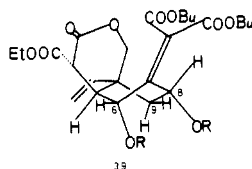
Commun., 1097 (1971); C. V. Grudzinshas and M. J. Weiss, *Tetrahedron Lett.*, 141 (1973).

(17) Actual conformation of the diacetate (**32**) [δ 2.19 (2H$_9$, d, J = 8.5 Hz), 5.63 ppm (H$_8$, t, J = 8.5 Hz)] could be the one inbetween IV and **39**, since A_2X-spin system in H-99–H-8 specifies only a twisted form in B ring around C-8 and C-9.



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(20) Melting points were determined on a hot stage apparatus (uncorrected). IR spectra were recorded on JASCO IR-G. ^{1}H NMR spectra were measured with JEOL MH-100 or FX-100 spectrometer, reporting chemical shifts in δ (ppm) by using Me_4Si as an internal standard. Low resolution electron impact (EI) mass spectra were recorded on JEOL D-100 instrument by using direct probe insertion. High resolution and field desorption (FD) mass spectra were determined on JEOL 01SG2 instrument. Tlc was performed on 0.25-mm pre-coated silica gel PF_{254} plates supplied by E. Merck (Art no. 5715). Preparative TLC separations were made on plates prepared with a 2-mm layer of silica gel PF_{254} obtained from E. Merck (Art no. 7747). Column chromatography was conducted on silica gel supplied by also E. Merck (Art no. 7734).

Studies on Polypeptides. 54. The Synthesis of a Peptide Corresponding to Positions 24–104 of the Peptide Chain of Ribonuclease T_1

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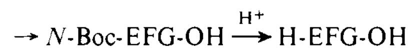
Abstract: A synthesis is described of a partially (S-cysteine and N^ϵ-lysine) protected peptide containing 81 amino acid residues corresponding to positions 24–104 of the peptide chain of the enzyme ribonuclease T_1. The peptide was assembled by condensing suitably protected fragments in a sequential manner by azide couplings. Every intermediate was purified, characterized, and analyzed. Small intermediates were characterized by thin-layer chromatography, elemental analysis, optical rotation, and amino acid analyses of acid and in some instances aminopeptidase M digests. Large fragments were characterized by thin-layer chromatography, amino acid analyses of acid, and aminopeptidase M digests (except for insoluble compounds) and dansyl end-group determinations. Certain sparingly soluble fragments obtained by azide coupling of acyl components not C-terminating in glycine or proline were acid hydrolyzed and the digests exposed to L-amino acid oxidase to assess the stereochemical homogeneity of certain amino acid residues. Based on the method of synthesis and the results of extensive analytical evaluation, it is concluded that the final peptide does not contain failure sequences and major backbone imperfections. However, the analytical methods employed are not sensitive enough to exclude some racemization.

In previous communications,[1-6] we have described syntheses of various fragments corresponding to sections of the polypeptide chain of the enzyme ribonuclease T_1 [ribonucleate guanine nucleotido-2′-transferase (cyclizing), EC 3.1.4.8] which served as intermediates in fragment condensation studies aimed at total synthesis of the entire peptide chain of the enzyme. Initially we subdivided the T_1 sequence into seven fragments (Figure 1) which were designated by the letters A to G.[3,7] These fragments were assembled from smaller subfragments designated as E_1, E_2, F_1, F_2, and G_1, G_2, G_3, etc. The larger fragments with the exception of A and G were N-protected by the benzyloxycarbonyl group and terminated in a Boc-hydrazide function. This combination of protecting groups made possible the selective deprotection of either the H_2N terminus or the HOOC terminus to generate fragments that could be coupled by the azide method to form larger peptide chains.[8] Using this approach, we succeeded in building up such sections of the T_1 chain as fragments ABCD,[4] BCD,[2] EF,[3] and FG.[6] In these studies we protected the sulfhydryl function of cysteine-103 by an ethylcarbamoyl group[9] and the carboxyl of threonine-104 by an amide function. The ϵ-amino group of lysine-41 was permanently protected by a formyl group.[10] This selection was based on results of chemical modifications of the N-terminal and the ϵ-amino group of lysine-41 which led to the conclusion that these groups are not essential for the activity of the enzyme. However, more recently it was shown that treatment of the enzyme with *cis*-aconitic anhydride brings about irreversible inactivation.[11]

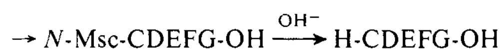
As we gained more experience it became apparent that the original combination of backbone protecting groups was not adequate for the construction of the entire peptide chain of the enzyme and consequently had to be modified. Hydrogenolysis, the method of choice for removing benzyloxycarbonyl groups, was not applicable to sections of the T_1 chain containing cysteine residues, i.e., fragments A and G. Here decarbobenzoxylation was effected by acidolytic cleavage with HBr/TFA. This technique was not likely to succeed with fragments incorporating the acid sensitive single tryptophan residue of the enzyme, i.e., those containing fragment E.

Assembly of Peptide H-CDEFG-OH. In the present communication, we describe experiments which enabled us to assemble a large section of the peptide chain of the enzyme (fragment H-CDEFG-OH (Figure 2) via the following steps:

N-Boc-E-azide + H-FG-OH


$\rightarrow$ N-Boc-EFG-OH $\xrightarrow{H^+}$ H-EFG-OH

N-Msc-CD-azide + H-EFG-OH


$\rightarrow$ N-Msc-CDEFG-OH $\xrightarrow{OH^-}$ H-CDEFG-OH

The amino acid sequences of these fragments are illustrated on Figure 3.

Since Boc groups can be cleaved under conditions that do not result in tryptophan destruction, N-Boc-E-hydrazide was

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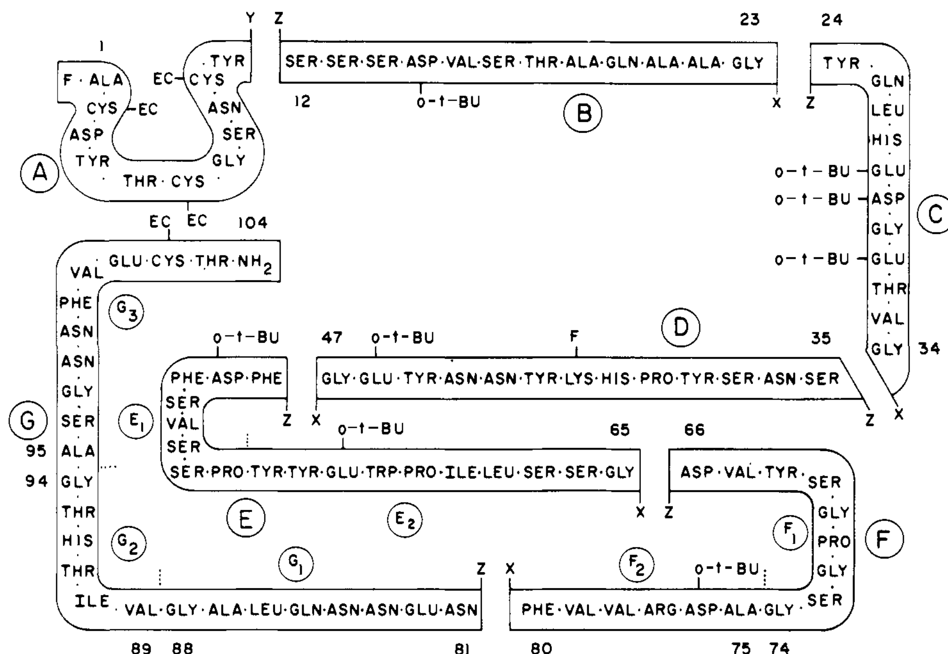


Figure 1. Designation of fragments used for assembly of fragment H-CDEFG-OH.

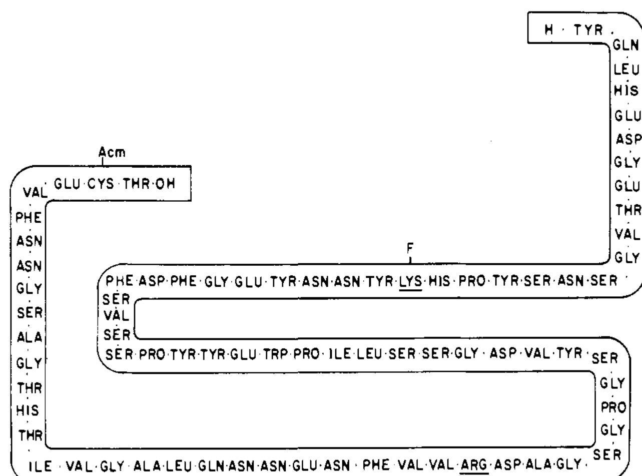


Figure 2. Primary structure of fragment H-CDEFG-OH.

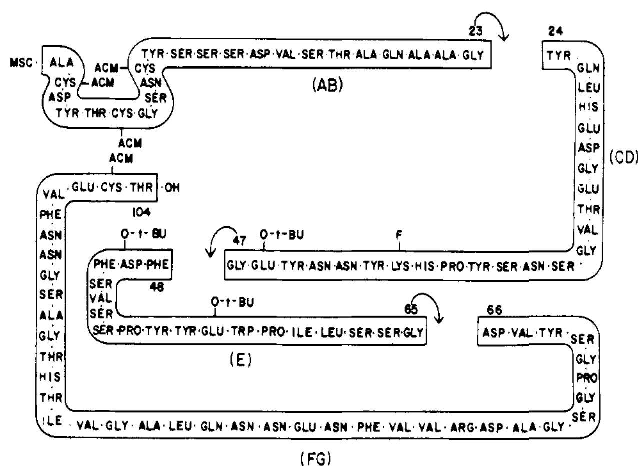


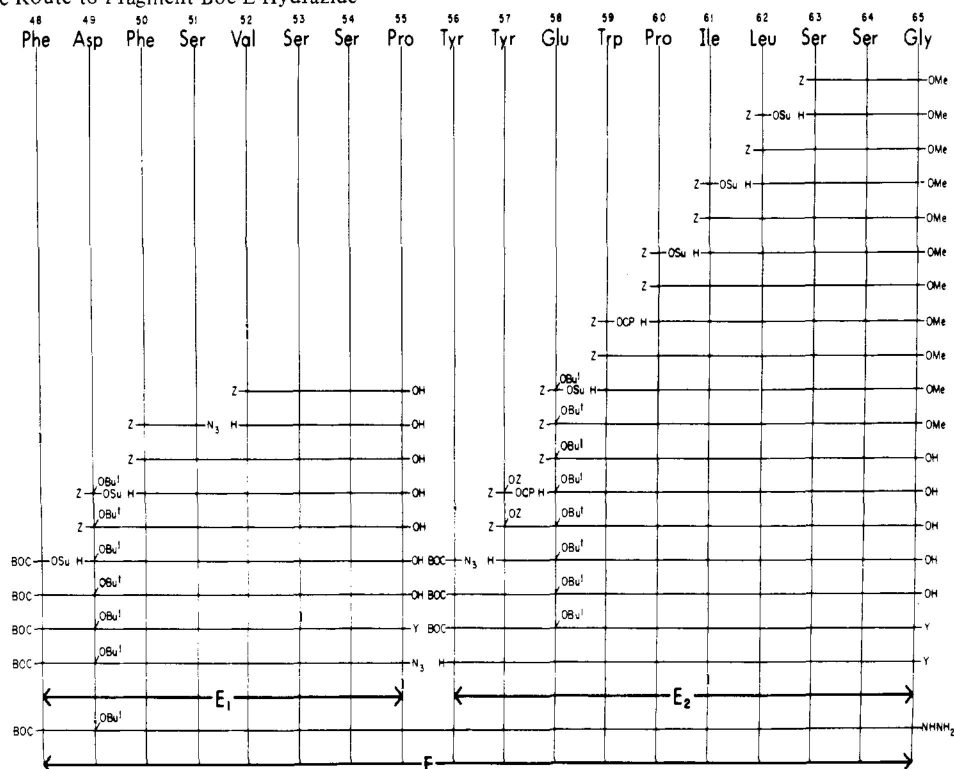
Figure 3. Primary structure of large fragments used in the final steps of the synthesis of fragment H-CDEFG-OH.

synthesized along the lines of a previous synthesis of *N*-Z-E-hydrazide.[3] Two subfragments E_1 and E_2 (Scheme I) were prepared and coupled by the azide procedure. For the synthesis of subfragment E_1, the C-terminal proline carboxyl in the starting Z-Val-Ser-Ser-Pro-OH was unprotected and the benzyloxycarbonyl group served for N protection during elongation of the peptide chain to the heptapeptide stage. Phenylalanine-48 was incorporated in the form of *N*-hydroxysuccinimido-*tert*-butoxycarbonylphenylalaninate. The resulting *N*-Boc-octapeptide was then coupled to benzyloxycarbonylhydrazine[12] by the method of mixed anhydrides. Hydrogenolysis of the ensuing Boc-E_1-Y afforded Boc-E_1-hydrazide.

For the synthesis of subfragment H-E_2-Y, the chain of methyl serylserylglycinate was elongated by stepwise addition of the desired benzyloxycarbonylamino acid active esters until the octapeptide stage was reached. At this point the C-terminal methyl ester group was removed by short exposure to alkali and the two tyrosine residues were introduced in the form of *N,O*-dibenzyloxycarbonyltyrosine 2,4,5-trichlorophenyl ester.[13] Hydrogenolysis removed the benzyloxycarbonyl groups to give the decapeptide. The *N*-Boc group was incorporated into this peptide by the use of di-*tert*-butyl dicarbonate.[14] Alternatively, only Tyr-57 was added to the growing chain and the benzyloxycarbonyl groups were cleaved by hydrogenolysis. Tyrosine-56 was then introduced in the form of *N*-Boc-tyrosine azide. Both methods afforded comparable yields and the optical rotations and R_f values of the two products were in good agreement. For conversion to H-E_2-Y, Boc-E_2-OH was reacted with benzyloxycarbonylhydrazine[12] by the method of mixed anhydrides and the Boc group of the resulting Boc-E_2-Y was cleaved with 90% TFA.

The final steps in the synthesis of Boc-E-hydrazide involved the coupling of Boc-E_1-azide with H-E_2-Y to give Boc-E-Y which was converted to the free hydrazide by hydrogenolysis. A sample of Boc-E-hydrazide was deprotected with 90% TFA containing 1% 1,2-ethanedithiol[15] and the ensuing product was digested with a mixture of aminopeptidase M and prolidase. Another sample of the deprotected material was subjected to acid hydrolysis to establish its peptide content. The average amino acid recovery in the acid hydrolysate was 68%, that in the aminopeptidase M digest was 77%. This result indicates that, within the limits of error of the amino acid analysis, the

Scheme I. Synthetic Route to Fragment Boc-E-Hydrazide



peptide was completely digestible by the enzymes. Trp recovery in the enzymic digest was 90% of theory. Recent studies with Trp containing peptides demonstrate that the acidolytic removal of Boc groups with TFA results in the formation of byproducts in which the indole portion of the Trp residue is tert-butylated.[16] It was also found that the tert-butylated Trp derivatives appear at a different elution volume from Trp on the amino acid analyzer[17] and that peptides containing modified Trp are not digestible by aminopeptidase M.[16] The finding that H-E-hydrazide was completely digestible by aminopeptidase M with a 90% recovery of Trp indicates that 10% or less of the Trp could have undergone tert-butylation during TFA deprotection. Sakakibara[17] noted a 4.5% tert-butylation when exposing Trp to TFA containing 1,2-ethanedithiol.

Boc-E-azide was coupled to fragment H-FG-OH to give Boc-EFG-OH. The cysteine sulfhydryl group in fragment H-FG-OH was protected by the acetamidomethyl (Acm) group;[18] the rest of the amino acid residues were unprotected. For conversion to fragment H-EFG-OH, the Boc derivative was deprotected with 90% TFA containing 1,2-ethanedithiol.[15] The sparing solubility of H-EFG-OH precluded an assessment of its Trp content by enzymic digestion. In a previous study,[2] we described syntheses of fragments *N*-benzyloxycarbonyl-CD-X and H-CD-X. For reasons stated previously, *N*-benzyloxycarbonyl-CD azide is not suitable for chain elongation of fragment H-EFG-OH. To overcome this difficulty, we exchanged the *N*-benzyloxycarbonyl group in *N*-benzyloxycarbonyl-CD-X by the Msc group.

Recently, Tesser[19] developed the methylsulfonylethyloxycarbonyl (Msc) group as a new N-protecting group in peptide synthesis. This group is very resistent to acidolytic conditions but is readily cleaved by very short exposure to alkali. The Msc group seemed suited for our purpose, i.e., the transient protection of the N terminal of large fragments during assembly of the T_1 chain. Since we were unable to secure information from the literature pertaining to the stability of the Msc group in Rudinger-type coupling reactions, we performed a model coupling reaction in which Msc-Ala-Ser-Gly azide was coupled to H-Asn-Asn-Phe-X. The ensuing product was deprotected by short exposure to alkali to afford H-Ala-Ser-Gly-Asn-Asn-Phe-X identical with the same material prepared by hydrogenolysis of the corresponding *N*-benzyloxycarbonyl derivative.[5] This result illustrates the usefulness of the Msc group in azide coupling reactions.

For the synthesis of *N*-Msc-CD-X, we subjected *N*-benzyloxycarbonyl-CD-X to hydrogenolysis and acylated the ensuing H-CD-X with Msc-OSu.[19] The Boc group was removed from the reaction product by exposure to 90% TFA to give the hydrazide trifluoroacetate of fragment Msc-CD. This compound was converted to the azide which served to acylate fragment H-EFG-OH with formation of fragment Msc-CDEFG-OH. The Msc group was cleaved by short exposure to dilute sodium hydroxide.

Of the 80 peptide bonds which constitute fragment H-CDEFG-OH, 36% were formed by azide couplings and 64% by stepwise active ester condensations.

Coupling Yields. Table I illustrates the yields that were realized in a number of fragment condensations by using Rudinger-type[20] azide coupling reactions. Because of the sparing solubility of the peptides in the usual organic solvents, coupling reactions were performed in DMF or mixtures of DMF and Me_2SO. In our previous studies, we employed triethylamine as the base; this has now been replaced by diisopropylethylamine (DIPEA) which is reported to minimize racemization.[21] Lack of material precluded systematic study of these reactions and for this reason the yields are not optimized. However, many of these couplings were repeated several times with comparable results. In Table I the acyl components (acylpeptide azides) are arranged according to chain length which varies from 8 to 24 amino acid residues. With one exception the yields declined with increasing chain length of the acylating component but other factors such as the conformations of both acylating and amino components may also be involved. The reactive ends of the peptides may become sterically hindered because of folding into the peptide chain. Since conformational stability can be expected to increase with increasing chain length, conformation may become the overriding factor influencing coupling yields.

Purification. As has been pointed out in earlier reports, the low solubility of various peptides in the T_1 series, particularly,

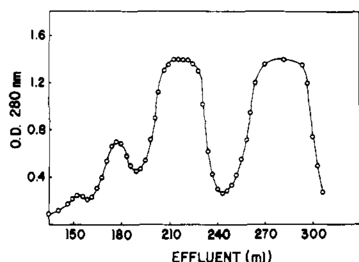


Figure 4. Gel filtration of the products resulting from the coupling of fragment Msc-CD azide with fragment H-EFG-OH. See Experimental Section for details.

Table I: Yields in Rudinger-Type Coupling Reactions (Based on Amount of Amino Component) Using N-Acylpeptide Azides of Varying Chain Lengths[a]

	coupling reaction	yield	ref
Z-E_1(8)	+ H-E_2-Y(10)—Z-E-Y(18)	42%	b
Z-B(12)	+ H-CD-X(24)—Z-BCD-X(36)	48%	2
Z-F(15)	+ H-G_1-X(8)—Z-$FG_1$$HNNH_2$, 2TFA(23)	74%[c]	b
Boc-E(18)	+ H-FG-OH(39)—Boc-EFG-OH(57)	33%[c]	b
Z-E(18)	+ H-F-X(15)—Z-EF-X(33)	39%	3
F-AB(23)	+ H-CD-X(42)—F-ABCD-X(65)	17%	4
Z-FG_1(23)	+ H-G_2G_3-OH(16)—Z-FG-OH(39)	22%[c]	b
Msc-CD(24)	+ H-EFG-OH(57)—Msc-CDEFG-OH(81)	5%	b

[a] Numbers in parentheses represent the number of amino acid residues in the acylating components, amino components, and coupling products. Yields refer to dried product. [b] This paper. [c] Yield of deprotected material.

fragments H-G_2G_3-OH, Z-FG_1-$HNNH_2$, H-FG-OH, and Boc-E-Y, created purification problems. Originally[6] we resorted to 8 M urea (pH 10.7) as a solvent for purification of some of these peptides, but this solvent has several disadvantages. Exposure to pH 10.7 for an extended period of time of peptides containing asparagine and glutamine residues may bring about partial deamidation and the removal of the urea from the purified products proved cumbersome. More recent experiences with some of the sparingly soluble peptides has led to purification techniques that are simpler and do not have the disadvantages of the earlier procedures. For example, fragment Z-G_2G_3-OH was readily purified by precipitation from glacial acetic acid with water. Fragment Z-FG_1-$HNNH_2$ trifluoroacetate was washed repeatedly with a mixture of DMF and ethyl acetate to give a chromatographically homogeneous product. For purification of fragment H-FG-OH the mixture of products resulting from the coupling of benzyloxycarbonyl-FG_1 azide with fragment H-G_2G_3-OH was washed with 5% acetic acid to remove unchanged amino component (fragment H-G_2G_3-OH). The washed material was deprotected with HBr/TFA. The resulting material was then subjected to gel filtration on Sephadex G-50 by using 45% formic acid as the solvent. Gel filtration on Sephadex LH-20 was used to purify fragment Boc-E-Y by using DMF as the solvent. This procedure was not very effective and the material had to be recycled three times to achieve chromatographic homogeneity. Fragment H-EFG-OH was purified by gel filtration on Sephadex G-50 with 45% formic acid as the solvent. Gel filtration on Bio-Gel P-100 in 50% acetic acid brought about a partial separation of the crude product mixture resulting from the reaction of Msc-CD azide with H-EFG-OH. The results of a typical experiment (Figure 4) show the presence of four not cleanly resolved peaks which represent from left to right a small amount of unidentified material, fragment Msc-CDEFG-OH (contaminated by unacylated H-EFG-OH), unacylated fragment H-EFG-OH, and rearrangement products of Msc-CD azide probably the amide. In certain experiments, the resolution between peaks 2 and 3 was not as favorable as shown and rechromatography of peak 3 material on Bio-Gel P-100 failed to separate the desired peptide from unacylated fragment H-EFG-OH. Further study of peak 3 material showed it to be readily soluble in 5 mM pH 8.4 disodium phosphate. This observation provided the key to the purification of Msc-CDEFG-OH particularly its clean separation from fragment H-EFG-OH. Separation was achieved by chromatography on DEAE-cellulose by using a gradient obtained by mixing 5 mM disodium phosphate (pH 8.4) with 0.25 M monosodium phosphate (pH 4.1), 0.25 M with respect to sodium chloride. Details of the various purification procedures are described in the Experimental Section. Regenerated fragment H-EFG-OH was recycled to prepare additional amounts of Msc-CDEFG-OH.

Table II: End-Group Identification in Synthetic T_1 Fragments by Dansylation

fragment	DNS-amino acids: expected	DNS-amino acids: found
H-D-X	N-DNS-Ser	N-DNS-Ser
	O-DNS-Tyr	O-DNS-Tyr
H-CD-X	O,N-BisDNS-Tyr	O,N-BisDNS-Tyr
	O-DNS-Tyr	O-DNS-Tyr
H-G_2G_3-OH	DNS-Val	DNS-Val
	N^{Im}-DNS-HIS	N^{Im}-DNS-His
H-FG-OH	DNS-Asp	DNS-Asp
	O-DNS-Tyr	O-DNS-Tyr
H-EFG-OH	DNS-Phe	DNS-Phe
	O-DNS-Tyr	O-DNS-Tyr
H-CDEFG-OH	O,N-BisDNS-Tyr	O,N-BisDNS-Tyr
	O-DNS-Tyr	O-DNS-Tyr
		DNS-Phe?[a]

[a] A weak fluorescent spot at the position of DNS-Phe was also observed during dansylation of H-Val-Ile-Thr-His-Thr-Gly-X and H-Val-Ile-Thr-His-Thr-Gly-Ala-Ser-Gly-X which correspond to positions 89–94 and 89–97 and do not contain Phe.

Assessment of Homogeneity. Elucidation of the homogeneity of complex synthetic peptides is a difficult task. Homogeneity can only be demonstrated in a negative sense, i.e., by failure to find imperfections by using various analytical techniques. The strength of a claim of homogeneity is, therefore, related to the number of independent techniques used for its evaluation. When the synthesis of a peptide exhibiting biological activity is the goal, then matching biological activity of the natural material with that of the synthetic product is usually accepted as proof of homogeneity despite the fact that the biological activity may not depend critically on one specific sequence (homology) and the bioassay may be inaccurate. In the present investigation we have synthesized some rather complex peptides, none of them exhibiting biological activity. Here the establishment of homogeneity rests solely on chemical and physical criteria which may not be sensitive enough to detect contamination.

It seems highly unlikely that the peptides described in this and previous communications[1-6] contain failure sequences since *the incorporation of every single amino acid residue into the growing peptide chain was carefully monitored by amino acid analysis*. Since the larger fragments are obtained by the linking of smaller segments of established composition, their sequence homogeneity also rests on a firm analytical basis. The ratio of the "diagnostic" amino acid residues[2] provides information regarding the presence of an excess of one of the coupling components in peptides obtained by the coupling of two fragments. These ratios were determined whenever possible and agreed, within the limit of error of amino acid analysis, with expected values.

The N-terminal amino acid residue in key fragments of the T_1 sequence was identified by dansylation.[22] The results (Table

Table III: Digestibility of Some Synthetic T_1 Fragments with AP-M

sequence	av recovery (%)	ref
fragment C		
H-Tyr-Gln-Leu-His-Glu-Asp-Gly-Glu-Thr-Val-Gly-HNNH$_2$ · 3TFA		
Tyr$_{1.2}$Gln$_{0.8}$Leu$_{1.0}$His$_{1.3}$Glu$_{2.1}$Asp$_{1.0}$Gly$_{1.8}$Thr$_{0.9}$Val$_{0.9}$	76	2
fragment D		
H-Ser-Asn-Ser-Tyr-Pro-His-Lys(F)-Tyr-Asn-Asn-Tyr-Glu(OBu^t)-Gly-X · acetate		
(Ser + Asn)$_{5.2}$Tyr$_{2.9}$Pro$_{1.0}$His$_{0.9}$Lys(F)$_{1.0}$Glu(OBu^t)$_{1.0}$Gly$_{1.0}$	84	2
fragment E$_1$		
H-Phe-Asp(OBu^t)-Phe-Ser-Val-Ser-Ser-Pro-X · acetate		
[Asp(OBu^t) + Asp]$_{1.2}$Phe$_{2.0}$Ser$_{3.1}$Val$_{1.0}$Pro$_{0.8}$	88	3
fragment E$_2$		
H-Tyr-Tyr-Glu-Trp-Pro-Ile-Leu-Ser-Ser-Gly-HNNH$_2$ · 2TFA		
Tyr$_{2.0}$Gly$_{1.0}$Trp$_{0.8}$Pro$_{1.0}$Ile$_{1.0}$Leu$_{1.0}$Ser$_{2.3}$Gly$_{1.0}$	82	3
fragment F$_1$		
H-Asp(OBu^t)-Val-Tyr-Ser-Gly-Pro-Gly-Ser-Gly-X		
[Asp(OBu^t) + Asp]$_{1.0}$Val$_{1.0}$Tyr$_{1.0}$Ser$_{2.0}$Gly$_{2.9}$Pro$_{1.1}$	83	3
fragment F$_2$		
H-Ala-Asp(OBu^t)-Arg-Val-Val-Phe-X · diacetate		
[Asp(OBu^t) + Ala]$_{1.7}$Asp$_{0.2}$Arg$_{1.1}$Val$_{2.0}$Phe$_{1.1}$	77	3
fragment G$_1$		
H-Glu(OBu^t)-Asn-Asn-Gln-Leu-Ala-Gly-X		
Glu(OBu^t)$_{1.0}$(Asn + Gln)$_{3.0}$Leu$_{1.0}$Ala$_{1.0}$Gly$_{1.0}$	88	5

II) show the presence of the expected DNS-amino acids in every instance. A weak fluorescent spot at the position of Phe was detected on plates derived from the dansylation of fragment H-CDEFG-OH in addition to the expected *O,N*-bis(DNS)-Tyr. Since we have observed a similar spot on plates derived from the dansylation of T_1 sequences not containing Phe,[6] we consider this spot to be an artifact.

For detection of contamination every peptide was examined by TLC whenever possible in several solvent systems. However, TLC becomes less meaningful when applied to larger T_1 fragments. For example, fragments H-EFG-OH and H-CDEFG-OH have identical R_f^{III} values and fail to run in other solvent systems.

A side reaction in azide coupling reactions is the formation of urea linkages. These arise by way of a Curtius rearrangement of the azide to form an isocyanate which in turn reacts with the amino component with formation of a urea rather than a peptide linkage. Backbone imperfection due to this side reaction is readily detectable in small peptides since the C-terminal amino acid residue of the acylating component is converted to the corresponding aldehyde during acid hydrolysis and escapes detection on amino acid analysis. We have observed one example of this side reaction when coupling Z-Ser-Tyr azide with H-Ser-Met-Gln-OH.[23] When carried out in DMF, the reaction proceeded in the desired manner to give the expected Z-Ser-Tyr-Ser-Met-Gln-OH. When the same reaction was performed in aqueous phase by adding the solid azide to the triethylammonium salt of the amino component, a crystalline product was obtained which was not identical with the desired product. Amino acid analysis of an acid hydrolysate of this compound showed the complete absence of tyrosine. In recent studies, Inouye and Watanabe[24,25] have shown that peptides containing urea linkages undergo fragmentation on exposure to formic acid, TFA, HF and HBr/acetic acid. These fragments differ in their properties from the original molecule and are removed during purification. Since the majority of the T_1 fragments were subjected to acidolytic deprotection prior to purification, major backbone imperfection due to urea linkages is unlikely but a small degree of contamination below the limits detectable by amino acid analysis cannot be ruled out.

Another side reaction that could result in backbone imperfection is the α–β shift of the aspartylglycine bond located in positions 29–30 of the T_1 sequence. This problem was investigated in a previous study[2] and no evidence for the presence of a β-aspartyl bond was obtained.

Although a number of highly sensitive racemization tests are available, none is applicable to large peptides. However, a major degree of racemization in large peptides can be detected by enzymatic methods.[26] We have subjected a great number of synthetic T_1 sequences to digestion by aminopeptidase M (AP-M) and have determined the amino acid ratios and average recovery of amino acids in the digests. The digestibility of some key fragments is illustrated in Table III. One may conclude from these results and additional data provided in the present communication that the digestible peptides are not grossly racemized. Unfortunately this technique is not applicable to sparingly soluble peptides.

In the course of our investigation with synthetic T_1 sequences we have used a variety of commercial AP-M preparations and found them to differ in their ability to cleave X–Pro bonds. The preparation used in the present study did not cleave such bonds and prolidase had to be added to achieve complete digestion of proline containing sequences.

As mentioned previously, 36% of the peptide bonds in fragment H-CDEFG-OH were formed by azide coupling steps. Most of these coupling reactions involved fragments C-terminating in glycine which eliminates the chance for racemization. However, the azide technique, long considered the standard for racemization-free coupling, has recently become suspect.[21] In particular it was observed that the phenylalanine in certain peptides C-terminating in phenylalanine azide can undergo racemization in Rudinger-type reactions[21] particularly in the presence of excess base. Two peptide bonds in our T_1 series of peptides were formed from acylating components C-terminating in phenylalanine azide. They are the Phe–Val bond (positions 100–101) and the Phe–Asn linkage (positions 80–81). Fragment H-G_2G_3-OH (positions 89–104) which contains the Phe–Val bond is completely digestible by AP-M. The average recovery of amino acids in the enzyme digest was 87% that obtained with an acid hydrolysate. The degree of racemization of Phe in the Phe–Asn linkage of fragment FG_1 could not be ascertained by AP-M digestion because of the low solubility of the peptide. However, incubation of an acid hydrolysate of fragment Z-FG_1 hydrazide with L-amino acid oxidase[27] resulted in a 94% deamination of Leu, Phe, and Tyr. These values are not corrected for racemization during acid hydrolysis.

The method of synthesis of fragment Msc-CDEFG-OH from Msc-CD azide and H-EFG-OH leaves little doubt regarding its sequential homogeneity. This conclusion is substantiated by the amino acid ratios in an acid hydrolysate. The ratio of 1.13/1.2 of the diagnostic amino acid residues (Lys from the acylating and Arg from the amino component) is particularly revealing. The low recoveries of His and Thr in the hydrolysate appears to reflect destruction during hydrolysis since these amino acids are present in the correct amounts of hydrolysates of Msc-CD hydrazide and H-EFG-OH. As concerns the loss of Acm-Cys, we have observed previously[6] that addition of Nps-Nle fails to protect this residue from destruction during acid hydrolysis of certain peptides. Msc-CDEFG-OH which should contain 10 amide groups per mol of peptide liberated 12.2 mol of ammonia per mol of peptide. The excess of ammonia appears to be derived from destruction of amino acids during acid hydrolysis. It seems reasonable to conclude that no major deamidation had occurred during the many steps involved in the assembly of fragment Msc-CDEFG-OH.

Based on the method of synthesis and the results of rather extensive analytical evaluation, we conclude that peptide H-CDEFG-OH does not contain failure sequences and major backbone imperfections. We realize, however, that the analytical methods employed are not sensitive enough to exclude some racemization.

Experimental[28] Section

General Procedures. Melting points are uncorrected. Rotations were determined with a Zeiss precision polarimeter. Measurements were carried out with a mercury lamp at 546 and 576 nm and extrapolated to the 589-nm sodium line. Elemental analyses were by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y. The amino acid composition of acid hydrolysates was determined with a Beckman-Spinco Model 120 amino acid analyzer according to the method of Moore et al.[29] with Nle and α-amino-β-guanidopropionic acid serving as internal standards. Peptides containing Cys (Acm) were hydrolyzed in the presence of Nps-Nle[30] (1 mol/mol of Cys(Acm) and 0.1% phenol). At the end of the hydrolysis (24 h at 110 °C except when noted otherwise), the HCl was evaporated, the residue was dissolved in 0.1 N NaOH (one-fifth the volume of the final diluted sample), and the tubes were kept at room temperature for 2 h. The samples were then made up to volume with diluter buffer. Trp was determined either spectrophotometrically[31] or in methanesulfonic acid hydrolysates or AP-M digests by amino acid analysis.[32] The enzyme digestions were performed with AP-M (Sigma) essentially as described.[33] For the digestion of proline containing peptides, prolidase (Sigma) (18 units) was added and the reaction mixture contained $MnCl_2$ (5 mM). Designation of solvent systems for ascending TLC on silica gel G (E. Merck and Co., Darmstadt, West Germany) are R_f^{I} 1-BuOH/AcOH/H_2O (60:20:20); R_f^{II} $CHCl_3$/MeOH/H_2O (8:3:1 lower phase); R_f^{III} 1-BuOH/pyridine/AcOH/H_2O (30:20:6:24). Peptides were visualized by the chlorine, fluorescamine, and ninhydrin reagents. A freshly prepared solution of fluorescamine[34] (3 mg) in acetone (20 mL) served as the spray reagent for visualization of unprotected peptides and hydrazides by fluorescence. See reference 4 for additional information regarding general experimental procedures.

(Positions 95–97) Msc-Ala-Ser-Gly-X (I). To a solution of H-Ala-Ser-Gly-X·AcOH (R_f^{I} 0.2, R_f^{II} 0.1) (276 mg, 0.68 mmol)[5] in DMF (1 mL) and 10% DIPEA in DMF (1.16 mL, 0.68 mmol) cooled at 0 °C was added Msc-OSu[19] (196 mg, 0.74 mmol). The reaction mixture was stirred at 0 °C for 1 h and at room temperature for 20 h. The bulk of the solvent was removed and the product precipitated with ether. The crude (slightly sticky) precipitate was reprecipitated from methanol with ethyl acetate/ether (2:1) and dried: 310 mg (92%); $[\alpha]^{22}{}_D$ −11.2° (*c* 1.01, MeOH) R_f^{I} 0.4; R_f^{II} 0.5; R_f^{III} 0.7.

(Positions 95–100) Msc-Ala-Ser-Gly-Asn-Asn-Phe-X (II). Msc-Ala-Ser-Gly-X (510 mg) was deprotected with 90% TFA in the usual manner and the product was precipitated with ether, washed with ether, and dried. For purification the material was dissolved in methanol and precipitated with ether, 448 mg (86%). *tert*-Butyl nitrite, 10% in DMF (0.56 mL, 0.48 mmol), was added to a stirred solution at −10 °C of Msc-Ala-Ser-Gly-HNNH$_2$·TFA (224 mg, 0.44 mmol) in DMF (2.5 mL) and 6.18 N HCl in dioxane (0.356 mL, 2.2 mmol) and the mixture was stirred at −10 °C for 20 min. The solution was cooled to −20 °C and DIPEA (0.445 mL, 2.6 mmol) was added followed by a solution of H-Asn-Asn-Phe-X (223 mg, 2.2 mmol)[5] in DMF (2 mL). The mixture was stirred at 4 °C for 90 h at pH 8.0, and the solvent was evaporated. The product was precipitated with ice-cold ether, triturated with ether, and dried. The precipitate was triturated with four 2-mL portions of methanol, washed with ether, and dried: 331 mg (86%); $[\alpha]^{24}{}_D$ −19.4° (*c* 1.02, DMF); R_f^{I} 0.4; R_f^{II} 0.1; R_f^{III} 0.6. Amino acid ratios in 24-h acid hydrolysate. Calcd: $Ala_{1.0}Ser_{1.0}Gly_{1.0}Asp_{2.0}Phe_{1.0}$. Found: $Ala_{1.0}Ser_{1.1}Gly_{1.0}Asp_{2.0}Phe_{1.0}$.

(Positions 95–100) H-Ala-Ser-Gly-Asn-Asn-Phe-X Acetate. a. From the Benzyloxycarbonyl Derivative. Z-Ala-Ser-Gly-Asn-Asn-Phe-X[5] (70 mg) was hydrogenated over Pd in isopropyl alcohol/methanol 1:1 (20 mL) and 0.09 M acetic acid (2 mL). The catalyst was removed, the solvents were evaporated, and the residue was dissolved in water. Insoluble material was removed by centrifugation and the solution was lyophilized: 37 mg; $[\alpha]^{27}{}_D$ −41.1° (*c* 0.56, 10% acetic acid); R_f^{I} 0.2; R_f^{III} 0.6.

b. From the Msc Derivative. Msc-Ala-Ser-Gly-Asn-Asn-Phe-X (132 mg, 0.15 mmol) was dissolved in DMF (3 mL) and methanol (3 mL) and dioxane (3 mL) were added. The solution was cooled at 0 °C and 4 N sodium hydroxide (0.6 mL, 2.4 mmol) was added. The mixture was kept at 0 °C for 5 min; then the pH was adjusted to 5.0 by addition of 50% acetic acid. The solution was evaporated, the residue was dissolved in a small volume of methanol, and the product was precipitated with ethyl acetate. The precipitate was dissolved in 10% acetic acid (3 mL) and desalted on a 1.2 × 155 cm column of Sephadex G-25 (fine). Fractions containing the desired material were pooled and lyophilized: 80 mg (68%); $[\alpha]^{27}{}_D$ −41.4° (*c* 0.92, 10% acetic acid); R_f^{I} 0.2; R_f^{III} 0.6.

(Positions 89–104) H-Val-Ile-Thr-His-Thr-Gly-Ala-Ser-Gly-Asn-Asn-Phe-Val-Glu-Cys(Acm)-Thr-OH (III). The synthesis of this fragment has been described,[6] but the method of purification has been improved and larger amounts of material have been prepared. The ethyl acetate precipitated dried material (1.0 g) obtained from the coupling of Z-Val-Ile-Thr-His-Thr-Gly-Ala-Ser-Gly-N$_3$ (from 731 mg (0.60 mmol) of the hydrazide ditrifluoro acetate)[5] with H-Asn-Asn-Phe-Val-Glu-Cys(Acm)-Thr-OH (449 mg, 0.5 mmol)[6] by using DIPEA in place of TEA was purified as follows: The material in batches of 250 mg was dissolved in glacial acetic acid (10 mL), and water (11 mL) was added. The suspension was placed in a freezer for 5 h, and the precipitate was collected by centrifugation, washed with ice-cold water, and dried. The compound was precipitated twice more in an identical manner: total yield 635 mg (69%); $[\alpha]^{27}{}_D$ −46.0° (*c* 1.05, 45% formic acid); R_f^{I} 0.3; R_f^{III} 0.6. This material (400 mg) was deprotected with HBr/TFA in the usual manner and HBr and TFA ions were exchanged for acetate ions: 347 mg (93%); $[\alpha]^{26}{}_D$ −45.2° (*c* 1.01, 50% acetic acid); R_f^{I} 0.1; R_f^{III} 0.4. Amino acid ratios in 48-h acid plus Nps-Nle and phenol hydrolysate (A) and AP-M digest (B). Calcd: $Val_{2.0}Ile_{1.0}Thr_{3.0}His_{1.0}Gly_{2.0}Ala_{1.0}Ser_{1.0}Asp_{2.0}Phe_{1.0}Glu_{1.0}$1/2-$Cys_{1.0}$. Found: (A) $Val_{2.2}Ile_{1.0}Thr_{2.9}His_{0.9}Gly_{2.1}Ala_{1.2}Ser_{0.8}Asp_{2.2}Phe_{1.0}Glu_{1.0}$1/2-$Cys_{1.0}$ (81%). Found: (B) $Val_{2.0}Ile_{1.0}Thr_{3.2}His_{1.0}Gly_{2.0}Ala_{1.0}(Ser + Asn)_{3.2}Phe_{0.9}Glu_{0.8}Cys(Acm)_{0.9}$ (70%).

(Positions 81–88) H-Asn-Glu(OBu^t)-Asn-Asn-Gln-Leu-Ala-Gly-X Acetate (IV). The protected peptide[5] (315 mg) was hydrogenated for 20 h in 70% aqueous DMF containing 0.07 mL of glacial acetic acid in the usual manner. The catalyst was removed by filtration through Filter-Cel and the filtrate was concentrated to a small volume in vacuo. The product was precipitated by addition of ethyl acetate, washed with ethyl acetate, and dried: 251 mg (86%); R_f^{I} 0.3; R_f^{III} 0.7. Amino acid analysis in 24-h acid hydrolysate. Calcd: $Asp_{3.0}Glu_{2.0}Gly_{1.0}Ala_{1.0}Leu_{1.0}$. Found: $Asp_{3.0}Glu_{2.0}Gly_{1.0}Ala_{1.0}Leu_{1.0}$.

(Positions 66–88) Z-Asp-Val-Tyr-Ser-Gly-Pro-Gly-Ser-Gly-Ala-Asp-Arg-Val-Val-Phe-Asn-Glu-Asn-Asn-Gln-Leu-Ala-Gly-HNNH$_2$ Ditrifluoroacetate (V). The synthesis of this fragment has been described[6] but the method of purification has been improved and larger amounts have been prepared. The ethyl acetate precipitated dried material obtained from the coupling of Z-Asp-Val-Tyr-Ser-Gly-Pro-Gly-Ser-Gly-Ala-Asp-Arg-Val-Val-Phe-N$_3$ (from 1.44 g, 0.756 mmol of the hydrazide ditrifluoroacetate)[3] and H-Asn-Glu(OBu^t)-Asn-Asn-Gln-Leu-Ala-Gly-X acetate (653 mg, 0.60 mmol)[5] by using DIPEA in place of TEA was washed with two 50-mL portions of 20% acetic acid followed by one 50-mL and three 20-mL portions of water and was dried: 1.63 g; R_f^{I} 0.3 with minor impurity at 0.2; R_f^{III} 0.7 with

minor impurity at 0.6. The protected hydrazide (1.35 g) was deprotected with 90% TFA in the usual manner and the product was washed with ether and dried, 1.33 g. This material was washed in suspension with one 52-mL and three 26-mL portions of DMF/ethyl acetate (1:1) and then with four 14-mL portions of ethyl acetate and was dried. After each washing, the suspension was placed in a freezer for 15 min prior to centrifugation: 1.22 g (74%); R_f^{I} 0.3, R_f^{III} 0.6, single chlorine and hydrazide positive spot. Amino acid ratios in 48-h acid hydrolysate. Calcd: $Asp_{5.0}Val_{3.0}Tyr_{1.0}Ser_{2.0}Gly_{4.0}Pro_{1.0}Ala_{2.0}Arg_{1.0}Phe_{1.0}Glu_{2.0}Leu_{1.0}$. Found: $Asp_{4.9}Val_{2.9}Tyr_{0.8}Ser_{2.0}Gly_{4.0}Pro_{1.1}Ala_{2.1}Arg_{1.0}Phe_{1.1}Glu_{1.9}Leu_{1.0}$. Incubation of a 48-h acid hydrolysate with L-amino acid oxidase[27] resulted in a 94% destruction of Leu, Phe, and Tyr. These values are not corrected for racemization during acid hydrolysis.

(Positions 66–104) H-Asp-Val-Tyr-Ser-Gly-Pro-Gly-Ser-Gly-Ala-Asp-Arg-Val-Val-Phe-Asn-Glu-Asn-Asn-Gln-Leu-Ala-Gly-Val-Ile-Thr-His-Thr-Gly-Ala-Ser-Gly-Asn-Asn-Phe-Val-Glu-Cys-(Acm)-Thr-OH Dihydrobromide (VI). *tert*-Butyl nitrite (10% in DMF) (0.28 mL, 0.24 mmol) was added with stirring to a solution cooled at −10 °C of Z-Asp-Val-Tyr-Ser-Gly-Pro-Gly-Ser-Gly-Ala-Asp-Arg-Val-Val-Phe-Asn-Glu-Asn-Asn-Gln-Leu-Ala-Gly-$HNNH_2$ ditrifluoroacetate (610 mg, 0.22 mmol) in Me_2SO (3.6 mL) and DMF (2.4 mL) containing 6.96 N HCl in dioxane (0.165 mL, 1.11 mmol) and the mixture was stirred at −10 °C for 20 min. The mixture was cooled at −20 °C and DIPEA (0.34 mL, 2.0 mmol) was added followed by a solution of H-Val-Ile-Thr-His-Thr-Gly-Ala-Ser-Gly-Asn-Asn-Phe-Val-Glu-Cys(Acm)-Thr-OH (318 mg, 0.19 mmol)[6] in Me_2SO (0.9 mL) and 10% DIPEA in DMF (0.32 mL, 0.19 mmol). The mixture was stirred for 100 h at 4 °C; then the product was precipitated by addition of ethyl acetate (200 mL). The precipitate was washed with ethyl acetate and ether and was dried. The product was then washed with eight 20-mL portions of water and seven 10-mL portions of 5% acetic acid (to remove the amino component) and was redried, 560 mg. This material was deprotected with TFA/HBr containing 5% anisole in the usual manner. The deprotected material (646 mg) in batches of 215 mg each was dissolved in 45% formic acid (2 mL) and the solution was applied to a Sephadex G-50 column (1.9 × 235 cm) which was eluted with 45% formic acid. Fractions (4 mL each) were collected at a flow rate of 20 mL/h and monitored by absorbancy measurements at 280 nm; fractions corresponding to the first of the two UV-absorbing peaks contained the desired material. The contents of these tubes were pooled and lyophilized: 165 mg (22%); $[\alpha]^{26}_{D}$ −45.7° (*c* 1.03, 45% HCOOH); lit.[6] $[\alpha]^{29}_{D}$ −43.5° (*c* 1.02, 50% CH_3COOH). R_f^{I} 0.1; R_f^{III} 0.5. Amino acid ratios in 48-h acid plus Nps-Nle and phenol hydrolysate. Calcd: $His_{1.0}Arg_{1.0}Asp_{7.0}Thr_{3.0}Ser_{3.0}Glu_{3.0}Pro_{1.0}Gly_{6.0}Ala_{3.0}1/2\text{-}Cys_{1.0}Val_{5.0}Ile_{1.0}Leu_{1.0}Tyr_{1.0}Phe_{2.0}$. Found: $His_{0.8}Arg_{1.0}Asp_{7.4}Thr_{2.7}Ser_{3.2}Glu_{3.0}Pro_{1.0}Gly_{6.1}Ala_{3.1}1/2\text{-}Cys_{0.9}Val_{4.6}Ile_{0.7}Leu_{1.0}Tyr_{1.0}Phe_{1.9}Cys(SO_3H)_{0.07}$.

(Positions 52–55) H-Val-Ser-Ser-Pro-OH (VII). Z-Val-Ser-Ser-Pro-OH[3] (2.56 g, 4.9 mmol) was hydrogenated in the usual manner in methanol (20 mL) and 10% acetic acid (65 mL). The solvents were evaporated, the residue was dissolved in 98% ethanol (25 mL), and the product was precipitated by the addition of ether. The product was washed with ethanol and ether and was dried: 1.90 g (99%); $[\alpha]^{28}_{D}$ −93.5° (*c* 1.0, water); R_f^{I} 0.2; R_f^{III} 0.3. Amino acid ratios in 24-h AP-M digest. Calcd: $Val_{1.0}Ser_{2.0}Pro_{1.0}$. Found: $Val_{1.0}Ser_{2.0}Pro_{1.0}$ (84%).

(Positions 50–55) Z-Phe-Ser-Val-Ser-Ser-Pro-OH (VIII). *tert*-Butyl nitrite (1.52 mL, 13.1 mmol) was added to a solution cooled at −20 °C of Z-Phe-Ser-$HNNH_2$[35] (4.78 g, 11.9 mmol) in DMF (60 mL) containing 6.18 N HCl in dioxane (7.75 mL, 47.8 mmol). The mixture was stirred at −20 °C until the hydrazide test was negative, cooled at −30 °C, and DIPEA (8.18 mL, 47.8 mmol) was added. This was followed by a solution of H-Val-Ser-Ser-Pro-OH (3.09 g, 7.96 mmol) and DIPEA (1.36 mL, 7.96 mmol) in DMF (31 mL) and Me_2SO (9.25 mL) and the mixture was stirred at 4 °C for 24 h. The solvents were removed and the resulting oil was added to a chilled, stirred solution of ethyl acetate. The gummy precipitate was collected, dried, washed with water, and redried. The solid was triturated with ethyl acetate containing a small amount of methanol and dried: 5.22 g (86%); mp 190–191 °C; $[\alpha]^{27}_{D}$ −21.9° (*c* 1.25, DMF); R_f^{I} 0.6; R_f^{III} 0.7. Amino acid ratios in 48-h acid hydrolysate. Calcd: $Phe_{1.0}Ser_{3.0}Val_{1.0}Pro_{1.0}$. Found: $Phe_{1.0}Ser_{3.1}Val_{1.0}Pro_{1.0}$. Anal. ($C_{36}H_{48}N_6O_{12}\cdot 2H_2O$): C, H, N.

(Positions 50–55) H-Phe-Ser-Val-Ser-Ser-Pro-OH (IX). Z-Phe-Ser-Val-Ser-Ser-Pro-OH (1.15 g) was hydrogenated in the usual manner in methanol (40 mL) and 10% acetic acid (160 mL). The solvents were evaporated, the residue was dissolved in hot water (50 mL), the solution was concentrated to a syrup, and the product precipitated by the addition of 3 volumes of ethanol. The precipitate was collected, washed with ethanol, and dried: 775 mg (83%); $[\alpha]^{28}_{D}$ −80.5° (*c* 1.05, water); R_f^{I} 0.3; R_f^{III} 0.5. Amino acid ratios in 24-h AP-M digest. Calcd: $Phe_{1.0}Ser_{3.0}Val_{1.0}Pro_{1.0}$. Found: $Phe_{1.0}Ser_{2.9}Val_{1.0}Pro_{1.1}$ (89%).

(Positions 49–55) Z-Asp(OBu^t)-Phe-Ser-Val-Ser-Ser-Pro-OH Dihydrate (X). Z-Asp(OBu^t)-OSu (2.58 g, 6.13 mmol)[36] in DMF (30 mL) was added to a stirred solution cooled at 0 °C containing H-Phe-Ser-Val-Ser-Ser-Pro-OH (3.47 g, 5.57 mmol) and DIPEA (0.95 mL, 5.57 mmol) in DMF (80 mL) and water (8 mL). The cloudy mixture was stirred at room temperature for 18 h (clear solution after 3 h of stirring). The solvents were evaporated and the residue was triturated with 1 N citric acid (15 mL) and ether (400 mL). The white solid was collected, washed with water, and dried: 4.13 g (80%); mp 175–177 °C dec; $[\alpha]^{25}_{D}$ −54.1° (*c* 0.71, MeOH); R_f^{I} 0.6; R_f^{III} 0.6. Amino acid ratios in 24-h acid hydrolysate. Calcd: $Asp_{1.0}Phe_{1.0}Ser_{3.0}Val_{1.0}Pro_{1.0}$. Found: $Asp_{1.0}Phe_{0.9}Ser_{3.0}Val_{1.1}Pro_{0.9}$. Anal. ($C_{44}H_{61}N_7O_{15}\cdot 2H_2O$): C, H, N.

(Positions 49–55) H-Asp(OBu^t)-Phe-Ser-Val-Ser-Ser-Pro-OH (XI). The above protected peptide (3.17 g) was hydrogenated in the usual manner in methanol (250 mL) containing 10% acetic acid (5 mL). The solvent was evaporated, the residue was evaporated twice with ethanol and dissolved in ethanol (30 mL), and the product was precipitated with ether (150 mL). After standing at 4 °C for 12 h, the product was collected and dried: 2.21 g (82%); $[\alpha]^{25}_{D}$ −44.5° (*c* 0.12, 20% acetic acid); R_f^{I} 0.5; R_f^{III} 0.6. Amino acid ratios in 24-h acid hydrolysate. Calcd: $Asp_{1.0}Phe_{1.0}Ser_{3.0}Val_{1.0}Pro_{1.0}$. Found: $Asp_{1.1}Phe_{1.0}Ser_{2.9}Val_{1.0}Pro_{1.0}$.

(Positions 48–55) Boc-Phe-Asp(OBu^t)-Phe-Ser-Val-Ser-Ser-Pro-OH (XII). Boc-Phe-OSu[37] (477 mg, 1.32 mmol) in DMF (15 mL) was added to a solution cooled at 5 °C of H-Asp(OBu^t)-Phe-Ser-Val-Ser-Ser-Pro-OH (950 mg, 1.2 mmol) in DMF (25 mL) containing DIPEA (0.21 mL, 1.2 mmol) and the solution was stirred at room temperature for 60 h. The solvents were removed, the residue was suspended in ethyl acetate, and the product was collected and dried. This material was triturated with water and precipitated twice from methanol with ether: 1.19 g (87%); $[\alpha]^{25}_{D}$ −25.4° (*c* 0.76, DMF); R_f^{III} 0.7. Amino acid ratios in 24-h acid hydrolysate. Calcd: $Phe_{2.0}Asp_{1.0}Ser_{3.0}Val_{1.0}Pro_{1.0}$. Found: $Phe_{1.9}Asp_{1.0}Ser_{3.0}Val_{1.1}Pro_{1.0}$. Anal. ($C_{50}H_{72}N_8O_{16}\cdot 3H_2O$): C, H, N, O.

(Positions 48–55) Boc-Phe-Asp(OBu^t)-Phe-Ser-Val-Ser-Ser-Pro-$HNNH_2$ Acetate (XIII). To a stirred solution of Boc-Phe-Asp(OBu^t)-Phe-Ser-Val-Ser-Ser-Pro-OH trihydrate (2.3 g, 2.23 mmol) and *N*-methylmorpholine (0.25 mL, 2.23 mmol) in THF (85 mL) at −15 °C was added isobutyl chloroformate (0.29 mL, 2.23 mmol). After 5 min, Z-hydrazide (370 mg, 2.23 mmol)[12] in THF (12 mL) was added and the solution was allowed to reach room temperature. The mixture was stirred for 14 h and concentrated to a small volume. The residue was dissolved in ethyl acetate (125 mL) and the solution was washed in countercurrent fashion with 1 N citric acid (3 × 50 mL), saturated NaCl (2 × 40 mL), saturated $NaHCO_3$ (2 × 40 mL), and saturated NaCl (2 × 40 mL). The combined organic layers were dried and evaporated to give an oily residue. This material was dissolved in ethanol, the solution was evaporated to a small volume, and the product was precipitated with ether. The ensuing precipitate was washed with petroleum ether (bp 30–60 °C) and dried: 1.92 g (72%); $[\alpha]^{24}_{D}$ −29.4° (*c* 1.20, DMF); R_f^{I} 0.7; R_f^{III} 0.7. Amino acid ratios in 24-h acid hydrolysate. Calcd: $Phe_{2.0}Asp_{1.0}Ser_{3.0}Val_{1.0}Pro_{1.0}$. Found: $Phe_{2.1}Asp_{1.0}Ser_{2.9}Val_{1.0}Pro_{1.0}$. Hydrogenolysis in the usual manner in methanol/acetic acid (2:1) afforded the free hydrazide acetate: yield 81%; R_f^{I} 0.4.

(Positions 63–65) Z-Ser-Ser-Gly-OMe (XIV). *tert*-Butyl nitrite (2.56 mL, 22.1 mmol) was added to a solution cooled at −10 °C of Z-Ser-Ser-$NHNH_2$[36] (6.84 g, 20.1 mmol) in DMF (55 mL) containing 6.8 N HCl in dioxane (11.82 mL, 80.4 mmol), and the solution was stirred at −10 to −15 °C for 20 min. The solution was cooled at −30 °C, the pH was adjusted to 8.0–8.5 by addition of DIPEA and a solution of H-Gly-OMe HCl (2.53 g, 20.1 mmol) in DMF (40 mL) containing DIPEA (3.44 mL, 20.1 mmol) was added. The mixture was stirred for 40 h at 4 °C, the pH being maintained at 8.0–8.5 by addition of DIPEA. The solvent was evaporated, the residue was dissolved in ethyl acetate, and the solution was washed in the usual

manner and dried. The solution was concentrated to a small volume, ether was added, and the ensuing crystals were collected, dried, and recrystallized from ethyl acetate/ether: 6.25 g (78%); mp 165-166 °C; $[\alpha]^{25}{}_D$ +14.6° (*c* 1.02, DMF); R_f^{I} 0.6; R_f^{III} 0.7. Amino acid ratios in 24-h acid hydrolysate. Calcd: $Ser_{2.0}Gly_{1.0}$. Found: $Ser_{2.0}Gly_{1.0}$. Anal. ($C_{17}H_{23}N_3O_8$): C, H, N, O.

(Positions 63-65) H-Ser-Ser-Gly-OMe Acetate (XV). The protected methyl ester (9.4 g) was hydrogenated for 7 h in methanol (150 mL) and 10% acetic acid (21 mL) in the usual manner. The catalyst was removed by filtration, the filtrate was evaporated, and the solid residue was recrystallized from methanol/ether: 7.46 g (97%); mp 134-136 °C; $[\alpha]^{27}{}_D$ +4.5° (*c* 1.03, DMF); R_f^{I} 0.2; R_f^{III} 0.5 (single fluorescamine and chlorine positive spot). Amino acid ratios in 24-h acid hydrolysate. Calcd: $Ser_{2.0}Gly_{1.0}$. Found: $Ser_{2.0}Gly_{1.0}$. Anal. ($C_9H_{17}N_3O_6{\cdot}CH_3COOH$): C, H, N, O.

(Positions 62-65) Z-Leu-Ser-Ser-Gly-OMe (XVI). Z-Leu-OSu (6.56 g, 18.2 mmol)[37] was added to a solution of H-Ser-Ser-Gly-OMe acetate (4.89 g, 15.1 mmol) and DIPEA (3.12 g, 18.2 mmol) in DMF (75 mL). The mixture was stirred at room temperature for 20 h, the solvent was evaporated, and the residue was triturated with ethyl acetate, washed with ethyl acetate, and dried: 7.14 g (93%); mp 144-145 °C; $[\alpha]^{25}{}_D$ +0.39° (*c* 1.08, DMF); R_f^{I} 0.7; R_f^{III} 0.7. Amino acid ratios in 24-h acid hydrolysate. Calcd: $Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Leu_{1.1}Ser_{1.9}Gly_{0.9}$. Anal. ($C_{23}H_{34}N_4O_9$): C, H, N, O.

(Positions 62-65) H-Leu-Ser-Ser-Gly-OMe Acetate (XVII). The protected tetrapeptide methyl ester (6.84 g) was hydrogenated in methanol (120 mL) and 10% acetic acid (21 mL) in the usual manner. The resulting product was recrystallized from methanol/ether: 5.5 g (95%); mp 158-160 °C; $[\alpha]^{26}{}_D$ +5.2° (*c* 1.0, DMF); R_f^{I} 0.3; R_f^{III} 0.6 (single fluorescamine and chlorine positive spot). Amino acid ratios in 24-h acid hydrolysate. Calcd: $Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Leu_{1.1}Ser_{1.9}Gly_{1.0}$. Anal. ($C_{15}H_{28}N_4O_7{\cdot}CH_3COOH$): C, H, N, O.

(Positions 61-65) Z-Ile-Leu-Ser-Ser-Gly-OMe (XVIII). Z-Ile-OSu (9.03 g, 24.9 mmol)[37] was added to a solution of H-Leu-Ser-Ser-Gly-OMe acetate (9.04 g, 20.7 mmol) in DMF (90 mL) and DIPEA (4.27 mL, 24.9 mmol). The mixture was stirred at room temperature for 17 h, ethyl acetate (500 mL) was added to the suspension, and the mixture was cooled in an ice bath. The precipitate was collected, washed with ethyl acetate, and dried: 12.0 g (93%); mp 224-225 °C dec; $[\alpha]^{24}{}_D$ −7.0° (*c* 1.0, DMF); R_f^{I} 0.7; R_f^{III} 0.8. Amino acid ratios in 48-h acid hydrolysate. Calcd: $Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Ile_{1.1}Leu_{1.1}Ser_{1.8}Gly_{1.0}$. Anal. ($C_{29}H_{45}N_5O_{10}$): C, H, N, O.

(Positions 61-65) H-Ile-Leu-Ser-Ser-Gly-OMe Acetate (XIX). The protected pentapeptide methyl ester (6.65 g) was hydrogenated in methanol (120 mL) and 10% acetic acid (14 mL) in the usual manner. The product was recrystallized from methanol/ether: 5.8 g (99%); mp 150-153 °C; $[\alpha]^{24}{}_D$ −8.7° (*c* 1.08, DMF); R_f^{I} 0.3; R_f^{III} 0.7; single fluorescamine and chlorine positive spot. Amino acid ratios in 48-h acid hydrolysate. Calcd: $Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Ile_{1.0}Leu_{1.2}Ser_{1.8}Gly_{1.0}$. Anal. ($C_{21}H_{39}N_5O_8{\cdot}CH_3COOH$): C, H, N, O.

(Positions 60-65) Z-Pro-Ile-Leu-Ser-Ser-Gly-OMe (XX). Z-Pro-OSu (4.2 g, 12.1 mmol)[37] was added to a solution of H-Ile-Leu-Ser-Ser-Gly-OMe acetate (5.57 g, 10.1 mmol) in DMF (75 mL) and DIPEA (2.08 mL, 12.1 mmol). The mixture was stirred at room temperature for 20 h, the solvent was evaporated, and the residue was triturated with ethyl acetate and the solid was collected. This material, without drying, was suspended in methanol/ethyl acetate 1:9 (200 mL) and the suspension was stirred for 2 h at room temperature and was then cooled in an ice bath. The solid was collected, washed with ethyl acetate, and dried: 6.84 g (94%); mp 233-235 °C dec; $[\alpha]^{27}{}_D$ −30.6° (*c* 1.0, DMF); R_f^{I} 0.6; R_f^{III} 0.7 (single chlorine positive spot). Amino acid ratios in 48-h acid hydrolysate. Calcd: $Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Pro_{1.2}Ile_{1.0}Leu_{1.1}Ser_{1.7}Gly_{1.0}$. Anal. ($C_{34}H_{52}N_6O_{11}$): C, H, N, O.

(Positions 60-65) H-Pro-Ile-Leu-Ser-Ser-Gly-OMe Acetate (XXI). The protected hexapeptide methyl ester (6.0 g) was hydrogenated for 6 h in the usual manner in methanol (130 mL) and 10% acetic acid (20 mL). The resulting product was precipitated from methanol with ether: 5.39 g (100%); mp 203-205 °C dec; $[\alpha]^{27}{}_D$ −23.8° (*c* 1.0, DMF); R_f^{I} 0.4; R_f^{III} 0.6 (single fluorescamine and chlorine positive spot). Amino acid ratios in 48-h acid hydrolysate. Calcd: $Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Pro_{1.2}Ile_{1.0}Leu_{1.0}Ser_{1.8}Gly_{1.0}$. Anal. ($C_{26}H_{46}N_6O_9{\cdot}CH_3COOH$): C, H, N, O.

(Positions 59-65) Z-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OMe (XXII). Z-Trp-OCP (2.49 g, 4.8 mmol)[13] was added to a stirred suspension of H-Pro-Ile-Leu-Ser-Ser-Gly-OMe acetate (2.59 g, 4.0 mmol) in DMF (80 mL) and DIPEA (0.82 mL, 4.8 mmol). The suspension was stirred at room temperature for 24 h (clear solution resulted after 2 h) and was then evaporated. The solid residue was triturated with ice-cold ethyl acetate, collected, and dried. This crude material was suspended in methanol/ethyl acetate 1:9 (200 mL) and the mixture was stirred for 1 h at room temperature and then cooled in an ice bath and filtered. The filter cake was washed with ethyl acetate and dried: 3.03 g (84%); mp 193-194 °C; $[\alpha]^{27}{}_D$ −33.7° (*c* 1.0, DMF); R_f^{I} 0.7; R_f^{III} 0.8 (single Ehrlich and chlorine positive spot). Amino acid ratios in 48-h acid hydrolysate. Calcd: $Trp_{1.0}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Pro_{0.9}Ile_{1.0}Leu_{1.1}Ser_{1.9}Gly_{1.2}$ (Trp destroyed). Anal. ($C_{45}H_{62}N_8O_{15}$): C, H, N, O.

(Positions 59-65) H-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OMe Acetate (XXIII). The protected heptapeptide methyl ester (3.02 g) was hydrogenated in the usual manner in methanol (150 mL) and 10% acetic acid (6 mL). The resulting product was precipitated from methanol with ether: 2.78 g (100%); mp 130-140 °C dec; $[\alpha]^{27}{}_D$ −24.6° (*c* 1.04, DMF); R_f^{I} 0.5; R_f^{III} 0.7 (single chlorine, fluorescamine and Ehrlich positive spot). Amino acid ratios in 48-h acid hydrolysate. Calcd: $Trp_{1.0}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Pro_{0.9}Ile_{1.0}Leu_{1.1}Ser_{1.9}Gly_{1.2}$ (Trp destroyed). Anal. ($C_{37}H_{56}N_8O_{10}{:}CH_3COOH$): C, H, N, O.

(Positions 58-65) Z-Glu(OBu^t)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OMe (XXIV). Z-Glu(OBu^t)-OSu (1.72 g, 3.96 mmol)[38] was added to a solution of H-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OMe acetate (2.75 g, 3.3 mmol) in DMF (50 mL) and DIPEA (0.68 mL, 3.96 mmol) and the mixture was stirred at room temperature for 40 h. *N,N*-Dimethyl-1,3-propanediamine (0.35 mL) was added and the mixture was stirred for an additional 1.5 h. The solvent was evaporated, the residue was extracted with three 300-mL portions of ethyl acetate, and the extracts were washed and dried in the usual manner. The solution was then concentrated to a small volume and the concentrate was poured into ether. The precipitate was collected, washed with ether, and dried. For further purification, this material was dissolved in methanol and precipitated with ether: 3.44 g (95%); mp 115-120 °C; $[\alpha]^{28}{}_D$ −31.5° (*c* 1.0, DMF); R_f^{I} 0.7 with trace impurity at 0.9; R_f^{II} 0.7 with trace impurity at 0.9; R_f^{III} 0.8. Amino acid ratios in 48-h acid hydrolysate. Calcd: $Glu_{1.0}Trp_{1.0}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Glu_{1.0}Pro_{1.1}Ile_{1.0}Leu_{1.0}Ser_{1.8}Gly_{1.0}$ (Trp destroyed). Anal. ($C_{54}H_{77}N_9O_{15}$): C, H, N, O.

(Positions 58-65) Z-Glu(OBu^t)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH (XXV). To a solution of the above methyl ester (3.46 g, 3.16 mmol) in methanol (12 mL) was added 1 N sodium hydroxide (3.8 mL) and the mixture was stirred at room temperature for 30 min. The solution was cooled in an ice bath; ice-water (200 mL) was added followed by 1 N HCl (5.7 mL, 5.7 mmol) to precipitate the product. The suspension was extracted with ice-cold ethyl acetate (three 400-mL portions), the extracts were washed with ice-cold 0.5 M sodium chloride, and dried. The solution was concentrated to a small volume, the product was precipitated with ether, and the precipitate was collected, washed with ether, and dried: 3.28 g (96%); mp 171-173 °C; $[\alpha]^{28}{}_D$ −29.0° (*c* 1.0, DMF); R_f^{I} 0.7; R_f^{III} 0.7 (single chlorine positive spot). Amino acid ratios in 48-h acid hydrolysate. Calcd: $Glu_{1.0}Trp_{1.0}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Glu_{1.1}Pro_{1.0}Ile_{0.9}Leu_{0.9}Ser_{2.0}Gly_{1.1}$ (Trp destroyed). Anal. ($C_{53}H_{75}N_9O_{15}$): C, H, N, O.

(Positions 58-65) H-Glu(OBu^t)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH (XXVI). The protected octapeptide (3.13 g) was hydrogenated in the usual manner in methanol (120 mL) and the product was purified by precipitation from methanol with ethyl acetate: 2.9 g (100%); mp 160-163 °C dec; $[\alpha]^{28}{}_D$ −32.3° (*c* 1.0, DMF); R_f^{I} 0.5 with trace impurity at 0.4; R_f^{III} 0.6 with trace impurity at 0.5. Amino acid ratios in 48-h acid hydrolysate. Calcd: $Glu_{1.0}Trp_{1.0}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Glu_{1.0}Pro_{1.1}Ile_{1.0}Leu_{1.1}Ser_{1.8}Gly_{1.1}$ (Trp destroyed). Anal. ($C_{45}H_{69}N_9O_{13}$): C, H, N, O.

(Positions 57-65) Z-Tyr(OZ)-Glu(OBu^t)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH (XXVII). Z-Tyr(OZ)-OCP (2.3 g, 3.7 mmol)[13] was added to a solution of H-Glu(OBu^t)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH (2.87 g, 3.0 mmol) in DMF (70 mL) and DIPEA (0.63 mL, 3.7 mmol) and the solution was stirred for 20 h at room temperature. The solvent was removed and the residue extracted with four 300-mL portions of ice-cold ethyl acetate. The ethyl acetate extracts were washed with 1 N citric acid and water and dried. The ethyl acetate solution was then concentrated to a volume of approximately 100 mL and the product was precipitated by addition of 100 mL of ether. The precipitate was collected, washed with ethyl acetate/ether 1:2 and with ether, and was dried. The material was then dissolved in methanol and

precipitated by addition of ether: 3.04 g (73%); mp 145–150 °C dec; $[\alpha]^{25}{}_D$ −29.5° (*c* 1.0, DMF); $R_f{}^I$ 0.7 with trace impurity at 0.8; $R_f{}^{III}$ 0.7 with trace impurity at 0.8. Amino acid ratios in 48-h acid hydrolysate. Calcd: $Tyr_{1.0}Glu_{1.0}Trp_{1.0}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Tyr_{1.0}Glu_{1.2}Pro_{1.0}Ile_{1.0}Leu_{1.1}Ser_{1.7}Gly_{1.0}$ (Trp destroyed). Anal. ($C_{70}H_{90}N_{10}O_{19}$): C, H, N, O.

(Positions 57–65) H-Tyr-Glu(OBu *t*)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH (XXVIII). The above protected nonapeptide (312 mg) was hydrogenated in the usual manner in methanol (30 mL) for 6 h. The ensuing peptide was precipitated from methanol with ethyl acetate: 240 mg (96%); mp 170–173 °C dec; $[\alpha]^{25}{}_D$ −41.1° (*c* 1.0, DMF); $R_f{}^I$ 0.6; $R_f{}^{III}$ 0.7 (single fluorescamine and chlorine positive spot). Amino acid ratios in 48-h acid hydrolysate. Calcd: $Tyr_{1.0}Glu_{1.0}Trp_{1.0}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Tyr_{0.9}Glu_{1.0}Pro_{1.2}Ile_{1.0}Leu_{1.1}Ser_{1.8}Gly_{1.0}$ (Trp destroyed). Anal. ($C_{54}H_{78}N_{10}O_{15}$): C, H, N, O.

(Positions 56–65) Z-Tyr(OZ)-Tyr-Glu(OBu *t*)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH (XXIX). Z-Tyr(OZ)-OCP (694 mg, 1.1 mmol)[13] was added to a solution of H-Tyr-Glu(OBu*t*)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH (1.1 g, 1.0 mmol) in DMF (15 mL) and the mixture was stirred at room temperature for 18 h. The solvent was evaporated and the residue was triturated with ethyl acetate/ether 1:1, collected, and dried. For purification, the material was precipitated from acetic acid with water and then from methanol with ether: 1.3 g (84%); mp 145–149 °C dec; $[\alpha]^{25}{}_D$ −29.8° (*c* 1.0, DMF); $R_f{}^I$ 0.7 with trace impurities at 0.6 and 0.9; $R_f{}^{III}$ 0.7 with trace impurities at 0.6 and 0.8. Amino acid ratios in 48-h acid hydrolysate. Calcd: $Tyr_{2.0}Glu_{1.0}Trp_{1.0}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Tyr_{1.8}Glu_{1.1}Pro_{1.1}Ile_{1.1}Leu_{1.0}Ser_{1.9}Gly_{0.9}$ (Trp destroyed). Anal. ($C_{79}H_{99}N_{11}O_{21}$): C, H, N, O.

(Positions 56–65) H-Tyr-Tyr-Glu(OBu *t*)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH (XXX). The protected decapeptide (1.24 g) was hydrogenated in methanol (120 mL) and 50% acetic acid (4 mL) for 16 h. The catalyst was removed by filtration and the filtrate was concentrated to a small volume. Methanol was added and the product was precipitated with ethyl acetate. For purification the material was dissolved in acetic acid/methanol (1:3) and precipitated with ethyl acetate: 1.05 g (100%); mp 170–178 °C dec; $[\alpha]^{26}{}_D$ −35.8° (*c* 1.0, DMF); $R_f{}^I$ 0.6; $R_f{}^{III}$ 0.7 (single fluorescamine, chlorine, Ehrlich and Pauly positive spot). Amino acid ratios in 48-h acid hydrolysate. Calcd: $Tyr_{2.0}Glu_{1.0}Trp_{1.0}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Tyr_{1.9}Glu_{1.0}Pro_{1.1}Ile_{1.0}Leu_{1.1}Ser_{2.0}Gly_{1.0}$ (Trp destroyed).

(Positions 56–65) Boc-Tyr-Tyr-Glu(OBu *t*)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH (XXXI). a. From H-Tyr-Glu(OBu *t*)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH and Boc-Tyr-NHNH$_2$. *tert*-Butyl nitrite (0.21 mL, 1.83 mmol) was added to a stirred solution cooled at −20 °C containing Boc-Tyr-NHNH$_2$ acetate, prepared by hydrogenolysis of the benzyloxycarbonylhydrazide[4] (640 mg, 1.8 mmol) and 6.18 N HCl in dioxane (1.46 mL, 9.0 mmol) in DMF (3 mL). The mixture was stirred at −20 °C for 10 min and was then cooled at −40 °C. DIPEA (1.85 mL, 10.8 mmol) was added followed by a solution of H-Tyr-Glu(OBu*t*)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH (1.33 g, 1.2 mmol) in DMF (3 mL) and DIPEA (0.2 mL, 1.2 mmol). The mixture was stirred at 4 °C for 20 h, the pH being maintained at 8–8.5 by addition of DIPEA. The solution was concentrated to a small volume, water was added, and the precipitate was collected by centrifugation, washed with 5% acetic acid and water, and dried. For purification the material was twice precipitated from methanol with ethyl acetate/ether (3:1) and washed with ether: 1.25 g (77%); $[\alpha]^{26}{}_D$ −49.4° (*c* 1.02, MeOH); $R_f{}^I$ 0.6; $R_f{}^{II}$ 0.15. Amino acid ratios in 24-h 4 N methanesulfonic acid hydrolysate. Calcd: $Tyr_{2.0}Glu_{1.0}Trp_{1.0}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Tyr_{2.0}Glu_{1.1}Trp_{0.9}Pro_{1.1}Ile_{1.0}Leu_{1.0}Ser_{1.9}Gly_{1.1}$.

b. From H-Tyr-Tyr-Glu(OBu *t*)-Trp-Pro-Ile-Leu-Ser-Gly and Di-*tert*-butyl Dicarbonate. To a solution of H-Tyr-Tyr-Glu(OBu*t*)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH (800 mg, 0.63 mmol) in DMF (7 mL) containing 10% DIPEA/DMF (1.1 mL, 0.63 mmol) di-*tert*-butyl dicarbonate (151 mg, 0.7 mmol)[14] was added. The reaction mixture was stirred at room temperature for 20 h; then the DMF was evaporated to a volume of approximately 3 mL and the product was precipitated by the addition of ice-cold 5% acetic acid (130 mL). The precipitate was washed with 5% acetic acid (four 20-mL portions) and water (two 20-mL portions) and was dried over P_2O_5 and KOH. This material was reprecipitated from MeOH with ethyl acetate/ether (3:1): yield 703 mg (82%); $[\alpha]^{26}{}_D$ −48.9° (*c* 1.03, MeOH); $R_f{}^I$ 0.7; $R_f{}^{II}$ 0.15.

(Positions 56–65) Boc-Tyr-Tyr-Glu(OBu *t*)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-Y (XXXII). A mixed anhydride was prepared in the usual manner from Boc-Tyr-Tyr-Glu(OBu*t*)-Trp-Pro-Ile-Leu-Ser-Ser-Gly-OH (510 mg, 0.37 mmol), in THF (9 mL) containing 10% *N*-methylmorpholine in THF (0.46 mL, 0.41 mmol), and 10% isobutyl chloroformate in THF (0.54 mL, 0.41 mmol). Reaction time was 4 min at −15 °C. A solution of Z-hydrazide (75 mg, 0.45 mmol)[12] in THF (1.0 mL) was then added and the mixture was stirred at room temperature for 20 h. At this point, the reaction mixture did not contain unreacted peptide by TLC in system II. The solvent was evaporated, the residue was dissolved in 1-butanol, and the solution was washed eight times with 5% acetic acid. The butanol layers were evaporated and the product was dissolved in methanol and precipitated with ether, 447 mg (79%). For purification this material (660 mg) was subjected to 170 transfers in the solvent system MeOH/ammonium acetate buffer, pH 4.5/$CHCl_3$/CCl_4.[39] Tubes containing the desired material (TLC) were pooled, the solvent was evaporated, and the residue washed with water and dried: yield 423 mg (64%); $[\alpha]^{24}{}_D$ −39.2° (*c* 1.04, MeOH); $[\alpha]^{28}{}_D$ −27.7° (*c* 1.06, DMF); $R_f{}^I$ 0.7; $R_f{}^{II}$ 0.6. Amino acid ratios in 48-h 4 N methanesulfonic acid hydrolysate. Calcd: $Tyr_{2.0}Glu_{1.0}Trp_{1.0}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.0}Gly_{1.0}$. Found: $Tyr_{2.1}Glu_{0.9}Trp_{1.2}Pro_{0.9}Ile_{1.0}Leu_{1.1}Ser_{2.0}Gly_{1.0}$.

(Positions 56–65) H-Tyr-Tyr-Glu-Trp-Pro-Ile-Leu-Ser-Ser-Gly-Y Trifluoroacetate (XXXIII). The above protected hydrazide (550 mg) was dissolved in ice-cold 90% TFA (6 mL) containing 0.06 mL of 1,2-ethanedithiol,[16] and the mixture was kept under nitrogen for 10 min at 0 °C and for 40 min at room temperature. Ether was added and the precipitate was washed with ether and dried: 524 mg (98%); $R_f{}^I$ 0.6 (fluorescamine, Ehrlich and chlorine positive single spot).

(Positions 48–65) Boc-Phe-Asp(OBu *t*)-Phe-Ser-Val-Ser-Ser-Pro-Tyr-Tyr-Glu-Trp-Pro-Ile-Leu-Ser-Ser-Gly-Y (XXXIV). *tert*-Butyl nitrite (0.07 mL, 0.59 mmol) was added with stirring to a solution cooled at −20 °C of Boc-Phe-Asp(OBu*t*)-Phe-Ser-Val-Ser-Ser-Pro-NHNH$_2$ acetate (600 mg, 0.54 mmol) in DMF (5 mL) and 6.69 N HCl in dioxane (0.4 mL, 2.7 mmol) and the mixture was stirred at −20 °C for 10 min. The solution was cooled at −40 °C and DIPEA (0.56 mL, 3.24 mmol) was added followed by a solution of H-Tyr-Tyr-Glu-Trp-Pro-Ile-Leu-Ser-Ser-Gly-Y trifluoroacetate (400 mg, 0.27 mmol) in DMF (2 mL) containing 10% DIPEA in DMF (0.92 mL). The mixture was stirred at 4 °C for 94 h and the solvent was evaporated. The product was precipitated with ice-cold water, and the precipitate, collected by centrifugation, was washed with two 15-mL portions of water, six 15-mL portions of 10% acetic acid, and two 15-mL portions of water and was dried, 900 mg. Unreacted amino component (51 mg) was recovered from the acetic acid and second water washes. The washed product (450 mg) was dissolved in DMF (2 mL) and the solution was applied to a Sephadex LH-20 column (2 × 150 cm) which was eluted with DMF. Fractions (3 mL each) were collected at a flow rate of 15 mL/h, and the desired material was located in the various fractions by absorbancy measurements at 280 nm and by TLC in system II (Ehrlich and chlorine reagents). Fractions containing the desired material ($R_f{}^{II}$ 0.3) were pooled and evaporated to a small volume, and the product was precipitated with ether, washed with ether, and dried. Material from two runs was combined and rechromatographed twice as described: 270 mg (42%); $[\alpha]^{25}{}_D$ −30.4° (*c* 1.01, DMF); $R_f{}^I$ 0.7; $R_f{}^{II}$ 0.3; $R_f{}^{III}$ 0.8. Amino acid ratios in 48-h methanesulfonic acid hydrolysate. Calcd: $Phe_{2.0}Asp_{1.0}Ser_{5.0}Val_{1.0}Pro_{2.0}Tyr_{2.0}Glu_{1.0}Trp_{1.0}Ile_{1.0}Leu_{1.0}Gly_{1.0}$. Found: $Phe_{2.0}Asp_{1.1}Ser_{5.3}Val_{0.9}Pro_{2.3}Tyr_{1.8}Glu_{0.9}Trp_{0.7}Ile_{0.8}Leu_{0.9}Gly_{0.9}$.

(Positions 48–65) Boc-Phe-Asp(OBu *t*)-Phe-Ser-Val-Ser-Ser-Pro-Tyr-Tyr-Glu-Trp-Pro-Ile-Leu-Ser-Ser-Gly-HNNH$_2$ Acetate (XXXV). The above protected hydrazide (90 mg) was hydrogenated in the usual manner in 50% acetic acid (15 mL) for 20 h. The catalyst was removed by filtration through Filter Cel, the filtrate was evaporated to small volume, and the product precipitated by addition of ice-cold water (100 mL). The product, collected by centrifugation, was washed with ice-cold water and dried: 62 mg (71%); $R_f{}^I$ 0.7; $R_f{}^{II}$ 0.2. Amino acid ratios in 48-h methanesulfonic acid hydrolysate. Calcd: $Phe_{2.0}Asp_{1.0}Ser_{5.0}Val_{1.0}Pro_{2.0}Tyr_{2.0}Glu_{1.0}Trp_{1.0}Ile_{1.0}Leu_{1.0}Gly_{1.0}$. Found: $Phe_{2.2}Asp_{1.1}Ser_{5.1}Val_{1.1}Pro_{2.0}Tyr_{1.9}Glu_{0.9}Trp_{0.9}Ile_{0.8}Leu_{0.9}Gly_{1.0}$.

(Positions 48–65) H-Phe-Asp-Phe-Ser-Val-Ser-Ser-Pro-Tyr-Tyr-Glu-Trp-Pro-Ile-Leu-Ser-Ser-Gly-HNNH$_2$ Ditrifluoroacetate. Boc-E-HNNH$_2$ acetate (20 mg) was dissolved in 90% TFA containing 1% of ethanedithiol (0.3 mL) and the solution was stirred under nitrogen at 0 °C for 10 min and at room temperature for 40 min. The product was precipitated with ether, washed with ether, and dried:

15 mg; R_f^{I} 0.6; R_f^{III} 0.6. Amino acid ratios in 48-h acid hydrolysate (A) and 72-h AP-M digest (B). Calcd: $Phe_{2.0}Asp_{1.0}Ser_{5.0}Val_{1.0}Pro_{2.0}Tyr_{2.0}Glu_{1.0}Trp_{1.0}Ile_{1.0}Leu_{1.0}Gly_{1.0}$. Found (A): $Phe_{2.0}Asp_{0.9}Ser_{4.7}Val_{1.0}Pro_{2.5}Tyr_{1.9}Glu_{0.9}Ile_{1.0}Leu_{1.1}Gly_{0.9}$ (66%) (Trp destroyed). Found (B): $Phe_{1.9}Asp_{0.9}Ser_{4.9}Val_{1.1}Pro_{2.5}Tyr_{2.0}Glu_{1.0}Trp_{0.9}Ile_{1.0}Leu_{1.0}Gly_{1.0}$ (72%).

(Positions 48–104) H-Phe-Asp-Phe-Ser-Val-Ser-Ser-Pro-Tyr-Tyr-Glu-Trp-Pro-Ile-Leu-Ser-Ser-Gly-Asp-Val-Tyr-Ser-Gly-Pro-Gly-Ser-Gly-Ala-Asp-Arg-Val-Val-Phe-Asn-Glu-Asn-Asn-Gln-Leu-Ala-Gly-Val-Ile-Thr-His-Thr-Gly-Ala-Ser-Gly-Asn-Asn-Phe-Val-Glu-Cys(Acm)-Thr-OH (XXXVI). *tert*-Butyl nitrite (10% in DMF) (0.094 mL, 0.081 mmol) was added to a stirred solution cooled at −10 °C of Boc-Phe-Asp(OBu^t)-Phe-Ser-Val-Ser-Ser-Pro-Tyr-Tyr-Glu-Trp-Pro-Ile-Leu-Ser-Ser-Gly-$HNNH_2$ acetate (190 mg, 0.082 mmol) in DMF (1.0 mL) and 6.18 N HCl in dioxane (0.066 mL, 0.405 mmol), and the mixture was stirred at −10 °C for 20 min. The solution was cooled at −30 °C, DIPEA (0.083 mL, 0.487 mmol) was added, and the mixture was allowed to reach −15 °C. A solution of H-Asp-Val-Tyr-Ser-Gly-Pro-Gly-Ser-Gly-Ala-Asp-Arg-Val-Val-Phe-Asn-Glu-Asn-Asn-Gln-Leu-Ala-Gly-Val-Ile-Thr-His-Thr-Gly-Ala-Ser-Gly-Asn-Asn-Phe-Val-Glu-Cys(Acm)-Thr-OH dihydrobromide (109 mg, 0.026 mmol) in Me_2SO (1.0 mL) and 10% DIPEA in DMF (0.185 mL, 0.108 mmol) was then added, the pH was adjusted to 8–8.5 by addition of DIPEA (10% in DMF), and the mixture was stirred at 4 °C for 100 h when the product was precipitated by addition of a 2:1 mixture of ethyl acetate/ether (200 mL). The precipitate was washed with ether and dried, 292 mg. This product was dissolved in ice-cold 90% TFA containing 1% of 1,2-ethanedithiol[15] (9 mL) and the solution was kept under nitrogen at 0 °C for 10 min and at room temperature for 40 min. The reaction product was precipitated with ether, and the precipitate, collected by centrifugation, was washed with ether and dried, 268 mg. This material, dissolved in 50% acetic acid (40 mL), was passed through a column of acetate cycle Amberlite IRA-400 (1.4 × 7 cm) and the chlorine positive effluents were pooled, evaporated to a small volume, and lyophilized, 266 mg. This material, dissolved in 45% formic acid (2.4 mL), was applied to a Sephadex G-50 column (1.9 × 235 cm) which was developed with 45% formic acid. Fractions (3.5 mL each) were collected at a flow rate of 16 mL/h. Absorbancy at 280 nm and TLC in system III (Ehrlich reagent) served to localize the desired product in the various fractions. Trp positive fractions corresponding to the first major UV peak were pooled, evaporated to a small volume, and lyophilized, 58 mg. This material, dissolved in 50% acetic acid (0.9 mL), was applied to a Bio-Gel P-100 column (2.0 × 160 cm) and the column was developed with 50% acetic acid. Fractions (3.2 mL each) were collected at a flow rate of approximately 6 mL/h. Single spot Trp positive fractions (R_f^{III} 0.5) were pooled and lyophilized: total yield 52 mg (33%); $[\alpha]^{27}{}_D$ −49.8° (*c* 0.92, 50% CH_3COOH); R_f^{III} 0.5; Tyr/Trp ratio 2.92:1.[31] Amino acid ratios in 48-h acid plus Nps-Nle and phenol hydrolysate. Calcd: $His_{1.0}Arg_{1.0}Asp_{8.0}Thr_{3.0}Ser_{8.0}Glu_{4.0}Pro_{3.0}Gly_{7.0}Ala_{3.0}1/2\text{-}Cys_{1.0}Val_{6.0}Ile_{2.0}Leu_{2.0}Tyr_{3.0}Phe_{4.0}Trp_{1.0}$. Found: $His_{0.7}Arg_{1.2}Asp_{8.7}Thr_{2.7}Ser_{8.1}Glu_{4.1}Pro_{2.9}Gly_{7.4}Ala_{3.3}1/2\text{-}Cys_{0.8}Val_{6.1}Ile_{1.5}Leu_{2.0}Tyr_{2.7}Phe_{3.8}Cys(SO_3H)_{0.05}$ (Trp destroyed).

(Positions 24–47) Msc-Tyr-Gln-Leu-His-Glu-Asp-Gly-Glu-Thr-Val-Gly-Ser-Asn-Ser-Tyr-Pro-His-Lys(F)-Tyr-Asn-Asn-Tyr-Glu-Gly-$HNNH_2$ Tritrifluoroacetate (XXXVII). To an ice-cold solution of H-CD-X acetate (140 mg, 0.046 mmol)[2] in DMF (3 mL) and 10% DIPEA in DMF (0.078 mL, 0.046 mmol) Msc-OSu (13.52 mg, 0.051 mmol) was added and the reaction mixture was stirred at 0 °C for 30 min, and at room temperature for 20 h. The solution was concentrated to a small volume and the product was precipitated with ethyl acetate, collected, dried, and reprecipitated from DMF (2 mL) with ethyl acetate: 138 mg (96%); $[\alpha]^{25}{}_D$ −25.2° (*c* 1.03, DMF); R_f^{I} 0.2; R_f^{III} 0.6 (ninhydrin and fluorescamine negative spot). This material (130 mg) was deprotected with 90% TFA (8 mL) in the usual manner: 125 mg (91%); R_f^{I} 0.1; R_f^{III} 0.5. Amino acid ratios in 24-h acid hydrolysate. Calcd: $Lys_{1.0}His_{2.0}Asp_{4.0}Thr_{1.0}Ser_{2.0}Glu_{4.0}Pro_{1.0}Gly_{3.0}Val_{1.0}Leu_{1.0}Tyr_{4.0}$. Found: $Lys_{1.0}His_{2.0}Asp_{4.0}Thr_{1.0}Ser_{2.1}Glu_{4.0}Pro_{1.1}Gly_{3.0}Val_{1.0}Leu_{1.0}Tyr_{3.8}$.

(Positions 24–104) H-Tyr-Gln-Leu-His-Glu-Asp-Gly-Glu-Thr-Val-Gly-Ser-Asn-Ser-Tyr-Pro-His-Lys(F)-Tyr-Asn-Asn-Tyr-Glu-Gly-Phe-Asp-Phe-Ser-Val-Ser-Ser-Pro-Tyr-Tyr-Glu-Trp-Pro-Ile-Leu-Ser-Ser-Gly-Asp-Val-Tyr-Ser-Gly-Pro-Gly-Ser-Gly-Ala-Asp-Arg-Val-Val-Phe-Asn-Glu-Asn-Asn-Gln-Leu-Ala-Gly-Val-Ile-Thr-His-Thr-Gly-Ala-Ser-Gly-Asn-Asn-Phe-Val-Glu-Cys(Acm)-Thr-OH (XXXVIII). *tert*-Butyl nitrite, 5% in DMF (0.10 mL, 0.043 mmol) was added to a stirred solution cooled at −15 °C of Msc-CD-$HNNH_2$·3TFA (137 mg, 0.041 mmol) in DMF (1.8 mL) containing 6.18 N HCl in dioxane (0.033 mL, 0.205 mmol). The mixture was stirred at −15 to −20 °C for 1 h and was then cooled to −25 °C and neutralized with DIPEA (0.084 mL, 0.42 mmol). A solution of H-EFG-OH (127 mg, 0.021 mmol) in Me_2SO (1 mL) and 10% DIPEA in DMF (0.25 mL, 0.144 mmol) was added and the mixture (pH 8.0–8.5) was stirred at 4 °C for 90 h when the products were precipitated by the addition of ice-cold ethyl acetate (70 mL). The precipitate was collected by centrifugation, washed with one portion of ethyl acetate and three portions of ether, and dried, yield 250 mg. This material was dissolved in 50% acetic acid (2.5 mL), and a small portion of insoluble residue was removed by centrifugation. The clear, slightly yellow solution was applied to a Bio-Gel P-100 column (100–200 mesh) (1.9 × 160 cm) which was equilibrated and eluted with 50% acetic acid. Fractions (3 mL each) were collected at a flow rate of 3.6 mL/h. Absorbancy measurements at 280 nm showed the presence of four peaks (Figure 4) corresponding to elution volumes of 134–180 mL (peak I), 181–227 mL (peak II), 228–272 mL (peak III), and 273–349 mL (peak IV). The fractions corresponding to each peak were pooled, evaporated to a small volume, and lyophilized to afford the following amounts of material: peak I, 13 mg; peak II, 50 mg; peak III, 113 mg; peak IV, 70 mg. Analysis of acid hydrolysates indicated that the material corresponding to peak II consisted of a mixture of Msc-CDEFG-OH and H-EFG-OH, that the material corresponding to peak III consisted mainly of H-EFG-OH (which was recycled), and the material corresponding to peak IV represented Msc-CD rearrangement products. The material corresponding to peak II was further purified by chromatography on DEAE-cellulose as follows: One vessel of a gradient maker was charged with 50 mL of 5 mM Na_2HPO_4, pH 8.4, the other with 50 mL of 0.25 M NaH_2PO_4, pH 4.1, containing 0.25 M NaCl. The two vessels were connected, and 20 mL of gradient was collected and discarded. The connection between the two vessels was then closed and the solution in the outlet chamber was used to elute a DEAE column (5 × 66 mm) which was equilibrated with the pH 8.4 buffer and charged with peak II material (50 mg) dissolved in 5 mL of the pH 8.4 buffer. This eluted unchanged H-EFG-OH. The column was then developed with the remaining buffer to elute Msc-CDEFG-OH. The column was monitored continuously by absorbancy at 280 nm. Tubes containing UV-absorbing material were pooled, desalted on a Sephadex G-25 column (0.9 × 160 cm), and lyophilized: yield 9.0 mg (5%). This material (4.15 mg) was suspended in methanol (0.05 mL), water (0.75 mL) was added, and the mixture was cooled at 0 °C. With stirring 0.1 N sodium hydroxide (0.20 mL) was added and stirring was continued for 2 min and then 0.1 N acetic acid (0.3 mL) was added to the clear solution. The solution was lyophilized and the residue desalted in 50% acetic acid on a column (0.9 × 160 cm) of Sephadex G-25 (fine). Fractions containing the peptide were pooled and lyophilized: 3.5 mg; Tyr/Trp ratio 7.2:1.[31] Amino acid ratios in 48-h acid plus Nps-Nle and phenol hydrolysate. Calcd: $Lys_{1.00}His_{3.0}Arg_{1.0}Asp_{12.0}Thr_{4.0}Ser_{10.0}Glu_{8.0}Pro_{4.0}Gly_{10.0}Ala_{3.0}Val_{7.0}Ile_{2.0}Leu_{3.0}Tyr_{7.0}Phe_{4.0}Trp_{1.0}1/2\text{-}Cys_{1.0}$. Found: $Lys_{1.13}His_{2.3}Arg_{1.2}Asp_{12.1}Thr_{3.2}Ser_{10.6}Glu_{7.6}Pro_{4.6}Gly_{9.9}Ala_{3.0}Val_{6.9}Ile_{1.7}Leu_{3.3}Tyr_{7.2}Phe_{4.4}$ (Trp and Acm-Cys destroyed). Ammonia corrected for ammonia content of 6 N HCl and diluter buffer 12.2 μmol/μmol of peptide.

Acknowledgments. The authors wish to express their appreciation to Michiko Kiso, Judith Montibeller, and William German for skillful technical assistance. This investigation was supported by grants from the U.S. Public Health Service (Grant AM-01128) and the National Science Foundation (Grant PCM75-01169).

Supplementary Material Available: Elemental analyses (3 pages). Ordering information is given on any current masthead page.

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^{1}H NMR Study of High-Spin Ferric Natural Porphyrin Derivatives as Models of Methemoproteins

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Abstract: High-field ^{1}H NMR spectra have been recorded and analyzed for a series of five- and six-coordinate, high-spin ferric complexes of natural porphyrin derivatives. Protoporphyrins with α- and β-deuterated vinyl groups have been synthesized that reveal the location of the elusive vinyl H_α resonances in the models. Deuterium labeling of individual methyl groups reveals that the increasing spread of the methyl isotropic shifts as the porphyrin 2,4 substituents are made more electron withdrawing is similar to that observed in low-spin ferric complexes. This supports a direct influence of the substituents on the asymmetry of the ligand molecular orbital and argues against a role of the raising of the orbital degeneracy in the low-spin species. Strongly electron-withdrawing 2,4 substituents induce a methyl spread similar to that observed in metaquomyoglobins, suggesting that the in-plane asymmetry in proteins arises primarily from peripheral heme–apoprotein interactions. Comparison of pyrrole substituent shift patterns in the five- and six-coordinate models suggests that the pyrrole proton/methyl shift ratio may serve as a useful indicator of the state of occupation of the sixth site in high-spin hemoproteins.

^{1}H NMR studies over the past decade have established the value of the heme hyperfine or isotropic shifts as sensitive structural probes in paramagnetic hemoproteins.[2] The interpretation of the protein shifts has been facilitated by the analysis of the influence of controlled perturbations in selected model compounds.[3,4] Of particular utility are the four heme methyl groups, one on each pyrrole, whose peaks are prominent in the NMR spectra and which provide a direct index of the degree of similarity of the environments of the individual pyrroles. The ubiquitous vinyl groups have also been shown to serve as sensitive probes of protein–heme interactions.[4]

A qualitative, though highly characteristic difference between the NMR spectra of hemes in models and proteins is that the rhombic or in-plane asymmetry is always much larger in the protein environment[4] and appears to be highly characteristic of the type of protein.[5] Studies[6-8] on the more frequently studied low-spin (LS) ferric models and proteins using isotope labeling have indicated[6] that peripheral perturbations are the source of the protein-induced in-plane asymmetry, and that the asymmetry is sufficiently characteristic of a protein conformation so as to permit determination[9] of the heme orientation in a protein by NMR.

Although several high-spin (HS) ferric models and proteins have been investigated[2-4,10-13] by NMR, unambiguous assignments of all resonances have not been made, and the origin of the shifts in various models and their sensitivity to axial and peripheral perturbations remain unexplored. In order to interpret the protein spectra, it is necessary to know the physical/electronic properties reflected in the shift patterns[14] in models. In some preliminary work[15] on deuterium labeling of

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