product mixtures were formed on irradiation. Small amounts of products with short retention times were observed, but these materials did not accumulate sufficiently to allow isolation. Addition of acetic acid after irradiation did not lead to any new product peaks.

A preparative scale photolysis was carried out on 0.76 g of **17** in 100 ml of nitrogen-purged *tert*-butyl alcohol. After irradiation for 24 hr, the solvent was removed *in vacuo* and the residue was subjected to preparative gc (B, 180°). The major product (0.10 g) appeared to be a mixture of cyclobutanols as evidenced by the presence of hydroxyl absorptions in the infrared spectrum at 2.90 μ: nmr δ 1.21 (s) and 1.40 (s); mass spectrum *m/e* (rel intensities) 180 (11), 162 (38), 122 (100), 43 (81).

Anal. Calcd for $C_{12}H_{20}O$: C, 79.94; H, 11.18. Found: C, 79.70; H, 11.03.

Several minor products were detected in quantities too small to isolate and characterize.

Acknowledgments. We are indebted to the National Institutes of Health (GM08701) and to the donors of the Petroleum Research Fund (GF #560), administered by the American Chemical Society, for financial support of this work. Funds for the purchase of the mass spectrometer were obtained from the National Science Foundation. We also wish to thank Dr. D. Z. Denney and Mr. S. Schutzbank for the 100-MHz spectra. We are grateful to Mr. Alan Rousseau for assistance with the quantum yield determinations.

Studies on Polypeptides. XLIX. Fragment Condensations with Peptide Derivatives Related to the Primary Structure of Ribonuclease T_1[1-4]

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Abstract: Syntheses are described of three *N*-benzyloxycarbonylpeptide *tert*-butoxycarbonylhydrazides which correspond to positions 12–23, 24–34, and 35–47, respectively, of the proposed primary structure of the enzyme ribonuclease T_1. Evidence is presented to indicate that these materials are sequentially homogeneous. These fragments were condensed to form an *N*-benzyloxycarbonylhexatricontapeptide *tert*-butoxycarbonylhydrazide corresponding to positions 12–47 of the primary sequence of the enzyme. Available techniques to evaluate homogeneity of complex peptides are critically discussed and the concept of "diagnostic" amino acid residues is introduced.

Ribonuclease T_1 [ribonucleate guanine nucleotido-2′-transferase (cyclizing), 2.7.7.26] is an acidic single-chain protein, 104 amino acid residues in length, cross-linked by two disulfide bridges.[5] The enzyme can be unfolded by reduction,[6,7] and such unfolding is accompanied by a complete loss of activity. Essentially complete reactivation occurs when the reduced enzyme is allowed to reoxidize.[6,7] Thus, assembly of the correct peptide chain will constitute synthesis of the enzyme.

Ribonuclease T_1 contains one residue each of lysine, arginine, and tryptophan, and no methionine; moreover, three of the four half-cystines are located in the N-terminal region of the peptide chain. From the point of view of synthesis these structural characteristics simplify the problem of side-chain protection and it was for this reason that this particular enzyme was selected for exploratory synthetic studies. The peptide chain was subdivided into a number of *N*-benzyloxycarbonylpeptide *tert*-butoxycarbonylhydrazide fragments B to F (Figure 1) which provide potential building blocks for the synthesis of larger sections of the polypeptide chain.

The present article describes syntheses of fragments B, C, and D and their assembly into the protected hexatricontapeptide hydrazide BCD (Figure 2, shaded section). This peptide derivative corresponds to positions 12–47 of the amino acid sequence of the enzyme.

Preparation of Fragments B, C, and D

Doubly protected peptide hydrazides, such as fragments B, C, and D, are desirable intermediates for con-

(1) See K. Hofmann, R. Andreatta, F. M. Finn, J. Montibeller, G. Porcelli, and A. J. Quattrone, *Bioorg. Chem.*, **7**, 66 (1971), for paper XLVIII in this series.

(2) Supported by grants from the U. S. Public Health Service and the Hoffmann-La Roche Foundation. The early phases of this investigation were supported by the Research Laboratories, Edgewood Arsenal, Contract DA-18-035-AMC-307 (A). The opinions expressed are those of the authors and do not reflect endorsement by the contractor.

(3) Preliminary communications of some of the results presented in this communication have appeared: (a) N. Yanaihara, C. Yanaihara, G. Dupuis, J. Beacham, R. Camble, and K. Hofmann, *J. Amer. Chem. Soc.*, **91**, 2184 (1969); (b) K. Hofmann in "Peptides 1969," E. Scoffone, Ed., North Holland Publishing Co., Amsterdam, 1971, p 130.

(4) The amino acid residues except glycine are of the L configuration. The following abbreviations are used: AP-M = aminopeptidase M; DCC = *N*,*N*′-dicyclohexylcarbodiimide; DMSO = dimethyl sulfoxide; DMF = dimethylformamide; EC = ethylcarbamyl; F = formyl; OCP = 2,4,5-trichlorophenyl ester; ONHS = *N*-hydroxysuccinimido ester; O-*t*-Bu = *tert*-butyl ester; TEA = triethylamine; TFA = trifluoroacetic acid; THF = tetrahydrofuran; tlc = thin-layer chromatography; X = *tert*-butoxycarbonylhydrazide; Y = benzyloxycarbonylhydrazide; Z = benzyloxycarbonyl. In order to simplify the designation of the complex products the following nomenclature is used: fragments B, C, D, CD, and BCD = the *N*-benzyloxycarbonyl *tert*-butoxycarbonylhydrazides: fragments B, C, D, CD, and BCD *tert*-butoxycarbonylhydrazides = the amino-deprotected *tert*-butoxycarbonylhydrazides; fragment B, C, D, CD, and BCD hydrazides = the free hydrazides of the *N*-benzyloxycarbonyl fragments.

(5) K. Takahashi, *J. Biol. Chem.*, **240**, 4117 (1965).

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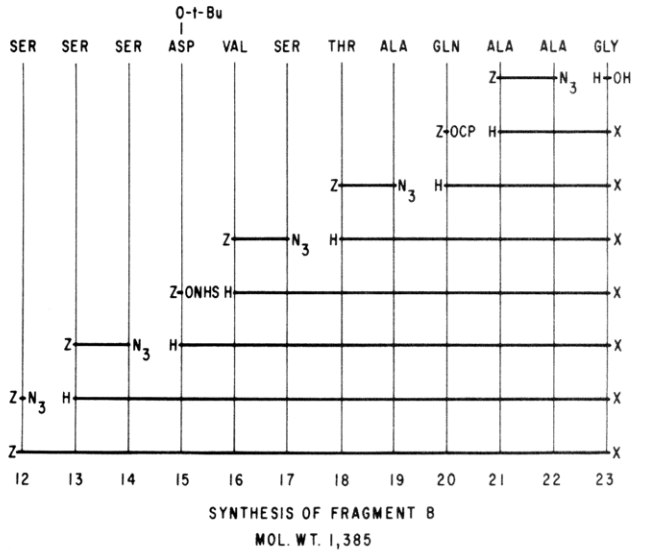
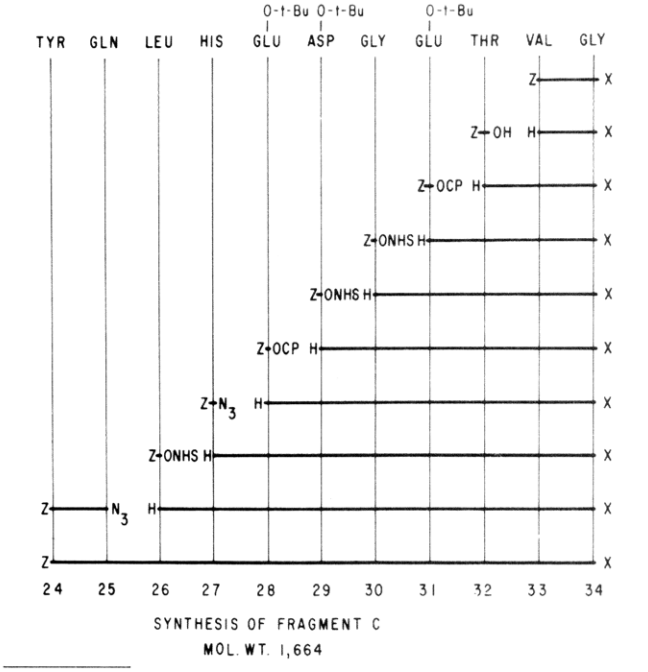
struction of large peptides *via* fragment condensation.[8] Hydrogenolysis removes the benzyloxycarbonyl group with formation of an "amino component" in terms of peptide terminology[9] while selective removal of the *tert*-butoxycarbonyl group affords an acylpeptide hydrazide which is readily converted into the corresponding azide for coupling to an "amino component."

Since fragments B, C, and D served as starting materials for an investigation of their suitability as building blocks for synthesis of larger segments of ribonuclease T_1 it was important to prepare gram quantities of these intermediates in a high state of purity within a reasonable span of time. The stepwise procedure[10] was selected for this purpose. The peptide chains of two di- and a tripeptide *tert*-butoxycarbonylhydrazide were extended from the amino end in a stepwise manner (Schemes I–III) using the benzyloxycarbonyl group for α-amino

Scheme I



Scheme II



(8) K. Hofmann, A. Lindenmann, M. Z. Magee, and N. H. Khan, *J. Amer. Chem. Soc.*, **74**, 470 (1952).
(9) K. Hofmann and P. G. Katsoyannis, *Proteins*, **1**, 53 (1963).
(10) (a) M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959); (b) J. S. Morley, *J. Chem. Soc. C*, 2410 (1967).

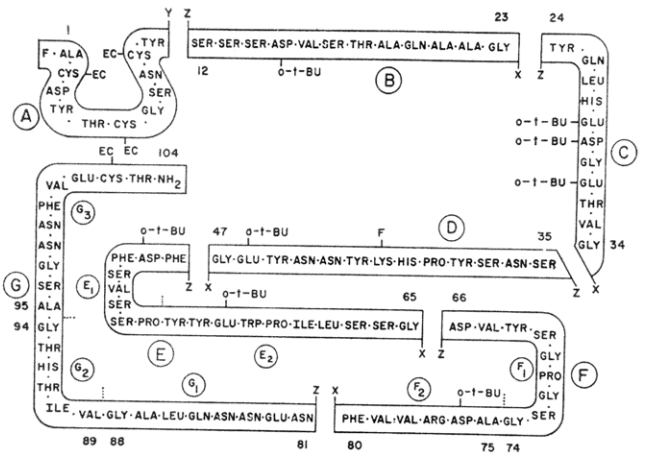


Figure 1. Protected peptide hydrazides corresponding to the sequence of ribonuclease T_1.

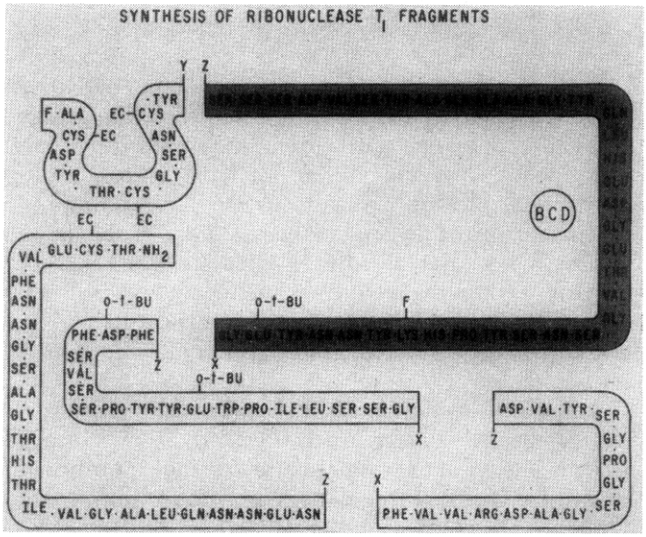
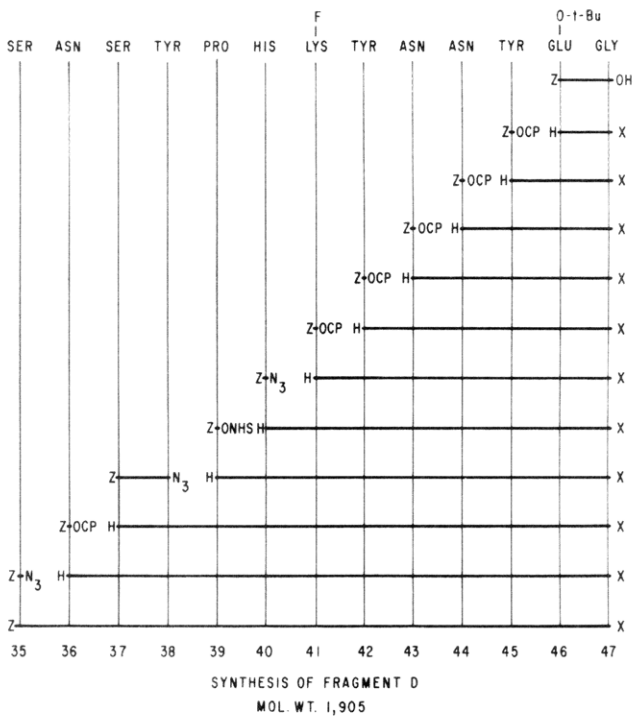


Figure 2. Location of fragment BCD within the sequence of ribonuclease T_1.

Scheme III



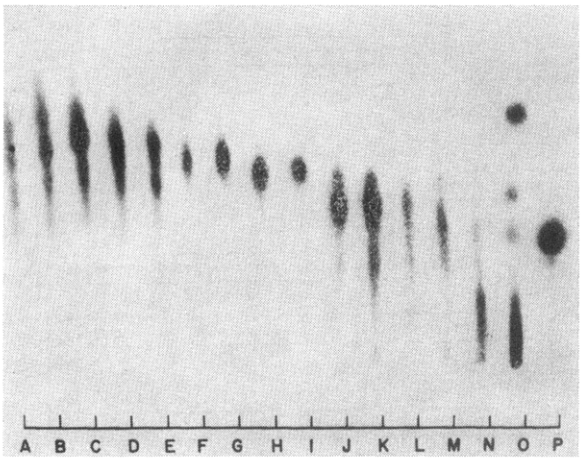


Figure 3. Thin-layer chromatogram of fractions obtained during purification of fragment D *tert*-butoxycarbonylhydrazide on Sephadex G-50. Solvent system I; chromatogram developed with chlorine reagent. See text for experimental details.

protection. Hydrogenolysis over spongy palladium was used exclusively to remove amino protecting groups. Certain dipeptide *tert*-butoxycarbonylhydrazides proved to be sensitive to acid and formed the corresponding diketopiperazines on exposure to methanol containing acetic acid during hydrogenolysis. This problem was avoided when the *tert*-butoxycarbonylhydrazides of benzyloxycarbonylvalylglycine and benzyloxycarbonyl-γ-*tert*-butylglutamylglycine were decarbobenzoxylated by hydrogenolysis in methanol without addition of acetic acid.

Serine and histidine residues were introduced *via* their N^{α}-benzyloxycarbonyl azides.[11,12] DCC served as the carboxyl-activating reagent for incorporation of threonine 32 into fragment C. Other amino acid residues were introduced in the form of benzyloxycarbonyl *N*-hydroxysuccinimido,[13] or 2,4,5-trichlorophenyl[14] esters. As concerns side-chain protection, the formyl group was used to protect the ε-amino group of lysine 41[15] and *tert*-butyl esters protected the β-carboxyl of aspartic acids 15 and 29 and the γ-carboxyl of glutamic acids 28, 31, and 46.[16] The side chains of histidines 27 and 40 and those of the various serine, threonine, and tyrosine residues were not protected. Certain sequences such as 13–14, 16–17, and 18–19 in fragment B and 37–38 in fragment D were introduced as *N*-benzyloxycarbonyldipeptide azides. In order to avoid lactam formation of glutamine 25 the design of the synthesis of fragment C involved coupling of benzyloxycarbonyltyrosylglutamine azide to the nonapeptide *tert*-butoxycarbonylhydrazide corresponding to positions 26–34.

The *tert*-butoxycarbonylhydrazide function was incorporated into fragment B at the tripeptide stage by treating a mixed anhydride of benzyloxycarbonylalanylalanylglycine with *tert*-butoxycarbonylhydrazine.[17] The benzyloxycarbonyldipeptide *tert*-butoxycarbonylhydrazides required in the synthesis of fragments C and D were obtained from the benzyloxycarbonyldipeptides and *tert*-butoxycarbonylhydrazine in the presence of DCC.

Attempts to introduce the three N-terminal amino acid residues (positions 12–14) into fragment B in the form of *N*-benzyloxycarbonyltriserine azide were unsuccessful; however, benzyloxycarbonylserylserine azide was used to incorporate serines 13 and 14 and benzyloxycarbonylserine azide served to add serine 12 to the peptide chain. Solubility problems arose during the synthesis of fragment B and it was found necessary to employ hydrogenolysis in DMF at 70° to remove the benzyloxycarbonyl group following the two final coupling steps.

Purification and Assessment of Homogeneity of Fragments B, C, and D

In many instances the crude coupling products were distributed between aqueous citric or acetic acid and a suitable organic solvent to remove unreacted amino component; then the material from the organic phase was purified by solvent precipitation. Crystallization was employed to purify intermediates whenever possible but unfortunately only a limited number of the protected peptides were obtained in crystalline form.

Protected intermediates were characterized by melting point, optical rotation, elemental analysis, and tlc. Optical rotation, tlc in at least two solvent systems, and digestibility by aminopeptidase M(AP-M)[18] served to assess the homogeneity of peptide *tert*-butoxycarbonylhydrazides. Buffer-insoluble peptide *tert*-butoxycarbonylhydrazides were deblocked with TFA and the ensuing soluble trifluoroacetate salts were subjected to AP-M digestion. The amino acid composition of acid hydrolysates of a number of the protected intermediates was also determined.

Many peptides are highly hydrated and undergo marked alterations when attempts are made to assess peptide content by drying to constant weight at elevated temperatures. However, average recovery of amino acids in acid hydrolysates and AP-M digests provides a satisfactory measure of peptide content and such figures are reported in the Experimental Section.

In the course of this investigation it became apparent that tlc of protected peptide hydrazides has limited value as an index of purity for the reason that many of these intermediates migrate close to the solvent front. However, tlc of the corresponding peptide *tert*-butoxycarbonylhydrazides revealed the presence of contaminants. These impurities accumulated with progressive chain elongation, and repeated precipitation failed, in certain instances, to yield a chromatographically acceptable material. The impurities do not necessarily appear in the form of distinct spots, but create the well-known streaking effect on the plates. Despite the fact that tlc demonstrated the presence of contaminants, the elemental analyses and amino acid compositions of acid or enzymic hydrolysates afforded values which agreed, within experimental error, with those expected by

(11) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).
(12) R. W. Holley and E. Sondheimer, *J. Amer. Chem. Soc.*, **76**, 1326 (1954).
(13) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *ibid.*, **86**, 1839 (1964).
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(16) (a) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1991 (1961); (b) R. Schwyzer and H. Dietrich, *ibid.*, **44**, 2003 (1961).
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theory. It follows that these analytical tools are not adequate to establish homogeneity.

The progressive accumulation of impurities was particularly apparent during the synthesis of fragment D which contains three unprotected tyrosine residues in positions 38, 42, and 45. O-Acylation of the tyrosine hydroxyls may contribute to the accumulation of contaminants. It should be noted that the amino acid ratios in acid hydrolysates and AP-M digests of crude fragment D *tert*-butoxycarbonylhydrazide were in good agreement with theory. The inhomogeneity of this material is shown in Figure 3, which illustrates a systematic evaluation by tlc of its purification on a Sephadex G-50 partition column with the solvent system 1-butanol–acetic acid–water (4:1:5) lower phase as the stationary and upper phase as the moving component. Channel I represents the desired homogeneous fragment D *tert*-butoxycarbonylhydrazide which was obtained in a yield of 55–60% based on the crude material applied to the column. No attempt was made to identify the nature of the various contaminants (channels A to H and J to P). The chromatogram magnifies the problem since the desired product (channel I) and the various side products in its synthesis were applied to the plate in comparable concentrations; each individual impurity was actually present in a small proportion (see Experimental Section).

The amino acid composition of acid hydrolysates and AP-M digests of column-purified material agreed closely with that observed with the crude product. This result supports the conclusion, stated above, that amino acid analyses of acid or enzymic hydrolysates of complex synthetic peptides which are not shown to be "pure" by other methods provide little support for homogeneity. *Amino acid analyses in agreement with theory are a necessary, but not sufficient, criterion of purity.*

As has been mentioned, fragment B and its protected precursor, undecapeptide, are very sparingly soluble in DMF. The fortuitous observation that serylseryl-β-*tert*-butylaspartylvalylserylthreonylalanylglutaminylalanylalanylglycine *tert*-butoxycarbonylhydrazide (positions 13–23) is soluble in aqueous pyridinium acetate buffers enabled us to evaluate its homogeneity by chromatography on a Dowex resin using the amino acid analyzer detecting system. The material contained only traces of ninhydrin-positive impurities. Dowex-purified material formed sharp spots on tlc in two solvent systems and its acid hydrolysate afforded the correct amino acid composition. The corresponding hydrazide was completely digestible by AP-M. Fragment B *tert*-butoxycarbonylhydrazide, also readily soluble in pyridinium acetate buffers, was obtained in a high state of chromatographic homogeneity by chromatography on a Dowex column. The purified material contained a trace impurity which may be completely deprotected fragment B hydrazide.

The synthesis of fragment C (Scheme II) proceeded smoothly and material of good quality was obtained by the purification steps described in the Experimental Section. The *tert*-butoxycarbonylhydrazides corresponding to the C sequence up to glycyl-γ-*tert*-butylglutamylthreonylvalylglycine (positions 30–34) were completely digested by AP-M under our standard conditions[19] with average recoveries of amino acids ranging from 89 to 99%. However, aspartylglycylglutamylthreonylvalylglycine hydrazide (positions 29–34), when digested under identical conditions, gave an average amino acid recovery of only 27%. Since this peptide derivative contains an aspartylglycine bond which can be subject to an $\alpha \rightarrow \beta$ shift[20] this problem received critical study. The model peptide γ-*tert*-butylglutamyl-β-*tert*-butylaspartylglycine *tert*-butoxycarbonylhydrazide (positions 28–30) was synthesized from identical starting materials by the procedures employed to prepare aspartylglycylglutamylthreonylvalylglycine hydrazide and was subjected to AP-M digestion under standard conditions; an average amino acid recovery of 80% was observed. The rate of amino acid liberation from aspartylglycylglutamylthreonylvalylglycine hydrazide, using twice the enzyme concentration employed in standard digests, was then investigated. From the results presented in Table I it is apparent that the rate of hydrolysis of the aspartyl–glycine bond is abnormally slow and thus represents the rate-limiting step. This same behavior has been previously observed with aspartylserine and peptides containing this linkage.[19] The evidence presented indicates that fragment C and intermediates in its synthesis are not contaminated by significant proportions of corresponding peptides containing the β-aspartyl–glycine bond.

Table I. Liberation of Amino Acids in AP-M Digests of Aspartylglycylglutamylthreonylvalylglycine Hydrazide Bis(trifluoroacetate) as a Function of Time

Amino acid	Recovery, % — Incubation time, hr				
	4	6	16	24	40
Asp	24	30	63	70	88
Gly[a]	9	14	38	47	69
Glu	13	23	57	67	88
Thr	9	17	52	58	80
Val	7	14	49	62	78

[a] Glycine values are based on a theoretical recovery of two glycines per mole of peptide hydrazide. See text for experimental details.

The experiments presented have shown that fragments B and D, synthesized by the stepwise method, were not pure. Purification of the amino-deprotected compounds by chromatography was possible since the chromatographic behavior of the contaminants differed significantly from that of the desired product. The chromatographically purified materials were homogeneous as judged by tlc and their acid hydrolysates exhibited, within the experimental error, the correct amino acid composition. Buffer-soluble derivatives of these compounds were completely digestible by AP-M and the ensuing digest possessed essentially the same amino acid composition as the acid hydrolysates. However, we recognize the fact that the analytical procedures used are not sensitive enough to detect minor degrees of racemization.

Assembly of Fragment BCD

Complex peptides in which all the side-chain functional groups are protected are frequently difficult to

(19) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Amer. Chem. Soc.*, **88**, 3633 (1966).

(20) (a) E. E. Haley and B. J. Corcoran, *Biochemistry*, **6**, 2668 (1967); (b) K. Weber and W. Konigsberg, *J. Biol. Chem.*, **242**, 3563 (1967); (c) M. A. Ondetti, A. Deer, J. T. Sheehan, J. Pluščec, and O. Kocy, *Biochemistry*, 7, 4096 (1968).

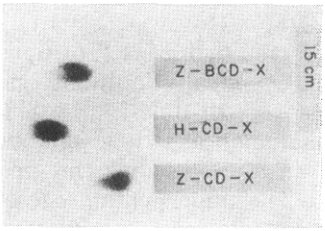

Figure 4. Thin-layer chromatogram of fragment CD, fragment CD *tert*-butoxycarbonylhydrazide, and fragment BCD. Plate developed for 16 hr in solvent system I. The 15-cm marker indicates the relative distance the peptides migrated and does not correspond to the solvent front. Peptides visualized with chlorine reagent.

purify. For this reason we employ a minimum of side-chain protection in our synthetic schemes. Of particular interest are the free β- and γ-carboxyls of aspartic and glutamic acid since these charged sites provide an added dimension for purification by ion exchange chromatography. Chromatography on the anion exchanger AG 1-X2[21] using increasing concentrations of acetic acid as the eluent provides a potent tool for the highly selective separation of peptides or peptide derivatives which differ in net negative charge. This principle, which was developed in connection with the synthesis of S-peptide[22] and a series of its analogs,[19,23] was successfully applied to the preparation of fragment BCD (Figure 2, shaded section).

For the synthesis of fragment CD (positions 24–47), fragment C was exposed to TFA and the ensuing trifluoroacetate salt converted to the azide. This azide served to acylate the *tert*-butoxycarbonylhydrazide of fragment D. In concert with the removal of the *tert*-butoxycarbonyl group, the TFA treatment liberates the carboxyls in positions 28, 29, and 31 which are critical for the successful isolation of fragment CD. The Rudinger modification of the azide reaction,[24] which gave excellent results during our synthetic studies in the S-peptide series,[19,22,23] was used exclusively for coupling of complex fragments.

Following completion of the coupling reaction the mixture was simply diluted with the solvent mixture 1-butanol–methanol–water (1:1:1) and the solution was applied to an AG 1-X2 column. Elution of the column with the same solvent system removed unchanged amino component; the desired fragment CD was released in sequentially homogeneous form (Figure 4) by the solvent mixture 1-butanol–methanol–0.09 *N* acetic acid (1:1:1). The average yield from several experiments was 50%. Inspection of Figure 1 will show that fragment D *tert*-butoxycarbonylhydrazide contains one terminal amino group and a histidine residue in position 40. The azide of fragment C and its rearrangement products (amide) contain one histidine in position 27 plus three free carboxyls in positions 28, 29, and 31. The desired coupling product CD contains two histidines in positions 27 and 40 and three carboxyls in positions 28, 29, and 31. The unreacted amino component (fragment D *tert*-butoxycarbonylhydrazide), which contains no net negative charge, has no affinity

(21) AG 1-X2 is an anion exchanger manufactured by Bio-Rad Laboratories, Richmond, Calif.

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(23) K. Hofmann, J. P. Visser, and F. M. Finn, *ibid.*, **92**, 2900 (1970).

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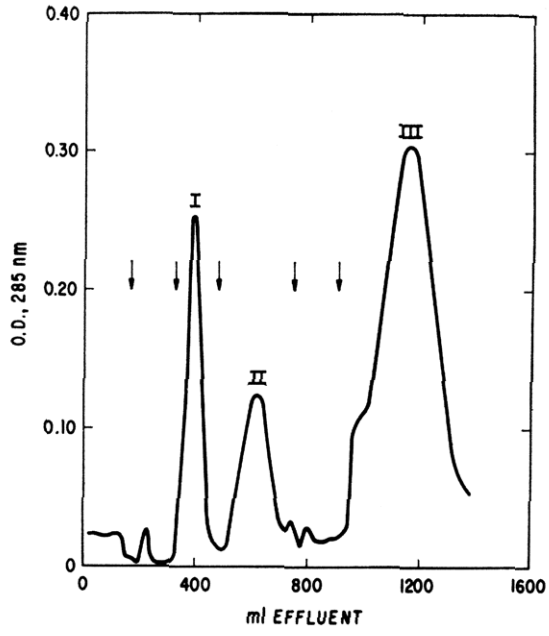

Figure 5. Purification of fragment BCD on AG 1-X2. Arrows designate solvent changes. Peak I, fragment H-CD-X; peak II, unknown; peak III, fragment BCD. See text for experimental details.

for the exchanger and is eluted with the neutral solvent. When the concentration of acetic acid reaches a certain level the acidic reaction product CD is displaced. The more acidic azide rearrangement products require still higher levels of acetic acid for elution. With some experience it is possible to predict the acetic acid concentration which will be required to elute a given peptide.

Hydrogenation of fragment CD over palladium in the solvent system 1-butanol–methanol–0.09 *N* acetic acid removed the benzyloxycarbonyl group. This experiment demonstrates that large sequential peptides can be decarbobenzoxylated by hydrogenolysis. The hydrogenation product was purified by chromatography on AG 1-X2 using the 1-butanol–methanol–acetic acid system for elution. The fact that the deprotected peptide was eluted with a lower concentration of acetic acid than fragment CD emphasizes the sensitivity of this convenient chromatographic technique. A chromatogram of fragment CD *tert*-butoxycarbonylhydrazide is shown on Figure 4.

The synthesis of fragment BCD (Figure 2, shaded section) was patterned according to the procedure used to synthesize fragment CD. Exposure of fragment B to TFA removed the *tert*-butoxycarbonyl group from the hydrazide and the β-*tert*-butyl ester from aspartic acid in position 15. The hydrazide TFA salt was converted to the azide in the solvent system DMSO–DMF. The salt was insoluble in DMF but dissolved readily in DMSO; when the peptide was completely dissolved in a small volume of this solvent the solution could be diluted with DMF without causing precipitation. The azide was prepared in the usual manner, coupled to fragment CD *tert*-butoxycarbonylhydrazide, and the desired product isolated by AG 1-X2 chromatography. A typical elution pattern is shown in Figure 5. The yield of chromatographically pure fragment BCD (Figure 4) averaged 45% in several experiments.

"Diagnostic" Amino Acid Residues

Determination of the ratios between certain diagnostic amino acid residues in a peptide which is obtained by condensation of two homogeneous fragments provides a valid criterion for assessment of the purity of the coupling product. Diagnostic residues are those which are stable to acid hydrolysis and which occur in one but not in the other of the reaction partners. Fragments CD and BCD may serve to illustrate the concept. The diagnostic residues in fragment C are leucine and valine; those in fragment D are lysine and proline. The diagnostic amino acid in fragment B is alanine which is absent from fragment CD. Since the amino acid composition of homogeneous fragments which are to be combined to form a larger chain is not likely to undergo change during the coupling step, it follows that the ratios between the diagnostic residues provide a convenient measure for the homogeneity of the coupling product. The focus is on but a few amino acid residues and deviation from the predicted ratio is indicative of contamination of the reaction product by one or the other of the components employed for coupling. In our fragment condensation studies we were guided by the ratios between diagnostic amino acids and critical evaluation of the large coupling products by tlc. Elemental analyses and optical rotations were not performed since these determinations provide little valid information.

Experimental Section[25]

Preparation of Amino Acid and Small Peptide Derivatives. 2,4,5-Trichlorophenyl Benzyloxycarbonylasparaginate. DCC (4.1 g) in DMF (10 ml) was added to an ice-cold solution of benzyloxycarbonylasparagine (5.3 g) and 2,4,5-trichlorophenol (4.4 g) in DMF (25 ml). The mixture was stirred for 3 hr at 0° and was filtered. Water was added to the filtrate and the precipitate was collected, dried, and recrystallized from THF–ether: 4.0 g (45%); mp 184–185°; $[\alpha]^{27}D$ −28.1° (*c* 3.60, DMF); R_f^{IV} 0.7. *Anal.* Calcd for $C_{18}H_{15}O_5N_2Cl_3$: C, 48.5; H, 3.4; N, 6.3. Found: C, 49.1; H, 3.4; N, 6.8.

α-*N*-Hydroxysuccinimido γ-*tert*-Butyl Benzyloxycarbonylglutamate. To an ice-cold solution of benzyloxycarbonyl-γ-*tert*-butylglutamic acid (4.4 g) and *N*-hydroxysuccinimide (1.52 g) in dioxane (50 ml) was added DCC (2.66 g), and the mixture was stirred for 14 hr at 4° and for 2 hr at room temperature. The suspension was filtered, and the filtrate was evaporated yielding an oil which crystallized on standing. Recrystallization from ethyl acetate–petroleum ether (bp 30–60°) gave needles: 3.23 g (57%); mp 101–103°; $[\alpha]^{26}D$ −29.9° (*c* 2.43, EtOH). *Anal.* Calcd for $C_{21}H_{26}O_8N_2$: C, 58.1; H, 6.0; N, 6.4. Found: C, 58.1; H, 6.0; N, 6.7.

Benzyloxycarbonylglutamine *tert*-Butoxycarbonylhydrazide. DCC (9.60 g) was added to a stirred solution of benzyloxycarbonylglutamine[26] (13.0 g) and *tert*-butoxycarbonylhydrazine (6.15 g) in DMF (30 ml) and THF (170 ml) at 0° and the mixture was stirred at 0° for 5 hr. The suspension was allowed to reach room temperature, the precipitate was removed by filtration, and the filtrate was concentrated to a small volume. Ethyl acetate (70 ml) was added, the suspension was filtered, and the ethyl acetate was evaporated. Ether and water were added to the residue and the resultant precipitate was collected, dried, and crystallized from MeOH–ethyl acetate (1:2): 12.18 g (67%); mp 193–194°; $[\alpha]^{26}D$ −33.0° (*c* 2.10, MeOH); R_f^{I} 0.7; R_f^{IV} 0.8. *Anal.* Calcd for $C_{18}H_{26}O_6N_4$: C, 54.8; H, 6.6; N, 14.2. Found: C, 54.8; H, 6.6; N, 14.1.

Glutamine *tert*-Butoxycarbonylhydrazide. Benzyloxycarbonylglutamine *tert*-butoxycarbonylhydrazide (11.52 g) was hydrogenated in MeOH. The catalyst was removed by filtration, the filtrate was evaporated to dryness *in vacuo*, and the residue was lyophilized: 7.63 g (99%); $[\alpha]^{25}D$ +15.1° (*c* 1.91, MeOH); R_f^{I} 0.4; R_f^{IV} 0.4. *Anal.* Calcd for $C_{10}H_{20}O_4N_4$: C, 46.2; H, 7.7; N, 21.5. Found: C, 46.0; H, 7.9; N, 21.6

***N*-Hydroxysuccinimido Benzyloxycarbonylglutaminate.** DCC (6.18 g) was added at −15° to a solution of benzyloxycarbonyl glutamine (8.41 g) and *N*-hydroxysuccinimide (3.45 g) in THF (100 ml) and the mixture was stirred at 0° for 18 hr. The precipitate was removed and the filtrate evaporated to dryness. The residue was crystallized from ethyl acetate: 7.40 g (65%); mp 134–136°; $[\alpha]^{26}D$ −31.4° (*c* 0.98, EtOH); $[\alpha]^{27}D$ −23.6° (*c* 2.37, DMF); R_f^{IV} 0.6 *Anal.* Calcd for $C_{17}H_{19}O_7N_3$: C, 54.1; H, 5.1; N, 11.1. Found: C, 54.2; H, 5.4; N, 11.0.

Benzyloxycarbonyltyrosylglutamine *tert*-Butoxycarbonylhydrazide. 2,4,5-Trichlorophenyl *N*-benzyloxycarbonyltyrosinate[14] (14.50 g) was added to a solution of glutamine *tert*-butoxycarbonylhydrazide (7.67 g) in THF (120 ml) and the solution was kept at room temperature for 18 hr. The solvent was removed, the residue dissolved in MeOH (80 ml), and the product was precipitated by addition of water (400 ml) and ether (400 ml). The material was washed with ether and water and dried: 14.18 g (86%); mp 145–149°; $[\alpha]^{27}D$ −23.1° (*c* 2.03, DMF); R_f^{I} 0.7; R_f^{IV} 0.8. *Anal.* Calcd for $C_{27}H_{33}O_8N_5$: C, 58.2; H, 6.3; N, 12.5. Found: C, 58.1; H, 6.1; N, 12.4.

Benzyloxycarbonyltyrosylglutamine Hydrazide. The protected hydrazide (20.75 g) was dissolved in TFA (100 ml) and the solution was kept at room temperature for 1 hr. Excess ether was added and the precipitate was collected, washed with ether, and dried. The precipitate was dissolved in hot MeOH (400 ml), and the solution was cooled and neutralized with TEA. Addition of water (600 ml) and refrigeration for 18 hr gave a granular gel which was collected, washed with water, and dried: 14.08 g (83%); mp 195–198° (soften); 202–206° dec; $[\alpha]^{26}D$ −15.5° (*c* 1.96, DMF); R_f^{I} 0.7; R_f^{III} 0.8. *Anal.* Calcd for $C_{22}H_{27}O_6N_5$: C, 57.8; H, 5.9; N, 15.3. Found: C, 58.0; H, 6.2; N, 14.8.

Methyl Benzyloxycarbonylvalylserinate. A mixed anhydride was prepared in the usual manner from benzyloxycarbonylvaline (31.2 g) in THF (70 ml) with tributylamine (29.4 ml) and ethyl chloroformate (11.0 ml). This solution was added slowly with stirring to a chilled solution of methyl serinate (17.1 g) and TEA (17.1 ml) in THF (100 ml) and water (30 ml). The mixture was stirred for 30 min in an ice bath and was then kept for 1 hr at room temperature. The resulting crystalline material was collected, and the filtrate was concentrated to obtain an additional quantity of

(25) 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.; oxygen values were actually determined and not computed by difference. Analytical samples were dried *in vacuo* over P_2O_5 at 50–60°. The amino acid composition of acid hydrolysates and AP-M digests were determined with a Beckman-Spinco Model 120 amino acid analyzer according to the method of S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958). The figures in parentheses are average recoveries of amino acids based on formula weight. Nle and α-amino-β-guanidopropionic acid served as internal standards. Acid hydrolyses were performed in constant-boiling HCl at 110° for 24 hr in evacuated tubes except with peptides containing valine or isoleucine which were hydrolyzed for 48 hr; values are not corrected for amino acid destruction. AP-M digests were prepared as described in ref 19 except when noted otherwise. Designations of solvent systems for descending paper chromatography on Whatman No. 1 filter paper are: R_f^1 1-butanol–glacial acetic acid–water (4:1:5) top layer; R_f^2 2-butanol–3% ammonium hydroxide (3:1); R_f^3 1-butanol–pyridine–water–glacial acetic acid (45:30:36:9); with this system R_f values are expressed as multiples of the distance traveled by a histidine marker. Designation of solvent systems for ascending tlc on silica gel G (E. Merck and Co., Darmstadt, West Germany) are: R_f^{I} 1-butanol–glacial acetic acid–water (60:20:20); R_f^{II} 95% EtOH–concd NH_4OH (100:27); R_f^{III} 1-butanol–pyridine–glacial acetic acid–water (30:20:6:24); R_f^{IV} MeOH–chloroform (20:80). Sparingly soluble compounds were prepared for spotting by dissolving them in a few drops of glacial acetic acid with slight warming and adding a few drops of water. Solvents were freshly distilled and evaporations were carried out *in vacuo* at a temperature of 40–45° in rotary evaporators. AG 1-X2 (chloride form, 200–400 mesh) was washed twice with 2 *N* KOH, then with water until neutral; this was followed by two washings with 10% acetic acid and water until neutral. Columns were equilibrated with the appropriate solvent before use. Except when noted otherwise, hydrogenations were performed at room temperature and atmospheric pressure. Routinely, catalyst was removed by filtering the hydrogenation mixture through a bed of Filter Cel. Yields are based on weight of vacuum-dried (over P_2O_5 and KOH) substance.

(26) R. Boissonnas, St. Guttmann, P. A. Jaquenoud, and J. P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955), report mp 135°; $[\alpha]^{20}D$ +5.8° (*c* 2, EtOH). We recorded mp 139–141°; $[\alpha]^{26}D$ −5.7° (*c* 5.01, EtOH).

the compound. The combined crops were washed with water and ether and recrystallized from ethyl acetate: needles; 30.5 g (69%); mp 171–172°; $[\alpha]^{23}D$ −19.2° (*c* 4.28, MeOH). *Anal.* Calcd for $C_{17}H_{24}O_6N_2$: C, 57.9; H, 6.9; N, 8.0. Found: C, 57.9; H, 6.9; N, 8.0.

Benzyloxycarbonylvalylserine Hydrazide. Hydrazine hydrate (11 ml) was added to a solution of methyl benzyloxycarbonylvalylserinate (35.2 g) in MeOH (600 ml) and the solution was kept at room temperature for 4 hr. The resulting crystalline product was collected and washed with MeOH: yield 33.2 g (94%); mp 245–247° dec. A sample for analysis was recrystallized from water: mp 246–248° dec; $[\alpha]^{26}D$ −43.4° (*c* 1.45, 1 *N* HCl). *Anal.* Calcd for $C_{16}H_{24}O_5N_4$: C, 54.5; H, 6.9; N, 15.9. Found: C, 55.1; H, 7.0; N, 15.5.

Benzyloxycarbonyl-γ-*tert*-butylglutamyl-β-*tert*-butylaspartylglycine Dicyclohexylammonium Salt. A solution of glycine (564 mg) and sodium bicarbonate (630 mg) in water (16 ml) was added to a solution of α-*N*-hydroxysuccinimido β-*tert*-butyl benzyloxycarbonylaspartate (2.1 g) in THF (20 ml). The mixture was stirred for 4 hr at room temperature, then the THF was removed. The solution was acidified with 1 *N* hydrochloric acid and extracted by ethyl acetate. The extract was dried over sodium sulfate and evaporated to give an oil which was hydrogenated in MeOH–10% acetic acid (1:1) for 3 hr. The catalyst was removed by filtration, the filtrate was evaporated, and the residue was precipitated from MeOH with ether. The precipitate was absorbed from water onto AG 50W-X2 (H+), which was washed with water. The resin was eluted with 3% aqueous pyridine and the eluate evaporated. The residue was crystallized from EtOH–water: 800 mg (65%); mp 179–180° dec; $[\alpha]^{25}D$ +61.7° (*c* 1.00, water); R_f^{I} 0.4; R_f^{III} 0.5; amino acid ratios in acid hydrolysate, $Asp_{1.0}Gly_{1.0}$.

α-*N*-Hydroxysuccinimido γ-*tert*-butyl benzyloxycarbonylglutamate (1.140 g) in THF (10 ml) was added to a solution of the above product (665 mg) and sodium bicarbonate (228 mg) in water (10 ml) and the reaction stirred at room temperature for 18 hr. The THF was removed, and the residue was acidified with 1 *N* hydrochloric acid and extracted by ethyl acetate. The extract was dried and evaporated to an oil. Dicyclohexylamine was added to a solution of the oil in ether and the resulting sticky solid crystallized from MeOH–ether: 824 mg (44%); mp 150–152°; $[\alpha]^{29}D$ −22.9° (*c* 1.97, MeOH); R_f^{I} 0.6; amino acid ratios in acid hydrolysate, $Glu_{1.0}Asp_{1.0}Gly_{1.0}$

γ-*tert*-Butylglutamyl-β-*tert*-butylaspartylglycine *tert*-Butoxycarbonylhydrazide Acetate. Benzyloxycarbonyl-γ-*tert*-butylglutamyl-β-*tert*-butylaspartylglycine dicyclohexylammonium salt (560 mg) was partitioned between aqueous citric acid and ethyl acetate. The ethyl acetate layer was washed with water and dried. *tert*-Butoxycarbonylhydrazine (99 mg) and DCC (155 mg) were added to this solution which was stirred at 0° for 18 hr. The suspension was filtered, and the filtrate was washed with 1 *N* hydrochloric acid, saturated sodium chloride, and saturated sodium bicarbonate, dried, and evaporated to an oil.

The oil was hydrogenated in MeOH containing acetic acid (0.2 ml). The catalyst was removed by filtration and the filtrate was evaporated to dryness. A solution of the residue in water was freed from a small amount of insoluble material by filtration and lyophilized: 384 mg (76%); $[\alpha]^{28}D$ −8.4° (*c* 2.21, MeOH); R_f^{I} 0.9; R_f^{IV} 0.8; amino acid ratios in AP-M digest, γ-O-*tert*-$Bu\text{-}Glu_{1.0}$β-O-*tert*-$BuAsp_{1.0}Gly_{0.9}$ (80%).

Preparation of Fragment B (Positions 12–23). (Positions 21–23) Benzyloxycarbonylalanylalanylglycine. Sodium nitrite (2.76 g) in water (10 ml) was added to an ice-cold solution of benzyloxycarbonylalanylalanine hydrazide[27] (12.32 g) in a mixture of water (90 ml), THF (110 ml), and 5 *N* hydrochloric acid (24 ml). The solution was kept at −3 to 0° for 15 min and then neutralized with TEA (12.4 ml). To this solution containing Z-Ala-Ala-azide was added an ice-cold solution of glycine (4.50 g) in a mixture of water (30 ml), THF (20 ml), and TEA (8.3 ml). The solution was kept at 4° for 20 hr, maintaining the pH at approximately 7.5 by addition of TEA. The bulk of the solvents was evaporated and the residue diluted with 1 *N* ammonium hydroxide. This solution was extracted with three portions of ethyl acetate which were discarded. The aqueous phase was acidified to Congo red with 5 *N* hydrochloric acid, and the ensuing suspension was kept at 4° for 1 hr when the precipitate was collected, washed with ice-cold water, and dried. The solid was then washed with boiling ethyl acetate, dried, and recrystallized from MeOH: needles; 10.11 g (72%); mp 210–211°; $[\alpha]^{27}D$ −46.4° (*c* 1.13, MeOH); R_f^{I} 0.7; R_f^{III} 0.6; R_f^{IV} 0.5. *Anal.* Calcd for $C_{16}H_{21}O_6N_3$: C, 54.7; H, 6.0; N, 12.0. Found: C, 54.9; H, 5.9; N, 12.3.

(Positions 21–23) Benzyloxycarbonylalanylalanylglycine *tert*-Butoxycarbonylhydrazide. Isobutyl chloroformate (1.31 ml) was added at −15° to a solution of benzyloxycarbonylalanylalanylglycine (3.51 g) in DMF (30 ml) containing *N*-methylmorpholine (1.12 ml). The mixture was kept at −15° for 15 min, then a solution of *tert*-butoxycarbonylhydrazine[17] (1.58 g) in DMF (10 ml) cooled at −15° was added, and the mixture was warmed to 40° and kept at that temperature for 3 min. The solvents were evaporated, the residue was dissolved in ethyl acetate, and the solution was washed successively with water, saturated sodium bicarbonate, 1 *N* citric acid, and saturated sodium chloride, and was dried over sodium sulfate, and evaporated. The solid residue which was obtained on addition of ether–petroleum ether (1:1), was collected, dried, and recrystallized from ethyl acetate: 4.24 g (91%); mp 151–153°; $[\alpha]^{27}D$ −27.8° (*c* 3.66, MeOH); R_f^{I} 0.8; R_f^{III} 0.8; R_f^{IV} 0.9. *Anal.* Calcd for $C_{21}H_{31}O_7N_5$: C, 54.2; H, 6.7; N, 15.1. Found: C, 54.1; H, 6.6; N, 15.1.

(Positions 21–23) Alanylalanylglycine *tert*-Butoxycarbonylhydrazide Monoacetate. Benzyloxycarbonylalanylalanylglycine *tert*-butoxycarbonylhydrazide (4.0 g) was hydrogenated over palladium in MeOH (150 ml) containing 10% acetic acid (8 ml). The catalyst was removed by filtration and the filtrate was evaporated. The residue was dissolved in a small volume of EtOH and ether was added. The precipitate was collected and dried: 3.20 g (95%); mp 110–112°; $[\alpha]^{25}D$ −17.4° (*c* 3.05, MeOH); R_f^{1} 0.6; R_f^{3} 3.5 × His; R_f^{I} 0.4; R_f^{III} 0.6; R_f^{IV} 0.4; amino acid ratios in AP-M digest, $Ala_{2.0}Gly_{1.0}$ (89%).

(Positions 20–23) Benzyloxycarbonylglutaminylalanylalanylglycine *tert*-Butoxycarbonylhydrazide. 2,4,5-Trichlorophenyl benzyloxycarbonylglutaminate[14] (8.28 g) in DMF (20 ml) was added at room temperature to a solution of alanylalanylglycine *tert*-butoxycarbonylhydrazide monoacetate (4.70 g) in DMF (150 ml) and TEA (1.66 ml). The mixture was kept at room temperature for 20 hr and then evaporated. To the residue was added ethyl acetate and the resulting precipitate was collected, washed with ethyl acetate, and dried. The material was precipitated from MeOH with water and was recrystallized from MeOH: needles; 6.30 g (88%); mp 227–228°; $[\alpha]^{27}D$ −37.1° (*c* 0.82, MeOH); $[\alpha]^{25}D$ −10.8° (*c* 3.7, DMF); R_f^{I} 0.6; R_f^{III} 0.8; R_f^{IV} 0.9; amino acid ratios in acid hydrolysate, $Glu_{1.0}Ala_{2.0}Gly_{1.0}$. *Anal.* Calcd for $C_{26}H_{39}O_9N_7$: C, 52.6; H, 6.6; N, 16.5; O, 24.3. Found: C, 52.4; H, 6 6; N, 16.2; O, 23.8.

(Positions 20–23) Glutaminylalanylalanylglycine *tert*-Butoxycarbonylhydrazide. The protected tetrapeptide hydrazide (6.71 g) was hydrogenated in MeOH (300 ml) for 5 hr. The catalyst was removed by filtration and the filtrate was evaporated. The solid residue which was obtained by addition of ether was collected and dried: 5.02 g (97%); $[\alpha]^{25}D$ −36.3° (*c* 4.00, MeOH); R_f^{1} 0.5; R_f^{3} 3.0 × His; R_f^{I} 0.4; R_f^{III} 0.6; R_f^{IV} 0.3; amino acid ratios in AP-M digest, $Gln_{1.0}Ala_{2.1}Gly_{1.0}$ (90%).

(Positions 18–23) Benzyloxycarbonylthreonylalanylglutaminylalanylalanylglycine *tert*-Butoxycarbonylhydrazide. *tert*-Butyl nitrite (1.72 ml) was added to a solution cooled at −20 to −25° of benzyloxycarbonylthreonylalanine hydrazide[27] (4.39 g) in DMF (20 ml) and THF (20 ml) containing 5.9 *N* hydrogen chloride in dioxane (8.8 ml). The mixture was stirred at −20 to −25° for 10 min, TEA (7.18 ml) was added dropwise followed by an ice-cold solution of glutaminylalanylalanylglycine *tert*-butoxycarbonylhydrazide (4.60 g) in DMF (15 ml). The mixture was stirred at 4° for 24 hr and ethyl acetate (approximately 200 ml) was added to the heavy suspension. The solid was collected, washed with a mixture of hot ethyl acetate and water, and dried. Additional material was obtained from the filtrate by evaporation and washing of the solid residue with hot ethyl acetate and water. Both batches were combined and the product was precipitated from DMF with ethyl acetate and from DMF with water: 6.00 g (78%); mp 267–270° dec; $[\alpha]^{23}D$ −14.2° (*c* 1.18, DMF); R_f^{I} 0.7; R_f^{III} 0 8; amino acid ratios in acid hydrolysate, $Thr_{1.0}Ala_{3.1}Glu_{1.0}Gly_{0.9}$. *Anal.* Calcd for $C_{33}H_{51}O_{12}N_9$: C, 51.8; H, 6.7; N, 16.5; O, 25.1. Found: C, 51.5; H, 6.8; N, 16.7; O, 24.5.

(Positions 18–23) Threonylalanylglutaminylalanylalanylglycine *tert*-Butoxycarbonylhydrazide Acetate. Benzyloxycarbonylthreonylalanylglutaminylalanylalanylglycine *tert*-butoxycarbonylhydrazide monohydrate (4.50 g) was hydrogenated in MeOH–water–acetic acid (20:10:1). The catalyst was removed by filtration and the filtrate evaporated to dryness. A solution of the residue in water

(27) K. Hofmann, R. Schmiechen, R. D. Wells, Y. Wolman, and N. Yanaihara, *J. Amer. Chem. Soc.*, **87**, 611 (1965).

was freed from a small amount of insoluble material by filtration and lyophilized: 3.88 g (95%); $[\alpha]^{25}D$ −73.0° (*c* 4.17, water); $[\alpha]^{28}D$ −52 3° (*c* 0.88, DMF–water 1:1); R_f^1 0.5; R_f^3 1.6 × His; R_f^{III} 0.7; amino acid ratios in AP-M digest, $Thr_{1.0}Ala_{3.1}Gln_{1.0}Gly_{0.9}$ (97%).

(Positions 16–23) Benzyloxycarbonylvalylserylthreonylalanylglutaminylalanylalanylglycine *tert*-Butoxycarbonylhydrazide. An ice-cold solution of sodium nitrite (1.24 g) in water (6 ml) was added at −3 to 0° to a solution of benzyloxycarbonylvalylserine hydrazide (6.34 g) in DMF (50 ml), THF (30 ml), and 2 *N* hydrochloric acid (27.0 ml). The resulting precipitate was dissolved by adding DMF (50 ml) and THF (70 ml) and the solution was kept at 0° for 10 min, when TEA (4.96 ml) was added dropwise. To this solution containing Z-Val-Ser-azide was added an ice-cold solution of threonylalanylglutaminylalanylalanylglycine *tert*-butoxycarbonylhydrazide acetate (4.15 g) in DMF (100 ml), THF (20 ml), water (65 ml), and TEA (0.83 ml). The mixture was kept at 4° for 24 hr and the bulk of the solvents was evaporated. Water (approximately 100 ml) was added to the residue and the resulting suspension was heated in a boiling water bath for approximately 3 min. The suspension was cooled; the solid was collected, washed with MeOH, and dried. The material was dissolved in warm DMF (approximately 300 ml), the solution was concentrated to a volume of approximately 30 ml, and MeOH (approximately 150 ml) was added to the residue. The precipitate was collected and dried: 3.72 g (65%); mp 281–283° dec; $[\alpha]^{27}D$ −13.3° (*c* 0.72, DMSO); R_f^I 0.5; R_f^{III} 0.8. *Anal.* Calcd for $C_{41}H_{65}O_{15}N_{11}$: C, 51.7; H, 6.9; N, 16.2; O, 25.2. Found: C, 51.5; H, 7.0; N, 16.1; O, 25.4.

(Positions 16–23) Valylserylthreonylalanylglutaminylalanylalanylglycine *tert*-Butoxycarbonylhydrazide. The protected octapeptide hydrazide (3.5 g) in DMF (300 ml), water (60 ml), and MeOH (60 ml) was hydrogenated over palladium. The catalyst was removed by filtration, the filtrate was evaporated, and the solid residue which was obtained on addition of ether was collected and dried: 2.8 g (93%); mp 280–285° dec; $[\alpha]^{29}D$ −14.6° (*c* 1.40, DMSO); R_f^1 0.3; R_f^3 2.9 x His; R_f^I 0 3; R_f^{III} 0.7; amino acid ratios in acid hydrolysate, $Val_{1.0}Ser_{0.9}Thr_{1.0}Ala_{3.2}Glu_{1.0}Gly_{1.0}$ (94%). A sample (50 mg) was dissolved in TFA (1 ml) and the solution was kept at room temperature for 40 min. The solvent was evaporated and the residue lyophilized three times from water; amino acid ratios in AP-M digest: $Val_{1.0}Ser + Gln_{2.1}Thr_{1.0}Ala_{3.0}Gly_{0.9}$ (88% based on bis(trifluoroacetate)).

(Positions 15–23) Benzyloxycarbonyl-β-*tert*-butylaspartylvalylserylthreonylalanylglutaminylalanylalanylglycine *tert*-Butoxycarbonylhydrazide Monohydrate. α-*N*-Hydroxysuccinimido β-*tert*-butyl benzyloxycarbonylaspartate[28] (1.87 g) was added to a solution of valylserylthreonylalanylglutaminylalanylalanylglycine *tert*-butoxycarbonylhydrazide (2.81 g) in DMF (200 ml) and water (40 ml). The mixture was kept at room temperature for 15 hr, when the solvents were evaporated and water was added to the residue. The ensuing precipitate was collected, dried, and washed three times with boiling ethyl acetate. The dried material was dissolved in DMF (approximately 300 ml), the solution was concentrated to a small volume (approximately 30 ml), and MeOH was added. The precipitate was collected, dried, and reprecipitated from DMF with water: 3.05 g (78%); mp 284–286° dec; $[\alpha]^{28}D$ −14.8° (*c* 1.03, DMSO); R_f^I 0.5; R_f^{III} 0.7. *Anal.* Calcd for $C_{49}H_{78}O_{18}N_{12}\cdot H_2O$: C, 51.6; H, 7.1; N, 14.7; O, 26.6. Found: C, 51.8; H, 6.8; N, 14.5; O, 26.8.

(Positions 15–23) β-*tert*-Butylaspartylvalylserylthreonylalanylglutaminylalanylalanylglycine *tert*-Butoxycarbonylhydrazide. The protected nonapeptide hydrazide (1.50 g) was hydrogenated in DMF (200 ml) for 20 hr at 70°. The catalyst was removed by filtration, the filtrate was concentrated to a volume of approximately 100 ml, and this solution was used for the next coupling reaction.

For analytical evaluation a 10-ml aliquot was evaporated, washed with ether, and dried: mp 279–280° dec; $[\alpha]^{28}D$ −15 4° (*c* 1.26, DMSO); R_f^1 0.5; R_f^3 3.1 × His; R_f^I 0.3; R_f^{III} 0.7; amino acid ratios in acid hydrolysate, $Asp_{1.0}Val_{1.0}Ser_{0.9}Thr_{1.0}Ala_{3.1}Glu_{1.0}Gly_{1.0}$ (95%). A sample (50 mg) was dissolved in TFA (0.5 ml) and the solution was kept at room temperature for 40 min. The solvent was evaporated and the residue lypohilized three times from water; amino acid ratios in AP-M digest: $Asp_{0.9}Val_{1.0}Ser + Gln_{2.0}Thr_{1.0}Ala_{3.2}Gly_{0.8}$ (99% based on bis(trifluoroacetate)).

(Positions 13–23) Benzyloxycarbonylserylseryl-β-*tert*-butylaspartylvalylserylthreonylalanylglutaminylalanylalanylglycine *tert*-Butoxycarbonylhydrazide. *tert*-Butyl nitrite (0.65 ml) was added at −20 to −25° to a solution of benzyloxycarbonylserylserine hydrazide[28] (1.70 g) in DMF (10 ml) and THF (10 ml) containing 5.9 *N* hydrogen chloride in dioxane (3.40 ml). The solution was kept at −20 to −25° for 20 min, was cooled at −35°, and TEA (2.80 ml) was added. To this solution containing Z-Ser-Ser-azide was added an ice-cold DMF solution of β-*tert*-butylaspartylvalylserylthreonylalanylglutaminylalanylalanylglycine *tert*-butoxycarbonylhydrazide obtained in the manner described above and the mixture was stirred at 4° for 20 hr. The solvents were evaporated, water was added to the residue, and the resulting precipitate was collected and dried. This material was dissolved in hot DMF (100 ml) and water (30 ml), and the solution was concentrated to 0.5 its volume and was extracted with six 250-ml portions of 1-butanol (saturated with 3% acetic acid). The extracts were washed in counter-current fashion with 3% acetic acid (saturated with 1-butanol) and evaporated. Addition of ether gave a solid which was collected, dried, and dissolved in DMF (100 ml) and water (30 ml). The solution was concentrated to a volume of approximately 30 ml and MeOH (approximately 150 ml) was added. The precipitate was collected and dried: 1.11 g (64%); mp 270–277° dec; $[\alpha]^{28}D$ −16.2° (*c* 1.81, DMSO); R_f^I 0.4; R_f^{III} 0.8. *Anal.* Calcd for $C_{55}H_{88}O_{22}N_{14}$: C, 50.9; H, 6.9; N, 15.1; O, 27.1. Found: C, 50.7; H, 6.8; N, 15.0; O, 27.8.

(Positions 13–23) Serylseryl-β-*tert*-butylaspartylvalylserylthreonylalanylglutaminylalanylalanylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected undecapeptide (502 mg) was dissolved in DMF (100 ml) at 70° and water (33 ml) was added. This mixture was hydrogenated over palladium at room temperature for 18 hr, then at 70° for 3 hr. The catalyst was removed by filtration, the filtrate was evaporated, and the residue was dissolved in 0.1 *M* acetic acid. The solution was lyophilized and the ensuing colorless fluffy material dried: 464 mg (98%); $[\alpha]^{26}D$ −18.7° (*c* 1.77 DMSO); R_f^I 0.2 with slight tailing; R_f^{III} 0.7 with slight tailing; amino acid ratios in acid hydrolysate, $Ser_{2.5}Asp_{1.0}Val_{1.0}Thr_{1.0}Ala_{3.1}Glu_{1.0}Gly_{1.0}$ (100%). A sample (50 mg) was dissolved in TFA (0.5 ml) and the solution was kept at room temperature for 1 hr. The solvent was removed and the residue was lyophilized from water; amino acid ratios in AP-M digest: $(Ser+Gln)_{4.0}Asp_{1.0}Val_{1.0}Thr_{1.0}Ala_{3.1}Gly_{0.9}$ (92% based on bis(trifluoroacetate)). A sample of the undecapeptide *tert*-butoxycarbonylhydrazide acetate (9.0 mg) was dissolved in 0.1 *M*, pH 3.1, pyridinium acetate buffer (0.5 ml) and the solution was centrifuged to remove a trace of insoluble material. The clear supernatant was then applied to a column (0.9 × 24 cm) of Beckman ion-exchange resin type 15A. The residue was resuspended in 0.25 ml of buffer, the solution was clarified by centrifugation, and the supernatant also was added to the column. The column was first eluted with 0.1 *M*, pH 3.1, pyridinium acetate for 125 min at a flow rate of approximately 15 ml/hr, then with a continuous linear gradient of pyridinium acetate between 0.1 *M*, pH 3.1 (150 ml), and 2.0 *M*, pH 5.0 (150 ml), for 600 min. The column was then flushed with 2.0 *M*, pH 5.0, buffer for 73 min. The eluates were fed continuously through a Beckman Model 120 B amino acid analyzer detector system. A large peak was eluted near the void volume; only small impurity peaks were observed.

Preparative Column Purification. The undecapeptide *tert*-butoxycarbonylhydrazide acetate (146 mg) was dissolved, with warming, in 0.1 *M*, pH 3.1, pyridinium acetate buffer (7 ml) and the solution was applied to a Beckman ion-exchange resin type 15A column (1.9 × 12 cm). The column was eluted at a flow rate of approximately 15 ml/hr with the pH 3.1 buffer and fractions (8 min each) were collected. Samples (2 μl) were withdrawn from each fraction and evaluated by tlc using the chlorine test for visualization. Chlorine-positive fractions were pooled, concentrated to a small volume, and lyophilized to constant weight: 132 mg; R_f^I 0.2; R_f^{III} 0.7; amino acid ratios in acid hydrolysate, $Ser_{3.0}Asp_{1.0}Val_{1.0}Thr_{1.0}Ala_{3.1}Glu_{1.0}Gly_{1.0}$ (80%). A sample (15.8 mg) was dissolved in 90% TFA (2 ml) and the solution was kept at room temperature for 30 min. The solvent was removed and the residue was lyophilized from water and dried; amino acid ratios in AP-M digest: $(Ser+Gln)_{4.0}Asp_{0.9}Val_{1.0}Thr_{1.0}Ala_{3.2}Gly_{1.0}$ (79%).

(Positions 12–23) Benzyloxycarbonylserylserylseryl-β-*tert*-butylaspartylvalylserylthreonylalanylglutaminylalanylalanylglycine *tert*-Butoxycarbonylhydrazide (Fragment B). A solution of benzyloxycarbonylserine hydrazide[11] (495 mg) in DMF (20 ml) was cooled at −5° and 4.36 *N* HCl (1.34 ml) was added followed by sodium nitrite (185 mg/ml; 0.79 ml). The mixture was stirred at −5° for 20 min (negative hydrazide test); then TEA was added until the pH of the solution reached 7.5–8.0 (approximately 0.55 ml required). To this solution was added a solution of the undeca-

(28) K. Hofmann, W. Haas, M. J. Smithers, and G. Zanetti, *J. Amer. Chem. Soc.*, 87, 631 (1965).

peptide *tert*-butoxycarbonylhydrazide acetate (395 mg) in DMF (20 ml), water (12 ml), and TEA (1:1 in DMF; 0.09 ml). The mixture was stirred for 2 hr at room temperature; then the pH was adjusted to 7.5–8.0 by addition of TEA. After stirring for 12 hr the solvents were evaporated and ethyl acetate (75 ml) was added to the residue. The mixture was kept in a refrigerator for 2 hr and the precipitate was collected and dried. The material was washed, in centrifuge tubes, with four 12-ml portions of water, two 12-ml portions of MeOH, and three 12-ml portions of ethyl acetate and was dried: 373 mg (83%) of a colorless powder was obtained; $[\alpha]^{26}D$ $-13.2°$ (*c* 0.92, DMSO); R_f^{I} 0.5 with trace impurity at 0.6; R_f^{III} 0.7; ninhydrin negative; amino acid ratios in acid hydrolysate, $Ser_{3.6}Asp_{1.0}Val_{1.0}Thr_{1.0}Ala_{3.0}Glu_{1.0}Gly_{1.0}$ (94%). *Anal.* Calcd for $C_{58}H_{93}O_{24}N_{15}$: C, 50.3; H, 6.8; N, 15.2; O, 27.7. Found: C, 50.2; H, 7.0; N, 15.0; O, 28.0.

Fragment B Hydrazide Trifluoroacetate. Fragment B (123 mg) was dissolved in cold aqueous 90% TFA (5.0 ml) and the solution kept at rt for 40 min. The solvent was evaporated, ether (50 ml) was added, and the suspension was stored at $-10°$ for 30 min. The material was collected, washed with ether, and dried: 120 mg (100%) of a colorless nonhygroscopic powder was obtained; amino acid ratios in AP-M digest, Ser + $Gln_{5.1}Asp_{1.0}Val_{0.9}Thr_{1.0}Ala_{3.0}$-$Gly_{0.8}$ (91% based on bis(trifluoroacetate)).

Fragment B *tert*-Butoxycarbonylhydrazide. Fragment B (506 mg) was dissolved in DMF (100 ml) by triturating and warming at 70° After 30 min a clear solution was obtained to which H_2O (33 ml) was added. This mixture was hydrogenated at rt for 15 hr and at 70° for 3 hr. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in 0.1 *M* aqueous acetic acid and lyophilized: 450 mg (94%); $[\alpha]^{26}D$ $-15.6°$ (*c* 1.88, DMSO); R_f^{I} 0.3 streaking with impurity at 0.1; R_f^{III} 0.7 with streaking; amino acid ratios in acid hydrolysate, $Ser_{3.0}Asp_{1.1}Val_{1.1}$-$Thr_{1.0}Ala_{3.0}Glu_{1.0}Gly_{1.1}$ (82%). A sample (12.5 mg) was dissolved in 0.1 *M* pyridinium acetate buffer (pH 3.1, 0.5 ml) and the solution was applied to a column (0.9 × 24 cm) of Beckman ion-exchange resin type 15A. The column was eluted with a linear buffer gradient obtained by mixing 100 ml of 0.1 *M* pyridinium acetate, pH 3.1, with 100 ml of 2.0 *M* pyridinium acetate, pH 5.0. The eluate was monitored with the Beckman Model 120 B amino acid analyzer detector system.

A large peak representing the desired product was eluted soon after initiation of ninhydrin color response. Four diffuse peaks were then observed followed by two sharp symmetric peaks and further minor peaks. A broad, low intensity peak was observed at approximately the midpoint of the buffer gradient. Eleven impurity peaks were distinguished clearly.

It was found that excellent separation of the major peak from all impurity peaks could be accomplished by delaying the application of the buffer gradient. However, recovery of product and impurities and recycling of the column could most readily be achieved by alternate elution with the two pyridinium acetate buffers described. This procedure was used in preparative experiments.

Preparative Column Purification. The crude hydrogenation product (157 mg) was dissolved at 40° in 0.1 *M* pyridinum acetate buffer (pH 3.1, 6.0 ml) and the solution was applied to a column of Beckman type 15A ion-exchange resin (1.9 × 12 cm). The column was eluted with the above buffer at a rate of 15 ml/hr while fractions were collected at intervals of 8 min. Fractions containing the desired product were identified by the chlorine test.

These fractions were pooled, concentrated to a small volume, and lyophilized to constant weight: 143 mg (91%). Analytical examination revealed the absence of impurity peaks excepting the broad, low-intensity peak located at approximately the midpoint of the buffer gradient. The proportion of this impurity was negligible: $[\alpha]^{27}D$ $-68.0°$ (*c* 1.015, 10% acetic acid); R_f^{I} 0 4; R_f^{III} 0 7; amino acid ratios in acid hydrolysate, $Ser_{4.2}Asp_{1.0}Val_{1.0}Thr_{0.9}Ala_{3.0}Glu_{1.0}$-$Gly_{1.0}$ (81%); amino acid ratios in AP-M digest, $(Ser + Gln)_{5.4}$-$Asp_{1.0}Val_{0.9}Thr_{1.1}Ala_{2.8}Gly_{0.9}$ (80%).

Preparation of Fragment C (Positions 24–34). (Positions 33–34) Methyl Benzyloxycarbonylvalylglycinate. TEA (5.60 ml) was mixed with a stirred suspension of methyl glycinate hydrochloride (2.51 g) in THF (20 ml). *N*-Hydroxysuccinimido benzyloxycarbonylvalinate[13] (6.97 g) was added, and the reaction was stirred for 6 hr at room temperature. The solvent was evaporated, and the residue was partitioned between ethyl acetate and 1 *N* HCl. The organic phase was washed with saturated sodium chloride and saturated sodium bicarbonate, dried, and evaporated. The residue was crystallized from ethyl acetate–ether: needles; 5.40 g (83%); mp 161–162°; $[\alpha]^{26}D$ $-26.7°$ (*c* 3.53 MeOH); R_f^{I} 0.8; R_f^{IV} 0.9; lit.[29] mp 160–161°; $[\alpha]^{24}D$ $-30.0°$ (*c* 1.85, MeOH). *Anal.* Calcd for $C_{16}H_{22}O_5N_2$: C, 59.6; H, 6.9; N, 8.7. Found: C, 59.8; H, 6.9; N, 8.6.

(Positions 33–34) Benzyloxycarbonylvalylglycine *tert*-Butoxycarbonylhydrazide. a. By the DCC Procedure. DCC (6.88 g) was added to a stirred solution of benzyloxycarbonylvalylglycine[30] (10.28 g), *tert*-butoxycarbonylhydrazine (4.41 g), and *N*-hydroxysuccinimide (1.90 g), in THF–ethyl acetate (1:1) (150 ml) at $-20°$ and the reaction was stirred at 0° for 20 hr. A few drops of acetic acid were added, and 15 min later the solution was filtered. The filtrate was evaporated to dryness and the residue was partitioned between ethyl acetate and 1 *N* HCl. The organic phase was washed with saturated sodium chloride and saturated sodium bicarbonate, dried, and evaporated. Trituration with ether–petroleum ether gave a crystalline product: 13.75 g (97%); mp 139–140°; $[\alpha]^{26}D$ $-9.5°$ (*c* 3.17, MeOH); R_f^{I} 0.8; R_f^{IV} 0.8. A sample for analysis was recrystallized from ethyl acetate–petroleum ether; mp 142–144°; $[\alpha]^{26}D$ $-9.8°$ (*c* 3.84, MeOH). *Anal.* Calcd for $C_{20}H_{30}O_6N_4$: C, 56.9; H, 7.2; N, 13.3. Found: C, 56.8; H, 7.3; N, 13.4.

b. By the Mixed Anhydride Procedure. A mixed anhydride was prepared, in the usual manner, from benzyloxycarbonylvalylglycine[30] (10.6 g) in THF (125 ml) with TEA (4.75 ml) and ethyl chloroformate (3.3 ml). This solution was added slowly with stirring to an ice-cold solu on of *tert*-butoxycarbonylhydrazine[17] (4.5 g) in THF (30 ml) and the mixture was stirred at ice-bath temperature for 1 hr and at room temperature for 4 hr. The suspension was filtered, the filtrate was evaporated, and the residue was dissolved in ethyl acetate (250 ml). This solution was washed successively with four 60-ml portions each of 1 *N* citric acid, water, 1 *N* sodium bicarbonate, and water and was dried over sodium sulfate. The oily residue which ensued when the solvent was evaporated crystallized when triturated with ether–petroleum ether. The compound was recrystallized from the same solvents: 11.0 g (76%); mp 89–90° A mixture of this material with the higher melting form melted at 138–140°: $[\alpha]^{22}D$ $-9.7°$ (*c* 3.26, MeOH); R_f^{1} 0.9; R_f^{I} 0.8; iodine- and chlorine-positive spot. *Anal.* Calcd for $C_{20}H_{30}O_6N_4$: C, 56.9; H, 7.2; N, 13.3. Found: C, 56.9; H, 7.2; N, 13.8.

(Positions 33–34) Valylglycine *tert*-Butoxycarbonylhydrazide. Benzyloxycarbonylvalylglycine *tert*-butoxycarbonylhydrazide (6.00 g) was hydrogenated in MeOH. The catalyst was removed by filtration and the filtrate was evaporated. The residue was crystallized from ethyl acetate (25 ml): needles; 3.19 g (77%); mp 143–145°; $[\alpha]^{28}D$ $+6.9°$ (*c* 3.76, MeOH); R_f^{IV} 0.6; amino acid ratios in AP-M digest, $Val_{1.0}Gly_{1.0}$ (94%). *Anal.* Calcd for $C_{12}H_{24}O_4N_4$: C, 50.0; H, 8.4; N, 19.4. Found: C, 50.3; H, 8.5; N, 19.2.

(Positions 32–34) Benzyloxycarbonylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide. DCC (4.8 g) in methylene chloride (10 ml) was added at room temperature to a methylene chloride solution (200 ml) containing valylglycine *tert*-butoxycarbonylhydrazide (6.6 g) and benzyloxycarbonylthreonine[31] (5.8 g). The mixture was kept at room temperature for 24 hr and filtered. The filtrate was evaporated, the residue was dissolved in ethyl acetate (500 ml), and the solution was washed successively with four 100-ml portions each of 1 *N* citric acid, water, 1 *N* sodium bicarbonate, and water. Following drying over sodium sulfate, the solvent was removed and the residue was crystallized from MeOH–water: 6.0 g (50%); mp 114–115°; $[\alpha]^{23}D$ $-24.0°$ (*c* 3.53, MeOH); R_f^{I} 0.8; R_f^{II} 0.8; amino acid ratios in acid hydrolysate, $Thr_{0.9}Val_{1.0}Gly_{1.1}$ (99%). *Anal.* Calcd for $C_{24}H_{37}O_8N_5$: C, 55.1; H, 7.1; N, 13.4. Found: C, 54.4; H, 7.1; N, 13.2.

(Positions 32–34) Threonylvalylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected tripeptide hydrazide (10.0 g) was hydrogenated in MeOH containing acetic acid (10 ml). The catalyst was removed by filtration and the filtrate was evaporated to dryness. The resultant froth after drying over P_2O_5 was solidified by refluxing with ethyl acetate containing a few drops of acetic acid: 7.1 g (83%); mp 140–142°; $[\alpha]^{26}D$ $-17.3°$ (*c* 2.77, MeOH); R_f^{1} 0.7; R_f^{IV} 0.6; amino acid ratios in AP-M digest, $Thr_{1.0}Val_{1.0}$-$Gly_{1.0}$ (99%).

(Positions 31–34) Benzyloxycarbonyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide. a. By the 2,4,5-Trichlorophenyl Ester Procedure. 2,4,5-Trichlorophenyl benzyloxycarbonyl-γ-*tert*-butylglutamate[14] (8.53 g) was added with stirring to a solution of threonylvalylglycine *tert*-butoxycarbonylhydrazide acetate (6.74 g) and TEA (1.46 ml) in DMF (70 ml) and the solution was kept at room temperature for 42 hr. 3-Dimethylaminopropyl-

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(30) W. Grassmann and E. Wünsch, *Chem. Ber.*, **91**, 449 (1958).
(31) R. B. Merrifield, *J. Biol. Chem.*, **232**, 43 (1958).

amine (0.5 ml) was added and after 1 hr the reaction mixture was poured into ether–water (1:1) (400 ml) containing 1 *N* HCl (10 ml). The precipitate was washed with ether–water, dried, and crystallized from MeOH (45 ml); ether (25 ml) was added after crystallization had begun: 8.70 g (82%); $[\alpha]^{26}D$ −28.8° (*c* 4.68, MeOH); $[\alpha]^{26}D$ −2.9° (*c* 3.32, DMF); R_f^I 0.8; amino acid ratios in acid hydrolysate, $Glu_{1.0}Thr_{0.9}Val_{1.0}Gly_{1.0}$. *Anal.* Calcd for $C_{38}H_{52}O_{11}N_6$: C, 55.9; H, 7.4; N, 11.9. Found: C, 55.8; H, 7.5; N, 12.0.

b. By the *N*-Hydroxysuccinimido Ester Procedure. α-*N*-Hydroxysuccinimido benzyloxycarbonyl-γ-*tert*-butylglutamate (10.0 g) was added to a solution of threonylvalylglycine *tert*-butoxycarbonylhyrazide acetate (7.61 g) and TEA (4.75 ml) in DMF (80 ml) and the solution was stirred for 20 hr at room temperature. Work-up as described under a gave 7.9 g (66%); $[\alpha]^{24}D$ −28.1° (*c* 4.03, MeOH).

(Positions 31–34) γ-*tert*-Butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected tetrapeptide hydrazide (7.0 g) was hydrogenated in MeOH containing acetic acid (5 ml). The catalyst was removed by filtration and the filtrate was evaporated. The residue was crystallized from MeOH (15 ml) with the addition of ethyl acetate (30 ml): 5.77 g (92%); mp 156–158°; $[\alpha]^{27}D$ −21.1° (*c* 3.22, MeOH); $[\alpha]^{24}D$ −26.4°; (*c* 4.22, water); R_f^I 0.8; R_f^{IV} 0.8; amino acid ratios in AP-M digest, $Glu_{1.0}Thr_{1.0}Val_{1.0}Gly_{1.0}$ (94%).

(Positions 30–34) Benzyloxycarbonylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide Monohydrate. *N*-Hydroxysuccinimido benzyloxycarbonylglycinate[13] (2.65 g) was added with stirring to a solution of γ-*tert*-butylglutamylthreonylvalylglycine *tert*-butoxycarbonylhydrazide acetate (4.50 g) and TEA (1.98 ml) in DMF (15 ml) and the mixture was stirred at room temperature for 20 hr. 3-Dimethylaminopropylamine (1 ml) was added and after 0.5 hr the reaction mixture was added to ether–water (1:1) (400 ml) containing acetic acid (5.5 ml). The precipitate was washed with ether–water and crystallized twice from MeOH with water (50 ml): 4.28 g (77%);[32] mp 203–205°; $[\alpha]^{27}D$ −29.5° (*c* 3.87, MeOH); $[\alpha]^{26}D$ −6.1° (*c* 3.11, DMF); R_f^I 0.8. *Anal.* Calcd for $C_{35}H_{55}O_{12}N_7 \cdot H_2O$: C, 53.6; H, 7.3; N, 12.5. Found: C, 53.6; H, 7.4; N, 12.5.

(Positions 30–34) Glycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected pentapeptide hydrazide (4.00 g) was hydrogenated in MeOH containing acetic acid (3 ml). The catalyst was removed by filtration, and the filtrate was evaporated to dryness. The residue was dissolved in water and the solution was lyophilized: 3.39 g (96%); mp 220–225° dec; $[\alpha]^{27}D$ −28.6° (*c* 3.03, MeOH); R_f^I 0.8; R_f^{IV} 0.5; amino acid ratios in AP-M digest, $Gly_{2.0}Glu_{1.0}Thr_{1.0}Val_{1.0}$ (89%).

(Positions 29–34) Benzyloxycarbonyl-β-*tert*-butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide. α-*N*-Hydroxysuccinimido β-*tert*-butyl benzyloxycarbonylaspartate[28] (3.88 g) was added with stirring to a solution of glycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-butoxycarbonylhydrazide acetate (5.20 g) and TEA (2.36 ml) in DMF (25 ml) and the mixture was stirred at room temperature for 20 hr. 3-Dimethylaminopropylamine (0.16 ml) was added and after 30 min the reaction mixture was poured into water (300 ml) containing 1 *N* HCl (21 ml). The resulting precipitate was washed with water and crystallized from MeOH–water (2:1) (50 ml): 6.13 g (87%); mp 184–186°; $[\alpha]^{28}D$ −23.8° (*c* 3.45, MeOH); $[\alpha]^{24}D$ −11.2° (*c* 4.19, DMF); R_f^I 0.8; R_f^{IV} 0.8 *Anal.* Calcd for $C_{43}H_{68}O_{15}N_8$: C, 55.1; H, 7.3; N, 12.0. Found: C, 54.9; H, 7.3; N, 11.6.

(Positions 29–34) β-*tert*-Butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected hexapeptide hydrazide (4.2 g) was hydrogenated in MeOH containing acetic acid. The catalyst was removed by filtration, the filtrate was evaporated, and the residue was precipitated from EtOH by ether: 3.52 g (91%); $[\alpha]^{27}D$ −24.2° (*c* 2.51, MeOH); R_f^1 0.9; R_f^{IV} 0.8.

A sample for AP-M digestion was deblocked with TFA for 1 hr at room temperature, followed by precipitation with ether; amino acid ratios in AP-M digest (twice the normal concentration of enzyme for 40 hr): $Asp_{1.0}Gly_{1.7}Glu_{1.0}Thr_{1.0}Val_{1.0}$ (83%). (See Table I for amino acid recoveries.)

(Positions 28–34) Benzyloxycarbonyl-γ-*tert*-butylglutamyl-β-*tert*-butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide. α-*N*-Hydroxysuccinimido γ-*tert*-butyl benzyloxycarbonylglutamate (8.17 g) was added with stirring to a solution of β-*tert*-butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-butoxycarbonylhydrazide acetate (16.20 g) and TEA (5.25 ml) in DMF (60 ml) and the mixture was stirred at room temperature for 20 hr. The reaction mixture was added to water (800 ml). The precipitate was washed with water and precipitated from MeOH with water: 17.33 g (82%); mp 204–206° dec; $[\alpha]^{27}D$ −30.7° (*c* 1.21, MeOH); R_f^I 0.8; R_f^{IV} 0.8. *Anal.* Calcd for $C_{52}H_{83}O_{18}N_9$: C, 55.7; H, 7.5; N, 11.2. Found: C, 55.5; H, 7.4; N, 11.4.

(Positions 28–34) γ-*tert*-Butylglutamyl-β-*tert*-butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected heptapeptide hydrazide (17.20 g) was hydrogenated in MeOH containing acetic acid. The catalyst was removed by filtration, the filtrate was evaporated, and the residue was precipitated from EtOH by ether: 16.50 g (102%); $[\alpha]^{28}D$ −23.0° (*c* 2.42, MeOH); R_f^1 0.9; R_f^{IV} 0.7.

A sample for AP-M digestion was deblocked with TFA for 1 hr at room temperature, followed by precipitation with ether; amino acid ratios in AP-M digest (twice the normal concentration of enzyme for 72 hr): $Glu_{2.1}Asp_{1.0}Gly_{1.9}Thr_{1.0}Val_{1.0}$ (78%).

(Positions 27–34) Benzyloxycarbonylhistidyl-γ-*tert*-butylglutamyl-β-*tert*-butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide. An ethyl acetate solution of benzyloxycarbonylhistidine azide, prepared from 3.04 g of the hydrazide,[12] was added with stirring to an ice-cold solution of γ-*tert*-butylglutamyl-β-*tert*-butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-butoxycarbonylhydrazide acetate (5.24 g) and TEA (0.70 ml) in DMF (50 ml). After stirring for 5 hr at 0° a fresh quantity of azide, prepared from 1.214 g of hydrazide, was added and stirring continued at 0° for 16 hr. The reaction mixture was concentrated to a small volume and the product was precipitated by addition of ethyl acetate; 6.77 g. The material was distributed between 1-butanol–ethyl acetate (1:1) and 6% ammonium hydroxide in six separatory funnels (120-ml upper and 50-ml lower phase). The butanol layers were then washed with two 75-ml portions of water and evaporated. The residue was dissolved in DMF (18 ml) and precipitated by addition of ethyl acetate (250 ml): 4.76 g (75%); mp 182–183° dec; $[\alpha]^{27}D$ −28.8° (*c* 2.17, MeOH); R_f^I 0.7. *Anal.* Calcd for $C_{58}H_{90}O_{19}N_{12}$: C, 55.3; H, 7.2; N, 13.3. Found: C, 55.4; H, 7.2; N, 13.1.

(Positions 27–34) Histidyl-γ-*tert*-butylglutamyl-β-*tert*-butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide Diacetate. The protected octapeptide hydrazide (4.70 g) was hydrogenated in MeOH containing acetic acid. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was precipitated from EtOH by ether: 4.20 g (90%); $[\alpha]^{27}D$ −28.3° (*c* 2.07, MeOH); R_f^1 0.8; R_f^I 0.5, trace 0.1; R_f^{IV} 0.7, trace 0.1. A sample was deblocked with TFA for 1 hr at room temperature, followed by precipitation with ether; amino acid ratios in AP-M digest (twice the normal concentration of enzyme for 72 hr): $His_{1.3}Glu_{2.0}Asp_{1.0}Gly_{1.0}Thr_{1.0}Val_{1.0}$ (64%).

(Positions 26–34) Benzyloxycarbonylleucylhistidyl-γ-*tert*-butylglutamyl-β-*tert*-butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide. *N*-Hydroxysuccinimido benzyloxycarbonylleucinate (2.33 g)[13] was added with stirring to a solution of histidyl-γ-*tert*-butylglutamyl-β-*tert*-butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-butoxycarbonylhydrazide diacetate (8.0 g) and TEA (2.70 ml) in DMF (60 ml) and the solution stirred at room temperature for 18 hr. The reaction mixture was poured into water and the precipitate was collected and reprecipitated from DMF (60 ml) with water (90 ml): 7.38 g (83%); mp 209–211° dec; $[\alpha]^{27}D$ −32.9° (*c* 1.03, MeOH); R_f^I 0.6; R_f^{IV} 0.8; amino acid ratios in acid hydrolysate, $Leu_{1.0}His_{1.0}Glu_{2.0}Asp_{1.0}Gly_{2.0}Thr_{1.0}Val_{1.0}$. *Anal.* Calcd for $C_{64}H_{101}O_{20}N_{13}$: C, 56.0; H, 7.4; N, 13.3. Found: C, 56.1; H, 7.3; N, 13.3.

(Positions 26–34) Leucylhistidyl-γ-*tert*-butylglutamyl-β-*tert*-butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide Diacetate. The protected nonapeptide hydrazide (7.50 g) was hydrogenated in MeOH containing acetic acid. The catalyst was removed by filtration and the filtrate was evaporated. The residue was distributed between 1-butanol and 7% aqueous acetic acid in five separatory funnels (80 ml of upper and 80 ml of lower phase). The butanol layers were washed with two 80-ml portions of water and were concentrated to a small volume. Water was added and the residue was lyophilized: 6.63 g (89%); $[\alpha]^{28}D$ −29.0° (*c* 1.67, 1-butanol–acetic acid–water, 3:1:1); R_f^1 0.8; R_f^I 0.5.

A sample was deblocked with TFA for 1 hr at room temperature, followed by precipitation with ether; amino acid ratios in acid

(32) Similar results were obtained when using 2,4,5-trichlorophenyl benzyloxycarbonylglycinate[14] as the acylating component.

hydrolysate, $Leu_{1.0}His_{0.9}Glu_{2.1}Asp_{1.0}Gly_{2.0}Thr_{1.0}Val_{1.1}$ (96%); amino acid ratios in AP-M digest (twice the normal concentration of enzyme for 72 hr), $Leu_{1.0}His_{1.1}Glu_{2.0}Asp_{1.0}Gly_{1.8}Thr_{1.0}Val_{1.0}$ (70%)

(Positions 24–34) Benzyloxycarbonyltyrosylglutaminylleucylhistidyl-γ-*tert*-butylglutamyl-β-*tert*-butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-Butoxycarbonylhydrazide (Fragment C). *tert*-Butyl nitrite (0.51 ml) was added to a solution of benzyloxycarbonyltyrosylglutamine hydrazide (2.061 g) and 4.8 *N* hydrogen chloride in dioxane (8.4 ml) in DMF (25 ml) cooled to −15°. The reaction was stirred for 20 min, cooled to −40°, and TEA (5.70 ml) was added. This azide solution was added to a mixture of leucylhistidyl-γ-*tert*-butylglutamyl-β-*tert*-butylaspartylglycyl-γ-*tert*-butylglutamylthreonylvalylglycine *tert*-butoxycarbonylhydrazide diacetate (4 077 g) and TEA (0.84 ml) in DMF (30 ml) at 0°. After 1 hr the pH of the reaction mixture was readjusted to 8 by addition of TEA. The mixture was stirred for 5 hr at 0°, then a fresh quantity of azide, prepared from 2.61 g of hydrazide as described, was added and stirring was continued for 16 hr at 0°. The reaction mixture was concentrated to a small volume and 1-butanol equilibrated with 5% aqueous acetic acid (250 ml) was added. The solution was washed with 5% aqueous acetic acid (equilibrated with 1-butanol) (150 ml) and water (150 ml). Addition of saturated aqueous sodium bicarbonate precipitated a gel. The aqueous phase was separated and the organic phase was washed repeatedly with water (approximately 3 l.) until only an aqueous suspension of granular gel remained. The combined washings were in turn extracted with 1-butanol (100 ml) and this was repeatedly extracted with water until only an aqueous suspension of granular gel remained. The two suspensions were combined and the granular gel collected by filtration, washed with MeOH (200 ml), and dried: 3.95 g; R_f^{I} 0.7; R_f^{III} 0.8, trace 0.7.

The granular solid was refluxed for 15 min with MeOH (100 ml), the solution was cooled, and the precipitate was collected and dried. This material was precipitated from DMF (50 ml) by water: 3.45 g (69%); mp 218–220° dec; $[\alpha]^{27}D$ −23.6° (*c* 1.72, DMF); R_f^{I} 0.7; R_f^{III} 0.8; single chlorine positive spot; amino acid ratios in acid hydrolysate, $Tyr_{0.9}Leu_{1.0}His_{1.0}Glu_{3.3}Asp_{1.0}Gly_{2.0}Thr_{0.9}Val_{1.0}$. *Anal.* Calcd for $C_{78}H_{118}O_{24}N_{16}$: C, 56.3; H, 7.1; N, 13.5. Found: C, 56.5; H, 7.3; N, 13.4.

Fragment C Hydrazide Trifluoroacetate. Fragment C (666 mg) was dissolved in chilled 90% TFA (12 ml) and the solution was kept at room temperature for 40 min. The solution was evaporated to a small volume at 20° and the TFA salt was precipitated with ice-cold ether, washed with ether, and dried over KOH pellets: colorless amorphous solid; 610 mg; R_f^{I} 0.4; R_f^{III} 0.6; amino acid ratios in acid hydrolysate, $Tyr_{0.9}Glu_{3.1}Leu_{1.0}His_{1.0}Asp_{1.0}Gly_{2.0}Thr_{1.0}Val_{1.0}$ (100%). A sample of this material was hydrogenated in water containing 2 drops of TFA, the catalyst was removed by filtration, and the filtrate was lyophilized: R_f^{I} 0.1; R_f^{III} 0.5; amino acid ratios in AP-M digest (twice the normal concentration of enzyme for 72 hr), $Tyr_{1.2}Gln_{0.8}Leu_{1.0}His_{1.3}Glu_{2.1}Asp_{1.0}Gly_{1.8}Thr_{0.9}Val_{0.9}$ (76%).

Preparation of Fragment D (Positions 35–47). (Positions 46–47) Benzyloxycarbonyl-γ-*tert*-butylglutamylglycine. A solution of α-*N*-hydroxysuccinimido benzyloxycarbonyl-γ-*tert*-butylglutamate (26.04 g) in THF (180 ml) was added to a solution of glycine (4. 44 g) and TEA (16.8 ml) in water (180 ml). The mixture was stirred for 5 hr at rt when the bulk of the solvents was evaporated. The residual solution was acidified with 1 *N* citric acid and extracted with three portions of ethyl acetate. The ethyl acetate layers were washed once with 1 *N* citric acid and three times with saturated sodium chloride and dried over sodium sulfate. Evaporation of the solvent gave an oily residue which solidified when triturated with a mixture of ether and petroleum ether. The compound was reprecipitated from ether with petroleum ether: 19.23 g (81%); mp 49–54°; $[\alpha]^{27}D$ −13.2° (*c* 3.57, MeOH); R_f^{I} 0.7; R_f^{III} 0.7. *Anal.* Calcd for $C_{19}H_{26}O_7N_2$: C, 57.9; H, 6.6; N, 7.1. Found: C, 57.8; H, 6.4; N, 7.3.

(Positions 46–47) Benzyloxycarbonyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide. DCC (12.36 g) was added to a solution cooled at −5° of benzyloxycarbonyl-γ-*tert*-butylglutamylglycine (20.51 g) and *tert*-butoxycarbonylhydrazine[17] (7.93 g) in THF (120 ml). The mixture was stirred for 1 hr at 0° and for 20 hr at room temperature and was filtered. The solvent was removed, the residue was dissolved in ethyl acetate, and the solution was washed three times with 1 *N* citric acid, once with saturated sodium chloride, three times with saturated sodium bicarbonate, and three times with saturated sodium chloride and dried over sodium sulfate. The solvent was evaporated to give a viscous oily residue, which was dissolved in ether and petroleum ether was added. The resulting oily precipitate solidified when triturated with petroleum ether: 24.40 g (92%; mp 68–73° (sintering at 60°); $[\alpha]^{28}D$ −8.8° (*c* 4.36, MeOH); R_f^{I} 0.7; R_f^{III} 0.8. *Anal.* Calcd for $C_{24}H_{36}O_8N_4$: C, 56.7; H, 7.1; N, 11.0. Found: C, 56.5; H, 7.4; N, 11.1.

(Positions 46–47) γ-*tert*-Butylglutamylglycine *tert*-Butoxycarbonylhydrazide. The protected dipeptide hydrazide (21.40 g) in MeOH (150 ml) was hydrogenated over palladium. The catalyst was removed by filtration and the solvent evaporated. Water (100 ml) was added to the residue and the resulting crystalline product was collected, washed with ether, and recrystallized from EtOH–water: needles; 14.41 g (91%); $[\alpha]^{26}D$ +5.6° (*c* 3.99, MeOH); R_f^{1} 0.8; R_f^{2} 0.9; R_f^{I} 0.5; amino acid ratios in AP-M digest, γ-O-*tert*-$BuGlu_{1.1}Gly_{0.9}$ (96%).

(Positions 45–47) Benzyloxycarbonyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide. 2,4,5-Trichlorophenyl benzyloxycarbonyltyrosinate[14] (18.80 g) was added to a solution of γ-*tert*-butylglutamylglycine *tert*-butoxycarbonylhydrazide (14.23 g) in DMF (30 ml) and THF (30 ml) containing TEA (5.32 ml). The mixture was allowed to stand for 15 hr at room temperature, when the bulk of the solvents was removed. The residue was dissolved in ethyl acetate, the solution was washed three times with 1 *N* citric acid, once with saturated sodium chloride, three times with saturated sodium bicarbonate, and three times with saturated sodium chloride, and dried over sodium sulfate. Evaporation of the solvent gave a crystalline residue, which was recrystallized from ethyl acetate: needles; 21.52 g (84%); mp 171–172°; $[\alpha]^{26}D$ −7.4° (*c* 4.53, MeOH); R_f^{I} 0.7. *Anal.* Calcd for $C_{33}H_{45}O_{10}N_5$: C, 59.0; H, 6.8; N, 10.4. Found: C, 58.9; H, 6.8; N, 10.6.

(Positions 45–47) Tyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected tripeptide hydrazide (20.15 g) was hydrogenated over palladium in MeOH (100 ml) and 10% acetic acid (50 ml). The catalyst was removed by filtration and the solvent was evaporated to dryness. The residue was dissolved in a small volume of EtOH and ether was added. The ensuing precipitate was collected and dried: 16.94 g (94%); $[\alpha]^{26}D$ +1.1° (*c* 3.63, MeOH); R_f^{1} 0.8; R_f^{2} 0.9; R_f^{I} 0.7; amino acid ratios in AP-M digest, $Tyr_{1.0}$γ-O-*tert*-$BuGlu_{1.0}Gly_{1.0}$ (95%).

(Positions 44–47) Benzyloxycarbonylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide. 2,4,5-Trichlorophenyl benzyloxycarbonylasparaginate (12.93 g) was added to a solution of tyrosyl-γ-*tert*-butylglutamylglycine *tert*-butoxycarbonylhydrazide acetate (16.96 g) in DMF (45 ml) and THF (45 ml) containing TEA (7.93 ml). The mixture was kept at room temperature for 20 hr; then the bulk of the solvents was evaporated, and the residue was diluted with ethyl acetate. The solution was washed three times with 1 *N* citric acid and three times with saturated sodium chloride and was concentrated to a small volume. The resulting amorphous product was collected by filtration and precipitated from MeOH with ethyl acetate: 16.5 g (74%); mp 174–176°; $[\alpha]^{28}D$ −21.1° (*c* 4.87, DMF); R_f^{I} 0.7; R_f^{III} 0.8. *Anal.* Calcd for $C_{37}H_{51}O_{12}N_7$: C, 56.6; H, 6.5; N, 12.5. Found: C, 56.4; H, 6.6; N, 12.3.

(Positions 44–47) Asparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected tetrapeptide hydrazide (15.7 g) was hydrogenated over palladium in MeOH (120 ml) and 10% acetic acid (35 ml). The catalyst was removed by filtration and the solution was evaporated to dryness. The residue was washed with ether and dried: 14.23 g (100%); $[\alpha]^{28}D$ −4.1° (*c* 4.07, MeOH); R_f^{1} 0.7; R_f^{2} 0.9; R_f^{I} 0.5; amino acid ratios in AP-M digest, $Asn_{1.0}Tyr_{1.0}$γ-O-*tert*-$BuGlu_{1.0}Gly_{1.0}$ (92%).

(Positions 43–47) Benzyloxycarbonylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide. 2,4,5-Trichlorophenyl benzyloxycarbonylasparaginate (13.38 g) was added to a solution of asparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-butoxycarbonylhydrazide acetate (14.23 g) in DMF (50 ml) and THF (50 ml) containing TEA (3.1 ml). The mixture was allowed to stand for 15 hr at room temperature, then ethyl acetate (300 ml) and 1 *N* citric acid (100 ml) were added, and the mixture was shaken. The resulting precipitate was collected by filtration, washed once with 1 *N* citric acid, three times with water, and twice with ethyl acetate and dried. This material was suspended in hot MeOH and water was added to obtain a clear solution which was placed in a refrigerator. The ensuing amorphous precipitate was collected and dried: 13.71 g (76%); mp 202–203° dec; $[\alpha]^{27}D$ −25.0° (*c* 3.43, DMF); R_f^{I} 0.7; R_f^{III} 0.7 *Anal.* Calcd for $C_{41}H_{57}O_{14}N_9$: C, 54.7; H, 6.4; N, 14.0. Found: C, 54.7; H, 6.7; N, 13.8.

(Positions 43–47) Asparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected pentapeptide hydrazide (20.0 g) was hydrogenated over palladium in MeOH (200 ml), water (100 ml), and glacial acetic acid (20 ml). The catalyst was removed by filtration and the solution was evaporated to dryness. The residue was washed with ether, collected, and dried: 18.05 g (98%); $[\alpha]^{24}D$ −14.8° (*c* 3.30, 50% acetic acid); R_f^1 0.5; R_f^2 0.9; R_f^I 0.4. A sample (50 mg) was dissolved in TFA (0.5 ml), the solution was kept at room temperature for 1 hr, and ether was added. The resulting precipitate was collected and dried: 46 mg; R_f^I 0.2; R_f^{III} 0.4; amino acid ratios in AP-M digest, $Asn_{2.0}Tyr_{1.0}Glu_{1.0}Gly_{1.0}$ (90% based on bis(trifluoroacetate)).

(Positions 42–47) Benzyloxycarbonyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide. 2,4,5-Trichlorophenyl benzyloxycarbonyltyrosinate[14] (7.42 g) was added to a solution of asparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-butoxycarbonylhydrazide acetate (9.09 g) in DMF (70 ml) containing TEA (2.8 ml). The mixture was allowed to stand for 20 hr at room temperature and water (700 ml) was added. The resulting precipitate was collected, washed with ether, and dried. This material was suspended in hot MeOH and water was added to obtain a clear solution which was placed in a refrigerator. The amorphous precipitate was collected and dried: 8.95 g (77%); mp 208–209° dec; $[\alpha]^{26}D$ −27.6° (*c* 4.23, DMF); R_f^I 0.8 with impurities at 0.9 and 0.7; R_f^{III} 0.8. *Anal.* Calcd for $C_{50}H_{66}O_{16}N_{10}$: C, 56.5; H, 6.3; N, 13.2. Found: C, 56.6; H, 6.5; N, 12.9.

(Positions 42–47) Tyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected hexapeptide hydrazide (6.0 g) was hydrogenated over palladium in MeOH (100 ml), 1-butanol (150 ml), water (100 ml), and glacial acetic acid (50 ml). The catalyst was removed by filtration, the solution was evaporated to dryness, and ether (150 ml) was added. The resulting amorphous product was collected and dried: 5.50 g (98%); $[\alpha]^{24}D$ −13.9° (*c* 3.44, 50% acetic acid); R_f^1 0.6; R_f^3 5.4 × His; R_f^I 0.5; R_f^{III} 0.8. A sample (50 mg) was dissolved in TFA (0.5 ml), the solution was kept at room temperature for 1 hr, and ether was added. The ensuing precipitate was collected and dried: 44 mg; R_f^I 0.2; R_f^{III} 0.5; amino acid ratios in AP-M digest, $Tyr_{2.1}Asn_{2.0}Glu_{1.1}Gly_{1.0}$ (90% based on bis(trifluoroacetate)).

(Positions 41–47) Benzyloxycarbonyl-N^ϵ-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide. 2,4,5-Trichlorophenyl benzyloxycarbonyl-N^ϵ-formyllysinate[14] (5.85 g) was added to a solution of tyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-butoxycarbonylhydrazide acetate (10.88 g) in DMF (200 ml) containing TEA (2.24 ml). The mixture was allowed to stand for 20 hr at room temperature when water (700 ml) was added. The amorphous precipitate was collected, washed with water, and dried. This material was suspended in hot MeOH and water was added to obtain a clear solution which was placed in a refrigerator. The resulting product was collected by filtration and dried: 9.14 g (68%); mp 210–211° dec; $[\alpha]^{26}D$ −25.4° (*c* 3.75, DMF); R_f^I 0.7 with impurities at 0.6 and 0.8; R_f^{III} 0.8. *Anal.* Calcd for $C_{57}H_{78}O_{18}N_{12}$: C, 56.2; H, 6.4; N, 13.8. Found: C, 56.1; H, 6.6; N, 13.5.

(Positions 41–47) N^ϵ-Formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected heptapeptide hydrazide (9.0 g) was hydrogenated over palladium in MeOH (200 ml), 1-butanol (100 ml), and 10% aqueous acetic acid (150 ml). The catalyst was removed by filtration, the solution was evaporated to dryness, and ether (300 ml) was added. The resulting amorphous product was collected by filtration and dried: 8.12 g (96%); $[\alpha]^{23}D$ −15.2° (*c* 2.48, 50% acetic acid); R_f^I 0.3 with impurities at 0.4, 0.2, and 0.1; R_f^{III} 0.7. A sample (50 mg) was dissolved in TFA (0.5 ml), the solution was kept at room temperature for 1 hr, and ether was added. The resulting precipitate was collected and dried, 48 mg; R_f^I 0.2; R_f^{III} 0.5; amino acid ratios in AP-M digest, $Formlys_{1.0}Tyr_{2.1}Asn_{2.0}Glu_{1.0}Gly_{1.0}$ (90% based on bis(trifluoroacetate)).

(Positions 40–47) N^α-Benzyloxycarbonylhistidyl-N^ϵ-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide. Sodium nitrite (0.52 g) dissolved in ice-cold water (7 ml) was added slowly to an ice-cold stirred solution of N^α-benzyloxycarbonylhistidine hydrazide[12] (2.25 g) in 4 *N* hydrochloric acid (5.55 ml) and THF (20 ml). The mixture was stirred at −5° for 10 min and TEA (2.1 ml) was added. To this solution containing the azide was added a solution of N^ϵ-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-butoxycarbonylhydrazide acetate (4.24 g) in DMF (40 ml) containing TEA (0.52 ml). The mixture was stirred at 4° for 20 hr and water (500 ml) was added. The resulting amorphous product was collected, washed with water, and ether, and dried. The compound was suspended in hot MeOH and water was added to obtain a clear solution which was placed in a refrigerator. The ensuing gelatinous mass was collected and dried: 3.51 g (63%); mp 215–217° dec; $[\alpha]^{27}D$ −28.8° (*c* 3.36, DMF); R_f^I 0.4 with impurities at 0.5 and 0.3; R_f^{III} 0.8. *Anal.* Calcd for $C_{63}H_{85}O_{19}N_{15}$: C, 55 8: H, 6.3; N, 15.5. Found: C. 55.8; H, 6.4; N, 15.6.

(Positions 40–47) Histidyl-N^ϵ-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide Diacetate. The protected octapeptide hydrazide (5.90 g) was hydrogenated over palladium in MeOH (100 ml), 1-butanol (30 ml), and 10% acetic acid (70 ml). The catalyst was removed by filtration, the solution was evaporated to dryness, and ether (50 ml) was added. The resulting amorphous material was collected and dried: 5.71 g (102%); $[\alpha]^{28}D$ −21.3° (*c* 2.73, 50% acetic acid); R_f^I 0.2 with impurities at 0.3 and 0.4; R_f^{III} 0.5. A sample (50 mg) was dissolved in TFA (0 5 ml), the solution was kept at room temperature for 1 hr, and ether was added. The resulting precipitate was collected and dried: 43 mg; amino acid ratios in AP-M digest, $His_{1.0}Formlys_{0.9}Tyr_{2.0}Asn_{2.0}Glu_{1.0}Gly_{0.9}$ (86% based on tris(trifluoroacetate)).

(Positions 39–47) Benzyloxycarbonylprolylhistidyl-N^ϵ-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide. *N*-Hydroxysuccinimido benzyloxycarbonylprolinate[13] (5.19 g) was added to a solution of histidyl-N^ϵ-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-butoxycarbonylhydrazide diacetate (13.42 g) in DMF (100 ml) containing TEA (2.8 ml). The mixture was allowed to stand at room temperature for 5 hr, then the solvent was evaporated, and water (500 ml) and ethyl acetate (150 ml) were added to the residue. The mixture was shaken and was kept in a refrigerator. The ensuing amorphous product was collected and dried. The compound was suspended in hot MeOH and water was added to obtain a clear solution which was kept in a refrigerator. The gelatinous mass thus obtained was collected and dried: 11.80 g (78%); mp 207–208° dec; $[\alpha]^{26}D$ −38.4° (*c* 1.18, DMF); R_f^I 0.4 with impurities at 0.5 and 0.3; R_f^{III} 0.6. *Anal.* Calcd for $C_{68}H_{92}O_{20}N_{16}$: C, 56.2; H, 6.4; N, 15.4. Found: C, 56.0; H, 6.7; N, 15.1.

(Positions 39–47) Prolylhistidyl-N^ϵ-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide Diacetate. The protected nonapeptide hydrazide (11.6 g) was hydrogenated over palladium in MeOH (150 ml) and 10% acetic acid (150 ml). The catalyst was removed by filtration, the solution was evaporated to dryness, and the residue was triturated with ether, collected by filtration, and dried: 10.8 g (94%); $[\alpha]^{26}D$ −32.8° (*c* 1.22, 50% acetic acid); R_f^1 0.3; R_f^3 6.0 × His; R_f^I 0.1 with trace impurities; R_f^{III} 0.5. A sample (50 mg) was dissolved in TFA (0.5 ml); the solution was kept at room temperature for 1 hr and ether was added. The ensuing precipitate was collected and dried: 48 mg; R_f^I 0.1; R_f^{III} 0.3; amino acid ratios in AP-M digest, $Pro_{1.2}His_{1.0}Formlys_{1.0}Tyr_{2.0}Asn_{1.9}Glu_{1.0}Gly_{0.9}$ (91% based on tris(trifluoroacetate)).

(Positions 37–47) Benzyloxycarbonylseryltyrosylprolylhistidyl-N^ϵ-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide. *tert*-Butyl nitrite (2.5 ml) was added to a solution cooled at −20° of benzyloxycarbonlyseryltyrosinehydrazide[33] (8.76 g) in DMF (80 ml) containing 5.5 *N* hydrogen chloride in dioxane (15.2 ml). The mixture was stirred at −20° for 30 min and then cooled to −40° and TEA (8.8 ml) was added. To this solution containing the azide of Z-Ser-Tyr was added an ice-cold solution of prolylhistidyl-N^ϵ-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-butoxycarbonylhydrazide diacetate (10.08 g) in DMF (80 ml) containing TEA (1.96 ml). The mixture was stirred for 1 hr at −20° and at 4° for 20 hr and the solvents were evaporated. To the residue was added water (500 ml) and ethyl acetate (250 ml) and the mixture was shaken and then placed in a refrigerator. The resulting amorphous material was collected and dried. This compound was reprecipitated from DMF with ethyl acetate, washed with MeOH, and dried: 11.81 g (99%); mp 210–211° dec; $[\alpha]^{27}D$ −25.9° (*c* 2.18, DMF); R_f^I 0.5 with major impurity at 0.6; R_f^{III} 0.8. *Anal.* Calcd for $C_{80}H_{106}O_{24}N_{18}$: C, 56.4; H, 6.3; N, 14.8. Found: C, 56.3; H, 6.2; N, 14.6.

(33) K. Hofmann, A. Jöhl, A. E. Furlenmeier, and H. Kappeler, *J. Amer. Chem. Soc.*, **79**, 1636 (1957).

(Positions 37–47) Seryltyrosylprolylhistidyl-N^{ϵ}-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide Diacetate. The protected undecapeptide hydrazide (11.75 g) was hydrogenated over palladium in MeOH (200 ml) and 10% acetic acid (200 ml). The catalyst was removed by filtration, the solvents were evaporated to dryness, and the residue was triturated with ether and collected by filtration. The product was washed with warm EtOH and dried: 10.61 g (94%); $[\alpha]^{27}D$ −38.9° (*c* 1.94, 50% acetic acid); R_f^1 0.3; R_f^2 0.6; R_f^3 4.9 × His; R_f^I 0.2 streaking; R_f^{III} 0.6; amino acid ratios in AP-M digest, $Ser_{1.0}Tyr_{3.0}Pro_{1.0}His_{0.9}Formlys_{1.0}Asn_{2.0}$γ-O-*tert*-$BuGlu_{1.0}Gly_{1.0}$ (88%).

(Positions 36–47) Benzyloxycarbonylasparaginylseryltyrosylprolylhistidyl-N^{ϵ}-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide. 2,4,5-Trichlorophenyl benzyloxycarbonylasparaginate (4.29 g) was added to a solution of seryltyrosylprolylhistidyl-N^{ϵ}-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-butoxycarbonylhydrazide diacetate (10.48 g) in DMF (100 ml) containing TEA (1.74 ml). The mixture was allowed to stand at room temperature for 15 hr when additional 2,4,5-trichlorophenyl benzyloxycarbonylasparaginate (1.38 g) was added. The mixture was kept for 15 hr at room temperature. The solvent was evaporated, water (500 ml) and ethyl acetate (300 ml) were added, and the mixture was shaken and placed in a refrigerator. The resulting amorphous compound was collected, washed with water, and dried. The compound was precipitated from DMF with ethyl acetate and washed with warm EtOH: 11.0 g (94%); mp 208–209° dec; $[\alpha]^{27}D$ −31.0° (*c* 1.87, DMF); R_f^I 0.4 with impurities at 0.7, 0.5, 0.3, and 0.2; R_f^{III} 0.7. *Anal.* Calcd for $C_{84}H_{112}O_{26}N_{20}$: C, 55.5; H, 6.2; N, 15.4; O, 22.9. Found: C, 55.5; H, 6.4; N, 15.2; O, 22.9.

(Positions 36–47) Asparaginylseryltyrosylprolylhistidyl-N^{ϵ}-formyllysyltyrosylasparaginylapsaraginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide Diacetate. The protected dodecapeptide hydrazide (10.70 g) was hydrogenated in MeOH (250 ml), water (200 ml), and 10% acetic acid (100 ml). The catalyst was removed by filtration, the solution was evaporated to dryness, and the residue was triturated with ether. The solid was collected, washed with warm EtOH, and dried: 9.40 g (92%); $[\alpha]^{22}D$ − 37.8° (*c* 1.24, 50% acetic acid); R_f^1 0.5; R_f^3 3.4 × His; R_f^I 0.2 with heavy streaking; R_f^{III} 0.6. A sample (45 mg) was kept in TFA (0.45 ml) at room temperature for 1 hr and ether was added. The precipitate was collected, washed with ether, and dried: 39 mg; R_f^I 0.1; R_f^{III} 0.5; amino acid ratios in AP-M digest, Asn + $Ser_{4.3}Pro_{1.0}His_{0.9}Formlys_{1.0}Tyr_{3.0}Glu_{1.0}Gly_{1.0}$ (83% based on tris(trifluoroacetate)).

(Positions 35–47) Benzyloxycarbonylserylasparaginylseryltyrosylprolylhistidyl-N^{ϵ}-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-Butoxycarbonylhydrazide (Fragment D). Sodium nitrite (2.52 g) in ice-cold water (15 ml) was added slowly to an ice-cold stirred solution of benzyloxycarbonylserine hydrazide[11] (9.12 g) in 4 *N* hydrochloric acid (27 ml) and THF (100 ml). The mixture was stirred at −5° for 10 min when TEA (10.1 ml) was added. To this solution containing the azide of Z-Ser was added an ice-cold solution of asparaginylseryltyrosylprolylhistidyl-N^{ϵ}-formyllysyltyrosylasparaginylasparaginyltyrosyl-γ-*tert*-butylglutamylglycine *tert*-butoxycarbonylhydrazide diacetate (10.46 g) in DMF (120 ml) containing TEA (1.68 ml). The mixture was stirred at 4° for 20 hr when the bulk of the solvents was evaporated. Water (500 ml) and ethyl acetate (250 ml) were added and the mixture was shaken and placed in a refrigerator. The amorphous material was collected, precipitated twice from DMF with ethyl acetate. For further purification the compound was suspended in hot EtOH, the solution was cooled, and ether was added. The amorphous product was collected and dried: 9.04 g (79%); mp 201–202° dec; $[\alpha]^{28}D$ −25.6° (*c* 1.63, DMF); R_f^I 0.5 with impurity at 0.3; R_f^{III} 0.8. *Anal.* Calcd for $C_{87}H_{117}O_{28}N_{21}$: C, 54.8; H, 6.2; N, 15.4; O, 23.5. Found: C, 54.9; H, 6.2; N, 15.1; O, 23.5.

Fragment D *tert*-Butoxycarbonylhydrazide. Crude fragment D (450 mg) was hydrogenated for 14 hr over palladium in a mixture of 50% aqueous MeOH (225 ml) and 10% acetic acid (75 ml). The catalyst was removed by filtration, the filtrate was evaporated to dryness, and the residue was washed with EtOH and dried to give 400 mg of a tan-colored solid: $[\alpha]^{27}D$ −40.8° (*c* 1.0, 50% acetic acid); streaks in solvent systems 1, 3, I, and III; amino acid ratios in hydrolysate, $Ser_{1.9}Asp_{3.1}Tyr_{2.5}Pro_{1.1}His_{0.8}Lys_{1.0}Glu_{1.0}Gly_{1.0}$ (100%). A sample (45 mg) was dissolved in TFA (0.45 ml) and the solution was kept at room temperature for 1 hr. The product was precipitated by addition of ether, washed with ether, and dried: 38 mg; streaks in solvent systems I and III; amino acid ratios in AP-M digest, (Ser + $Asn)_{5.4}Tyr_{3.1}Pro_{1.1}His_{1.0}Formlys_{0.9}Glu_{1.0}Gly_{0.6}$ (85%).

A typical purification was carried out as follows: crude fragment D *tert*-butoxycarbonylhydrazide (716 mg) dissolved in 6 ml of the lower phase of the solvent system 1-butanol–acetic acid–water (4:1:5) was applied to a column (1.9 × 150 cm) of Sephadex G 50 which was poured with lower and equilibrated with upper phase of the same solvent. The column was then eluted with upper phase solvent at a flow rate of approximtely 3 ml/hr. Chlorine-positive material appeared after some 150–200 ml of solvent had passed through the column. Chlorine-positive tubes were analyzed by tlc and fractions containing single spot material in solvent system I (R_f 0.2) were pooled and evaporated. The residue was washed by centrifugation with ethyl acetate and dried: 400 mg of a colorless powder was obtained; $[\alpha]^{28}D$ −37.9° (*c* 1.04, 50% acetic acid); R_f^I 0.2, sharp single spot (Figure 3, channel I); amino acid ratios in acid hydrolysate, $Ser_{1.8}Asp_{3.1}Tyr_{2.7}Pro_{1.1}His_{1.0}Lys_{1.1}Glu_{1.2}Gly_{1.1}$; amino acid ratios in AP-M digest, Ser + $Asn_{5.2}Tyr_{2.9}Pro_{1.0}His_{0.9}Formlys_{1.0}$γ-O-*tert*-$BuGlu_{1.0}Gly_{1.0}$ (84%). Eight to ten days were required to operate the column. The chromatogram shown on Figure 3 was obtained as follows: starting with the first chlorine positive tube and eliminating tubes containing homogeneous material (channel I) eluents corresponding to ten tubes each were pooled and evaporated to dryness, and their weights were recorded. In a typical experiment these weights and the amounts spotted were as follows: channel A, tubes 1–10 (11 mg; 50 μg); channel B, tubes 11–20 (8 mg; 30 μg); channel C, tubes 21–30 (9 mg; 30 μg); channel D, tubes 31–40 (12 mg; 30 μg); channel E, tubes 41–50 (12 mg; 30 μg); channel F, tubes 51–60 (15 mg; 30 μg); channel G, tubes 61–70 (17 mg; 10 μg); channel H, tubes 71–80 (19 mg; 10 μg); channel I, tubes 81–120 (400 mg; 5 μg); channel J, tubes 121–130 (25 mg; 10 μg); channel K, tubes 131–140 (18 mg; 10 μg); channel L, tubes 141–150 (18 mg; 10 μg); channel M, tubes 151–160 (17 mg; 5 μg); channel N, tubes 161–170 (70 mg; 5 μg); channel O, tubes 171–180 (50 mg; 5 μg); channel P, tubes 181–190 (12 mg; 5 μg). The chromatogram was developed with the chlorine reagent

Preparation of Fragment CD (Positions 24–47). A solution of the TFA salt of fragment C hydrazide (245 mg) in DMF (4 ml) was cooled at −10 to −12° and 6.91 *N* hydrogen chloride in dioxane (0.108 ml) followed by 10% *tert*-butyl nitrite in DMF (0.19 ml) were added. The solution was stirred at −10 to −12° for 20 min and was then cooled to −25°. TEA (0.209 ml) was added followed by a solution of fragment D *tert*-butoxycarbonylhydrazide (189 mg) in DMF (2 ml) and 10% TEA in DMF (0.28 ml). The mixture was stirred at 4° for 66 hr and was then diluted with 150 ml of a 1:1:1 mixture of 1-butanol–MeOH–water.[34] This solution was added to an AG 1-X2 column (2 × 10 cm) which was eluted with 50 ml of the same solvent followed by 1-butanol–MeOH–0.03 *M* acetic acid (100 ml), 1-butanol–MeOH–0.06 *M* acetic acid (100 ml), 1-butanol–MeOH–0.09 *M* acetic acid (200 ml), and finally 1-butanol–MeOH–0.12 *M* acetic acid (100 ml). Fractions of 10 ml each were collected. The desired material was located in the 0.09 *M* eluates by absorbancy measurements at 280 nm and tlc. Tubes containing single spot material were pooled and concentrated to a small volume, and the residue was lyophilized:[35] average yield in five experiments, 206 mg (63%); R_f^I 0.4; R_f^{III} 0 6 (see Figure 4 for tlc); amino acid ratios in acid hydrolysate, $Try_{3.3}Glu_{4.1}Leu_{1.0}His_{1.9}Asp_{4.1}Gly_{3.0}Thr_{0.9}Val_{1.0}Ser_{1.7}Pro_{1.0}Lys_{1.0}$; ratios of "diagnostic" amino acid residues Leu,Val/Lys, Pro = 1.0. Evaporation of the 1-butanol–MeOH–water eluates afforded 70 mg of fragment D *tert*-butoxycarbonylhydrazide. The 0.12 *M* acetic acid eluates contained azide rearrangement products.

Fragment CD *tert*-Butoxycarbonylhydrazide (Positions 24–47). The benzyloxycarbonyl derivative (400 mg) was hydrogenated for 20 hr over palladium in 1-butanol–MeOH–0.09 *M* acetic acid (1:1:1) (240 ml). The catalyst was removed by filtration, and the solvents were evaporated. The residue was dissolved in 10% acetic acid (50 ml) and the solution was lyophilized. The dried residue dissolved in 1-butanol–MeOH–water (100 ml) was then added to a column (2 × 10 cm) of AG 1-X2 which was eluted with 1-butanol–MeOH–water (50 ml), 1-butanol–MeOH–0.1 *M* acetic acid (150 ml), 1-butanol–MeOH–0.02 *M* acetic acid (150 ml), and 1-butanol–MeOH–0.03 *M* acetic acid (350 ml). The desired material

(34) The ratios of 1-butanol–MeOH–water (or acetic acid) were 1:1:1 in all instances.

(35) Since fragment CD has a tendency to precipitate on standing in the eluant the tubes were rinsed with 50% acetic acid.

was located in the 0.03 *M* acetic acid eluates by absorbancy measurements at 280 nm and tlc. Fractions containing homogenous material were pooled, the solvents were evaporated, and the residue was lyophilized from 5% acetic acid: fluffy colorless powder; 290 mg; R_f^I 0.1; R_f^{III} 0.6 (see Figure 4 for tlc); amino acid ratios in acid hydrolysate, $Tyr_{3.7}Glu_{4.1}Leu_{1.0}His_{1.9}Asp_{4.4}Gly_{3.1}Thr_{1.0}Val_{1.0}Ser_{1.9}Pro_{1.0}Lys_{1.0}$ (77%); amino acid ratios in AP-M digest, $Tyr_{4.0}(Glu + Asn + Ser)_{5.8}Leu_{1.1}His_{1.9}Glu_{2.1}Asp_{1.0}Gly_{3.0}Thr_{1.0}Val_{1.1}Pro_{1.1}Formlys_{1.1}\gamma$-O-*tert*-$BuGlu_{1.0}$ (81%); ratios of "diagnostic" amino acid residues Leu, Val/Pro, Formlys = 1.0.

Preparation of Fragment BCD (Positions 12–47). The trifluoroacetate salt of fragment B hydrazide (104 mg) was dissolved in DMSO (1 ml) and DMF (1 ml) was added to the clear solution. The solution was cooled at −10°, then 6.91 *N* hydrogen chloride in dioxane diluted 1:10 with DMF (0.58 ml) was added followed by 10% *tert*-butyl nitrite in DMF (0.11 ml). The solution was stirred at −10 to −15° for 15 min and was then cooled at −25°. A 10% solution of TEA in DMF (0.78 ml) was then added and the mixture was stirred at −25° for 5 min. A solution of fragment CD *tert*-butoxycarbonylhydrazide (75 mg) in DMF (2 ml) and 10% TEA in DMF (0.11 ml) was then added to the suspension which was stirred at −4° for 66 hr. TEA (10% in DMF) was added from time to time to adjust the pH of the mixture to 7.5–8.0. The reaction mixture was diluted with 100 ml of 1-butanol–MeOH–water and the solution was added to an AG 1-X2 column (1.9 × 11 cm) which was eluted with 1-butanol–MeOH–water (50 ml), 1-butanol–MeOH–0.01 *M* acetic acid (150 ml), 1-butanol-MeOH–0.03 *M* acetic acid (150 ml), 1-butanol–MeOH–0.06 *M* acetic acid (250 ml), 1-butanol–MeOH–0.09 *M* acetic acid (150 ml), and 1-butanol–MeOH–0.12 *M* acetic acid (450 ml). The desired product was located in the 0.12 *M* acetic acid eluates by absorbancy measurements at 280 nm and tlc. Fractions containing homogeneous material were pooled and evaporated to dryness, and the residue was lyophilized from 50% acetic acid: 49 mg (48%); R_f^I 0.4; R_f^{III} 0.6; amino acid ratios in acid hydrolysate, $Ser_{6.0}Asp_{5.4}Val_{2.0}Thr_{1.9}Ala_{2.9}Glu_{5.2}Gly_{4.1}Tyr_{3.8}Leu_{1.0}His_{1.8}Pro_{1.5}Lys_{0.9}$ (94%); ratios of "diagnostic" amino acid residues Ala/Leu, Lys = 3.05 (see Figure 4 for tlc).

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Synthesis and Pharmacological Properties of Deaminotocinamide and a New Synthesis of Tocinamide[1-3]

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Abstract: Deaminotocinamide (the 20-membered disulfide pentapeptide amide ring of deamino-oxytocin) and tocinamide (the corresponding disulfide pentapeptide amide ring of oxytocin) were synthesized by the stepwise *p*-nitrophenyl ester method using the *p*-nitrobenzyl ester for C-terminal carboxyl protection. *p*-Nitrobenzyl *S*-benzyl-β-mercaptopropionyltyrosylisoleucylglutaminylasparaginyl-*S*-benzylcysteinate and *p*-nitrobenzyl *N*-benzyloxycarbonyl-*S*-benzylcysteinyltyrosylisoleucylglutaminylasparaginyl-*S*-benzylcysteinate were converted to the corresponding C-terminal amide compounds in liquid ammonia. The polypeptide amides were then converted to the corresponding ring compounds by treatment with sodium in liquid ammonia followed by oxidation and purification. Deaminotocinamide was found to possess 34.2 ± 3.0 units/mg of oxytocic activity, but no detectable avian vasodepressor activity. Tocinamide possessed 3.2 ± 0.2 units/mg of oxytocic activity, but no detectable avian vasodepressor activity.

The synthesis of oxytocin (Figure 1) established the presence of a 20-membered disulfide ring in the hormone.[6] To investigate whether the ring structure itself of oxytocin possesses intrinsic biological activity, Ressler[1] synthesized the cyclic disulfide of cysteinyltyrosylisoleucylglutaminylasparaginylcysteinamide.[7] This cyclic pentapeptide amide, tocinamide,[1] was found by Ressler to have low but significant oxytocic (3.3 units/mg) and milk-ejecting (0.5 unit/mg) potencies, but no detectable avian vasodepressor activity, whereas oxytocin possesses 546 ± 18 units/mg oxytocic,[8] 410 ± 16 units/mg milk-ejecting,[9] and 507 ± 23 units/mg avian vasodepressor[9] potencies.

It may be recalled that deamino-oxytocin possesses 803 ± 36 units/mg of oxytocic activity and 975 ± 24 units/mg of avian vasodepressor activity.[10] This paper presents the synthesis and some of the pharmacological activities of the deamino analog of the cyclic

(1) *Tocinamide* refers here to the cyclic disulfide of cysteinyltyrosylisoleucylglutaminylasparaginylcysteinamide originally synthesized by C. Ressler (*Proc. Soc. Exp. Biol. Med.*, **92**, 725 (1956)). It represents the amide of the cyclic moiety of oxytocin. Since this compound and various analogs thereof will be receiving considerable attention in the future for various chemical, biological, and particularly physical studies, we suggest referring to it as *tocinamide* and the free acid as *tocinoic acid*. The deamino analogs would thus be *deaminotocinamide* and *deaminotocinoic acid*. Likewise, the corresponding compounds in the vasopressin series would be called *pressinamide* and *pressinoic acid*, respectively. It may be recalled that in a previous communication (R. Walter and V. du Vigneaud, *Biochemistry*, **5**, 3720 (1966)) the ring of oxytocin has been referred to as the tocin ring and that of the vasopressins as the pressin ring.

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(3) All optically active amino acid residues are of the L variety.

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