to an overall contraction of the central core region. This can be seen from Figure 5 which compares the geometry of the central core region of TPrP and CuTPrP. From Figure 5, it can be seen that in CuTPrP the arrangement of the pyrrolic nitrogen atoms "square-up" with an accompanying overall contraction (average ~0.12 Å). Since the central core radius of 2.01 Å corresponds to the minimization of radial strain in the porphine macrocycle of a metalloporphine,[2] the observed value of 2.000 ± 0.005 Å in CuTPtP would seem to suggest that the Cu(II) ion is accommodated with a minimal perturbation to the system. A similar but more pronounced contraction of the central core region is observed with NiOEP.[12]

The bond distances of the propyl groups are not affected by the Cu(II) substitution. As in TPrP, the distances tend to be smaller than expected (second and third atoms of the side chains). In fact, the distributions of the bond distances in the propyl groups of TPrP and CuTPrP are remarkaly similar. Since the structures are isomorphous, this reproducibility would seem to suggest that the shortening in some of these distances might be related to packing effects noted in the TPrP structure (close intermolecular contacts).[1]

(12) D. L. Cullen and E. F. Meyer, Jr., private communication.

Acknowledgment. We would like to take the opportunity here to gratefully acknowledge the support of this work by the National Science Foundation, Molecular Biology Section (Grant No. GB-3372XX).

Supplementary Material Available. A table of $|F_o|$ and F_c (in electrons) will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 20× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number JACS-73-6811.

Studies on Polypeptides. LII. Synthesis of a Cysteine Protected Peptide Amide Corresponding to Positions 81–104 of the Ribonuclease T_1 Sequence[1-3]

Koichi Kawasaki, Roger Camble, Gilles Dupuis, Hana Romovacek, Harold T. Storey, Chizuko Yanaihara, and Klaus Hofmann*

Contribution from the Protein Research Laboratory, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15261. *Received May 9, 1973*

Abstract: Syntheses are described of a cysteine protected tetracosapeptide amide which corresponds to positions 81–104 of the proposed primary sequence of the enzyme ribonuclease T_1. The peptide amide was constructed by the protected hydrazide approach and was shown to be sequentially homogeneous. The low solubility of the tetracosapeptide and several subfragments used in its synthesis created problems as concerns purification and these are discussed. Some racemization may have occurred during formation of the phenylalanyl–valine bond, but its level was below that detectable by enzymatic digestion.

We have reported syntheses of two protected peptide hydrazides corresponding to positions 1–47 (fragment ABCD)[1] and 48–80 (fragment EF)[4] of the proposed amino acid sequence of the enzyme ribonuclease T_1 (ribonucleate guanine nucleotido-2′-transferase (cyclizing) 2.7.7.26).[5] Our fundamental studies on fragment condensation have been continued and the present communication describes experimental details for the preparation of a cysteine protected tetracosapeptide amide (fragment G) corresponding to positions 81–104 of the enzyme's peptide chain. The synthesis of three large fragments spanning the entire peptide chain of the T_1 enzyme (Figure 1) has now been completed.

The overall scheme of the present synthesis is based on the protected hydrazide approach[6] and involved the synthesis of five subfragments (G_1–G_5) and their assembly by the azide procedure[7] to form the tetracosapeptide amide G. Three subfragment combinations, *i.e.*, $G_1G_2 + G_3$, $G_4 + G_5$, and $G_1 + G_2G_3$ (Scheme I), were explored.

As in our previous syntheses of T_1 fragments, a

(1) See H. T. Storey, J. Beacham, S. F. Cernosek, F. M. Finn, C. Yanaihara, and K. Hofmann, *J. Amer. Chem. Soc.*, **94**, 6170 (1972), for paper LI in this series. A preliminary communication of some of the results presented in this paper has appeared: N. Yanaihara, C. Yanaihara, G. Dupuis, J. Beacham, R. Camble, and K. Hofmann, *ibid.*, **91**, 2184 (1969).

(2) Supported by grants from the U. S. Public Health Service, the National Science Foundation, and the Hoffmann-La Roche Foundation.

(3) The amino acid residues except glycine are of the L configuration. The following abbreviations are used: AP-M = aminopeptidase M [G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964)]; Boc = *tert*-butoxycarbonyl; DCC = *N,N′*-dicyclohexylcarbodiimide; DCU = *N,N′*-dicyclohexylurea; DMSO = dimethyl sulfoxide; DMF = dimethylformamide; EC = ethylcarbamoyl; EtOH = ethanol; F = formyl; HOSU = *N*-hydroxysuccinimide; MeOH = methanol; 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; Z = benzyloxycarbonyl. See ref 1 for nomenclature of complex peptide derivatives.

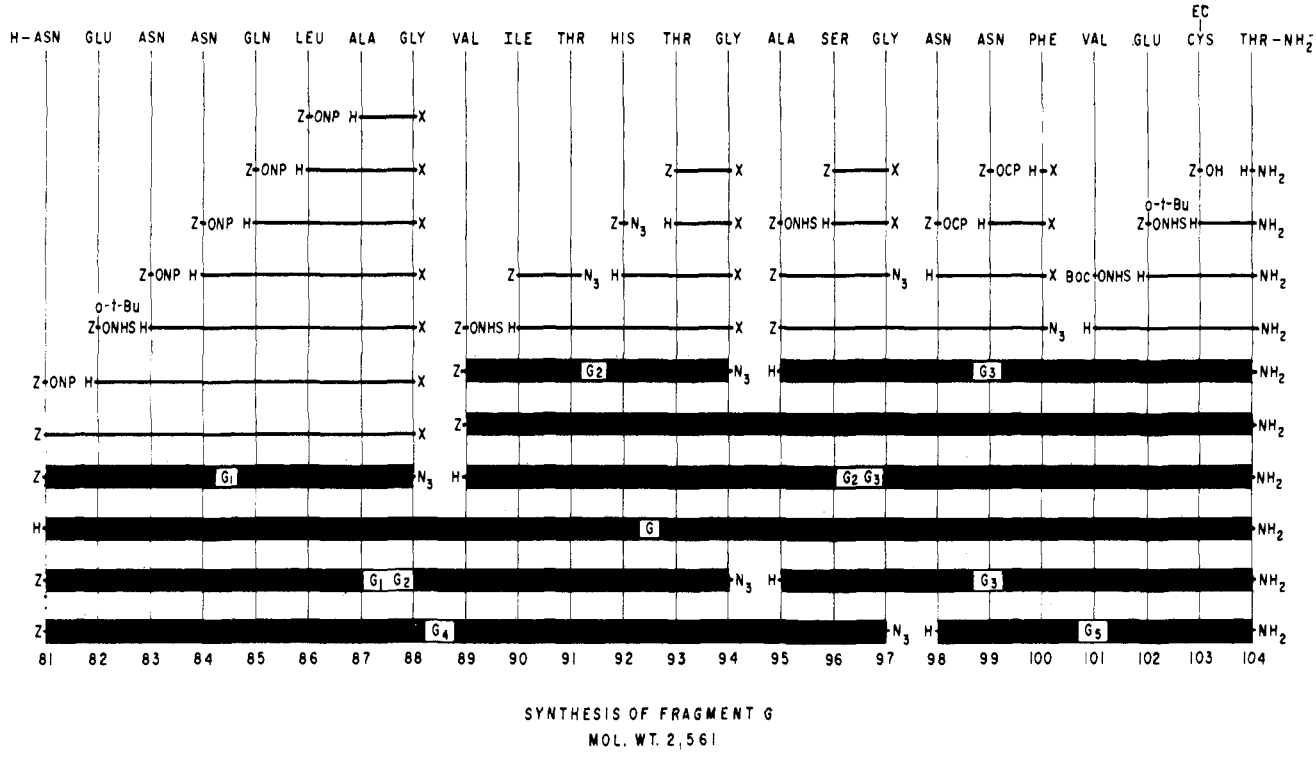
(4) R. Camble, G. Dupuis, K. Kawasaki, H. Romovacek, N. Yanaihara, and K. Hofmann, *J. Amer. Chem. Soc.*, **94**, 2091 (1972).

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

(6) K. Hofmann, A. Lindenmann, M. Z. Magee, and N. H. Khan, *J. Amer. Chem. Soc.*, 74, 470 (1952).

(7) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

Scheme I



SYNTHESIS OF FRAGMENT G
MOL. WT. 2,561

SYNTHESIS OF RIBONUCLEASE T_1 FRAGMENTS

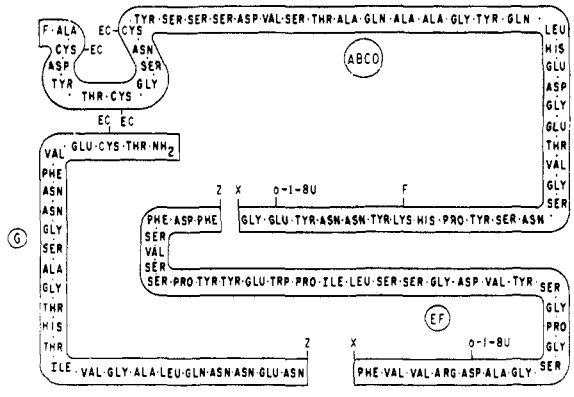


Figure 1.

minimum of side-chain protection was employed for reasons that have been discussed.[8] The sulfhydryl group of the cysteine residue was protected by the ethylcarbamoyl group.[9]

Preparative Aspects

Subfragment G_1. Starting with alanylglycine *tert*-butoxycarbonylhydrazide, subfragment G_I was constructed entirely by the stepwise active ester method in solution[10] using catalytic hydrogenolysis for removal of benzyloxycarbonyl groups. Sparingly soluble protected intermediates were purified by washing in suspension. The final product was homogeneous as judged by thin-layer chromatography and acid hydrolysates of the protected peptide contained the constituent amino acids in the ratios predicted by theory. Amino deprotected fragment G_1 acetate obtained from the protected compound by hydrogenolysis was completely digestible by aminopeptidase M (AP-M) with an 87% average recovery of the constituent amino acids and formed a single spot on thin-layer chromatography. This finding and the fact that all intermediates were carefully characterized point to the sequential homogeneity of subfragment G_1.

Subfragment G_2. For the synthesis of this subfragment benzyloxycarbonyl threonylglycine was converted to the *tert*-butoxycarbonylhydrazide and this compound was amino deprotected by hydrogenolysis. The histidine residue was added in the form of *N*-benzyloxycarbonylhistidine azide[11] and the peptide chain was completed by consecutive additions of benzyloxycarbonylisoleucylthreonine azide followed by *N*-hydroxysuccinimido benzyloxycarbonylvalinate. As was the case with subfragment G_I, hydrogenolysis was employed exclusively for amino deprotection. Prior to the final acylation step, the intermediate isoleucylthreonylhistidylthreonylglycine *tert*-butoxycarbonylhydrazide was purified by chromatography on Dowex 50 using pyridinium acetate buffers for column elution. Subfragment G_2 was homogeneous as judged by thin-layer chromatography and subfragment G_2 *tert*-butoxycarbonylhydrazide obtained from subfragment G_2 by hydrogenolysis was completely digestible by AP-M with a practically quantitative average recovery of the constituent amino acids.

Subfragment G_3. Numerous problems were encountered during the development of a synthetic route to subfragment G_3. Among these were low coupling yields, low solubility, and difficulties in purification.

Threonine amide, the starting material, was obtained from *N*-benzyloxycarbonylthreonine amide by hydrogenolysis. The latter compound was prepared

(8) J. Beacham, G. Dupuis, F. M. Finn, H. T. Storey, C. Yanaihara, N. Yanaihara, and K. Hofmann, *J. Amer. Chem. Soc.*, **93**, 5526 (1971).
(9) St. Guttmann, *Helv. Chim. Acta*, **49**, 83 (1966).
(10) (a) M. Bodansky and V. du Vigneaud, *J. Amer. Chem. Soc.*, 81, 5688 (1959); (b) J. S. Morley, *J. Chem. Soc. C*, 2410 (1967).
(11) R. W. Holley and E. Sondheimer, *J. Amer. Chem. Soc.*, 76, 1326 (1954).

in good yield by exposing a mixed anhydride of benzyloxycarbonylthreonine to ammonia in tetrahydrofuran. The literature synthesis of threonine amide[12] affords very poor yields.

Valylglutamyl - *S* - ethylcarbamoylcysteinylthreonine amide was obtained from threonine amide by the active ester procedure *via* the crystalline benzyloxycarbonyl-γ-*tert*-butylglutamyl-*S*-ethylcarbamoylcysteinylthreonine amide and was purified by Dowex 50 chromatography. Attempts to elongate the peptide chain further by the active ester method proved unsatisfactory, presumably because of the presence of the alkali labile *S*-ethylcarbamoyl group.[1] Consequently, the synthesis of fragment G_3 was completed by fragment condensation, an approach which offered the distinct advantage that hydrogenolysis could be employed for amino deprotection during preparation of a large segment of the peptide chain.

Asparaginylasparaginylphenylalanine *tert*-butoxycarbonylhydrazide prepared by the active ester or DCC–HOSU[13] procedures was acylated with benzyloxycarbonylalanylserylglycine azide to give benzyloxycarbonylalanylserylglycylasparaginylasparaginylphenylalanine *tert*-butoxycarbonylhydrazide. The corresponding hydrazide trifluoroacetate obtained by exposure of the protected peptide hydrazide to 90% trifluoroacetic acid was then coupled *via* the azide with valylglutamyl-*S*-ethylcarbamoylcysteinylthreonine amide to afford the benzyloxycarbonyl derivative of subfragment G_3 which was amino deprotected with HBr in anhydrous trifluoroacetic acid. The ensuing fragment G_3 hydrobromide was purified by gel filtration on Sephadex G-25 using 45% formic acid as the solvent.

Subfragments G_4 and G_5. Subfragment G_4 was prepared by coupling the azide corresponding to subfragment G_2 with alanylserylglycine *tert*-butoxycarbonylhydrazide to give the *tert*-butoxycarbonylhydrazide of benzyloxycarbonylvalylisoleucylthreonylhistidylthreonylglycylalanylserylglycine. This material was amino deprotected by hydrogenolysis and acylated with the azide corresponding to subfragment G_1. Following removal of the *tert*-butoxycarbonyl group, the hydrazide trifluoroacetate was purified by gel filtration on Sephadex G-50. Here again aqueous 45% formic acid served as the eluent.

The preparation of subfragment G_5, which requires little comment, was readily achieved by coupling the azide of benzyloxycarbonylasparaginylasparaginylphenylalanine with valylglutamyl-*S*-ethylcarbamoylcysteinylthreonine amide followed by amino deprotection with HBr in anhydrous trifluoroacetic acid. Purification was achieved by chromatography on Biorex-70.

Subfragments G_1G_2 and G_2G_3. The synthesis of these two subfragments offers little novelty and involved in each case an azide coupling step which was performed in a mixture of DMSO and DMF. The solubility characteristics of the protected reaction products were unfavorable and 45% aqueous formic acid had to be used as the solvent for purification. Since these strongly acidic conditions could be expected to bring about the partial acidolytic cleavage of the *tert*-butoxycarbonyl protecting group, the crude reaction products were deprotected with 90% aqueous trifluoroacetic acid prior to purification on Sephadex G-50. Coupling yields of 54 and 36%, respectively, were realized.

Fragment G. Three routes (Scheme I) were explored for the synthesis of fragment G. The molar ratio of azide to amino component was usually 1.5:1 and 10–20 min at −10° were allowed for azide formation. Mixtures of DMSO and DMF had to be used as the solvent, since the subfragments were sparingly soluble in DMF alone. The azide of subfragment G_4 was isolated and added to a solution of subfragment G_5 in DMSO. The Honzl–Rudinger procedure[7] was employed for the coupling of subfragment G_1G_2 to subfragment G_3 and for the combination of subfragment G_1 with subfragment G_2G_3. The benzyloxycarbonyl derivative of fragment G is extremely insoluble and procedures could not be developed for its purification. Amino deprotection markedly increased its solubility in aqueous formic or acetic acids and in aqueous DMF and purification by chromatography on Biorex-70, Sephadex, or AG 1-X2 columns became feasible.

Discussion

The low solubility of fragment G and several intermediates used in its preparation appears to be largely responsible for the problems that were encountered in this investigation. This low degree of solubility which may be attributed to the high asparagine and glutamine content dictated the use of mixtures of DMSO and DMF as the solvent for many of the coupling reactions and severely limited the choice of purification procedures. The value of thin-layer chromatography as an analytical tool for evaluating homogeneity was also restricted for the reason that many of the peptides in this series were either insoluble or remained at the origin in several solvent systems. The more complex intermediates and fragment G migrated only in the not very discriminating pyridine system (system III). The coupling yields in the final step which ranged from 13 to 28% in the three approaches investigated must be regarded as minimum values since the problem of securing the required subfragments necessitated working with small amounts of material and precluded systematic exploration of optimum coupling conditions.

We have found consistently that the removal of the N-terminal benzyloxycarbonyl group exerts a striking effect on the solubility and it was for this reason that in many instances the deprotected materials were purified rather than the protected coupling products. Aqueous formic acid proved to be superior to aqueous acetic acid as a solvent for the sparingly soluble peptides. Since fragment G was obtained by azide coupling of various subfragments, which individually were shown to be sequentially homogeneous, the ratio of the diagnostic amino acid residues in acid hydrolysates provided a significant criterion for sequential homogeneity.[8] These ratios, recorded in the Experimental Section, are in agreement with theory. Diagnostic amino acids in fragment G are Phe (subfragment G_3), Ile (subfragment G_2), and Leu (subfragment G_1).

As a further check of the homogeneity of fragment

(12) C. A. Dekker, S. P. Taylor, Jr., and J. S. Fruton, *J. Biol. Chem.*, 180, 155 (1949).

(13) F. Weygand, D. Hoffmann, and E. Wünsch, *Z. Naturforsch. B*, 21, 426 (1966).

G, the compound was subjected to the dansyl procedure[14] and Asp was identified as the sole N-terminal residue.

Acid hydrolysates of peptides containing *S*-ethylcarbamoylcysteine contain varying proportions of cysteine and cystine and do not provide an accurate measure of the actual cysteine content. These peptides were oxidized with performic acid[1] prior to acid hydrolysis since the cysteic acid content of the hydrolysates could be determined more accurately. Although the analytical data presented leave little doubt regarding the sequential homogeneity of fragment G, the data do not allow a categorical statement to be made pertaining to its stereochemical purity. The observation that the *tert*-butoxycarbonylhydrazides of subfragments G_1 and G_2 are digestible with AP-M and that the digests contained the constituent amino acid residues in the theoretically expected ratios with a high average recovery supports their stereochemical purity within the limits of error of this technique. Insolubility prevented digestion of fragment G_3 by AP-M but the more soluble *S*-sulfonate was digested, and the digest contained the constituent amino acid residues in the ratios predicted by theory.

Problems were encountered when peptides containing the sequence *S*-ethylcarbamoylcysteinylthreonine amide were digested with AP-M. As is apparent from inspection of the pertinent Experimental Sections, low recoveries of threonine are frequently observed. This could be the result of a slow hydrolysis rate of the cysteinyl–threonine bond or may be attributable to resistance of threonine amide to enzymatic cleavage. Threonine amide can be determined on the 6-cm column of the amino acid analyzer and was present in digests containing low threonine. The presence in AP-M digests of peptides containing *S*-ethylcarbamoylcysteine of a substance that elutes from the 60-cm column of the analyzer at 47 min preceding threonine was consistently observed. The nature of this component has not been determined. The *S*-ethylcarbamoyl group is largely cleaved during incubation of the peptides with AP-M (pH 7.75).

Inspection of Scheme I shows that with the exception of the threonylhistidyl and phenylalanylvalyl bonds glycine occupied the C-terminus of the protected peptides that were employed as acylating components. Since azide couplings involving C-terminal phenylalanine can be subject to racemization,[15] a small degree of racemization may have occurred during formation of the phenylalanyl–valine bond. If it had occurred, racemization was below the level detectable by AP-M digestion.

Experimental Section[16]

Synthesis of Subfragment G_1 (Positions 81–88). (Positions 87 and 88) Benzyloxycarbonylalanylglycine *tert*-Butoxycarbonylhydrazide. a. By the Mixed Anhydride Procedure. A mixed anhydride was prepared, in the usual manner, from benzyloxycarbonylalanylglycine[17] (28.4 g) in THF (200 ml) with TEA (13.8 ml) and ethyl chloroformate (9.6 ml). This solution was added to an ice-cold solution of *tert*-butoxycarbonylhydrazine[18] (13.2 g) in THF (50 ml) and the mixture was stirred for 1 hr at 0° and for 12 hr at room temperature. The mixture was filtered, the filtrate was evaporated *in vacuo*, and the residue was dissolved in ethyl acetate (150 ml). The ethyl acetate solution was washed in the usual manner, dried, and evaporated. The product was recrystallized from aqueous MeOH (needles): 21.0 g (53%); mp 92–95°; $[\alpha]^{23}D$ −8.3° (*c* 5.91, MeOH); R_f^{I} 0.7; R_f^{II} 0.8.

Anal. Calcd for $C_{18}H_{28}N_4O_6$: C, 54.8; H, 6.6; N, 14.2. Found: C, 54.8; H, 6.6; N, 14.2.

b. By the DCC–HOSU Procedure. DCC (20.6 g) in THF (50 ml) was added to an ice-cold solution of benzyloxycarbonylalanylglycine[17] (28.0 g), *tert*-butoxycarbonylhydrazine[18] (13.2 g), and HOSU (2.3 g) in THF (200 ml). The mixture was stirred for 1 hr at 0° and for 3 hr at room temperature and was then filtered. The filtrate was evaporated *in vacuo*, the residue was dissolved in ethyl acetate (250 ml), and the product was isolated in the manner described under a. The compound crystallized from aqueous MeOH (needles): 32.1 g (81%); mp 93–94°; $[\alpha]^{28}D$ −8.6° (*c* 4.36, MeOH); R_f^{I} 0.7; R_f^{IV} 0.8.

(Positions 87 and 88) Alanylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected dipeptide hydrazide was hydrogenated, in the usual manner, over palladium in MeOH (150 ml) containing 10% (v/v) aqueous acetic acid (25 ml). The catalyst was removed by filtration and the solvent was evaporated. The residue was dissolved in water–dioxane (5:1) and lyophilized: 13.0 g (100%); mp 235–236° dec; $[\alpha]^{28}D$ +14.9° (*c* 3.43, H_2O); R_f^{I} 0.4.

(Positions 86–88) Benzyloxycarbonylleucylalanylglycine *tert*-Butoxycarbonylhydrazide. a. By the *p*-Nitrophenyl Ester Method. A solution of *p*-nitrophenyl benzyloxycarbonylleucinate[10a] (15.4 g) in DMF (40 ml) was added to a DMF solution (80 ml) containing alanylglycine *tert*-butoxycarbonylhydrazide acetate (13.0 g) and TEA (5.5 ml). The mixture was stirred for 12 hr at room temperature and the solvent evaporated. The residue was dissolved in ethyl acetate (150 ml), the solution was washed in the usual manner and dried, and the solvent was evaporated. The residue was crystallized from aqueous MeOH (needles): 14.6 g (72%); mp 187°; $[\alpha]^{26}D$ −29.0° (*c* 4.55, MeOH); R_f^{I} 0.8; R_f^{II} 0.8; R_f^{IV} 0.9; amino acid ratios in acid hydrolysate, $Leu_{1.0}Ala_{1.0}Gly_{1.0}$ (97%).

Anal. Calcd for $C_{24}H_{37}N_5O_7$: C, 56.8; H, 7.4; N, 13.8. Found: C, 56.9; H, 7.4; N, 13.2.

b. By the *N*-Hydroxysuccinimido Ester Method. *N*-Hydroxysuccinimido benzyloxycarbonylleucinate[19] (18.1 g) was added to a solution of alanylglycine *tert*-butoxycarbonylhydrazide acetate (16.2 g) and TEA (6.0 ml) in DMF (150 ml). The solution was stirred for 6 hr at room temperature and the solvent was evaporated. The residue was dissolved in ethyl acetate (300 ml) and the solution was washed in the usual manner, dried, and evaporated. The residue was crystallized from ethyl acetate (needles): 19.8 g (78%); mp 187°; $[\alpha]^{26}D$ −29.0° (*c* 3.40, MeOH); R_f^{I} 0.8.

(Positions 86–88) Leucylalanylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected tripeptide hydrazide (3.7 g) was hydrogenated, in the usual manner, over palladium in MeOH (75 ml) containing 10% (v/v) aqueous acetic acid (4.5 ml). After 3 hr, the catalyst was removed by filtration and the solvent was evaporated. The residue was lyophilized from water: 3.16 g (100%); $[\alpha]^{24}D$ −13.8° (*c* 2.86, H_2O); R_f^{I} 0.4; amino acid ratios in AP-M digest, $Leu_{1.0}Ala_{1.0}Gly_{1.0}$ (97%).

(Positions 85–88) Benzyloxycarbonylglutaminylleucylalanylglycine *tert*-Butoxycarbonylhydrazide Hemihydrate. a. By the *p*-Nitrophenyl Ester Method. A solution of *p*-nitrophenyl benzyloxycarbonylglutaminate[10a] (12.0 g) in DMF (50 ml) was added to a DMF solution (50 ml) containing leucylalanylglycine *tert*-butoxycarbonylhydrazide acetate (13.0 g) and TEA (4.2 ml). The reaction mixture was stirred for 24 hr at room temperature, the solvent was evaporated, and the material precipitated by addition of ice-cold 2 *N* NH_4OH (100 ml). The precipitate was washed in suspension with eight 75-ml portions of ice-cold 2 *N* NH_4OH, three 100-ml portions of ice-cold water, four 75-ml portions of ice-cold 1 *N* citric acid, and three 100-ml portions of ice-cold water and dried. The material was suspended in boiling EtOH and water was added to obtain a clear solution which was kept at room temperature.

(14) B. S. Hartley, *Biochem. J.*, **119**, 805 (1970).

(15) (a) G. W. Anderson, J. E. Zimmerman, and F. Callahan, *J. Amer. Chem. Soc.*, 88, 1338 (1966); (b) P. Sieber, B. Riniker, M. Brugger, B. Kamber, and W. Rittel, *Helv. Chim. Acta*, **53**, 2135 (1970).

(16) See ref 1 for general experimental and analytical procedures. The solvent systems for ascending tlc on silica gel G (E. Merck and Co., Darmstadt, West Germany) are: R_f^{I} 1-butanol–acetic acid–water (60:20:20), R_f^{II} 2-butanol–3% ammonium hydroxide (3:1); R_f^{III} 1-butanol–pyridine–acetic acid–water (30:20:6:24); R_f^{IV} MeOH–chloroform (2:1); R_f^{V} 1-butanol–50% formic acid (1:1).

(17) W. Grassmann and E. Wünsch, *Chem. Ber.*, **91**, 449 (1958).

(18) L. A. Carpino, *J. Amer. Chem. Soc.*, **79**, 98 (1957).

(19) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **86**, 1839 (1964).

The resulting gelatinous material was collected and dried: 12.3 g (63%); mp 217–220°; $[\alpha]^{25}D$ −16.9° (*c* 5.21, DMF); R_f^{I} 0.7; R_f^{II} 0.7; R_f^{IV} 0.8; amino acid ratios in acid hydrolysate, $Glu_{1.0}$-$Leu_{1.0}Ala_{1.0}Gly_{1.0}$ (99%).

Anal. Calcd for $C_{29}H_{45}N_7O_9 \cdot {}^1/_2H_2O$: C, 54.0; H, 7.2; N, 15.2; O, 23.6. Found: C, 53.8; H, 7.3; N, 14.9; O, 23.6.

b. By the 2,4,5-Trichlorophenyl Ester Method. 2,4,5-Trichlorophenyl benzyloxycarbonylglutaminate[20] (18.4 g) in DMF (100 ml) was added to a solution of leucylalanylglycine *tert*-butoxycarbonylhydrazide acetate (17.3 g) and TEA (5.5 ml) in DMF (60 ml). The reaction mixture was stirred for 20 hr at room temperature, the solution was concentrated *in vacuo*, and ether was added. The precipitate was washed and purified in the manner described under a: 15.6 g (60%); mp 217–219°; $[\alpha]^{25}D$ −17.5° (*c* 3.70, DMF); R_f^{I} 0.7.

(Positions 85–88) Glutaminylleucylalanylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected tetrapeptide hydrazide (9.2 g) was suspended in MeOH (125 ml) containing 10% (v/v) aqueous acetic acid (8.7 ml) and hydrogenated over palladium. The catalyst was removed by filtration and the solvent was evaporated. The residue was dissolved in a small volume of EtOH and the product was precipitated with ether: 8.0 g (98%); $[\alpha]^{25}D$ −39.9° (*c* 3.22, H_2O); R_f^{I} 0.6; amino acid ratios in AP-M digest, $Gln_{0.9}Leu_{1.0}Ala_{1.1}Gly_{1.0}$ (82%).

(Positions 84–88) Benzyloxycarbonylasparaginylglutaminylleucylalanylglycine *tert*-Butoxycarbonylhydrazide. *p*-Nitrophenyl benzyloxycarbonylasparaginate[10a] (7.7 g) was added to a solution of glutaminylleucylalanylglycine *tert*-butoxycarbonylhydrazide acetate (11.2 g) and TEA (2.76 ml) in DMF (50 ml) at 50°. The reaction mixture was stirred for 7 hr at 50°, then an additional portion of "active" ester (2.5 g) was added and stirring at 50° was continued for 17 additional hr. The mixture was then diluted with ether, and the precipitate was collected by filtration and washed in suspension with ten 150-ml portions of 2 *N* NH_4OH, three 150-ml portions of 1 *N* sodium bicarbonate, water, four 150-ml portions of 1 *N* citric acid, and water and dried *in vacuo*. The residue was dissolved in hot DMF–water, and the solution was concentrated to a small volume and water was added to precipitate the product: 9.0 g (60%); mp 251–252° dec; $[\alpha]^{28}D$ −19.6° (*c* 1.80, DMSO); R_f^{I} 0.7; amino acid ratios in acid hydrolysate, $Asp_{1.1}Glu_{1.1}Leu_{1.0}Ala_{1.0}$-$Gly_{0.9}$ (99%).

Anal. Calcd for $C_{33}H_{51}N_9O_{11}$: C, 52.9; H, 6.9; N, 16.8. Found: C, 52.2; H, 7.1; N, 16.6.

(Positions 84–88) Asparaginylglutaminylleucylalanylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected pentapeptide hydrazide (7.6 g) was hydrogenated over palladium in 1-butanol–MeOH–water (1:1:1; 150 ml) containing 10% (v/v) aqueous acetic acid (12 ml). The catalyst was removed by filtration and the filtrate was evaporated. The residue was suspended in a small volume of EtOH and the material was precipitated by addition of ether: 5.9 g (76%); $[\alpha]^{27}D$ −49.6° (*c* 1.89, H_2O); R_f^{I} 0.3; amino acid ratios in AP-M digest, $(Asn + Gln)_{2.0}Leu_{1.0}Ala_{1.0}Gly_{1.0}$ (100%).

(Positions 83–88) Benzyloxycarbonylasparaginylasparaginylglutaminylleucylalanylglycine *tert*-Butoxycarbonylhydrazide. A solution of *p*-nitrophenyl benzyloxycarbonylasparaginate[10a] (2.00 g) in DMF (15 ml) was added to a DMF solution (45 ml) containing asparaginylglutaminylleucylalanylglycine *tert*-butoxycarbonylhydrazide acetate (3.46 g) and TEA (0.71 ml). The mixture was stirred for 10 hr at 50°, then additional "active" ester (1.0 g) was added and stirring at 50° was continued for 14 hr. The mixture was then poured into ether (1000 ml) and the precipitate was collected and washed in suspension with three 30-ml portions of boiling ethyl acetate, four 30-ml portions of 1 *N* citric acid, and three 30-ml portions of water. The dried material was dissolved in hot aqueous DMF (300 ml), and the solution was filtered, concentrated to a small volume, and water was added to precipitate the product: 3.0 g (68%); mp 251° dec; $[\alpha]^{23}D$ −22.7° (*c* 2.15, DMSO); R_f^{I} 0.5; amino acid ratios in acid hydrolysate, $Asp_{2.0}Glu_{1.0}Leu_{1.1}Ala_{0.9}$-$Gly_{1.0}$ (98%).

Anal. Calcd for $C_{37}H_{57}N_{11}O_{13}$: C, 51.4; H, 6.7; N, 17.8. Found: C, 51.2; H, 6.9; N, 17.4.

(Positions 83–88) Asparaginylasparaginylglutaminylleucylalanylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected hexapeptide hydrazide (4.3 g) was hydrogenated over palladium in 1-butanol–MeOH–water (1:1:1; 150 ml) containing 10% (v/v) aqueous acetic acid (4.0 ml). The catalyst was removed by filtration and the filtrate was evaporated. The residue was dissolved in water and the solution was evaporated. The solid was dissolved in a small volume of EtOH and precipitated by addition of ether: 3.3 g (84%); $[\alpha]^{26}D$ −24.0° (*c* 1.52, DMSO); $[\alpha]^{28}D$ −53.9° (*c* 2.38, H_2O); R_f^{I} 0.2; R_f^{III} 0.6; amino acid ratios in AP-M digest, $(Asn + Gln)_{3.0}Leu_{1.0}Ala_{1.0}Gly_{1.0}$ (100%).

(Positions 82–88) Benzyloxycarbonyl-γ-*tert*-butylglutamylasparaginylasparaginylglutaminylleucylalanylglycine *tert*-Butoxycarbonylhydrazide. *N*-Hydroxysuccinimido benzyloxycarbonyl-γ-*tert*-butylglutamate[21] (2.1 g) was added to a solution of asparaginylasparaginylglutaminylleucylalanylglycine *tert*-butoxycarbonylhydrazide acetate (2.5 g) in 80% aqueous DMF (150 ml). The reaction mixture was stirred for 5 hr at room temperature at a pH of 7.5–8.0 which was maintained by addition of TEA. The solvent was removed and water was added to the residue. The precipitate was distributed between 1-butanol and 5% acetic acid in the usual manner, the butanol layers were pooled and evaporated to dryness, and ethyl acetate (250 ml) was added to the residue. The ensuing precipitate was washed in suspension with five 50-ml portions of ethyl acetate and three 50-ml portions of THF and dried. The material was dissolved in DMF, the solution was concentrated and water was added to precipitate the product: 2.6 g (80%); mp 247–249° dec; $[\alpha]^{27}D$ −25.4° (*c* 2.10, DMSO); R_f^{I} 0.6; R_f^{III} 0.7; amino acid ratios in acid hydrolysate, $Glu_{2.0}Asp_{2.0}Leu_{1.1}Ala_{1.0}Gly_{1.0}$ (100%).

Anal. Calcd for $C_{46}H_{72}N_{12}O_{16}$: C, 52.7; H, 6.9; N, 16.0. Found: C, 52.0; H, 7.1; N, 16.1.

(Positions 82–88) γ-*tert*-Butylglutamylasparaginylasparaginylglutaminylleucylalanylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected heptapeptide hydrazide (1.1 g) was hydrogenated over palladium in 1-butanol–MeOH–water (1:1:1; 180 ml) containing 10% (v/v) aqueous acetic acid (7.0 ml). The catalyst was removed by filtration and the filtrate was evaporated. The residue was suspended in EtOH and ether was added to precipitate the product: 0.91 g (91%); $[\alpha]^{26}D$ −26.7° (*c* 2.17, DMSO); R_f^{I} 0.4; R_f^{III} 0.7; amino acid ratios in AP-M digest, $Glu(O\text{-}t\text{-}Bu)_{1.0}$-$(Asn + Gln)_{3.0}Leu_{1.0}Ala_{1.0}Gly_{1.0}$ (88%).

(Positions 81–88) Benzyloxycarbonylasparaginyl-γ-*tert*-butylglutamylasparaginylasparaginylglutaminylleucylalanylglycine *tert*-Butoxycarbonylhydrazide Monohydrate (Subfragment G_1). a. By the 2,4,5-Trichlorophenyl Ester Procedure. 2,4,5-Trichlorophenyl benzyloxycarbonylasparaginate[20] (1.1 g) in DMF (15 ml) was added to a DMF solution (60 ml) containing γ-*tert*-butylglutamylasparaginylasparaginylglutaminylleucylalanylglycine *tert*-butoxycarbonylhydrazide acetate (2.4 g) and TEA (0.35 ml) and the reaction mixture was stirred at room temperature. Additional amounts of "active" ester and TEA were added after 12 and 24 hr of stirring. Stirring was continued for 20 hr, then the solvent was removed and water was added to the residue. The ensuing precipitate was collected, dried, and washed in suspension, with four 25-ml portions each of ethyl acetate, boiling THF, 1 *N* citric acid, and water. The dried material was suspended in hot aqueous DMF, the solution was concentrated to a small volume, and water was added to precipitate the product: 1.45 g (50%); mp 254–255° dec; $[\alpha]^{28}D$ −25.6° (*c* 1.92, DMSO); R_f^{I} 0.6.

Anal. Calcd for $C_{50}H_{78}N_{14}O_{18} \cdot H_2O$: C, 50.8; H, 6.8; N, 16.6. Found: C, 51.0; H, 6.9; N, 16.5.

b. By the *p*-Nitrophenyl Ester Procedure. A solution of *p*-nitrophenyl benzyloxycarbonylasparaginate[10a] (1.7 g) in DMF (10 ml) was added to a suspension of γ-*tert*-butylglutamylasparaginylasparaginylglutaminylleucylalanylglycine *tert*-butoxycarbonylhydrazide acetate (2.75 g) in DMF (50 ml) and 10% (v/v) TEA in DMF (3.05 ml). The reaction mixture was stirred for 24 hr at 50° when an additional amount of "active" ester (0.43 g) was added. After 24 hr at 50°, the solvent was removed and water was added to precipitate the product. The precipitate was dried and was washed by decantation in the manner described under a. The solid was then distributed in countercurrent fashion between 1-butanol and 5% acetic acid, the 1-butanol layers were pooled and evaporated, and water was added to precipitate the product: 1.94 g (76%); mp 256° dec; $[\alpha]^{27}D$ −25.2° (*c* 1.52, DMSO); R_f^{I} 0.6; amino acid ratios in acid hydrolysate, $Asp_{3.0}Glu_{2.1}Leu_{1.0}Ala_{1.0}Gly_{1.0}$ (96%).

(Positions 81–88) Subfragment G_1 *tert*-Butoxycarbonylhydrazide Acetate. Subfragment G_1 (1.94 g) was hydrogenated for 24 hr over palladium in 1-butanol–MeOH–water (1:1:1; 150 ml) containing 10% (v/v) aqueous acetic acid (1.0 ml). The catalyst was removed by filtration and the filtrate was evaporated. A small volume of EtOH was added to the residue and the product was

(20) J. Pless and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1609 (1963).

(21) R. Zabel and H. Zahn, *Z. Naturforsch. B*, 20, 650 (1965).

precipitated by addition of ether: 1.44 g (81%); $[\alpha]^{27}D$ −26.8° (*c* 1.48, DMSO); R_f^I 0.5; R_f^{II} 0.5; R_f^{III} 0.7; amino acid ratios in AP-M digest, $Glu(O\text{-}t\text{-}Bu)_{1.0}(Asn + Gln)_{4.2}Leu_{1.0}Ala_{1.0}Gly_{0.8}$ (87%).

(Positions 81–88) Subfragment G_1 Hydrazide Trifluoroacetate. Subfragment G_1 (300 mg) was dissolved in 90% TFA (3 ml) and the solution was kept for 1 hr at room temperature. Excess TFA was removed *in vacuo* and ether was added to the residue. The precipitate was collected by filtration and dried *in vacuo* over P_2O_5 and KOH pellets: 280 mg (97%); $[\alpha]^{28}D$ −24.9° (*c* 0.98, DMSO); R_f^I 0.4; R_f^{III} 0.5; amino acid ratios in acid hydrolysate: $Asp_{3.0}Glu_{2.2}Leu_{1.0}Ala_{1.0}Gly_{1.0}$ (100%).

Synthesis of Subfragment G_2 (Positions 89–94). (Positions 93 and 94) Benzyloxycarbonylthreonylglycine. Sodium nitrite (7.3 g) in ice-cold water (30 ml) was added dropwise to an ice-cold solution of benzyloxycarbonylthreonine hydrazide[22] (26.7 g) in 50% aqueous THF (300 ml) and 5 *N* hydrochloric acid (60 ml). The mixture was stirred at 0° for 10 min and TEA (29 ml) was added followed by glycine (9.0 g) in water (100 ml), THF (50 ml), and TEA (16.6 ml). Following stirring the mixture at 5° for 24 hr the THF was evaporated, the pH was adjusted to 8–9 by addition of 1 *N* NH_4OH, and the solution was extracted with three portions of ethyl acetate. The aqueous phase was acidified to Congo red with 2 *N* hydrochloric acid and the resulting suspension was extracted three times with ethyl acetate. The extracts were washed in the usual manner, the solvent was evaporated, and the residue was recrystallized from ethyl acetate: 16.3 g (53%); mp 148–150°; $[\alpha]^{23}D$ −12.6° (*c* 4.00, MeOH); R_f^I 0.6; R_f^{III} 0.6.

Anal. Calcd for $C_{14}H_{18}N_2O_6$: C, 54.2; H, 5.9; N, 9.0. Found: C, 54.3; H, 6.1; N, 9.1.

(Positions 93 and 94) Benzyloxycarbonylthreonylglycine *tert*-Butoxycarbonylhydrazide. A solution of benzyloxycarbonylthreonylglycine (18.6 g) and *tert*-butoxycarbonylhydrazine[18] (7.8 g) in dioxane (400 ml) was cooled to 10° and DCC (12.3 g) in dioxane (50 ml) was added. The mixture was kept at 10° for 1 hr and at room temperature for 20 hr when it was filtered. The filtrate was evaporated, the residue was dissolved in ethyl acetate, and the solution was washed in the usual manner and dried. The solvent was evaporated and petroleum ether (bp 30–60°) was added to the residue. The ensuing solid material was reprecipitated from ethyl acetate with petroleum ether: 23.3 g (93%); mp 133–135° dec; $[\alpha]^{23}D$ −10.4° (*c* 3.32, MeOH); R_f^I 0.6; R_f^{III} 0.7.

Anal. Calcd for $C_{19}H_{28}N_4O_7$: C, 53.8; H, 6.7; N, 13.2. Found: C, 53.7; H, 6.9; N, 13.0.

(Positions 93 and 94) Threonylglycine *tert*-Butoxycarbonylhydrazide. Benzyloxycarbonylthreonylglycine *tert*-butoxycarbonylhydrazide (8.5 g) in MeOH (50 ml) was hydrogenated over palladium. The catalyst was removed by filtration, the filtrate evaporated, and the residue lyophilized from dioxane: colorless powder; 5.2 g (89%); $[\alpha]^{26}D$ −11.5° (*c* 1.74, MeOH); R_f^I 0.3; R_f^{III} 0.7.

(Positions 92–94) Benzyloxycarbonylhistidylthreonylglycine *tert*-Butoxycarbonylhydrazide Hemihydrate. N^{α}-Benzyloxycarbonylhistidine hydrazide[11] (6.06 g) was dissolved in DMF (50 ml) and the solution cooled at −20°; then 6.91 *N* HCl in dioxane (14.5 ml) was added dropwise with stirring and the solution was cooled to −20 to −30°. To this solution *tert*-butyl nitrite (2.55 ml) was added and after 5 min of stirring the mixture was cooled to −50° and TEA (13.9 ml) was slowly added followed after 5 min by a solution of threonylglycine *tert*-butoxycarbonylhydrazide (5.8 g) in DMF (20 ml). Stirring was continued for 20 hr at 4° and the solution was evaporated. The residue was dissolved in ethyl acetate and the solution was washed in the usual manner. Evaporation of the solvent gave an oil which solidified on addition of ether. This crude product (7.9 g) was distributed between 1-butanol and 3% acetic acid. The butanol layers, containing the desired product, were pooled and evaporated and the residue was solidified on addition of ether. The material was collected and dried: 6.4 g (57%); mp 143–148° dec; $[\alpha]^{25}D$ −12.7° (*c* 4.02, MeOH); R_f^I 0.6; R_f^{III} 0.7.

Anal. Calcd for $C_{25}H_{35}N_7O_8 \cdot {}^1/_2H_2O$: C, 52.6; H, 6.4; N, 17.2; O, 23.8. Found: C, 52.3; H, 6.4; N, 16.8; O, 23.4.

(Positions 92–94) Histidylthreonylglycine *tert*-Butoxycarbonylhydrazide Acetate. Benzyloxycarbonylhistidylthreonylglycine *tert*-butoxycarbonylhydrazide (10.5 g) in MeOH (80 ml) containing 10% acetic acid (26 ml) was hydrogenated over palladium. The catalyst was removed and the filtrate evaporated. The residue was dissolved in a small amount of EtOH, and ethyl acetate was added to give a precipitate which was collected and dried: 10.0 g (98%); mp 130–134° dec; $[\alpha]^{28}D$ −12.6° (*c* 4.01, MeOH); R_f^I 0.2; R_f^{II} 0.5; amino acid ratios in AP-M digest, $His_{1.0}Thr_{1.0}Gly_{1.0}$ (98%).

(Positions 90 and 91) Methyl Benzyloxycarbonylisoleucylthreoninate. DCC (8.3 g) in methylene chloride (30 ml) was added to an ice-cold solution of benzyloxycarbonylisoleucine (10.6 g) and methyl threoninate hydrochloride (6.8 g) in methylene chloride (300 ml) and TEA (5.5 ml). The mixture was stirred in an ice bath for 1 hr and at room temperature for 15 hr and filtered. The filtrate was washed in the usual manner and evaporated. The solid residue was recrystallized from ethyl acetate (needles): 12.9 g (85%); mp 152–154°; $[\alpha]^{25}D$ −20.5° (*c* 3.34, MeOH); R_f^I 0.8; R_f^{III} 0.8.

Anal. Calcd for $C_{19}H_{28}N_2O_6$: C, 60.0; H, 7.4; N, 7.4. Found: C, 60.2; H, 7.6; N, 7.7.

(Positions 90 and 91) Benzyloxycarbonylisoleucylthreonine Hydrazide. Methyl benzyloxycarbonylisoleucylthreoninate (9.5 g) was dissolved in MeOH (100 ml) and hydrazine hydrate (3.0 ml) was added. The mixture was kept at room temperature for 2 hr and at 5° for an additional 15 hr. The crystalline precipitate was collected, washed several times with cold MeOH, and dried: 8.0 g (84%); mp 255–257° dec; $[\alpha]^{24}D$ +5.3° (*c* 0.72, DMF); $[\alpha]^{24}D$ +6.1° (*c* 1.38, DMF); R_f^I 0.7; R_f^{III} 0.7.

Anal. Calcd for $C_{18}H_{28}N_4O_5$: C, 56.8; H, 7.4; N, 14.7. Found: C, 56.7; H, 7.6; N, 14.5.

(Positions 90–94) Benzyloxycarbonylisoleucylthreonylhistidylthreonylglycine *tert*-Butoxycarbonylhydrazide. *tert*-Butyl nitrite (0.79 ml) was added at −20 to −25° to a solution of benzyloxycarbonylisoleucylthreonine hydrazide (2.28 g) in DMF (20 ml) and THF (10 ml) containing 5.9 *N* hydrogen chloride in dioxane (4.1 ml). The mixture was kept at −20 to −25° for 10 min when TEA (3.4 ml) was added dropwise. A solution of histidylthreonylglycine *tert*-butoxycarbonylhydrazide acetate (2.74 g) in DMF (10 ml) and TEA (1.4 ml) was added and the mixture was stirred at 4° for 24 hr. The solvents were evaporated, the residue was extracted four times with 1-butanol, the extracts were washed eight times with water, and the butanol was evaporated. The residue solidified on addition of ether. The material was dried and precipitated from MeOH with ethyl acetate: 3.22 g (83%); mp 177–181°; $[\alpha]^{26}D$ −5.0° (*c* 1.23, DMF); R_f^I 0.5; R_f^{III} 0.8; amino acid ratios in 24 hr acid hydrolysate, $Ile_{1.0}Thr_{2.0}His_{1.0}Gly_{1.0}$ (84%).

Anal. Calcd for $C_{35}H_{53}N_9O_{11}$: C, 54.2; H, 6.9; N, 16.3. Found: C, 54.0; H, 6.8; N, 16.0.

(Positions 90–94) Isoleucylthreonylhistidylthreonylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected pentapeptide hydrazide (2.33 g) in MeOH (30 ml) containing 10% acetic acid (5 ml) was hydrogenated over palladium. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in EtOH and the product precipitated by addition of ether, 1.91 g (84%). The crude product (200 mg) was dissolved in 0.1 *M* (pH 3.1) pyridinium acetate buffer (5 ml) and the solution was added to a column of Beckman ion-exchange resin Type 15A (18 × 155 mm). The column was eluted at a flow rate of approximately 18 ml/hr with a linear gradient obtained by mixing 250 ml of 0.1 *M* (pH 3.1) pyridinium acetate with 250 ml of 2 *M* (pH 5) pyridinium acetate. Fractions (9 ml each) were collected and those containing homogeneous material by tlc were pooled and evaporated to a small volume and lyophilized: 195 mg (95%); $[\alpha]^{25}D$ −22.3° (*c* 1.52, H_2O); R_f^I 0.3; R_f^{III} 0.6; amino acid ratios in 24 hr acid hydrolysate, $Ile_{1.0}Thr_{2.0}His_{1.0}Gly_{1.0}$ (77%); amino acid ratios in AP-M digest, $Ile_{1.1}Thr_{2.1}His_{1.1}Gly_{0.7}$ (74%).

(Positons 89–94) Benzyloxycarbonylvalylisoleucylthreonylhistidylthreonylglycine *tert*-Butoxycarbonylhydrazide Monohydrate (Subfragment G_2). *N*-Hydroxysuccinimido benzyloxycarbonylvalinate[19] (3.63 g) was added to a solution of isoleucylthreonylhistidylthreonylglycine *tert*-butoxycarbonylhydrazide acetate (5.33 g) in DMF (100 ml) and TEA (1.82 ml). The mixture was stirred at room temperature for 16 hr, the DMF was evaporated, the residue was precipitated with ethyl acetate, and the precipitate was collected, dried, washed with water, and redried. The compound was precipitated twice from DMF with ethyl acetate: 4.52 g (72%); mp 222° dec; $[\alpha]^{29}D$ −6.0° (*c* 1.61, DMF); R_f^I 0.5; R_f^{III} 0.8; amino acid ratios in 24 hr acid hydrolysate, $Val_{1.1}Ile_{1.0}Thr_{1.9}His_{1.0}Gly_{1.1}$ (92%).

Anal. Calcd for $C_{40}H_{62}N_{10}O_{12} \cdot H_2O$: C, 53.8; H, 7.2; N, 15.7; O, 23.3. Found: C, 53.7; H, 7.4; N, 15.9; O, 22.9.

(Positions 89–94) Subfragment G_2 *tert*-Butoxycarbonylhydrazide Acetate. The protected hexapeptide hydrazide (267 mg) was hydrogenated over palladium in MeOH (20 ml) containing 10% acetic acid (0.5 ml). The catalyst was removed by filtration, the filtrate was evaporated, and the residue lyophilized: 240 mg (93%);

(22) E. Schröder and H. Gibian, *Justus Liebigs Ann. Chem.*, **656**, 190 (1962).

$[\alpha]^{25}D$ −27.2° (*c* 0.39, H_2O); R_f^I 0.2; R_f^{III} 0.6; amino acid ratios in AP-M digest, $Val_{1.0}Ile_{1.0}Thr_{2.0}His_{1.2}Gly_{1.0}$ (100%).

(Positions 89–94) Subfragment G_2 Hydrazide Bistrifluoroacetate. The protected hexapeptide hydrazide (561 mg) was dissolved in 90% TFA (4 ml) and the solution was kept at 0° for 10 min and at room temperature for 30 min. Ether was added and the resulting precipitate was collected, washed with ether, and dried: 600 mg (94%); $[\alpha]^{25}D$ −8.3° (*c* 1.27, DMF); R_f^I 0.4; R_f^{III} 0.7.

Synthesis of Subfragment G_3 (Positions 89–94). Benzyloxycarbonylthreonine Amide. A solution of benzyloxycarbonylthreonine (15.18 g) in THF (100 ml) was chilled to −18° and *N*-methylmorpholine (7.26 ml) was added. Isobutyl chloroformate (8.04 ml) was added and the mixture stirred for 3 min. Ammonia gas was passed through the mixture for 15 min at −18° and 15 min while warming to room temperature. The solvent was evaporated and a solution of the residue in ethyl acetate was washed in the usual manner and dried. The solution was evaporated and the residue reprecipitated from ethyl acetate with petroleum ether (bp 30–60°): 12 g (84%); sinters at 82–84°, melts at 98–100° (Lit.[12] mp 82–83°); $[\alpha]^{26}D$ +6.3° (*c* 1.83, MeOH); R_f^I 0.6; R_f^{III} 0.8; R_f^{IV} 0.1.

Anal. Calcd for $C_{12}H_{16}N_2O_4$: C, 57.1; H, 6.4; N, 11.1. Found: C, 57.0; H, 6.7; N, 11.2.

Threonine Amide. A solution of benzyloxycarbonylthreonine amide (41 g) in MeOH (400 ml) was hydrogenated over palladium. The catalyst was removed by filtration and the solution evaporated to a syrup which crystallized on trituration with ethanol. The solid was collected and washed with ethanol and petroleum ether (bp 30–60°), 15.7 g (82%). The product was dissolved in MeOH, the solution was evaporated, and the residual syrup was triturated with ethanol to give a white crystalline solid: 13.5 g (70%); mp 105–106°; $[\alpha]^{26}D$ −15.0° (*c* 2.51, H_2O); R_f^I 0.3; R_f^{III} 0.5; elutes from the 6-cm column of the Beckman Amino Acid Analyzer at 23 min (position of His).

Anal. Calcd for $C_4H_{10}N_2O_2$: C, 40.7; H, 8.5; N, 23.7. Found: C, 40.8; H, 8.3; N, 24.0.

(Positions 103 and 104) *N*-Benzyloxycarbonyl-*S*-ethylcarbamoylcysteinylthreonine Amide. To a solution of threonine amide (2.36 g) in DMF (30 ml) was added at 6° *N*-benzyloxycarbonyl-*S*-ethylcarbamoylcysteine[9] (6.52 g) in THF (50 ml) and DCC (4.12 g) in THF (15 ml) and the mixture was stirred for 16 hr at room temperature. The suspension was filtered, the filtrate was evaporated, the residue was dissolved in ethyl acetate, and the solution was washed with ice-cold 1 *N* potassium bicarbonate, saturated sodium chloride, 1 *N* sulfuric acid, and saturated sodium chloride and dried. Evaporation of the solvent gave a solid which was recrystallized from MeOH: 5.19 g (61%); mp 161–164°; $[\alpha]^{25}D$ −13.7° (*c* 0.92, DMF); R_f^I 0.6; R_f^{III} 0.7.

Anal. Calcd for $C_{18}H_{26}N_4O_6S$: C, 50.7; H, 6.1; N, 13.1; S, 7.5. Found: C, 50.7; H, 5.9; N, 13.5; S, 7.9.

(Positions 103 and 104) *N*-*tert*-Butoxycarbonyl-*S*-ethylcarbamoylcysteinylthreonine Amide. 2,4,5-Trichlorophenyl *N*-*tert*-butoxycarbonyl-*S*-ethylcarbamoylcysteinate[1,23] (2.48 g) in DMF (25 ml) was added at −10° to a solution of threonine amide (0.59 g) in DMF (15 ml) and the mixture was stirred at −10° for 30 min and at room temperature for 28 hr. The solvent was evaporated, the residue was dissolved in ethyl acetate, and the solution was washed in the usual manner and dried. The residue which remained after evaporation of the solvent was precipitated from ethyl acetate with petroleum ether (bp 30–60°): 630 mg (32%); $[\alpha]^{27}D$ −31.7° (*c* 2.0, MeOH); R_f^I 0.6; R_f^{III} 0.7; amino acid ratios in 24 hr acid hydrolysate, $Cys_{1.1}Thr_{0.9}$ (100%).

Anal. Calcd for $C_{13}H_{28}N_4O_6S$: C, 45.9; H, 7.2; N, 14.3; S, 8.2. Found: C, 45.3; H, 7.2; N, 13.8; S, 8.1.

(Positions 103 and 104) *S*-Ethylcarbamoylcysteinylthreonine Amide Hydrobromide. *N*-Benzyloxycarbonyl-*S*-ethylcarbamoylcysteinylthreonine amide (7.0 g) was dissolved in an ice-cold saturated solution of anhydrous HBr in anhydrous TFA (50 ml). Anhydrous HBr was passed through the solution for 30 min at 0° and then for 30 min at room temperature. The solution was evaporated to a small volume and peroxide-free ether (450 ml) added to give a white precipitate which was collected, washed thoroughly with ether, and dried over P_2O_5 and KOH. The product was twice reprecipitated from ethanol with ether and dried: 5.86 g (96%); $[\alpha]^{31}D$ +5.0° (*c* 1.62, H_2O); R_f^I 0.3, with faint traces at 0.2 and 0.5; R_f^{III} 0.6; amino acid ratios in 24 hr acid hydrolysate of performic acid oxidized sample, $Cys(SO_3H)_{1.0}Thr_{1.0}$. No evidence of an S → N shift could be detected by tlc when solutions of the dipeptide hydrobromide or acetate in DMF containing TEA were kept at room temperature for several hours.

(Positions 103 and 104) *S*-Ethylcarbamoylcysteinylthreonine Amide Trifluoroacetate. *N*-*tert*-Butoxycarbonyl-*S*-ethylcarbamoylcysteinylthreonine amide (544 mg) was dissolved in ice-cold 90% TFA (5 ml) and the solution was kept at room temperature for 30 min. The solvent was removed *in vacuo* and ether (100 ml) was added to the residue. The ensuing solid was collected, washed with ether, and dried over P_2O_5 and KOH pellets: 550 mg (98%); $[\alpha]^{27}D$ + 14.3° (*c* 2.2, MeOH); R_f^I 0.7; R_f^{III} 0.8; amino acid ratios in 24 hr acid hydrolysate, $Cys_{1.1}Thr_{0.9}$ (81%); amino acid ratios in 24 hr AP-M digest, $Cys(EC)_{0.2}Cys_{0.8}Thr_{1.0}$ (extra peak preceding Thr).

(Positions 102–104) Benzyloxycarbonyl-γ-*tert*-butylglutamyl-*S*-ethylcarbamoylcysteinylthreonine Amide. TEA (8.7 ml of a 25% solution in DMF) was added dropwise at room temperature over 7 min to a stirred solution of *S*-ethylcarbamoylcysteinylthreonine amide hydrobromide (5.86 g) and α-succinimido benzyloxycarbonyl-γ-*tert*-butylglutamate[21] (6.82 g) in DMF (16 ml). The mixture was stirred at room temperature for 22 hr and diluted with ethyl acetate (1500 ml), and the solution was washed in the usual manner. Evaporation of the solvent gave a gelatinous precipitate which was dissolved in hot MeOH (10 ml) and the solution was refrigerated for 40 hr. The ensuing white crystalline solid was collected, washed with small volumes of ice-cold MeOH and ether, and dried, 6.98 g (73%). The material was recrystallized from MeOH: 6.50 g (68%); mp 134–138°; $[\alpha]^{30}D$ −22.1° (*c* 1.49, DMF); R_f^I 0.7; R_f^{III} 0.8.

Anal. Calcd for $C_{27}H_{41}N_5O_8S$: C, 53.0; H, 6.8; N, 11.5; S, 5.2. Found: C, 52.9; H, 6.4; N, 12.0; S, 5.8.

(Positions 102–104) Glutamyl-*S*-ethylcarbamoylcysteinylthreonine Amide. The benzyloxycarbonyl derivative (314 mg) was dissolved in anhydrous TFA saturated with HBr at 0° and HBr was passed through the solution for 15 min at 0°, then for 45 min at room temperature. The solvents were evaporated and ether (70 ml) was added. The precipitate was collected, dissolved in TFA (2 ml), precipitated with ether, washed with ether, and dried over P_2O_5 and KOH: 266 mg (100%); $[\alpha]^{27}D$ −1.7° (*c* 1.40, H_2O); R_f^I 0.3; R_f^{III} 0.5; amino acid ratios in 24 hr acid hydrolysate, $Glu_{0.9}Cys_{1.2}Thr_{0.9}$ (79%); amino acid ratios in 24 hr AP-M digest, $Glu_{1.3}(Cys(EC) + Cys)_{1.0}Thr_{0.64}$ (extra peak preceding Thr).

(Positions 101–104) Valylglutamyl-*S*-ethylcarbamoylcysteinylthreonine Amide. *N*-Hydroxysuccinimido *tert*-butoxycarbonylvalinate[19] (158 mg) in DMF (7 ml) was added at −10° to a solution of glutamyl-*S*-ethylcarbamoylcysteinylthreonine amide hydrobromide (230 mg) in DMF (5 ml) and this was followed by TEA (1:10) in DMF (0.61 ml). The mixture was stirred for 4 hr at 0° and for 8 hr at room temperature, then the solvents were evaporated and the residue was distributed between 1-butanol and 3% acetic acid in the usual manner. The butanol layers containing homogeneous material on tlc were pooled and evaporated and the residue was precipitated from MeOH with ether: 186 mg (81%); $[\alpha]^{25}D$ −45.7° (*c* 1.41, MeOH); R_f^I 0.7; R_f^{III} 0.7.

The crude protected peptide amide (452 mg) was dissolved in ice-cold aqueous 90% TFA (10 ml) and the clear solution was kept at room temperature for 30 min. The solvent was removed *in vacuo*, ether was added, and the resultant precipitate was collected, washed with ether, and dried over KOH and P_2O_5, 433 mg; R_f^I 0.3; R_f^{III} 0.5, containing lower running impurities.

This crude tetrapeptide amide (200 mg) was dissolved in 0.1 *M* pyridinium acetate buffer (pH 3.1) (5 ml) and applied to a column (1.9 × 16 cm) of Beckman ion-exchange resin Type 15A. The column was eluted at approximately 20 ml/hr with a buffer gradient formed by mixing 0.1 *M* pyridinium acetate (pH 3.1) (230 ml) with 2.0 *M* pyridinium acetate buffer (pH 5.0) (230 ml). Fractions (10 ml each) were collected. Samples (10 μl) were withdrawn from each fraction and evaluated by tlc using the chlorine test for visualization. Fractions containing homogeneous material were pooled, concentrated to a small volume, and lyophilized to constant weight: 153 mg (76%); $[\alpha]^{25}D$ −19.7° (*c* 0.97, 50% acetic acid); R_f^I 0.3; R_f^{III} 0.5, single ninhydrin, chlorine, and Ellman + NH_3 positive spot; amino acid ratios in 24 hr acid hydrolysate, $Val_{1.0}Glu_{1.0}(CysSH + Cys)_{1.1}Thr_{0.9}$ (89%); amino acid ratios in 24 hr hydrolysate of performic acid oxidized sample, $Val_{1.0}Glu_{1.1}Cys(SO_3H)_{1.0}Thr_{1.0}$; amino acid ratios in AP-M digest, $Val_{1.3}Glu_{1.2}(Cys(EC) + Cys)_{0.8}Thr_{0.7}$ (extra peak preceding Thr); amino acid ratios in AP-M digest of performic acid oxidized sample, $Val_{1.4}Glu_{1.3}Cys(SO_3H)_{0.8}Thr_{0.5}$ (extra peak preceding $Cys(CO_3H)$; *S*-ethylcarbamoylcysteine content by PCMB titration,[1] 87%.

(23) H. Zahn and K. Hammerström, *Chem. Ber.*, 102, 1048 (1969).

(Positions 99 and 100) Benzyloxycarbonylasparaginylphenylalanine *tert*-Butoxycarbonylhydrazide. a. By the Active Ester Method. 2,4,5-Trichlorophenyl benzyloxycarbonylasparaginate[20] (3.26 g) in DMF (20 ml) was added at room temperature to a solution of phenylalanine *tert*-butoxycarbonylhydrazide[18] (1.7 g) in DMF (5 ml) and the pH was adjusted to 7.5–8.0 by addition of TEA. The mixture was stirred at room temperature for 48 hr. At this point an additional amount of the "active" ester (0.26 g) was added and the pH was adjusted to 7.5–8.0 by addition of TEA. Stirring at room temperature was continued for 48 hr when 3-dimethylaminopropylamine (1 ml) was added and the solution was stirred for 2 additional hr. The solution was concentrated *in vacuo* to approximately 1/4 of the original volume, diluted with 150 ml of 1-butanol, and washed with 5% acetic acid in the usual manner. The butanol layers containing homogeneous material by tlc were pooled and evaporated and the residue was triturated with ethyl acetate. The ensuing solid was recrystallized twice from MeOH–ethyl acetate: 2.26 g (70%); mp 236–237° dec; $[\alpha]^{29}D$ −39.1° (*c* 1.01, MeOH); R_f^{I} 0.8; R_f^{III} 0.8 (with chlorine reagent on wet plate a white spot is formed which disappears on drying); amino acid ratios in 24 hr acid hydrolysate, $Asp_{1.0}Phe_{1.0}$ (94%).

Anal. Calcd for $C_{26}H_{33}N_3O_7$: C, 59.2; H, 6.3; N, 13.3. Found: C, 59.1; H, 6.2; N, 13.3.

b. By the DCC–HOSU Procedure. To an ice-cold solution of phenylalanine *tert*-butoxycarbonylhydrazide (2.59 g) and benzyloxycarbonylasparagine (2.68 g) in DMF (20 ml) was added a solution of *N*-hydroxysuccinimide (2.14 g) in THF (3 ml) followed by DCC (1.9 g) in DMF (5 ml). The mixture was stirred at 0° for 1 hr and at 4° for 24 hr. The DCU was removed by filtration and the filtrate was evaporated. The residue was solidified with ethyl acetate and recrystallized from MeOH–ethyl acetate: 3.1 g (56%); $[\alpha]^{25}D$ −38.8° (*c* 1.02, MeOH); R_f^{I} 0.8; R_f^{III} 0.8; amino acid ratios in 24 hr acid hydrolysate, $Asp_{1.0}Phe_{1.0}$ (90%).

(Positions 99 and 100) Asparaginylphenylalanine *tert*-Butoxycarbonylhydrazide Acetate. The benzyloxycarbonyl derivative (1.06 g) was hydrogenated over Pd in MeOH (60 ml) and 10% acetic acid (1.2 ml) in the usual manner. The product was lyophilized to constant weight from water: 822 mg (91%); $[\alpha]^{27}D$ −17.7° (*c* 1.00, MeOH); R_f^{I} 0.5; R_f^{III} 0.7; hydrogenation in the absence of acetic acid gave the free peptide *tert*-butoxycarbonylhydrazide in a yield of 85%, $[\alpha]^{24}D$ −20.5° (*c* 1.0, MeOH).

(Positions 98–100) Benzyloxycarbonylasparaginylasparaginylphenylalanine *tert*-Butoxycarbonylhydrazide. a. By the Active Ester Method. 2,4,5-Trichlorophenyl benzyloxycarbonylasparaginate[20] (410 mg) in DMF (4 ml) was added with stirring to an ice-cold solution of asparaginylphenylalanine *tert*-butoxycarbonylhydrazide acetate (350 mg) in DMF (4 ml) and TEA (10% in DMF) (1.56 ml). The mixture was stirred for 72 hr at room temperature, additional active ester (50 mg) was added, and the pH was adjusted to 7.5 by addition of TEA (10% in DMF). Stirring was continued for 48 hr, 3-dimethylaminopropylamine (0.2 ml) was added and the mixture was stirred for 2 hr and was concentrated to 1/4 of the original volume. The residue was diluted with 1-butanol and the butanol solution extracted with 5% acetic acid. The butanol layers containing homogeneous material by tlc were pooled and evaporated and ethyl acetate was added to the residue. The ensuing solid was precipitated twice from DMF solution with ethyl acetate: 420 mg (67%); $[\alpha]^{25}D$ −24.9° (*c* 1.04, DMF); R_f^{I} 0.7; R_f^{III} 0.8.

Anal. Calcd for $C_{30}H_{39}N_7O_9$: C, 56.2; H, 6.1; N, 15.3. Found: C, 56.0; H, 6.4; N, 15.4.

b. By the DCC–HOSU Procedure. A solution of asparaginylphenylalanine *tert*-butoxycarbonylhydrazide (985 mg), benzyloxycarbonylasparagine (665 mg), and HOSU (288 mg) in DMF (20 ml) was cooled at 0° and an ice-cold solution of DCC (525 mg) in DMF (5 ml) was added with stirring. The suspension was stirred at 0° for 2 hr and at room temperature for 12 hr when the DCU was removed by filtration. The filtrate was concentrated to a small volume and the product precipitated by addition of ethyl acetate and ether. For purification the product was precipitated three times from DMF with ethyl acetate: 1.2 g (74%); $[\alpha]^{27}D$ −24.4° (*c* 1.04, DMF); R_f^{I} 0.7; R_f^{III} 0.8; amino acid ratios in 24 hr acid hydrolysate, $Asp_{2.0}Phe_{1.0}$ (84%).

(Positions 98–100) Asparaginylasparaginylphenylalanine *tert*-Butoxycarbonylhydrazide. The protected tripeptide hydrazide (1.2 g) was hydrogenated in aqueous MeOH in the usual manner and the product lyophilized from water: 930 mg (98%); $[\alpha]^{26}D$ −40.4° (*c* 1.00, MeOH); R_f^{I} 0.4; R_f^{III} 0.7; amino acid ratios in AP-M digest, $Asn_{2.0}Phe_{1.0}$ (81%).

(Positions 98–100) Benzyloxycarbonylasparaginylasparaginylphenylalanine Hydrazide Trifluoroacetate. The protected tripeptide hydrazide (240 mg) was dissolved in ice-cold 90% TFA (6 ml) and the solution was kept at 0° for 10 min and at room temperature for 30 min. The solvent was removed, and the product was precipitated with ether, washed with ether, and dried: 194 mg (79%); R_f^{I} 0.5; R_f^{III} 0.7; amino acid ratios in 24 hr acid hydrolysate, $Asp_{2.0}Phe_{1.0}$ (100%).

(Positions 95–97) Benzyloxycarbonylalanylserylglycine *tert*-Butoxycarbonylhydrazide. *N*-Hydroxysuccinimido benzyloxycarbonylalaninate[19] (840 mg) in dioxane (5 ml) was added to a solution of serylglycine *tert*-butoxycarbonylhydrazide[4]) (713 mg) in dioxane (5 ml) containing TEA (0.18 ml). The mixture was stirred at room temperature for 20 hr when water (5 ml) was added. The solvents were evaporated, the residue was dissolved in ethyl acetate, the solution was washed in the usual manner, and the solvent was evaporated. The residue was dissolved in MeOH and precipitated by addition of ethyl acetate: 853 mg (69%); mp 157–158° dec; $[\alpha]^{25}D$ −17.3° (*c* 3.70, MeOH); R_f^{I} 0.7; R_f^{III} 0.8.

Anal. Calcd for $C_{21}H_{31}N_5O_8$: C, 52.4; H, 6.5; N, 14.5. Found: C, 52.5; H, 6.8; N, 14.2.

(Positions 95–97) Alanylserylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected tripeptide hydrazide (500 mg) was hydrogenated in MeOH (50 ml) containing 10% acetic acid (1.5 ml) in the usual manner and the product was lyophilized from water: 400 mg (95%); $[\alpha]^{25}D$ −10.4° (*c* 1.29, water); R_f^{I} 0.4; R_f^{III} 0.6; amino acid ratios in AP-M digest, $Ala_{1.0}Ser_{1.0}Gly_{1.0}$ (100%).

(Positions 95–97) Benzyloxycarbonylalanylserylglycine Hydrazide Trifluoroacetate. The protected tripeptide hydrazide (800 mg) was deblocked with 90% TFA (12 ml) in the usual manner and the product was precipitated with ether and dried over KOH and P_2O_5: 620 mg (76%); R_f^{I} 0.5; R_f^{III} 0.7, with some tailing.

(Positions 95–100) Benzyloxycarbonylalanylserylglycylasparaginylasparaginylphenylalanine *tert*-Butoxycarbonylhydrazide, The above hydrazide trifluoroacetate (395 mg) was dissolved in DMSO–DMF (1:1; 6 ml) and the solution was cooled at −10°. To this solution was added 5.6 *N* hydrogen chloride in dioxane (0.72 ml) followed by 10% *tert*-butyl nitrite in DMF (1.02 ml) and the solution was stirred at −10° for 10 min. The reaction mixture was cooled at −20° and TEA (0.67 ml) was added, followed by a solution of asparaginylasparaginylphenylalanine *tert*-butoxycarbonylhydrazide (405 mg) in DMF (3 ml). The reaction mixture was stirred for 48 hr at 4° and the products were precipitated by addition of ethyl acetate. The solid was washed in a centrifuge tube with ethyl acetate, dried, washed with water, and redried. Finally, the material was precipitated from DMSO with ethyl acetate: 485 mg (71%); $[\alpha]^{26}D$ −14.6° (*c* 1.01, DMSO); R_f^{I} 0.6; R_f^{III} 0.7 (with chlorine reagent on wet plate a white spot is formed, which disappears on drying); amino acid ratios in 24 hr acid hydrolysate, $Ala_{1.0}Ser_{1.0}Gly_{1.0}Asp_{2.1}Phe_{1.0}$ (74%).

Anal. Calcd for $C_{38}H_{52}N_{10}O_{13}$: C, 53.3; H, 6.1; N, 16.4. Found: C, 53.0; H, 6.3; N, 16.1.

(Positions 95–100) Benzyloxycarbonylalanylserylglycylasparaginylasparaginylphenylalanine Hydrazide Trifluoroacetate. The protected hexapeptide hydrazide (527 mg) was dissolved in 90% TFA (5 ml) at 0° and the solution was kept at 0° for 10 min and at room temperature for 30 min. The product was precipitated and washed with ether and dried: 474 mg (90%); R_f^{I} 0.5; R_f^{III} 0.7.

(Positions 95–104) Alanylserylglycylasparaginylasparaginylphenylalanylvalylglutamyl-*S*-ethylcarbamoylcysteinylthreonine Amide Hydrobromide (Subfragment G_3). The above hydrazide trifluoroacetate (130 mg) was dissolved in DMSO (1.2 ml) and DMF (0.8 ml) and the solution was cooled at −10°. To this solution was added 7.7 *N* HCl in dioxane (0.097 ml) followed by 10% *tert*-butyl nitrite in DMF (0.2 ml) and the solution was stirred at −15° for 10 min. The reaction mixture was cooled at −20°, TEA (0.12 ml) was added, followed by a solution of valylglutamyl-*S*-ethylcarbamoylcysteinylthreonine amide (73 mg) in DMF (1 ml), and the solution was stirred at 4° for 48 hr; then the products were precipitated with ethyl acetate. The precipitate was collected, washed with ethyl acetate and water, and dried. This material (168 mg) was dissolved in anhydrous TFA saturated with HBr (2.5 ml) and containing anisole (0.1 ml) and HBr was passed through the solution for 20 min at 0° and for 30 min at room temperature. Ether was added and the precipitate was collected, washed with ether, and dried, yield 165 mg. This material was dissolved in 45% formic acid (4 ml) and the solution was added to a Sephadex G-25 column (2 × 135 cm) which was developed with 45% formic acid at a flow rate of approximately 3.5 ml/10 min. Individual fractions (4.5 ml each) were collected and small aliquots were subjected to tlc

using the chlorine test for visualization. Fractions containing single spot material (R_f^{III} 0.5) were pooled and lyophilized: 110 mg (63%); $[\alpha]^{26}D$ −28.8° (*c* 0.97, 90% HCOOH); amino acid ratios in 24 hr acid hydrolysate, $Ala_{1.0}Ser_{0.9}Gly_{1.0}Asp_{2.1}Phe_{1.0}Val_{1.0}Glu_{1.0}$-(Cys + CySH)$_{1.0}Thr_{0.9}$ (98%); amino acid ratios in 24 hr acid hydrolysate of performic acid oxidized sample, $Ala_{1.0}Ser_{0.9}Gly_{1.0}$-$Asp_{2.2}Phe_{1.1}Val_{1.0}Glu_{1.0}Cys(SO_3H)_{0.9}Thr_{0.9}$ (92%); amino acid ratios in AP-M digest of sulfitolized sample, $Ala_{1.1}$(Ser + Asn)$_{3.0}$-$Gly_{1.1}Phe_{1.0}Val_{1.0}Glu_{1.0}Cys(SO_3H)_{0.7}Thr_{0.7}ThrNH_{2_{0.2}}$.

Synthesis of Subfragment G_4 (Positions 81–97). (Positions 89–97) Benzyloxycarbonylvalylisoleucylthreonylhistidylthreonylglycylalanylserylglycine *tert*-Butoxycarbonylhydrazide. Subfragment G_2 hydrazide bistrifluoroacetate (912 mg) was dissolved in DMF (25 ml) and the solution was cooled at −10°. To this solution was added 7.7 *N* hydrogen chloride in dioxane (0.59 ml) followed by 10% *tert*-butyl nitrite in DMF (1.16 ml) and the mixture was stirred at −10° for 5 min when the hydrazide test was negative. The mixture was cooled at −25° and TEA (0.88 ml) was added followed by an ice-cold solution of alanylserylglycine *tert*-butoxycarbonylhydrazide acetate (740 mg) in DMF (5 ml) containing 10% TEA in DMF (0.25 ml). The solution was stirred at 4° for 22 hr, the pH being maintained at 8.0–8.5 by the addition of 10% TEA in DMF. The solvents were removed, the residue was dissolved in 20% acetic acid (10 ml), and this solution was added to a separatory funnel and equilibrated with 1-butanol. Four additional separatory funnels, each containing 1-butanol, were prepared and the butanol layers were equilibrated with 30 180-ml portions of water. The butanol layers containing the desired homogeneous peptide by tlc were pooled and evaporated and the residue was solidified by addition of ethyl acetate. The material was lyophilized from 10% acetic acid: 885 mg (89%); $[\alpha]^{26}D$ −6.8° (*c* 1.35, DMF); R_f^{I} 0.4; R_f^{III} 0.7; amino acid ratios in 48 hr acid hydrolysate, $Val_{1.0}$-$Ile_{1.0}Thr_{2.0}His_{1.1}Gly_{2.1}Ala_{1.0}Ser_{0.8}$ (84%).

Anal. Calcd for $C_{48}H_{75}N_{13}O_{16}$: C, 52.9; H, 6.9; N, 16.7. Found: C, 52.8; H, 6.7; N, 16.8.

(Positions 89–97) Valylisoleucylthreonylhistidylthreonylglycylalanylserylglycine *tert*-Butoxycarbonylhydrazide Acetate Tetrahydrate. The protected nonapeptide hydrazide (550 mg) was hydrogenated over palladium in 50% aqueous MeOH (150 ml) and 10% acetic acid (10 ml). The product (520 mg) isolated in the usual manner was impure as judged by tlc. A sample of this material (210 mg) was dissolved in 0.1 *M* (pH 3.1) pyridinium acetate buffer (5 ml) and this solution was added to a column (2 × 13 cm) of Beckman ion-exchange resin Type 15A. The column was eluted at approximately 18 ml/hr with a buffer gradient formed by mixing 0.1 *M* pyridinium acetate (pH 3.1) (225 ml) with 2.0 *M* pyridinium acetate buffer (pH 5.0) (225 ml). Fractions (9 ml each) were collected and the product was located in the various fractions by tlc. Fractions containing homogeneous product (R_f^{III} 0.7) were pooled, the solvents were evaporated and the residue was lyophilized from 10% acetic acid: 135 mg (64%); $[\alpha]^{26}D$ −30.3° (*c* 1.02, MeOH); R_f^{I} 0.2; R_f^{III} 0.7; amino acid ratios in 48 hr acid hydrolysate, $Val_{1.2}Ile_{1.0}Thr_{1.9}His_{1.0}Gly_{2.0}Ala_{1.1}Ser_{0.9}$ (90%); amino acid ratios in AP-M digest, $Val_{1.0}Ile_{1.0}Thr_{2.0}His_{1.1}Gly_{2.0}Ala_{1.0}Ser_{1.0}$ (90%).

Anal. Calcd for $C_{40}H_{69}N_{13}O_{14}AcOH \cdot 4H_2O$: C, 46.4; H, 7.5; N, 16.7; O, 29.4. Found: C, 46.1; H, 7.1; N, 16.2; O, 30.2.

(Positions 89–97) Benzyloxycarbonylvalylisoleucylthreonylhistidylthreonylglycylalanylserylglycine Hydrazide Bistrifluoroacetate. The protected nonapeptide *tert*-butoxycarbonyl hydrazide (200 mg) was dissolved in 3 ml of 90% TFA at 0° and the solution was kept for 40 min at room temperature. The solution was concentrated to a small volume and ether was added. The precipitate was collected and dried over P_2O_5 and KOH, 180 mg (81%), R_f^{I} 0.3, R_f^{III} 0.6.

(Positions 81–97) Benzyloxycarbonylasparaginylglutamylasparaginylasparaginylglutaminylleucylalanylglycylvalylisoleucylthreonylhistidylthreonylglycylalanylserylglycine Hydrazide Bistrifluoroacetate (Subfragment G_4). Subfragment G_1 hydrazide trifluoroacetate (200 mg) was dissolved in DMSO (5 ml) and DMF (5 ml) was added. The solution was cooled at −10° and 7.7 *N* hydrogen chloride in dioxane (0.12 ml) was added followed by 10% *tert*-butyl nitrite in DMF (0.22 ml). The reaction mixture was stirred at −10° for 20 min and cooled at −25° and TEA (0.19 ml) was added followed by a solution of valylisoleucylthreonylhistidylthreonylglycylalanylserylglycine *tert*-butoxycarbonylhydrazide acetate tetrahydrate (203 mg) in DMSO (1 ml) and DMF (3 ml) containing TEA (0.055 ml). The reaction mixture was stirred for 48 hr at 4° and turned into a viscous gel. Ethyl acetate was added and the precipitate was collected, washed with ethyl acetate and water, and dried (270 mg). This material was dissolved in 90% TFA (10 ml) containing anisole (0.2 ml) and the solution was kept at 0° for 5 min and at room temperature for 40 min. The product was precipitated with ether and dried (278 mg). The desired product was isolated from the crude product by gel filtration on a column (2 × 150 cm) of Sephadex G-50 using 45% formic acid as the eluent. The contents of tubes containing single spot material by tlc (R_f^{V} 0.5) were pooled and lyophilized: 170 mg (45%); $[\alpha]^{25}D$ −42.9° (*c* 1.0, 90% formic acid); R_f^{V} 0.5; amino acid ratios in 48 hr acid hydrolysate, $Asp_{2.9}Glu_{2.0}Leu_{1.06}Ala_{2.0}Gly_{2.9}Val_{1.1}Ile_{1.03}Thr_{2.0}His_{1.06}Ser_{1.1}$ (91%); ratio of Leu/Ile = 1.03, Leu/His = 1.0.

Synthesis of Subfragment G_5 (Positions 98–104). (Positions 98–104) Asparaginylasparaginylphenylalanylvalylglutamyl-*S*-ethylcarbamoylcysteinylthreonine Amide. Benzyloxycarbonylasparaginylasparaginylphenylalanine hydrazide trifluoroacetate (169 mg) was dissolved in DMF (14 ml) and the solution cooled at −10°. To this solution was added 7.7 *N* hydrogen chloride in dioxane (0.17 ml) followed by 10% *tert*-butyl nitrite in DMF (0.33 ml) and the mixture was stirred at −10° for 12 min. The reaction mixture was cooled at −25° and TEA (0.22 ml) was added followed by a solution of valylglutamyl-*S*-ethylcarbamoylcysteinylthreonine amide (134 mg) in DMF (4 ml). The reaction mixture was stirred at 4° for 24 hr when the product was precipitated by the addition of ethyl acetate. The material was washed with water and ethyl acetate and dried. For partial purification the material was precipitated from DMF with ethyl acetate: 250 mg (95%); $[\alpha]^{24}D$ −28.8° (*c* 0.71, DMF); R_f^{I} 0.6; R_f^{III} 0.7. The protected heptapeptide amide (253 mg) was deprotected by exposure to HBr in anhydrous TFA in the manner described and the product was precipitated by addition of ether (225 mg). Bromide ions were exchanged for acetate ions on Amberlite IRA-400 and the lyophilized product dissolved in water (70 ml) was applied to a Biorex 70 (mesh 50–100) column (2 × 13 cm) which was eluted with water (600 ml) and 0.005 *N* acetic acid (1000 ml). Individual fractions (10 ml each) were collected at a flow rate of approximately 5 ml/min. Fractions containing homogeneous material by tlc were pooled, evaporated to a small volume, and lyophilized: 118 mg (54%); $[\alpha]^{26}D$ −26.4° (*c* 0.53, 90% formic acid); R_f^{I} 0.3; R_f^{III} 0.6; amino acid ratios in 24 hr acid hydrolysate, $Asp_{2.2}Phe_{1.1}Val_{0.9}Glu_{1.0}Cys_{0.5}$-$Thr_{0.9}$ (87%); amino acid ratios in 24 hr AP-M digest, $Asp_{2.1}$-$Phe_{1.1}Val_{0.9}Glu_{0.9}Cys_{0.6}Thr_{0.1}ThrNH_{2_{1.0}}$.

(Positions 81–94) Benzyloxycarbonylasparaginylglutamylasparaginylasparaginylglutaminylleucylalanylglycylvalylisoleucylthreonylhistidylthreonylglycine Hydrazide Bistrifluoroacetate (Subfragment G_1G_2). Subfragment G_1 hydrazide trifluoroacetate(56 mg) was dissolved in a mixture of DMSO (0.8 ml) and DMF (0.8 ml) and the solution was cooled at −10°. To this solution was added 7.7 *N* hydrogen chloride in dioxane diluted (1:10) with DMF (0.33 ml) followed by 10% *tert*-butyl nitrite in DMF (0.07 ml) and the mixture was stirred at −10° for 10 min. The reaction mixture was cooled at −20° and 10% TEA in DMF (0.48 ml) was added followed by a solution of subfragment G_2 *tert*-butoxycarbonylhydrazide diacetate (43 mg) dissolved in DMF (0.5 ml) and 10% TEA in DMF (0.14 ml). The reaction mixture was stirred for 48 hr at 4° and ethyl acetate was added. The precipitate was collected, washed with ethyl acetate and water, and dried (87 mg). This material was dissolved in ice-cold 90% TFA (1.2 ml) and the solution was kept at 0° for 10 min and at room temperature for 30 min when ether was added. The precipitate was washed with ether and dried (86 mg). The material was dissolved in 45% formic acid (2.5 ml) and the solution added to a Sephadex G-25 column (1 × 120 cm) which was developed with the same solvent. Fractions (1.5 ml each) were collected at a flow rate of 1.5 ml/10 min and evaluated by tlc. Fractions containing homogeneous material (R_f^{III} 0.5) were pooled and lyophilized: 50 mg (54%); R_f^{III} 0.5; amino acid ratios in 48 hr acid hydrolysate, $Asp_{3.2}$-$Glu_{2.2}Leu_{1.1}Ala_{1.0}Gly_{1.8}Val_{0.9}Ile_{0.9}Thr_{2.0}Thr_{1.0}His_{1.0}$ (90%); ratio of Ile/Leu = 0.8.

(Positions 89–104) Valylisoleucylthreonylhistidylthreonylglycylalanylserylglycylasparaginylasparaginylphenylalanylvalylglutamyl-*S*-ethylcarbamoylcysteinylthreonine Amide (Subfragment G_2G_3). Fragment G_2 hydrazide bistrifluoroacetate (150 mg) was dissolved in DMF (1.5 ml) and the solution was cooled at −10°. To this solution was added 6.7 *N* hydrogen chloride in dioxane diluted 1:10 with DMF (0.67 ml) followed by 10% *tert*-butyl nitrite in DMF (0.20 ml) and the mixture was stirred at −10° for 15 min. The reaction mixture was cooled at −25° and TEA (0.10 ml) was added followed by a solution of subfragment G_3 hydrobromide (116 mg) in DMSO (1.5 ml) and TEA 10% in DMF (0.14 ml). The reaction mixture was stirred at 4° for 72 hr; then ethyl acetate was added and the precipitate was collected and washed with ethyl

acetate and water and dried, yield 221 mg. This material was deblocked in anhydrous TFA saturated with HBr (2 ml) in the usual manner and the reaction product was precipitated with ether, washed with ether, and dried, yield 238 mg. This material was dissolved in 40% formic acid (3 ml) and the solution was applied to a Sephadex G-25 column (2 × 150 cm) which was developed with 40% formic acid. Fractions (7 ml each) were collected at a flow rate of approximately 7 ml/15 min and small aliquots of individual fractions were subjected to tlc using the chlorine test for visualization. The content of tubes containing single spot material (R_f^{III} 0.6) were pooled and lyophilized: 69 mg (36%); $[\alpha]D^{29}$ −33.7° (*c* 1.0, 90% formic acid); amino acid ratios in 48 hr acid hydrolysate, $Val_{2.0}Ile_{1.0}Thr_{3.1}His_{1.0}Gly_{2.1}Ala_{1.0}Ser_{0.8}Asp_{2.0}Phe_{1.0}Glu_{1.0}Cys_{0.9}$ (82%); ratio Ile/Phe = 1.0; amino acid analysis in performic acid oxidized sample, $Val_{2.0}Ile_{1.0}Thr_{1.9}His_{1.1}Gly_{2.1}Ala_{1.1}Ser_{0.8}Asp_{2.1}Phe_{1.0}Glu_{1.0}Cys(SO_3H)_{0.8}$ (92%); ratio Ile/Phe = 1.0.

Synthesis of Fragment G (Positions 81–104). Route a. From Subfragment G_4 Azide and Subfragment G_5. Subfragment G_4 hydrazide bistrifluoroacetate (50 mg) was dissolved in 90% TFA (1 ml) and the solution was cooled at −10°. A 1% solution of *tert*-butyl nitrite in DMF (0.3 ml) was added and the solution was stirred at −10° for 10 min when ether was added. The precipitate was collected, washed with ice-cold ether, and dried at 4°. The azide (46 mg) was added to a solution of subfragment G_5 (25 mg) in DMSO (1 ml) and 10% TEA in DMF (0.12 ml) and the mixture was stirred at room temperature for 24 hr. Ethyl acetate was added and the precipitate was collected, washed with ethyl acetate and water, and dried (70 mg). This material was dissolved in anhydrous TFA saturated with HBr (3 ml) containing anisole (0.1 ml) and HBr was passed through the solution at 0° for 5 min and at room temperature for 40 min. Ether was added and the precipitate was washed with ether and dried (69 mg). This material was stirred for 12 hr at room temperature in water (70 ml) and the insoluble material was removed by centrifugation. The clear supernatant was added to a column of Biorex 70 H^+ form (1.5 × 5 cm) which was eluted with water (350 ml), 0.5 *N* acetic acid (280 ml), 1 *N* acetic acid (280 ml), 2 *N* acetic acid (70 ml), and 5 *N* acetic acid (280 ml). The 5 *N* eluates were evaporated to a small volume and lyophilized: 14 mg (22%); R_f^{III} 0.5; amino acid ratios in 48 hr acid hydrolysate, $Asp_{5.0}Glu_{3.1}Leu_{1.05}Ala_{2.0}Gly_{3.1}Val_{1.8}Ile_{0.98}Thr_{3.0}His_{1.0}Ser_{1.0}Phe_{0.95}Cys_{0.6}$ (76%); ratio Ile/Phe = 1.03.

Route b. From Subfragment G_1G_2 Azide and Subfragment G_3. Subfragment G_1G_2 hydrazide bistrifluoroacetate (60 mg) was dissolved in 90% TFA (1.2 ml) and the solution was cooled at −20°. A 10% solution of *tert*-butyl nitrite in DMF (0.042 ml) was added and the solution was stirred at −20° for 10 min when ether was added. The precipitate was collected, washed with ether and 1% TEA in ether, and dried at 4°. This azide dissolved in DMSO (0.8 ml) was added at 4° to a solution of subfragment G_3 hydrobromide (37 mg) in DMSO (1 ml), DMF (0.8 ml), and 10% TEA in DMF (0.044 ml). The mixture was stirred for 36 hr at 4° and ethyl acetate was added. The precipitate was washed with ethyl acetate and water and dried (90 mg). The material was deprotected with HBr in TFA in the manner described above and the reaction product dissolved in 20% acetic acid (60 ml) was applied to a column (1 × 12 cm) of Amberlite IRA-400 and the column was eluted with 20% acetic acid. Chlorine positive eluates were pooled and lyophilized (92 mg). The desired product was isolated by Sephadex G-50 chromatography using 30% formic acid as the solvent: 11 mg (13%) of the lyophilized product was obtained; R_f^{III} 0.5; amino acid ratios in 48 hr acid hydrolysate, $Asp_{4.9}Glu_{3.0}Leu_{1.06}Ala_{2.1}Gly_{3.0}Val_{2.0}Ile_{1.03}Thr_{2.9}His_{1.0}Ser_{1.0}Phe_{1.06}Cys_{0.7}$ (92%); ratio Leu/Phe = 1.0.

Route c. From Subfragment G_1 Azide and Subfragment G_2G_3. Subfragment G_1 hydrazide trifluoroacetate (84 mg) was dissolved in DMSO (1.0 ml) and DMF (0.7 ml) was added. The clear solution was cooled at −10° and 6.7 *N* hydrogen chloride in dioxane diluted (1:10) with DMF (0.34 ml) was added followed by 10% *tert*-butyl nitrite in DMF (0.11 ml). The reaction mixture was stirred at −10° for 15 min and cooled at −20° and TEA (10% in DMF, 0.44 ml) was added followed by a solution of subfragment G_2G_3 hydrobromide (94 mg) in DMSO (0.7 ml) and 10% TEA in DMF (0.14 ml). The precipitate was collected, washed with ethyl acetate and water, and dried (131 mg). This material was deprotected in 2 ml of anhydrous TFA–HBr in the manner described above, and the product was precipitated with ether, washed with ether, and dried (138 mg). The desired product was isolated by gel filtration on a column of Sephadex G-50 (1.8 × 145 cm) using 30% formic acid as the solvent. The contents of tubes containing single spot material by tlc (R_f^{III} 0.5) were pooled and the product was isolated by lyophilization: 38 mg (28%); $[\alpha]^{25}D$ −42.5° (*c* 0.98, 90% HCOOH); R_f^{III} 0.5, ninhydrin, Pauly, Ellman + NH_3 positive spot; amino acid ratios in 48 hr acid hyrolysate, $Asp_{5.0}Glu_{2.9}Leu_{1.05}Ala_{2.1}Gly_{3.0}Val_{2.0}Ile_{0.99}Thr_{2.9}His_{1.0}Ser_{1.0}Phe_{1.01}Cys_{0.5}$ (92%); ratio Ile/Phe = 0.98.

Fragment G was also isolated by chromatography on AG1-X2 in the following manner. Crude fragment G (294 mg) prepared from 168 mg of G_1 hydrazide and 188 mg of G_2G_3 in the manner described above was lyophilized twice from aqueous formic acid and dissolved in DMF–H_2O (3:2; 60 ml). The solution was applied to an AG1-X2 column (2 × 7 cm) that was developed with the following solvents: DMF–H_2O (150 ml), DMF–0.01 *N* AcOH (150 ml), DMF–0.02 *N* AcOH (100 ml), DMF–0.05 *N* AcOH (150 ml), and DMF–1 *N* AcOH (100 ml). The ratio of DMF to aqueous AcOH was 3:2. Fractions (5 ml each) were collected and chlorine positive tubes from the DMF–0.05 *N* AcOH eluates containing the desired material were pooled, evaporated, and lyophilized from aqueous formic acid: 50 mg (19%); R_f^{III} 0.5; amino acid ratios in 48 hr acid hydrolysate, $Asp_{4.8}Glu_{3.1}Leu_{1.02}Ala_{2.1}Gly_{3.2}Val_{1.8}Ile_{0.93}Thr_{3.2}His_{0.9}Ser_{0.9}Phe_{1.03}Cys_{0.9}$ (88%); ratio Leu/Phe = 0.97; amino acid ratios in 48 hr acid hydrolysate of performic acid oxidized sample, $Asp_{4.8}Glu_{2.9}Leu_{1.01}Ala_{2.0}Gly_{3.1}Val_{2.1}Ile_{1.14}Thr_{3.1}His_{1.1}Ser_{0.8}Phe_{1.01}Cys(SO_3H)_{0.9}$ (92%); ratio Leu/Phe = 1.0.

Acknowledgment. The authors wish to express their appreciation to Mr. Guirguis Rizk for the amino acid analyses.

Communications to the Editor

Charge-Transfer Complexing between Permethylpolysilanes and Tetracyanoethylene[1]

Sir:

Excited-state charge transfer (CT) complexes between aromatic hydrocarbons and π acceptors are well known,[2] and CT complexes are also formed between lone pair donors and π acceptors. In studying the spectral properties of permethylpolysilanes we find that compounds with Si–Si bonds appear to form CT complexes with the π acceptor tetracyanoethylene (TCNE), even though these silanes lack either π bonds or lone electron pairs.

Solutions of permethylpolysilanes, 0.1–0.5 *M* in chloroform containing 0.015 *M* TCNE, are colored, and the color deepens as the number of silicon atoms in the chain increases. In the visible spectrum new CT bands appear, sometimes rather broad and unsymmetrical

(1) This work was supported in part by the Air Force Office of Scientific Research (NC) O.A.R., USAF, Grant No. AF-AFOSR, 70-1904.

(2) R. Foster, "Organic Charge-Transfer Complexes," Academic Press, New York, N. Y., 1969; R. S. Mulliken and W. Person, "Molecular Complexes," Wiley, New York, N. Y., 1969.