presumed receptors[2]. These findings permitted design of an *in vitro* PTH antagonist, [Nle-8,Nle-18,Tyr-34]bPTH-(3-34)-amide, which can completely inhibit PTH-stimulated increases in cAMP in canine renal membranes[3] or human cells of skeletal origin[4]. The inhibitor competes for PTH binding sites in a radioligand receptor assay with avidity corresponding to its inhibitory potency[5]. Because the inhibitor is potent and oxidation-stable, it is ideally suited for exploring the 3-34 region for a binding domain. Fragments of the inhibitor, truncated at the NH_2 terminus, were synthesized: 7-34, 10-34, 15-34, 20-34, and 25-34. Binding properties were compared to inhibitory potency for each fragment.

Methods

Preparation of Hormone Analogs. Peptides were prepared by Merrifield solid-phase technique[6,7,8]. Details of synthesis procedure, purification, and analysis have been reported previously[3,8]. To permit valid comparison of affinities, fragments were derived from a single synthesis.

Radioligand Binding Assay. A binding assay based on an ^{125}I-labeled hormone analog was employed[5]. The assay is specific and saturable with regard to PTH binding, and binding of hormone agonists correlates with biologic activity.

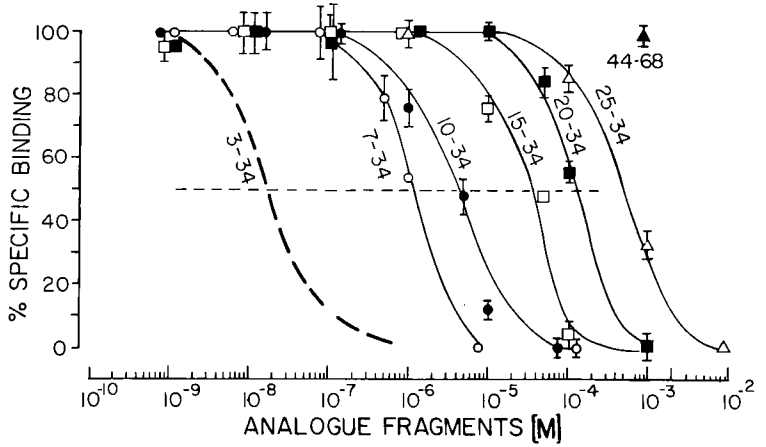

Fig. 1. Radioligand receptor assay: inhibition of radioligand binding by fragments. K_B = 50% inhibition of specific binding.

Results

Fragment binding was tested over a concentration range of 10^{-10}M to 10^{-2}M. Inhibition of specific binding of radioligand for each fragment is shown in Figure 1. The concentration causing half-maximal specific binding of radioligand (K_B) was compared to apparent inhibitory constants (K_i) observed in the renal adenylate cyclase assay[9] (Table I).

Discussion

In order to identify the structural requirements for PTH receptor occupancy, we synthesized and studied binding properties of fragments of a PTH inhibitor. Deletion of NH_2-terminal residues caused progressive decline in binding avidity and, when present in sufficiently high concentration, each fragment inhibited completely radioligand-specific binding. Hence, even the fragment 25-34 contains all the structural determinants needed for receptor occupancy, indicating that a sequence 10 amino acids in length or smaller may be the principal binding region of PTH.

Table I. Comparison of Inhibitory and Binding Properties of Analog Fragments.

COMPOUND	K_B BINDING CONSTANT (Renal Membranes)	K_i INHIBITORY CONSTANT (Adenylate Cyclase)
3—Nle^{8}—Nle^{18}—Tyr^{34}-amide	1.4×10^{-8}M	1.2×10^{-8}M
7-Nle^{8}—Nle^{18}—Tyr^{34}-amide	1.4×10^{-6}M	3×10^{-6}M
10—Nle^{18}—Tyr^{34}-amide	3×10^{-6}M	7×10^{-6}M
15-Nle^{18}—Tyr^{34}-amide	3.8×10^{-5}M	3.7×10^{-5}M
20—Tyr^{34}-amide	7×10^{-5}M	7×10^{-5}M
25—Tyr^{34}-amide	2.5×10^{-4}M	no inhibition*

*Not tested at a concentration higher than 5.0×10^{-4}M.

Relative order and magnitude of the binding constants observed in this study correspond closely with the apparent inhibitory constants derived previously[9]. Hence, the mechanism of antagonism caused by these peptides can be attributed to direct competition for binding sites.

Since bPTH-(1-25) is bioactive, regions of the molecule other than 25-34 must also satisfy receptor binding requirements. Further studies are necessary to determine the relative importance of sequences within the region 1-34 for receptor interaction. Delineation of the structural determinants of receptor binding will facilitate PTH analog design: small peptides containing modifications of features essential for receptor interaction can now be synthesized.

Acknowledgements

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GASTROINTESTINAL HORMONE-RECEPTOR RECOGNITION: POSITION THREE IS A DETERMINING RESIDUE FOR GLUCAGON, SECRETIN, AND VIP

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Glucagon, secretin, and vasoactive intestinal polypeptide (VIP) have a high degree of sequence homology. Of the three hormones, glucagon is the most effective on hepatic adenylate cyclase and appears to react through a receptor distinct from those for secretin and VIP[1,2,3]. Indeed, secretin and VIP do not compete with ^{125}I-glucagon at the glucagon receptor in liver membranes. The question arises as to the basis of this remarkable specificity especially when comparing the N-terminal region where changes in the primary sequence of these hormones is slight.

Recently, we have shown that glucagon$_{1-6}$ competes with ^{125}I-glucagon at the hepatic glucagon receptor and stimulates through this receptor the adenylate cyclase system[4]. Glucagon$_{1-6}$ is a partial agonist yielding about 80% of the maximal activity given by the native hormone. To determine whether the N-terminal region may impart the specificity for distinguishing glucagon, secretin and VIP, we have studied the interaction of the N-terminal fragments of these three hormones on hepatic adenylate cyclase and for competition with ^{125}I-glucagon for the glucagon receptor.

The primary sequence of the first seven residues for these hormones is: Glucagon$_{1-7}$ His-Ser-Gln-Gly-Thr-Phe-Thr

Secretin$_{1-7}$ His-Ser-Asp-Gly-Thr-Phe-Thr

VIP$_{1-7}$ His-Ser-Asp-Ala-Val-Phe-Thr

For test purposes, glucagon$_{1-6}$ was obtained from Chemical Dynamics; secretin$_{1-6}$amide was a gift from M. Ondetti (Squibb Institute for Medical Research), and VIP$_{1-7}$ was synthesized by the solid phase procedure and purified by HPLC. The biological assays were performed as described elsewhere[4].

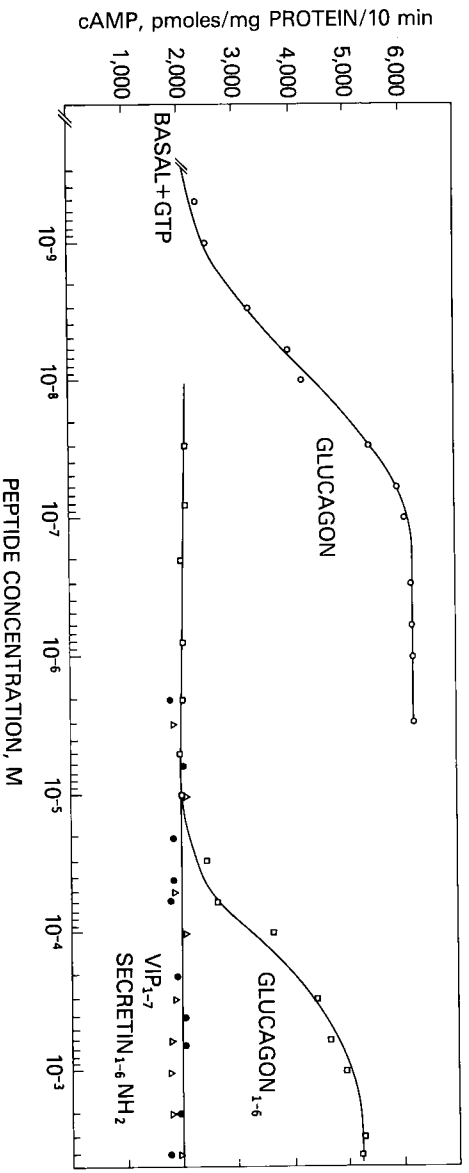

Fig. 1. Log concentration activity curves for porcine glucagon (O), glucagon$_{1-6}$ (□), VIP$_{1-7}$ (△) and secretin$_{1-6}$amide (●).

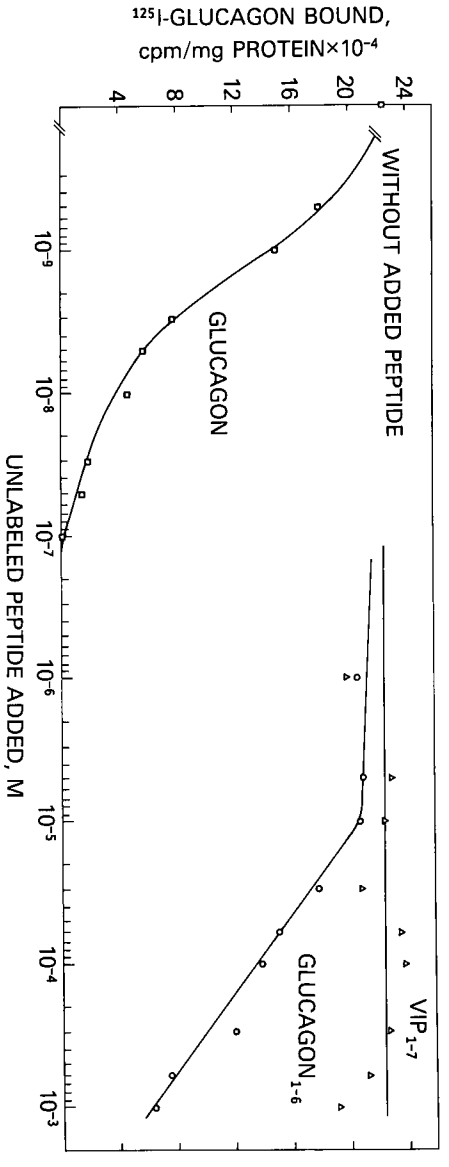

Fig. 2. Effect of addition of unlabeled peptides on the binding of ^{125}I-glucagon.

As shown in Figure 1, glucagon$_{1-6}$ displays its typical activating effect on adenylate cyclase; both secretin$_{1-6}$amide and VIP$_{1-7}$ were inactive. In competition binding with ^{125}I-glucagon (Figure 2), VIP$_{1-7}$ failed to compete indicating that it does not interact with the glucagon receptor. For lack of sufficient material the effects of secretin$_{1-6}$amide on binding could not be tested. However, like native secretin, secretin$_{1-6}$amide did not inhibit the actions of glucagon on adenylate cyclase (not shown).

Since glutamine at position three of glucagon essentially differentiates it from the other two hormones, it would appear that specific biological recognition by the glucagon receptor involves this position. By contrast, it appears that VIP and secretin have sufficient sequence homology to be recognized by each other's receptors[2,3].

Although the first six residues of glucagon is all that is required for biological recognition and action, the affinity of the hormone for the receptor is largely determined by residues toward the C-terminal end. Since the potency of native glucagon is about five orders of magnitude greater than that of glucagon$_{1-6}$, the possibility is raised that the C-terminal region of glucagon binds preferentially to a separate domain on the glucagon receptor. To test this, we investigated the ability of glucagon$_{13-29}$ to compete with ^{125}I-glucagon for the glucagon receptor and activate adenylate cyclase. It failed to activate. However, preliminary experiments show that it causes 50% inhibition of ^{125}I-glucagon binding at a concentration of 5 x 10^{-6}M. Thus it appears that the glucagon receptor contains domains that can distinguish between the N-terminal activating region and the remaining parts of the molecule that are responsible for potency of binding.

In conclusion, it now appears that the glutamine residue at position three of glucagon is an arbiter of the hormone's recognition by the receptor. This may have important meaning in the development of glucagon as a distinct hormone from secretin and VIP.

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STUDIES ON TYROSINASE STIMULATION, BINDING AND DEGRADATION OF α-MSH INTERACTING WITH NON-SYNCHRONIZED MOUSE MELANOMA CELLS IN CULTURE

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Introduction

α-MSH (α-melanotropin) is a pleiotropic effector which exhibits strong effects on a variety of tissues of lower vertebrates, mammals, and to some extent also of man[1]. At least partially, this diversity of actions of α-MSH seems to be due to the fact that non-identical hormonal message sequences are responsible for eliciting the stimulus in different target cells[2]. Our finding that the amphibian melanophore α-MSH receptor contains two message-recognizing sites, one for -Glu-His-Phe-Arg-Trp- and one for —Gly-Lys-Pro-Val·NH_2[3,4], has raised the question as to whether or not mammalian pigment cells are stimulated by the same hormonal sites. We have therefore extended our studies on a mouse melanoma cell line in culture in order to learn more about how hormone-cell interaction is related to the biological response.

Cloudman S-91 mouse melanoma cells were shown to respond dramatically to MSH with increases in intracellular cyclic AMP, tyrosinase activity, and melanin deposition[5,6]. Although the response to the hormone seems to be restricted to the G-2 phase of the cell cycle[7], non-synchronized cells proved to be suitable for studying structure-activity relationships of MSH-peptides.

Tyrosinase Stimulation by α-MSH, β-MSH, and Fragments

Cloudman S-91 Clone M-3 (CCL 53.1) mouse melanoma cells were maintained in a rapidly growing monolayer culture (doubling time: 24 hours) in 25 cm^2 Falcon tissue culture flasks (inoculation: 10^6 cells per flask in 10 ml of NCTC-135 medium with 10% horse serum). Sterile solutions with 4-9 different concentrations of each peptide were added

together with 10 μCi of ^{3}H-tyrosine to the flasks when they contained 2 x 10^6 cells. The activity of tyrosinase was determined by measuring the release of ^3H_2O after 12, 24, and 48 hours, respectively (elimination of ^{3}H-tyrosine from the samples by treatment with charcoal). A maximal response of about 200% above the basal level of ^3H_2O of non-stimulated cells was obtained with a 10^{-7} M α-MSH solution. This value was arbitrarily set as 100% of hormonal tyrosinase-stimulating potency. To achieve a better comparison of these figures, reciprocal EC_{50} values were multiplied with a factor of 100. With this, the relative molar tyrosinase-stimulating potency of α-MSH amounts to 4 x 10^{10}.

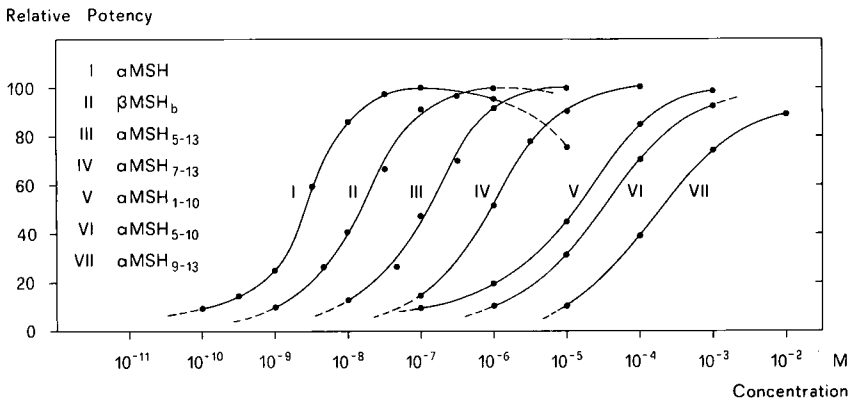

Fig. 1. Tyrosinase stimulation by α-MSH, β-MSH, and fragments. Each point of the log dose-response curves is the mean of 12 (I-III) or 8 (IV-VII) measurements.

Table I.

	Relative molar tyrosinase-stimulating potency ($1/EC_{50}$ x 100)	Melanophore-stimulating activity (Units/mmol)
α-MSH	4 x 10^{10}	4 x 10^{10}
β-MSH	6 x 10^9	5 x 10^9
α-MSH$_{5-13}$	8 x 10^8	5 x 10^8
α-MSH$_{7-13}$	9 x 10^7	5 x 10^6
α-MSH$_{1-10}$	8 x 10^6	1 x 10^7
α-MSH$_{5-10}$	3 x 10^6	4 x 10^5
α-MSH$_{9-13}$	5 x 10^5	6 x 10^5

Figure 1 shows log dose-response curves for α-MSH, β-MSH, and some N^{α}-acetylated fragments. The curves for peptides I-IV are fairly parallel, but differ slightly from those of the less potent analogues V-VII. Bovine β-MSH is about 6-7 times less active than α-MSH. A comparison of the relative molar tyrosinase-stimulating potencies with melanophore-stimulating activities reveals that there is a good correspondence of the values for the most active peptides (Table I). Whether the discrepancy in the case of the smaller fragments indicates any differences in the recognition pattern at the level of the receptors is an open question at present.

Binding and Degradation of Specifically Labelled α-MSH Derivatives

For the purpose of binding and degradation studies we have synthesized three α-MSH derivatives containing one or two labels at defined sites:

I [Tyr($^{3}H_2$)^{2}]α-MSH

II [Nva($^{3}H_4$)^{13}]α-MSH

III [Tyr(^{125}I)^{2}, Nva($^{3}H_4$)^{13}]α-MSH

I (34 Ci/mmol) and II (118 Ci/mmol) were used to determine specific binding of α-MSH to melanoma cells. Figure 2 displays a typical binding curve using peptide II. ('Specific' binding was regarded as the difference between total binding with and without a 1000-fold excess of α-MSH.) The SEM values increased towards higher concentrations because of increasingly higher (80 → 95%) 'non-specific' binding. Nevertheless, a rough calculation reveals that 2 x 10^6 cells per flask can bind about 5-10 x 10^9 α-MSH molecules (1000 cpm ≅ 6 x 10^9 molecules). As only a part of the whole cell population is in the G-2 phase during the incubation with the peptide (15 min), the number of receptors per cell is around n x 10^4 with n = 1-3. This would correspond approximately with the value already known from binding studies with ^{125}I-β-MSH[9].

Hormone degradation is negligible during the incubation time used for determining the binding. Electrophoretic analysis of culture medium containing peptide III (10^{-8}M) proved that even after 24 hours > 50% of the peptide is intact. However, incorporation of rather small amounts of radioactivity into the cell is increasing during the same period. Interest-

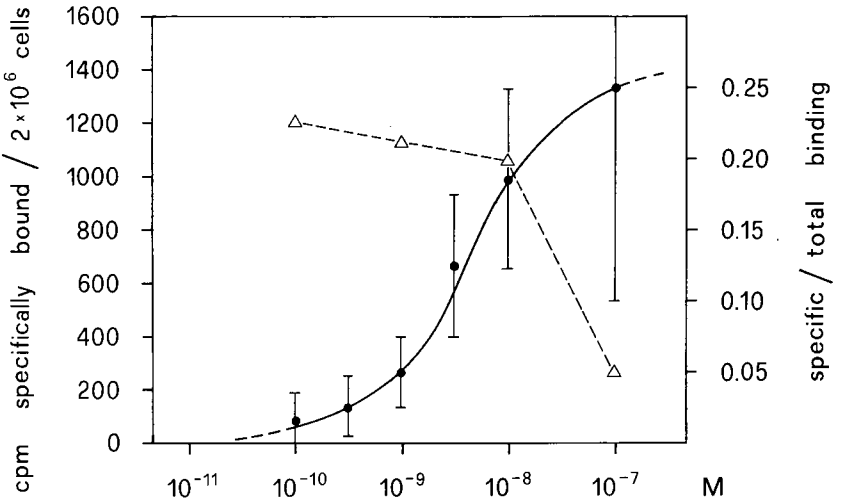

Fig. 2. Specific binding (●——●) of ^{3}H-α-MSH (peptide II) to 2 x 10^6 mouse melanoma cells (incubation: 15 min at 37°). Each point represents the mean of 8 measurements ± SEM. Specific/total binding: △-----△.

ingly, the uptake of ^{3}H and ^{125}I is not absolutely parallel indicating that MSH may be partially fragmented before incorporation. It is, however, too early to rule out an incorporation of the intact hormone as it has been postulated for FITC-β-MSH by Varga *et al*[10].

Acknowledgment

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