Studies on Polypeptides. XXXII. Synthetic Peptides Related to the N-Terminus of Bovine Pancreatic Ribonuclease A (Positions 1–13)[1-4]

Klaus Hofmann, Ralph Schmiechen, Michael J. Smithers, Robert D. Wells, Yecheskel Wolman, and Guido Zanetti

Contribution from the Biochemistry Department of the University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. *Received August 14, 1964*

Syntheses are described of the following seven peptides all related to the N-terminus of bovine pancreatic ribonuclease A: N^{ϵ}-formyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine, alanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine, threonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine, lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutamine, lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidine amide, lysylglutamylthreonylalanylalanylalanyllysylglutamylarginylglutaminylhistidylmethionine, and lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidyl-α-amino-n-butyric acid. Evidence is presented for the homogeneity of these compounds.

Tryptic digestion destroys the ability of S-peptide to generate ribonuclease activity with S-protein.[5] One of the two trypsin labile bonds (see arrows in Scheme I), presumably the one linking arginine 10 to glutamine 11, is split by trypsin at a significantly higher rate than the lysine 7 to phenylalanine 8 linkage, and rupture of this more labile bond appears to be responsible for deactivation of S-peptide. This evidence indicates that fragments corresponding to positions 1–10 and 11–20 of S-peptide lack the ability to activate S-protein but the enzymic digest was tested only at low peptide–S-protein ratios. In view of the possibility that specific segments of S-peptide, not obtainable by enzymic means, could activate S-protein, we prepared peptides I–VII (Scheme I) for enzymic evaluation. Since the synthetic peptides are not contaminated by S-peptide their ability to activate S-protein can be tested over a wide range of concentrations.

This communication describes synthetic routes to these peptides; their ability to generate ribonuclease activity with S-protein will be the subject of a later study.

The tridecapeptide VI and its α-amino-*n*-butyric acid analog (VII) constitute the largest peptides in this series. The other compounds arise from VI by successive shortening of the peptide chain both from the N-terminal (peptides I–III) and from the C-terminal end (peptides IV and V). Peptide VII is an analog of peptide VI in which α-amino-*n*-butyric acid (But) replaces the methionine residue.

In previous communications[6,7] we have described synthetic routes to three protected peptide hydrazides (VIII–X), three peptides (XI, XIII, and XIV), and the pentapeptide amide (XII). These compounds were linked in the manner illustrated in Scheme II to give six protected peptides (XVIII–XXIII). In essence all these syntheses involve formation of the peptide bond between lysine 7 and phenylalanine in position 8 of S-peptide *via* an azide coupling step.

The solid azide XVII was used for preparation of the protected peptides XVIII, XX, and XXI; the Rudinger modification[8] of the azide procedure was employed for the synthesis of compounds XIX, XXII, and XXIII with the azides XV–XVII as intermediates. The partially protected peptides XVIII–XXIII were separated from unreacted free peptides by distribution between aqueous acetic acid and 1-butanol. With exception of XIX the butanol-soluble materials were subjected to chromatography on Dowex 1-X2 columns. The ensuing, still inhomogeneous materials were deblocked by exposure to trifluoroacetic acid and the trifluoroacetate salts were converted to the corresponding acetates. Chromatography on carboxymethylcellulose with ammonium acetate solutions of increasing ionic strength serving as the eluting agents[9] was used to purify the free peptides. For the synthesis of N^{ϵ}-formyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine (I) peptide XIII[7] was acylated with succinimido N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-formyllysinate[6] and the ensuing protected heptapeptide was deblocked with trifluoroacetic acid at 0°. Experiments with N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-formyllysine indicated that the N^{ϵ}-formyl group undergoes significant cleavage on exposure to trifluoroacetic acid at 20° for 10 min.

The highly purified methionine-containing peptides were treated with thioglycolic acid to reduce contaminating sulfoxides. Except for minor contamination by the corresponding sulfoxides the resulting peptides were homogeneous as judged by paper chromatography. Compounds II, III, V, VI, and VII

(1) The authors wish to express their appreciation to the U. S. Public Health Service, the National Science Foundation, and the American Cancer Society for generous support of this investigation.

(2) The peptides and peptide derivatives mentioned are of the L-configuration. In the interest of space conservation the customary L-designations for individual amino acid residues are omitted.

(3) See K. Hofmann, W. Haas, M. J. Smithers, and G. Zanetti, *J. Am. Chem. Soc.*, **87**, 631 (1965), for paper XXXI in this series.

(4) A preliminary communication describing some of the results presented in this study has appeared: *ibid.*, **85**, 833 (1963).

(5) J. E. Allende and F. M. Richards, *Biochemistry*, **1**, 295 (1962).

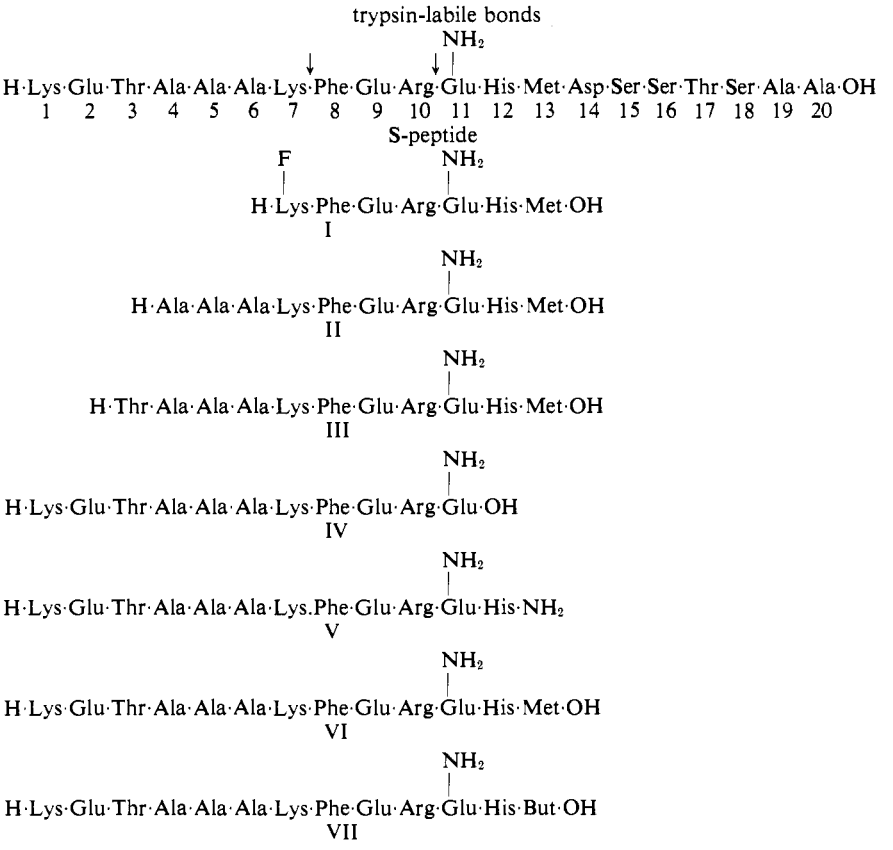
(6) K. Hofmann, R. Schmiechen, R. D. Wells, Y. Wolman, and N. Yanaihara, *J. Am. Chem. Soc.*, **87**, 611 (1965).

(7) K. Hofmann, W. Haas, M. J. Smithers, R. D. Wells, Y. Wolman, N. Yanaihara, and G. Zanetti, *ibid.*, **87**, 620 (1965).

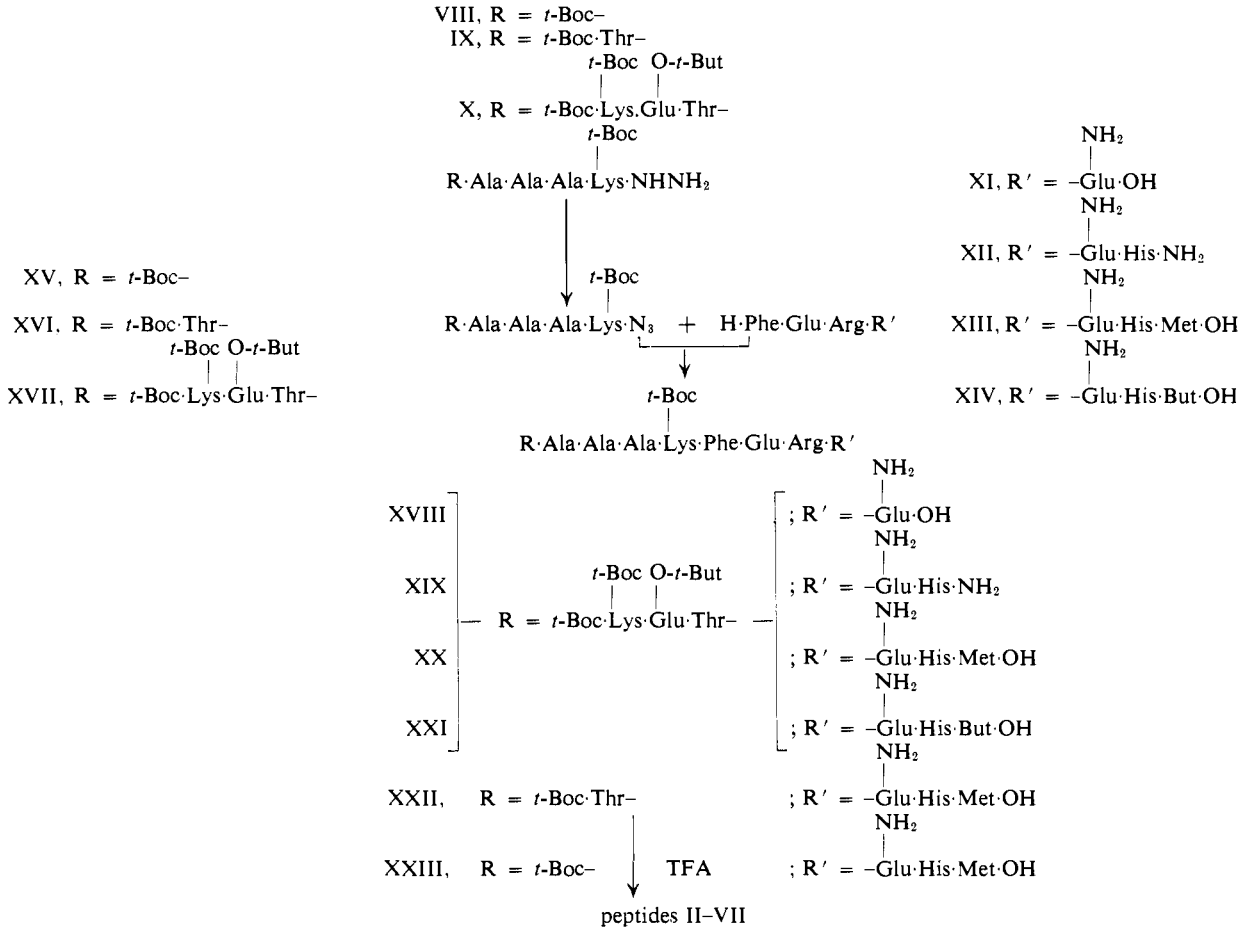
(8) J. Honzl and J. Rudinger, *Collection Czech. Chem. Commun.*, **26**, 2333 (1961).

(9) K. Hofmann, T. Y. Liu, H. Yajima, N. Yanaihara, and S. Lande, *J. Am. Chem. Soc.*, **83**, 2294 (1961).

Scheme I



Scheme II



formed a single spot on paper electrophoresis at various pH values. All the peptides afforded correct elemental analyses and their degree of hydration was verified by oxygen determinations. Acid hydrolysates showed the theoretically expected amino acid ratios and the peptides were completely digestible by leucine aminopeptidase.

In a previous communication[7] dealing with related peptides we have noted deamidation of glutamine in the leucine aminopeptidase digests. This was attributed to the presence of glutaminase in the enzyme preparation employed. The same behavior has now been observed with all of the peptides recorded in this communication. Their leucine aminopeptidase digests contained at best trace quantities of glutamine and the glutamic acid content was consistently high. Careful ammonia determinations[7] in acid hydrolysates of the peptides gave the values expected by theory, thus demonstrating unequivocally that one glutamine residue was present in these compounds.

Experimental[10]

Purification and Deblocking of Peptides. The residue from the azide coupling reaction was distributed between 1-butanol (equilibrated with 2% acetic acid) and 2% acetic acid (equilibrated with 1-butanol). The aqueous phase was extracted in countercurrent fashion with five portions of 1-butanol (equilibrated with 2% acetic acid) and the organic phases were washed with nine portions of 2% acetic acid (equilibrated with 1-butanol). The combined butanol phases were evaporated to dryness *in vacuo* and the residue was lyophilized from 90% aqueous dioxane. This material was subjected to chromatography on Dowex 1-X2 in the acetate cycle with 2-propanol–water–methanol (1:1:1) followed by 2-propanol–0.1 *M* acetic acid–methanol (1:1:1) serving as eluting agents. Histidine-containing peptides were located in the eluates by the Pauly reaction, and peptide XVIII was located by the Sakaguchi reaction. Fractions containing the desired material were pooled, evaporated to dryness *in vacuo*, and lyophilized from 90% aqueous dioxane. In all cases material at this stage of purification was rechromatographed on Dowex 1-X2 as described. Except when noted otherwise the protected peptides were deblocked by exposure to trifluoroacetic acid at room temperature for 20 min. and the trifluoroacetic acid was removed *in vacuo*. The residue was dissolved in a small volume of water and the solution was lyophilized.

The trifluoroacetate salt was dissolved in water and the solution was applied to an Amberlite IRA-400 column in the acetate cycle which was eluted with 2% acetic acid. Eluates containing the desired material were pooled, evaporated to a small volume *in vacuo*, and lyophilized. The peptides in water were applied to a CMC column which was eluted with pH 6.9 ammonium acetate solutions of increasing ionic strength essentially in the manner previously described.[9] For reduction, methionine-containing peptides were incubated under nitrogen at 45–50° with 1% aqueous thioglycolic acid for 24 hr. Thioglycolate ions were exchanged for acetate ions on Amberlite IRA-400 (acetate cycle) in the usual manner and the final product was lyophilized to constant weight from water.

N^{α},N^{ϵ}-Di-t-butyloxycarbonyllysyl-γ-t-butylglutamylthreonylalanylalanylalanyl-N-t-butyloxycarbonyllysine Azide (XVII). Sodium nitrite (26 mg.) in water (1.5 ml.) was added with stirring to an ice-cold solution of the hydrazide X[6] (330 mg.) in glacial acetic acid (10 ml.), water (5 ml.), and 1 *N* hydrochloric acid (0.60 ml.), and the mixture was kept at ice-bath temperature for 5 min. Ice-cold saturated sodium chloride (25 ml.) was added and the precipitate was collected, washed with saturated sodium chloride, saturated sodium bicarbonate, and water, and dried *in vacuo* over P_2O_5 and potassium hydroxide pellets at 5°; yield 315 mg.

N^{ϵ}-Formyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine Diacetate Trihydrate (I). Succinimido N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-formyllysinate[6] (320 mg.) in tetrahydrofuran (5 ml.) was added to a solution of phenylalanylglutamylarginylglutaminylhistidylmethionine monoacetate trihydrate (XIII)[7] (180 mg.) in water (3 ml.) containing triethylamine (0.12 ml.), and the mixture was stirred for 24 hr. at room temperature when the solvents were removed *in vacuo*. The residue was dissolved in water (10 ml.), acetic acid (0.2 ml.) was added, and the solution was lyophilized. The material dissolved in water (250 ml.) was added to a CMC column (1.5 × 20 cm.) which was eluted successively with water (100 ml.) and the following pH 6.9 ammonium acetate buffers: 0.005 (100 ml.) and 0.01 *M* (200 ml.). Individual fractions (6 ml. each) were collected at a flow rate of approximately 2 ml./min. The desired material was located by the Pauly reaction in the 0.01 *M* eluates which were pooled, concentrated *in vacuo*, and lyophilized to constant weight from small volumes of water; yield 210 mg.; R_f^{VI} 0.25; R_f^3 3.0 × His; contaminated by the sulfoxide R_f^{VI} 0.35; R_f^3 2.1 × His; both spots Pauly and Sakaguchi positive.

The above protected peptide (205 mg.) was dissolved in ice-cold trifluoroacetic acid (1 ml.) and the solution was kept in an ice bath for 20 min. Ice-cold ether (50 ml.) and ice–water (35 ml.) were added and the aqueous phase was extracted with two 50-ml. portions of ice-cold ether which were washed with two 35-ml. portions of ice–water. The combined aqueous phases were lyophilized and the free peptide was purified by chromatography on CMC. The peptide was eluted from the CMC column with 0.025 *M* ammonium acetate; yield 150 mg. (68%); $[\alpha]^{28}D$ −20.0° (*c* 0.30, 10% acetic acid); R_f^3 1.59 × His; contaminated with a trace of the sulfoxide R_f^3 0.67 × His; ninhydrin-, Sakaguchi-, Pauly-, and methionine-positive spots; amino acid ratios in acid hydrolysate $lys_{0.98}phe_{1.00}glu_{2.00}arg_{1.04}met_{0.92}his_{1.00}$; amino acid ratios in LAP digest N^{ϵ}-$formlys_{1.18}phe_{1.03}glu_{1.92}arg_{0.79}met_{1.04}his_{0.94}$; amide nitrogen 1.05 μmoles/μmole.

Anal. Calcd. for $C_{43}H_{66}N_{14}O_{12}S \cdot 2CH_3COOH \cdot 3H_2O$: C, 48.1; H, 6.9; N, 16.6; O, 25.8. Found: C, 47.8; H, 6.9; N, 16.7; O, 26.0.

Alanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine Octahydrate (II). A 10%

(10) The experimental and analytical procedures used were those described in papers XXIX and XXX in this series (see ref. 6 and 7). The following abbreviations are used: DMF = dimethylformamide; THF = tetrahydrofuran; *t*-Boc = *t*-butyloxycarbonyl; O-*t*-But = *t*-butyl ester; But = α-amino-*n*-butyric acid; Gluta = glutamine; N^{ϵ}-formlys = N^{ϵ}-formyllysine; LAP = leucine aminopeptidase.

solution of *t*-butyl nitrite in DMF (0.25 ml.) was added to a stirred solution cooled at −20° of *t*-butyloxycarbonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysine hydrazide (VIII)[6] (115 mg.) in DMF (3.0 ml.) containing 0.10 ml. of 6.1 *N* hydrogen chloride in dioxane. The solution was stirred at −20° for 20 min., then cooled at −40°, and triethylamine (0.08 ml.) was added. To this solution containing the azide XV was added a solution of phenylalanylglutamylarginylglutaminylhistidylmethionine monoacetate trihydrate (XIII)[7] (131 mg.) in water (0.8 ml.) and triethylamine (0.06 ml.), and the mixture was stirred at 4° for 24 hr. Additional azide (prepared from 115 mg. of hydrazide) was added and stirring was continued at 4° for 24 hr. The solvents were removed *in vacuo* and the crude protected peptide purified in the manner described above; yield 80 mg.; R_f[2] 0.82; contaminated by the sulfoxide R_f[2] 0.67. The protected peptide was deblocked and the free peptide purified in the manner described. The compound was eluted from the CMC column with 0.05 *M* ammonium acetate; yield 57 mg. (29%); $[\alpha]^{27}D$ −57.7° (*c* 0.21, 10% acetic acid); R_f[3] 0.78 × His; slightly contaminated with the sulfoxide R_f[3] 0.33 × His; ninhydrin-, Pauly-, and Sakaguchi-positive spots: single ninhydrin-, Pauly-, and chlorine-positive spot on paper electrophoresis at pH 1.9, 3.5, 6.5, and 8.0; amino acid ratios in acid hydrolysate $ala_{3.14}lys_{0.87}phe_{0.97}glu_{2.24}arg_{0.95}his_{0.93}met_{0.91}$; amino acid ratios in LAP digest $(lys + orn + arg)_{1.85}ala_{3.46}phe_{0.96}glu_{1.61}his_{1.02}met_{1.09}$; amide nitrogen 1.00 μmole/μ mole.

Anal. Calcd. for $C_{51}H_{81}N_{17}O_{14}S \cdot 8H_2O$: C, 46.0; H, 7.3; N, 17.9; O, 26.4; S, 2.4. Found: C, 45.3; H, 7.6; N, 17.3; O, 26.2; S, 2.3.

Threonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine Monoacetate Trihydrate (III). A 50% *t*-butyl nitrite solution in DMF (0.10 ml.) was added to a stirred suspension cooled at −20° of *t*-butyloxycarbonylthreonylalanylalanylalanyllysine hydrazide (X,[6] 270 mg.) in DMF (4 ml.) containing 0.20 ml. of 6.1 *N* hydrogen chloride in dioxane. The mixture was stirred at −20° for 20 min., then cooled at −40°, and triethylamine (0.17 ml.) was added. To this solution containing the azide XVIII was added phenylalanylglutamylarginylglutaminylhistidylmethionine monoacetate trihydrate (XIII,[7] 362 mg.) in 50% aqueous DMF (5 ml.) containing triethylamine (0.17 ml.). After stirring for 30 min. at −20° and 24 hr. at 4° a further batch of azide (prepared from 79 mg. of the hydrazide) was added and the solution was stirred an additional 24 hr. at 4° and 12 hr. at room temperature. The solvents were removed *in vacuo* and the crude protected peptide was purified in the manner described; yield 202 mg.; R_f[1] 0.75; R_f[2] 0.28; contaminated by the sulfoxide R_f[1] 0.58, R_f[2] 0.10. The protected peptide was deblocked and the free peptide purified in the manner described. The peptide was eluted from the CMC column with 0.05 *M* ammonium acetate; yield 95 mg. (18%); $[\alpha]^{28}D$ −65.0° (*c* 0.21, water); $[\alpha]^{28}D$ −64.6° (*c* 0.21, 10% acetic acid); R_f[3] 0.79 × His; slightly contaminated by the sulfoxide R_f[3] 0.36 × His; ninhydrin-, Pauly-, Sakaguchi-, and chlorine-positive spots; single ninhydrin-, Pauly-, and chlorine-positive spot on paper electrophoresis at pH 1.9, 3.5, 6.5, and 8.0; amino acid ratios in acid hydrolysate $thr_{0.98}ala_{3.08}lys_{0.98}phe_{1.02}glu_{1.97}arg_{1.12}his_{0.98}met_{0.88}$; amino acid ratios in LAP digest $thr_{1.14}ala_{3.28}(lys + orn + arg)_{1.95}phe_{0.98}glu_{1.37}his_{1.09}met_{1.02}$; amide nitrogen 1.05 μmoles/μmole.

Anal. Calcd. for $C_{55}H_{88}O_{16}N_{18}S \cdot CH_3COOH \cdot 3H_2O$: C, 48.8; H, 7.0; N, 18.0; O, 23.9. Found: C, 48.5; H, 7.0; N, 18.0; O, 24.0.

Lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutamine Diacetate Heptahydrate (IV). N$^{\alpha}$,N$^{\epsilon}$-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysine azide (XVII) (prepared from 330 mg. of the hydrazide X[6]) in ice-cold DMF (10 ml.) was added to an ice-cold 50% aqueous DMF solution (10 ml.) containing triethylamine (0.09 ml.) and phenylalanylglutamylarginylglutamine (XI[7] 180 mg.) and the mixture was stirred at 5° for 24 hr. An additional quantity of azide (prepared from 110 mg. of the hydrazide) dissolved in DMF (5 ml.) was added and stirring was continued for another 24 hr. at 5°. The solvents were evaporated *in vacuo* and the crude protected peptide was purified in the manner described above; yield 160 mg.; R_f[2] 0.54; Sakaguchi- and chlorine-positive spot contaminated by a substance of R_f[2] 0.35. The protected peptide was deblocked and the free peptide was purified in the manner described; yield 120 mg. (26%); $[\alpha]^{27}D$ −78.5° (*c* 0.23, 10% acetic acid); R_f[3] 0.48 × His; single ninhydrin- and Sakaguchi-positive spot; amino acid ratios in acid hydrolysate $lys_{2.10}glu_{2.96}thr_{0.95}ala_{3.00}phe_{0.95}arg_{1.03}$; amino acid ratios in LAP digest $(lys+orn+arg)_{2.78}glu_{3.03}thr_{1.19}ala_{3.36}phe_{0.72}$; amide nitrogen 1.00 μmole/μmole.

Anal. Calcd. for $C_{55}H_{91}O_{18}N_{17} \cdot 2CH_3COOH \cdot 7H_2O$: C, 46.5; H, 7.5; N, 15.6; O, 30.4. Found: C, 47.3; H, 7.8; N, 15.5; O, 31.0.

Lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidine Amide Triacetate Tetrahydrate (V). *t*-Butyl nitrite (0.03 ml.) was added to a stirred suspension cooled at −20° of N$^{\alpha}$,N$^{\epsilon}$-di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysine hydrazide (X,[6] 275 mg.) in DMF–THF (1:1, 20 ml.) containing 0.13 ml. of 6.1 *N* hydrogen chloride in dioxane. The mixture was stirred at −20° for 45 min. when a clear solution resulted which was then cooled at −40° and triethylamine (0.10 ml.) was added. To this solution containing the azide XVII was added a solution of phenylalanylglutamylarginylglutaminylhistidine amide acetate tetrahydrate (XII,[7] 210 mg.) in 50% aqueous DMF (6 ml.) containing triethylamine (0.07 ml.), and the mixture was stirred at 4° for 40 hr. Additional azide (prepared from 275 mg. of the hydrazide) was then added and stirring was continued at 4° for 24 hr. The solvents were removed *in vacuo* and the residue was dissolved in 1-butanol (50 ml.) and water (50 ml.). The solution was acidified with acetic acid and extracted with six 80-ml. portions of 1-butanol (equilibrated with 2% acetic acid). The butanol phases were washed with ten 50-ml. portions of 2% acetic acid (equilibrated with 1-butanol) and the organic phases were combined and evaporated to dryness; yield 690 mg.

The protected dodecapeptide (XIX, 690 mg.) which was contaminated with azide rearrangement products

was dissolved in anhydrous trifluoroacetic acid (10 ml.) and the solution was kept at room temperature for 30 min. Ice-cold ether (500 ml.) was added and the ensuing suspension was kept at −10° for 30 min. when the precipitate was collected and washed with ether. Two chromatographic steps on CMC were required to obtain a homogeneous sample of this peptide which was eluted from the column with 0.10 *M* ammonium acetate; yield 150 mg. (36%); $[\alpha]^{26}D$ −49.1° (*c* 0.29, 10% acetic acid); R_f[3] 0.27 × His; single ninhydrin-, Sakaguchi-, and Pauly-positive spot; single ninhydrin-, Pauly-, and Sakaguchi-positive spot on paper electrophoresis at pH 1.9 and 3.5; amino acid ratios in acid hydrolysate $lys_{1.91}glu_{3.30}thr_{0.95}ala_{3.12}phe_{0.93}arg_{0.91}his_{0.91}$; amino acid ratios in LAP digest $(lys+orn+arg)_{3.06}glu_{2.94}thr_{1.03}ala_{3.00}phe_{1.06}his_{1.06}$; amide nitrogen 2.08 μmoles/μmole.

Anal. Calcd. for $C_{61}H_{99}O_{18}N_{21}\cdot 3CH_3COOH\cdot 4H_2O$: C, 48.3; H, 7.2; N, 17.6; O, 26.9. Found: C, 48.3; H, 7.0; N, 17.6; O, 27.0.

Lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine Diacetate Tetrahydrate (VI). N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-N^{ϵ}-*t*-butyloxycarbonyllysine azide (XVII) (prepared from 305 mg. of the hydrazide X)[6] was dissolved in ice-cold DMF (10 ml.) and the solution was added to phenylalanylglutamylarginylglutaminylhistidylmethionine monoacetate trihydrate (XIII,[7] 254 mg.) in 50% aqueous DMF (6.0 ml.) containing 10% triethylamine in DMF (0.78 mg.). The mixture was stirred at 4° for 24 hr., then additional azide (prepared from 305 mg. of the hydrazide) was added and stirring was continued at 4° for 24 hr. and at room temperature for 12 hr. The solvents were removed *in vacuo*, and the crude protected peptide was purified as described; yield 300 mg.; R_f[1] 0.88; R_f[2] 0.90; contaminated by the sulfoxide R_f[1] 0.75, R_f[2] 0.83. The protected peptide XX was deblocked and the free peptide was purified in the manner described. The peptide was eluted from the CMC column with 0.05 *M* ammonium acetate; yield 158 mg. (34%); $[\alpha]^{27}D$ −62.5° (*c* 0.21, 10% acetic acid); R_f[3] 0.50 × His; slightly contaminated with the sulfoxide R_f[3] 0.25 × His; ninhydrin-, Pauly-, Sakaguchi-, and methionine-positive spots. Single ninhydrin-, Pauly-, Sakaguchi-, and chlorine-positive component on paper electrophoresis at pH 1.9, 3.5, 6.5, and 8.0; amino acid ratios in acid hydrolysate $lys_{2.09}glu_{3.09}thr_{0.95}ala_{2.95}phe_{1.04}arg_{1.00}his_{1.04}met_{0.95}$; amino acid ratios in LAP digest $(lys+orn+arg)_{3.25}glu_{2.46}thr_{1.14}ala_{3.25}phe_{0.92}his_{1.04}met_{1.07}$; amide nitrogen 1.13 μmoles/μmole.

Anal. Calcd. for $C_{66}H_{107}N_{21}O_{20}S\cdot 2CH_3COOH\cdot 4H_2O$: C, 48.4; H, 7.1; N, 16.9; O, 25.8. Found: C, 48.0; H, 7.3; N, 16.8; O, 26.0.

Lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidyl-α-amino-n-butyric Acid Diacetate Tetrahydrate (VII). N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamyl-threonylalanylalanylalanyl-N^{ϵ}-*t*-butyloxycarbonyllysine azide (XVII) (prepared from 428 mg. of the hydrazide, X)[6] was dissolved in ice-cold DMF (15 ml.) and was added to a solution of phenylalanylglutamylarginylglutaminylhistidyl-α-amino-*n*-butyric acid monoacetate tetrahydrate (XIV,[7] 250 mg.) in 50% aqueous DMF (6.0 ml.) containing 10% triethylamine in DMF (1.09 ml.). The mixture was stirred at 4° for 24 hr., then additional azide (prepared from 143 mg. of the hydrazide) was added and stirring was continued at 4° for 24 hr. and at room temperature for 12 hr. The solvents were removed *in vacuo*, and the crude protected peptide was purified in the manner described above; yield 306 mg.; R_f[1] 0.89; R_f[2] 0.67. The protected peptide XXI was deblocked and the free peptide was purified in the manner described. The peptide was eluted from the CMC column with 0.05 *M* ammonium acetate solution; yield 157 mg. (36%); $[\alpha]^{29}D$ −61.3° (*c* 0.20, 10% acetic acid); R_f[3] × His; single ninhydrin-, Pauly-, Sakaguchi-, and chlorine-positive spot; single ninhydrin-, Pauly-, Sakaguchi-, and chlorine-positive component on paper electrophoresis at pH 1.9, 3.5, 6.5, and 8.0; amino acid ratios in acid hydrolysate $lys_{1.89}glu_{3.04}thr_{0.94}ala_{3.08}phe_{1.00}arg_{1.00}his_{0.94}but_{1.00}$; amino acid ratios in LAP digest $(lys+orn+arg)_{2.98}glu_{2.48}thr_{1.12}ala_{3.20}phe_{1.00}his_{1.00}but_{1.09}$; amide nitrogen 1.36 μmoles/μmole.

Anal. Calcd. for $C_{63}H_{105}N_{21}O_{20}\cdot 2CH_3COOH\cdot 4H_2O$: C, 49.0; H, 7.2; N, 17.4; O, 26.5. Found: C, 48.6; H, 7.3; N, 17.2; O, 25.7.

Acknowledgment. The authors wish to express their appreciation to Mrs. Jemele Hudson and Miss Judy Montibeller for skillful technical assistance. They wish to thank Dr. Frances M. Finn for the ammonia determinations.