Studies on Polypeptides. XXX. Synthetic Peptides Related to the N-Terminus of Bovine Pancreatic Ribonuclease (Positions 8–13)[1-4]

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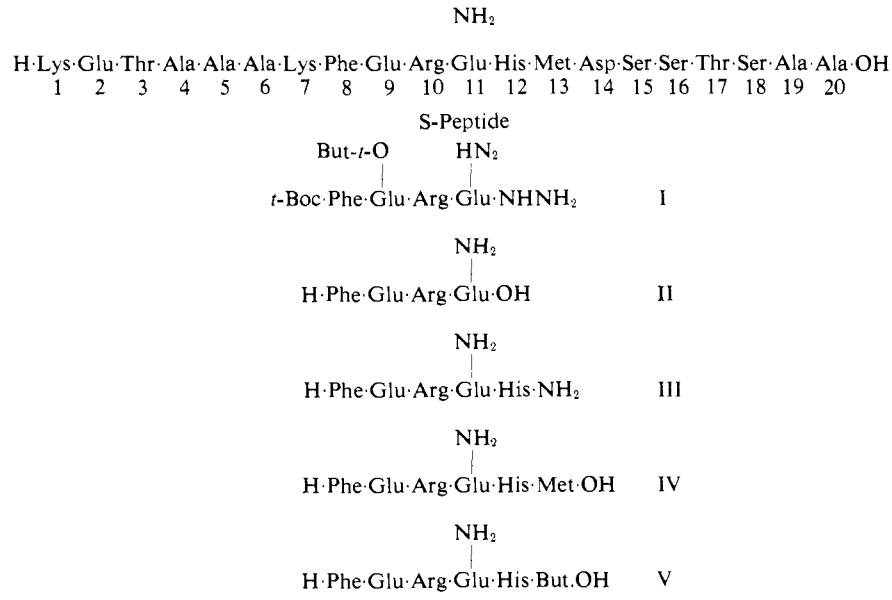
Syntheses are described of a series of peptides and peptide derivatives related to positions 8–13 in the amino acid sequence of bovine pancreatic ribonuclease A. In particular, the preparation is given of phenylalanylglutamylarginylglutamine, phenylalanylglutamylarginylglutaminylhistidine amide, phenylalanylglutamylarginylglutaminylhistidylmethionine, phenylalanylglutamylarginylglutamylhistidylmethionine, and phenylalanylglutamylarginylglutaminylhistidyl-α-amino-n-butyric acid. The hydrazide of t-butyloxycarbonylphenylalanyl-γ-t-butylglutamylarginylglutamine served as the key intermediate in the preparation of some of the above mentioned compounds. "Carbobenzoxyhydrazides" played an important role as intermediates.

The sequence about the histidine residue in position 12 of bovine pancreatic ribonuclease poses a challenge from the point of view of synthesis because residues derived from such polyfunctional amino acids as glutamic acid, glutamine, arginine, methionine, aspartic acid, and serine are interlinked in this section of the enzyme molecule. In this communication we describe syntheses of a number of peptides (II to IV, Scheme I) corresponding to this section of ribonuclease (S-peptide) and relate a method for preparation of the hydrazide (I) which provides a convenient subunit for construction of more complex fragments of the ribonuclease molecule. The preparation of the α-amino-*n*-butyric acid analog (V) of IV is also given.

Arginylglutamine, prepared according to Berse, *et al.*,[5] served as starting material for the synthesis of phenylalanylglutamylarginylglutamine (II). Interaction in aqueous methanol of triethylammonium arginylglutaminate with the *p*-nitrophenyl ester of benzyloxycarbonyl-γ-benzylglutamate[6] gave benzyloxycarbonyl-γ-benzylglutamylarginylglutamine which was converted into glutamylarginylglutamine by hydrogenolysis. As is frequently observed when using *p*-nitrophenyl esters in aqueous media the yield in the coupling reaction was low. Recently, Anderson[7,8] introduced the N-hydroxysuccinimide esters of acyl-amino acids as convenient reagents for peptide bond formation, particularly in aqueous systems. We employed the N-hydroxysuccinimide ester of benzyloxycarbonylphenylalanine[7b] for introducing the phenyl-

Scheme I



(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-designation for individual amino acid residues is omitted.

(3) See *J. Am. Chem. Soc.*, **87**, 611 (1965), for paper XXIX in this series.

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

(5) C. Berse, L. Piché, and A. Uchiyama, *Can. J. Chem.*, **38**, 1946 (1960).

(6) Ed. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1637 (1963).

(7) (a) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **85**, 3039 (1963); (b) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *ibid.*, **86**, 1839 (1964).

(8) We wish to express our appreciation to Dr. Anderson for making available to us a preprint of his detailed paper and a sample of N-hydroxysuccinimide.

alanine moiety into glutamylarginylglutamine and obtained benzyloxycarbonylphenylalanylglutamylarginylglutamine in analytically pure form in a yield of 67%. A mixture of tetrahydrofuran and water served as the solvent for this reaction. Following decarbobenzoxylation by hydrogenolysis the tetrapeptide (II) was obtained in crystalline form (Figure 1). The intramolecularly compensated zwitterionic form of the tetrapeptide appears to contribute to its pronounced tendency to crystallize. The peptide crystallizes from aqueous alcohol as fine needles or small prisms. Crystals in the latter habit were used in preliminary X-ray diffraction studies. The dried prisms, although seemingly single crystals, did not yield an X-ray diffraction pattern characteristic of single crystals. However, wet crystals in the presence of mother liquor yielded single crystal diffraction patterns. Crystal data obtained from rotation and Weissenberg photographs are as follows: monoclinic with $a = 20.00$, $b = 14.80$, $c \sin \beta = 14.05$ Å.; $\beta = 102°$; v = 4160 Å.^{3}; density, wet = 1.315 g. cm.^{3}, dry = 1.335 g. cm^3; space group P2.

Density of the wet crystals was measured by the flotation method in aqueous alcohol solutions of barium bromide. Density of dry crystals was determined in carbon tetrachloride and benzene solutions at ambient temperature. The molecular weight of the unit cell content of the wet crystals is 3294. Since the formula weight of one tetrapeptide molecule is 578.5, a reasonable interpretation of these data is that four tetrapeptide molecules make up 70.3% of the unit cell weight, the remainder consisting of water, alcohol, or both. However, the possibility of a fifth disordered peptide molecule on a twofold axis with 12% solvation is not excluded.[9]

Attempts to prepare II by condensing the azide of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamic acid (XV) with arginylglutamine followed by trifluoroacetic acid deblocking failed to provide homogeneous samples.

In 1950[10] we devised a route to protected hydrazides ("carbobenzoxyhydrazides") of amino acids and peptides which involves the use of benzyloxycarbonylhydrazide (VII). These "carbobenzoxyhydrazides" are of considerable interest from the point of view of synthesis of complex peptides since they allow the stepwise construction of peptide chains from the carboxyl end with retention of a protected hydrazide function. The hydrazide is readily liberated once assembly of a desired subunit has been achieved. In our initial work we made use of the differential stability between the phthaloyl and benzyloxycarbonyl groups for preparation of "carbobenzoxyhydrazides." Since then various amino protecting groups have been devised which can be cleaved by manipulations differing from those necessary to bring about decarbobenzoxylation. Particularly attractive in this connection is the *t*-butyloxycarbonyl group[11] which resists hydrogenolysis but can be cleaved under conditions which do not affect the benzyloxycarbonyl function. Conversely, hydrogenolysis splits the benzyloxycarbonyl group but leaves the *t*-butyloxycarbonyl function unaltered.

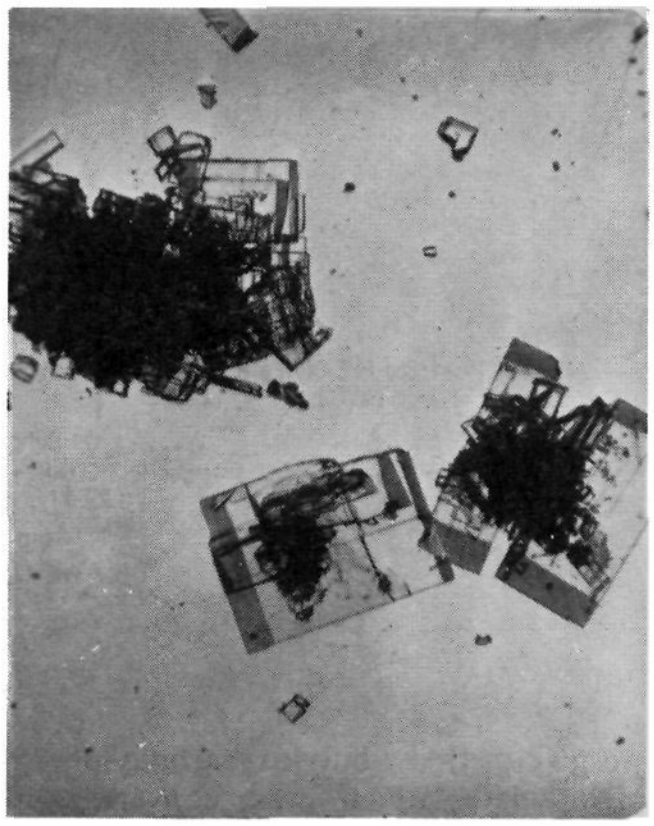


Figure 1. Crystals of H·Phe·Glu·Arg·Gluta·OH from aqueous ethanol: 87.5 times magnification.

Based on these principles two routes (Scheme II) were developed for synthesis of the "carbobenzoxyhydrazide" of N$^\alpha$-*t*-butyloxycarbonylnitroarginylglutamine (XI).

t-Butyloxycarbonylglutamine (VI) in the form of a mixed anhydride was allowed to react with benzyloxycarbonylhydrazide (VII) to give *t*-butyloxycarbonylglutamine "carbobenzoxyhydrazide" (VIII) which was partially deblocked by exposure to trifluoroacetic acid[11a,12] to form glutamine "carbobenzoxyhydrazide" (IX). Reaction of IX with a mixed anhydride of N$^\alpha$-*t*-butyloxycarbonylnitroarginine (X) afforded the "carbobenzoxyhydrazide" of N$^\alpha$-*t*-butyloxycarbonylnitroarginylglutamine (XI). Alternately, compound XI was obtained by treating N$^\alpha$-*t*-butyloxycarbonylnitroarginylglutamine (XIII), prepared from a mixed anhydride of N$^\alpha$-*t*-butyloxycarbonylnitroarginine (X) and glutamine (XII), with benzyloxycarbonylhydrazide (VII). N,N′-Carbonyldiimidazole[13] served as the condensing agent for this reaction. Although the optical rotations of XI prepared by these two procedures differed somewhat, the compound obtained by either route on partial deblocking with trifluoroacetic acid afforded apparently identical "carbobenzoxyhydrazides" of nitroarginylglutamine (XIV) in comparable yields. These observations suggest that significant racemization had not taken place when coupling the protected dipeptide XIII with benzyloxycarbonylhydrazide (VII), but the former, less ambiguous procedure was employed for routine preparation of XI.

Hydrogenation of α-methyl benzyloxycarbonyl-γ-*t*-butylglutamate[14] in the presence of acetic acid afforded α-methyl-γ-*t*-butylglutamate (XVI) which was coupled with *p*-nitrophenyl *t*-butyloxycarbonylphenylalaninate

(9) We wish to express our appreciation to Drs. Martin Sax and George A. Jeffrey of the Crystallography Laboratory, University of Pittsburgh, for these results.

(10) (a) K. Hofmann, M. Z. Magee, and A. Lindenmann, *J. Am. Chem. Soc.*, **72**, 2814 (1950); (b) K. Hofmann, A. Lindenmann, M. Z. Magee, and N. H. Khan, *ibid.*, **74**, 470 (1952).

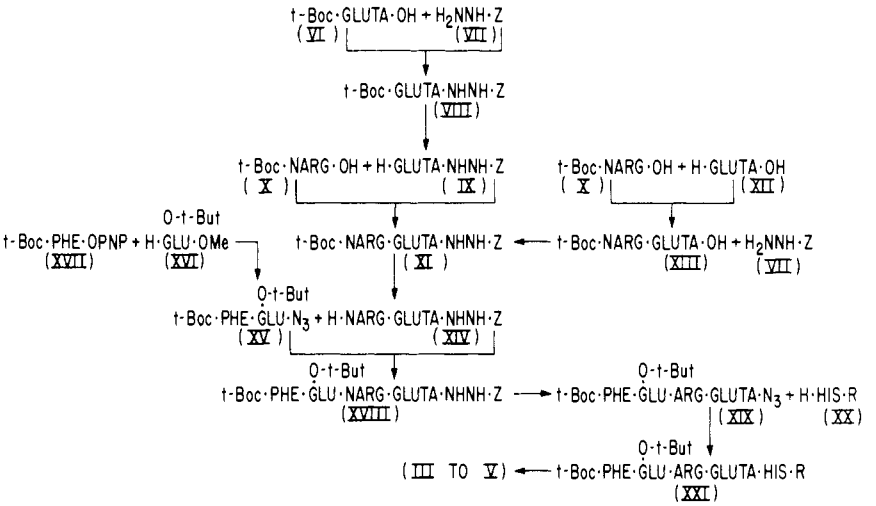
(11) (a) L. A. Carpino, *ibid.*, **79**, 98 (1957); (b) F. C. McKay and N. F. Albertson, *ibid.*, **79**, 4686 (1957); (c) G. W. Anderson and A. C. McGregor, *ibid.*, **79**, 6180 (1957).

(12) R. Schwyzer, W. Rittel, H. Kappeler, and B. Iselin, *Angew. Chem.*, **72**, 915 (1960).

(13) R. Paul and G. W. Anderson, *J. Am. Chem. Soc.*, **82**, 4596 (1960).

(14) E. Klieger and H. Gibian, *Ann.*, **655**, 195 (1962).

Scheme II



(XVII) to give methyl *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamate which was converted into the hydrazide in the usual manner. The corresponding azide (XV) was then treated with the "carbobenzoxyhydrazide" of nitroarginylglutamine (XIV) with formation of the "carbobenzoxyhydrazide" of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylnitroarginylglutamine (XVIII).

Both the standard azide procedure and the Rudinger modification[15] were employed. However, the ease of operation and the superior yields of product obtained prompted use of the Rudinger modification for routine preparation of XVIII which was isolated as a crystalline hydrate. Exhaustive hydrogenation of XVIII over palladium in methanol containing acetic acid liberated the hydrazide function and converted the nitroarginine moiety into an arginine residue with formation of the hydrazide (I) which was obtained in the form of its diacetate trihydrate. This compound was used for azide coupling reactions without purification since marked losses occurred in attempts to chromatograph the compound on carboxymethylcellulose (CMC). Synthesis of the α-hydrazide of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamic acid was achieved by a route similar to that employed for synthesis of I. N^{α}-*t*-Butyloxycarbonylnitroarginine (X) was coupled with γ-*t*-butylglutamate[16] to give N^{α}-*t*-butyloxycarbonylnitroarginyl-γ-*t*-butylglutamic acid, an oily compound which was purified *via* its crystalline dicyclohexylammonium salt. The protected acyldipeptide was converted into the "α-carbobenzoxyhydrazide" with N,N′-carbonyldiimidazole as the condensing agent. The "carbobenzoxyhydrazide" was partially deblocked by exposure to trifluoroacetic acid and the ensuing acyldipeptide "α-carbobenzoxyhydrazide" coupled with the azide (XV) to give *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylnitroarginylglutamic acid "α-carbobenzoxyhydrazide." Hydrogenolysis over palladium converted this compound into *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamic acid α-hydrazide.

The utility of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamine hydrazide (I) as a subunit for construction of larger sections of the S-peptide sequence (Scheme I) was investigated by the synthesis of three model peptides (III to V). The Rudinger modification[15] of the azide procedure was employed for conversion of the protected hydrazide (I) into the azide (XIX) for peptide bond formation with histidine amide (XX, R = NH_2). Distribution between 1-butanol and 20% acetic acid served to purify the reaction product, *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutaminylhistidine amide (XXI, R = NH_2), which was obtained in the form of the acetate hydrate. The protecting groups were removed with trifluoroacetic acid[11a,12] and the free pentapeptide amide (III) was purified by chromatography on CMC using pH 6.9 ammonium acetate buffers of increasing ionic strength as eluting agents.

Histidylmethionine (XX, R = Met·OH) was synthesized according to a procedure which we developed for the preparation of histidylphenylalanine.[17] N^{α}-Benzyloxycarbonylhistidine azide[18] was allowed to react with methyl methioninate to give methyl N^{α}-benzyloxycarbonylhistidylmethioninate which was saponified. The ensuing N^{α}-benzyloxycarbonylhistidylmethionine was decarbobenzoxylated by reduction with sodium in liquid ammonia. Histidyl-α-amino-*n*-butyric acid (XX, R = But·OH) was prepared in an analogous manner except that decarbobenzoxylation of the acyldipeptide was by hydrogenolysis. Preparation of the solid azide of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamine (XIX) and a procedure for its coupling with histidylmethionine (XX, R = Met·OH) to give *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutaminylhistidylmethionine (XXI, R = Met·OH) are described in the Experimental section. But the Rudinger modification[15] of the azide procedure was selected for routine synthesis of XXI (R = Met·OH). This protected hexapeptide was purified extensively, first by distribution between 2% acetic acid–1-butanol, then by chromatography on CMC. It was observed that in this and similar peptides related to this section of S-peptide the methionine residue exhibits a great tendency to undergo partial oxidation to the sulfoxide during the manipulations necessary for purification

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

(16) E. Schröder and E Klieger, *Ann.*, **673**, 196 (1964).

(17) K. Hofmann, H. Kappeler, A. Furlenmeier, M. E. Woolner, E. T. Schwartz, and T. A. Thompson, *J. Am. Chem. Soc.*, 79, 1641 (1957).

(18) R. W. Holley and E. Sondheimer, *ibid.*, **76**, 1326 (1954).

and isolation. Thus all samples of XXI (R = Met·OH) were more or less contaminated by the sulfoxide which is retarded on paper chromatograms with respect to the unoxidized compound. Paper chromatography of hydrogen peroxide oxidized samples of XXI (R = Met·OH) served to establish R_f values for the sulfoxide. Whether or not this sensitivity toward oxidation has a concrete basis in terms of the particular arrangement and nature of amino acid residues involved remains to be established. No attempts were made to isolate sulfoxide-free samples of XXI (R = Met·OH), since further oxidation was likely to take place during its conversion into the homogeneous hexapeptide IV. Following trifluoroacetic acid deblocking, the crude free hexapeptide (IV) was incubated with freshly distilled thioglycolic acid and the reduced material was purified on CMC. The amorphous hexapeptide was obtained in the form of the monoacetate trihydrate. With the exception of trace contaminations by sulfoxide the analytical criteria employed support the presence of a homogeneous compound.

Our route to peptide V followed the one described for synthesis of IV. The protected intermediate (XXI, R = But·OH), which could not be obtained in pure form by the methods employed for purification of XXI (R = Met·OH), was deblocked and the homogeneous free peptide isolated by CMC chromatography. Since presence of a glutamine residue in position 11 of S-peptide appears essential for its ability to activate S-protein,[19] peptides or peptide derivatives related to S-peptide used in structure–function studies or as intermediates for the ultimate synthesis of S-peptide must contain this residue intact. Evaluation by the technique of Spackmann, *et al.*,[20] of leucine aminopeptidase (LAP) digests of peptides II to V afforded low recoveries of glutamine and high recoveries of glutamic acid. This suggested the possibility that our procedures may have brought about some deamidation of the glutamine residue.

Incubation of glutamine with the LAP preparation used to digest the peptides demonstrated some conversion to glutamic acid. With previously prepared enzyme preparations this reaction was not observed. Careful ammonia analyses of the intact peptides and of their acid hydrolysates showed unequivocally that hydrolysates of peptide III contained 2 and hydrolysates of peptides II, IV, and V contained 1 equiv. of ammonia. These results demonstrate that the synthetic peptides contain an intact glutamine residue. The glutamic acid analog of peptide IV was prepared from *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamic acid α-azide plus histidylmethionine followed by deblocking with trifluoroacetic acid. The final product which was contaminated with the sulfoxide was completely reduced by incubation with thioglycolic acid.

Experimental[21]

t-Butyloxycarbonylglutamine (VI). A mixture of glutamine (14.6 g.), water (100 ml.), dioxane (100 ml.), triethylamine (41.7 ml.), and *t*-butyl azidoformate[22] (28.6 g.) was stirred for 24 hr. at 45–50°, water (100 ml.) was then added, and the dioxane was removed *in vacuo*. The solution was extracted with three 100-ml. portions of ethyl acetate and the extracts were washed with three 50-ml. portions of 0.1 *N* ammonium hydroxide. The organic phase was discarded, the combined aqueous phases were concentrated *in vacuo* to approximately 200 ml., and the concentrate was cooled in an ice-bath and acidified with 10% acetic acid. The solution was extracted with six 100-ml. portions of 1-butanol (equilibrated with 2% acetic acid) and the butanol extracts were washed with ten 100-ml. portions of 2% acetic acid (equilibrated with 1-butanol). The butanol extracts were evaporated *in vacuo* and the residue was dried to give a hydroscopic foam; yield 13.9 g. (58%); a sample for analysis was precipitated from ethyl acetate by petroleum ether; m.p. 114–118° dec.; $[\alpha]^{28}D$ −3.0° (*c* 1.93, ethanol); R_f^1 0.80; R_f^2 0.62; single ninhydrin-negative, chlorine-positive spot.

Anal. Calcd. for $C_{10}H_{18}O_5N_2$: C, 48.7; H, 7.4; N, 11.4. Found: C, 48.6; H, 7.6; N, 11.5.

t-Butyloxycarbonylglutamine Carbobenzoxyhydrazide (VIII). A mixed anhydride was prepared, in the usual manner, from *t*-butyloxycarbonylglutamine (7.15 g.) in tetrahydrofuran (40 ml.) with tri-*n*-butylamine (5.25 g.) and ethyl chloroformate (3.14 g.). This solution was added slowly with stirring to a chilled solution of benzyloxycarbonyl hydrazide (VII,[10] 4.82 g.) in tetrahydrofuran (30 ml.) and the mixture was stirred at ice-bath temperature for 1 hr. and at room temperature for another hour. The solvent was removed *in vacuo*, the residue was distributed between ethyl acetate (300 ml.) and water (100 ml.), and the aqueous layer was extracted with two additional 300-ml. portion of ethyl acetate. The organic layers were extracted in countercurrent fashion with the following solutions: 5% citric acid (100 ml.), 5% sodium bicarbonate (two 100-ml. portions), and water (two 100-ml. portions). The solution was dried over sodium sulfate and concentrated to a small volume *in vacuo*. The concentrate was kept at 0° for 4 hr. when the crystalline product was collected and recrystallized from ethyl acetate (approximately 700 ml.); yield 7.48 g. (65%); m.p. 159–161°; $[\alpha]^{29}D$ −31.9° (*c* 2.89, methanol); R_f^1 0.94; R_f^2 0.97; single ninhydrin-negative, chlorine-positive spot.

Anal. Calcd. for $C_{18}H_{26}O_6N_4$: C, 54.8; H, 6.6; N, 14.2. Found: C, 54.8; H, 6.7; N, 14.5.

N^{α}-t-Butyloxycarbonylnitroarginine (X). A solution of *t*-butyl azidoformate[22] (28.6 g.) and nitroarginine (21.9 g.) in dioxane (100 ml.) and water (100 ml.) containing suspended magnesium oxide (8.0 g.) was

(19) P. J. Vithayathil and F. M. Richards, *J. Biol. Chem.*, **236**, 1380 (1961).

(20) D. H. Spackmann, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(21) General experimental and analytical procedures used were those described in paper XXIX (see ref. 3). Amide nitrogen was determined as the difference between the ammonia content of the peptides and their acid hydrolysates. The values were corrected for the ammonia contributed by the aliquot of 6 *N* hydrochloric acid used for hydrolysis. Ammonia determinations were performed on the short column of the amino acid analyzer. Carboxymethylcellulose (CMC) was obtained from Biorad Laboratories, Richmond, Calif. The following abbreviations are used: DMF = dimethylformamide; THF = tetrahydrofuran; OPNP = *p*-nitrophenyl ester; *t*-Boc = *t*-butyloxycarbonyl; O-*t*-But = *t*-butyl ester; Z = benzyloxycarbonyl; But = α-amino-*n*-butyric acid; Narg = nitroarginine; Gluta = glutamine; LAP = leucine aminopeptidase.

(22) *t*-Butyl azidoformate was prepared from the hydrazide as described by L. A. Carpino, C. A. Giza, and B. A. Carpino, *J. Am. Chem. Soc.*, **81**, 955 (1959), and was used directly without distillation.

stirred at 45–50° for 24 hr., then water (100 ml.) was added, the solution was filtered, and the dioxane was removed *in vacuo*. The filtrate was extracted with three 100-ml. portions of ethyl acetate and the organic phases were washed with three 50-ml. portions of 0.1 *N* ammonium hydroxide. The combined aqueous layers were cooled at 0° in an ice bath, acidified with 1 *M* citric acid, and extracted with three 250-ml. portions of ethyl acetate which were washed with five 75-ml. portions of water. Evaporation of the sodium sulfate dried ethyl acetate solution gave a yellow oil which was dissolved in hot ethyl acetate. The solution was kept at 0° for 12 hr. when crystallization occurred. The material was recrystallized from ethyl acetate; yield 26.8 g. (84%); m.p. 115–116° dec.; softens at 106°; $[\alpha]^{28}D$ −5.9° (*c* 2.46, DMF); R_f^1 0.84; R_f^2 0.52; single ninhydrin-negative, chlorine- and ultraviolet-positive spot.

Anal. Calcd. for $C_{11}H_{21}O_6N_5$: C, 41.4; H, 6.6; N, 21.9. Found: C, 41.2; H, 6.6; N, 21.7.

p-Nitrophenyl t-Butyloxycarbonylphenylalaninate (XVII). This compound was prepared essentially as described by Sandrin and Boissonnas[6]; m.p. 126–128°; $[\alpha]^{28}D$ −13.6° (*c* 0.65, methanol); $[\alpha]^{28}D$ −20.8° (*c* 1.45, DMF); lit. m.p. 132°, $[\alpha]^{22}D$ −17.5° (methanol), $[\alpha]^{22}D$ −21.0° (DMF).

α-Methyl t-Butyloxycarbonylphenylalanyl-γ-t-butylglutamate. α-Methyl benzyloxycarbonyl-γ-*t*-butylglutamate[14] (2.10 g.) was hydrogenated over palladium in methanol (50 ml.) containing glacial acetic acid (0.5 ml.). The catalyst was removed by filtration and the filtrate was evaporated to give a sirup (XVI); yield 1.57 g. (95%); R_f^1 0.77; R_f^2 0.87. To a solution of the oil in ethyl acetate (50 ml.) containing triethylamine (0.78 ml.) was added *p*-nitrophenyl *t*-butyloxycarbonylphenylalaninate (2.16 g.), and the mixture was kept at room temperature for 15 hr. The solution was washed with 1 *N* ammonium hydroxide (until the yellow color was removed), four 30-ml. portions of 1 *N* citric acid, and four 30-ml. portions of saturated sodium chloride, and was dried over sodium sulfate. The solvent was removed *in vacuo* and the residue was recrystallized from petroleum ether; needles; yield 2.02 g. (73%); m.p. 89–93°. A sample for analysis was recrystallized from ethyl acetate–petroleum ether; m.p. 98–100°; $[\alpha]^{27}D$ −15.3° (*c* 0.81, methanol); R_f^1 0.92; ninhydrin negative, chlorine positive.

Anal. Calcd. for $C_{24}H_{36}O_7N_2$: C, 62.1; H, 7.8; N, 6.0. Found: C, 62.2; H, 7.9; N, 6.3.

t-Butyloxycarbonylphenylalanyl-γ-t-butylglutamic Acid Hydrazide. Hydrazine hydrate (2.0 ml.) was added to a methanol solution (20 ml.) containing methyl *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamate (1.96 g.) and the solution was kept at room temperature for 20 hr. when the methanol was removed *in vacuo*. The residue was dried over concentrated sulfuric acid to give a solid which crystallized from methanol–water in needles; yield 1.37 g. (70%); m.p. 123–125°. A sample for analysis was recrystallized from the same solvents with no change in melting point.

Anal. Calcd. for $C_{23}H_{36}O_6N_4$: C, 59.5; H, 7.8; N, 12.1. Found: C, 59.6; H, 8.0; N, 12.0.

Arginylglutamine Acetate Hemihydrate. N^{α}-Benzyloxycarbonylnitroarginylglutamine [m.p. 176–179°; $[\alpha]^{28}D$ −0.2° (*c* 3.24, DMF); $[\alpha]^{25}D$ −1.7° (*c* 1.83, dichloroacetic acid); R_f^1 0.73; R_f^2 0.46; lit.[5] m.p. 168°, $[\alpha]^{25}D$ −0.5° (DMF). *Anal.* Calcd. for $C_{19}H_{27}O_8N_7$: C, 47.4; H, 5.7; N, 20.4. Found: C, 47.2; H, 5.9; N, 20.4] was prepared and reduced essentially as described by Berse, *et al.*[5] Arginylglutamine acetate hemihydrate had m.p. 173–175°; $[\alpha]^{30}D$ +18.5° (*c* 1.83, water); R_f^1 0.09; R_f^3 0.79 × His; lit.[5] m.p. 160°; $[\alpha]^{25}D$ +19.2°, (water).

Anal. Calcd. for $C_{11}H_{22}O_4N_6 \cdot CH_3COOH \cdot 0.5H_2O$: C, 42.0; H, 7.3; N, 22.6; O, 28.0. Found: C, 41.8; H, 7.5; N, 22.5; O, 28.2.

N^{α}-t-Butyloxycarbonylnitroarginylglutamine Ethanol Solvate (XIII). A mixed anhydride was prepared in the usual manner from N^{α}-*t*-butyloxycarbonylnitroarginine (X, 9.84 g.) in freezing dioxane (50 ml.) and DMF (10 ml.) with tri-*n*-butylamine (7.28 ml.) and ethyl chloroformate (2.96 ml.). This solution was added slowly with stirring to a chilled solution of glutamine (4.53 g.) and triethylamine (4.31 ml.) in water (50 ml.). The mixture was stirred at ice-bath temperature for 1 hr. and at room temperature for another hour. The dioxane was removed *in vacuo*, the aqueous phase was adjusted to pH 8 by addition of 1 *N* ammonium hydroxide, and the solution was extracted with three 40-ml. portions of ethyl acetate which were washed with three 20-ml. portions of water. The ethyl acetate extracts were discarded, and the aqueous phases were combined, acidified at ice-bath temperature with 10% acetic acid, and extracted with six 100-ml. portions of 1-butanol (equilibrated with 2% acetic acid). The butanol extracts were washed with five 25-ml. portions of water (equilibrated with 1-butanol) and evaporated to give an oil which was dissolved in ethanol and precipitated by ether. This procedure was repeated several times to give a hydroscopic solid; yield 6.41 g. (42%); m.p. 95–100°; $[\alpha]^{28}D$ −11.8° (*c* 1.68, water); R_f^1 0.79; R_f^2 0.58; single ninhydrin-negative, chlorine- and ultraviolet-positive spot.

Anal. Calcd. for $C_{16}H_{29}O_8N_7 \cdot C_2H_5OH$: C, 43.8; H, 7.1; N, 19.9; O, 29.2. Found: C, 43.9; H, 7.2; N, 19.8; O, 29.5.

N^{α}-t-Butyloxycarbonylnitroarginyl-γ-t-butylglutamic Acid. A mixed anhydride was prepared in the usual manner from N^{α}-*t*-butyloxycarbonylnitroarginine (X, 7.9 g.) in tetrahydrofuran (100 ml.) with triethylamine (3.5 ml.) and ethyl chloroformate (2.3 ml.). This solution was added slowly with stirring to a chilled solution of γ-*t*-butylglutamate[16] (5.6 g.) in 60% aqueous tetrahydrofuran (70 ml.) containing triethylamine (3.8 ml.), and the mixture was stirred in an ice-bath for 30 min. and at room temperature for another hour. The organic solvent was removed *in vacuo*, water (100 ml.) was added, and the solution was acidified at 0° with solid citric acid. The aqueous solution was extracted with three 75-ml. portions of ethyl acetate, the ethyl acetate extract was washed with three 50-ml. portions of water and dried over sodium sulfate, and the solvent was removed *in vacuo*. The resulting oil was dissolved in ethyl acetate (300 ml.) and dicyclohexylamine (12.5 g.) was added. After standing overnight the dicyclohexylammonium salt was collected and recrystallized from ethyl acetate; yield 7.6 g. (40%); m.p. 125–128°; $[\alpha]^{26}D$ +3.2° (*c* 1.10, meth-

anol); R_f^I 0.63; R_f^{VI} 0.72; ninhydrin-negative, chlorine-positive spot.

Anal. Calcd. for $C_{32}H_{59}O_9N_7$: C, 56.0; H, 8.7; N, 14.3. Found: C, 55.9; H, 9.1; N, 14.1.

The free dipeptide was obtained by suspending the dicyclohexylammonium salt (0.685 g.) in ethyl acetate (20 ml.) and adding 0.16 ml. of 6.1 *N* HCl in dioxane. The suspension was stirred for 1 hr., and the dicyclohexylammonium chloride was removed by filtration and washed with ethyl acetate (20 ml.). The combined filtrate and washings were washed with three 20-ml. portions of water and two 20-ml. portions of saturated sodium chloride, dried over sodium sulfate, and evaporated *in vacuo* to yield an oil; yield 0.45 g. (89%); R_f^I 0.63; R_f^V 0.74; R_f^{VI} 0.74; ninhydrin-negative, chlorine-positive spot.

Anal. Calcd. for $C_{20}H_{36}O_9N_6$: C, 47.6; H, 7.2; N, 16.7. Found: C, 47.3; H, 7.3; N, 16.7.

N^α-t-Butyloxycarbonylnitroarginylglutamine "Carbobenzoxyhydrazide" Hemihydrate (XI). (a) From N^α-t-Butyloxycarbonylnitroarginylglutamine (XIII) and Benzyloxycarbonyl Hydrazide (VII). N,N'-Carbonyldiimidazole[13] (2.4 g.) was added to an ice-cold, stirred solution of N^α-*t*-butyloxycarbonylnitroarginylglutamine ethanol solvate (XIII, 6.20 g.) in DMF (25 ml.) and the mixture was stirred with cooling until the evolution of carbon dioxide had ceased (approximate time required 1 hr.). Benzyloxycarbonyl hydrazide (VII, 2.30 g.) was then added and the solution was stirred for 12 hr. at room temperature. The solvent was removed *in vacuo* and the resulting oil was distributed between 1-butanol (equilibrated with 2% acetic acid) (60 ml.) and 2% acetic acid (equilibrated with 1-butanol) (40 ml.). The aqueous phase was passed through six additional separatory funnels each containing 60 ml. of 1-butanol (equilibrated with 2% acetic acid) and the butanol phases were washed with ten 40-ml. portions of 2% acetic acid (equilibrated with 1-butanol). The combined butanol phases were evaporated to dryness *in vacuo*, the resulting oil was dissolved in ethanol, and the product was precipitated by addition of ether. The compound was purified by a second precipitation from ethanol with ether; yield 4.05 g. (53%); $[\alpha]^{28}D$ −19.5° (*c* 3.09, ethanol); $[\alpha]^{28}D$ −11.5° (*c* 3.90, DMF); R_f^I 0.84; single ninhydrin-negative, chlorine- and ultraviolet-positive spot.

Anal. Calcd. for $C_{24}H_{37}O_9N_9 \cdot 0.5H_2O$: C, 47.7; H, 6.3; N, 20.8; O, 25.1. Found: C, 47.8; H, 6.4; N, 20.5; O, 25.6.

(b) From N^α-t-Butyloxycarbonylnitroarginine (X) plus Glutamine "Carbobenzoxyhydrazide" (IX). N^α-*t*-Butyloxycarbonylglutamine "carbobenzoxyhydrazide" (VIII, 15.0 g.) was dissolved in ice-cold, anhydrous trifluoroacetic acid (25 ml.) and the solution was kept at room temperature for 10 min. Ice-cold ether (1 l.) was added and the ensuing suspension was kept at −10° for 30 min. when the precipitate was collected and washed with ether. The hydroscopic residue was dissolved in water (150 ml.), Amberlite IRA-400 (acetate cycle) (100 ml. settled in water) was added, and the suspension was stirred for 30 min., then the resin was removed by filtration. The resin was washed with water (50 ml.), the combined filtrate and washings were adjusted to pH 8 with 2 *N* ammonium hydroxide, and the solution was extracted with six 100-ml. portions of 1-butanol (equilibrated with 0.01 *N* ammonium hydroxide). The butanol extracts were washed with ten 50-ml. portions of 0.01 *N* ammonium hydroxide (equilibrated with 1-butanol) and the butanol phases were combined and evaporated to dryness *in vacuo*. The residue was lyophilized to constant weight from small volumes of water; yield 7.9 g. (71%) of glutamine "carbobenzoxyhydrazide" (IX); R_f^1 0.59; single ninhydrin- and chlorine-positive spot. This compound is unstable and was used for the following step without further purification. A mixed anhydride was prepared, in the usual manner, from N^α-*t*-butyloxycarbonylnitroarginine (X) (8.6 g.) in dioxane (100 ml.) and DMF (20 ml.) with tri-*n*-butylamine (6.4 ml.) and ethyl chloroformate (2.57 ml.). This solution was added slowly with stirring to a chilled solution of glutamine "carbobenzoxyhydrazide" (IX, 7.9 g.) in tetrahydrofuran (100 ml.) and DMF (20 ml.) and the mixture was stirred for 1 hr. at ice-bath temperature and at room temperature for 2 hr. Water (100 ml.) was added and the solvents were removed *in vacuo*. The residue was dissolved in water (100 ml.), the solution was extracted with six 100-ml. portions of 1-butanol (equilibrated with 2% acetic acid), and the butanol phases were washed with eight 50-ml. portions of 2% acetic acid (equilibrated with 1-butanol). The combined organic phases were evaporated to dryness *in vacuo*, the residue was dissolved in a minimal amount of ethanol, and ether was added to precipitate the product. The material was three times reprecipitated from ethanol with ether and was dried; white amorphous solid; yield 10.3 g. (63%); $[\alpha]^{29}D$ −23.4° (*c* 3.87, ethanol); $[\alpha]^{27}D$ −12.7° (*c* 3.68, DMF); R_f^1 0.86; single ninhydrin-negative, chlorine- and ultraviolet-positive spot.

Anal. Calcd. for $C_{24}H_{37}O_9N_9 \cdot 0.5\ H_2O$: C, 47.7; H, 6.3; N, 20.8. Found: C, 47.4; H, 6.6; N, 20.8.

N^α-t-Butyloxycarbonylnitroarginyl-γ-t-butylglutamic Acid "Carbobenzoxyhydrazide." N,N'-Carbonyldiimidazole (1.7 g.) was added to an ice-cold stirred solution of N^α-*t*-butyloxycarbonylnitroarginyl-γ-*t*-butylglutamic acid (5.1 g.) in methylene chloride (50 ml.) and the mixture was stirred with cooling for 1 hr. Benzyloxycarbonylhydrazide (VII, 1.65 g.) was added and the reaction mixture was stirred at room temperature for 5 hr. The solvent was removed *in vacuo*, the resulting oil was dissolved in ethyl acetate (75 ml.), and the solution was washed with four 25-ml. portions of 2 *N* citric acid, four 25-ml. portions of saturated sodium bicarbonate, four 25-ml. portions of water, and two 25-ml. portions of saturated sodium sulfate. The solution was dried over sodium sulfate and evaporated to dryness *in vacuo*. The resulting oil was dissolved in ethyl acetate (10 ml.) and the product was precipitated as an amorphous powder by addition of petroleum ether; yield 3.9 g. (59%); $[\alpha]^{28}D$ −14.7° (*c* 2.1, methanol); R_f^I 0.85; ninhydrin-negative, ultraviolet- and chlorine-positive spot.

Anal. Calcd. for $C_{28}H_{44}O_{10}N_8$: C, 51.5; H, 6.8; N, 17.2. Found: C, 51.8; H, 7.0; N, 17.0.

Nitroarginylglutamine "Carbobenzoxyhydrazide" Hydrate (XIV). N^α-*t*-Butyloxycarbonylnitroarginylglutamine "carbobenzoxyhydrazide" hemihydrate (XI), prepared according to method a (10.3 g.), was dissolved in ice-cold, anhydrous trifluoroacetic acid (20 ml.) and the solution was kept at room temperature

for 10 min. Ice-cold ether (200 ml.) was then added and the ensuing suspension was kept at −10° for 1 hr. when the precipitate was collected and washed with ether. The hydroscopic residue was dissolved in water (150 ml.), Amberlite IRA-400 (acetate cycle) (approximately 100 ml. settled in water) was added, and the suspension was stirred for 30 min., then the resin was removed by filtration. The resin was washed with water (100 ml.), the combined filtrate and washings were adjusted to pH 8 with 1 *N* ammonium hydroxide, and the solution was extracted with six 100-ml. portions of 1-butanol (equilibrated with 0.01 *N* ammonium hydroxide). The butanol extracts were washed with ten 90-ml. portions of 0.01 *N* ammonium hydroxide (equilibrated with 1-butanol) and the butanol phases were combined and evaporated to dryness. The residue was dissolved in a small volume of dioxane–water (1:1, v./v.) and the solution was lyophilized to constant weight; yield 6.61 g. (76%). A sample for analysis was dissolved in hot 1-butanol saturated with water and the solution was kept at 4° for 12 hr.; hydroscopic solid; $[\alpha]^{28}D$ −11.2° (*c* 3.02, DMF); R_f^1 0.46; R_f^2 0.75; single ninhydrin-, chlorine- and ultraviolet-positive spot; single ninhydrin- and chlorine-positive spot on paper electrophoresis at pH 1.9, 3.5, 6.5, and 8.0. Compound XIV prepared by deblocking XI obtained by method b showed $[\alpha]^{26}D$ −11.2° (*c* 1.49, DMF).

Anal. Calcd. for $C_{19}H_{29}O_7N_9 \cdot H_2O$: C, 44.4; H, 6.1; N, 24.5; O, 24.9. Found: C, 44.7; H, 6.3; N, 24.7; O, 25.1.

Nitroarginylglutamic Acid "α-Carbobenzoxyhydrazide" Monoacetate. A solution of N^{α}-*t*-butyloxycarbonylnitroarginyl-γ-*t*-butylglutamic acid "α-carbobenzoxyhydrazide" (3.3 g.) in anhydrous trifluoroacetic acid (5 ml.) was kept at room temperature for 20 min. The trifluoroacetic acid was removed *in vacuo*, the residue was dissolved in water (10 ml.), and the solution was lyophilized. The residue was dissolved in water (20 ml.) and the solution was added to an Amberlite IRA-400 (acetate cycle) column (1.5 × 10 cm.). The column was eluted with 2% acetic acid (150 ml.) and the combined eluates were concentrated *in vacuo* to a small volume and lyophilized; yield 2.2 g. (78%); $[\alpha]^{28}D$ −5.4° (*c* 1.43, water); R_f^1 0.58; R_f^2 0.55; R_f^3 3.8 × His; ninhydrin-, chlorine-, ultraviolet-positive spot.

Anal. Calcd. for $C_{19}H_{28}O_8N_8 \cdot CH_3COOH$: C, 45.3; H, 5.8; N, 20.1; O, 28.8. Found: C, 45.1; H, 6.0; N, 20.3; O, 28.4.

Methyl N^{α}-Benzyloxycarbonylhistidylmethioninate. An ice-cold ethyl acetate solution (approximately 150 ml.) containing N^{α}-benzyloxycarbonylhistidine azide (prepared from the hydrazide) (17.3 g.)[18] was added to an ice-cold ethyl acetate solution (approximately 150 ml.) containing methionine methyl ester (prepared in the usual manner from 11.4 g. of the hydrochloride with triethylamine (7.9 ml.)). The mixture was kept in a refrigerator for 22 hr., and the crystalline precipitate was collected, washed with cold ethyl acetate, and dried; yield 13.6 g.; m.p. 110–113°. Mother liquors and washings were combined and washed successively with 10% potassium bicarbonate (200 ml.) and water (200 ml.) and the solution was dried over sodium sulfate. Evaporation of the solvent gave an oil which crystallized when placed in a refrigerator. The crystals were collected, washed with cold ethyl acetate, and dried; yield 3.9 g.; m.p. 114–115°. This material was combined with the first crop and recrystallized from ethyl acetate; yield 14.9 g. (58%); m.p. 114–116°; $[\alpha]^{29}D$ −24.0° (*c* 1.28, methanol).

Anal. Calcd. for $C_{20}H_{26}O_5N_4S$: C, 55.3; H, 6.0; N, 12.9; S, 7.4. Found: C, 55.3; H, 6.3; N, 13.0; S, 7.5.

N^{α}-Benzyloxycarbonylhistidylmethionine Hydrate. The above methyl ester (19.35 g.) was dissolved in methanol (120 ml.) and 1 *N* sodium hydroxide (49.0 ml.) was added. The solution was kept at room temperature for 1 hr. when it was cooled in an ice-bath and 1 *N* hydrochloric acid (49.0 ml.) was added slowly with stirring. Crystals formed within a few minutes. The mixture was placed in a refrigerator for 3 hr. when the product was collected and recrystallized from water; yield 14.8 g. (76%); m.p. 197–198°; $[\alpha]^{27}D$ −7.1° (*c* 2.01, 95% acetic acid).

Anal. Calcd. for $C_{19}H_{24}O_5N_4S \cdot H_2O$: C, 52.0; H, 6.0; N, 12.8; O, 21.9; S, 7.3. Found: C, 52.3; H, 6.1; N, 12.6; O, 22.3; S, 7.2.

Histidylmethionine (XX, R = Met·OH). N^{α}-Benzyloxycarbonylhistidylmethionine monohydrate (15.6 g.) was dissolved in liquid ammonia (250 ml.) and sodium was added in small pieces until the blue color remained for 1 min. (amount required approximately 3.2 g.). Dry Dowex-50 (ammonium cycle) (35 g.) was then added, the ammonia was allowed to evaporate, and the residue was kept in a desiccator over sulfuric acid overnight. The residue was suspended in water (150 ml.), the resin was removed by filtration and washed with water (three 100-ml. portions), and the combined filtrate and washings were evaporated to dryness *in vacuo* to give an oil. The oil was dissolved in water (25 ml.) and the solution was acidified with glacial acetic acid and lyophilized. The residue was dissolved in water (25 ml.), ethanol (450 ml.) was added, and the mixture was kept in a refrigerator overnight. The crystals were collected and recrystallized from aqueous ethanol to give 9.7 g. (97%); m.p. 188–190°; $[\alpha]^{27}D$ −29.8° (*c* 1.76, 10% acetic acid); $[\alpha]^{26}D$ −14.5° (*c* 2.17, water); R_f^1 0.31; R_f^3 2.1 × His; single ninhydrin-, Pauly-, and methionine-positive spot; completely digestible by LAP.

Anal. Calcd. for $C_{11}H_{18}O_3N_4S$: C, 46.1; H, 6.3; N, 19.6. Found: C, 46.1; H, 6.4; N, 19.4.

Methyl N^{α}-Benzyloxycarbonylhistidyl-α-amino-n-butyrate Hydrate. An ice-cold ethyl acetate solution (approximately 80 ml.) containing N^{α}-benzyloxycarbonylhistidine azide (prepared from the hydrazide) (6.9 g.)[18] was added to an ether solution (approximately 70 ml.) of methyl α-amino-*n*-butyrate (prepared from 4.18 g. of the hydrochloride[23] with potassium carbonate). The mixture was stirred at 4° for 24 hr. when the crystalline precipitate was collected and dried; yield 7.02 g. (76%). The compound was recrystallized from aqueous methanol; m.p. 110–114°; $[\alpha]^{30}D$ −25.1° (*c* 2.42, methanol); R_f^1 0.85; R_f^2 0.97; single ninhydrin-negative, Pauly- and chlorine-positive spot.

Anal. Calcd. for $C_{19}H_{24}O_5N_4 \cdot H_2O$: C, 56.2; H, 6.4; N, 13.8; O, 23.6. Found: C, 56.4; H, 6.3; N, 13.8; O, 23.5.

(23) E. Klieger and H. Gibian, *Ann.* **649**, 183 (1961).

N^{α}-Benzyloxycarbonylhistidyl-α-amino-n-butyric Acid. The above methyl ester hydrate (1.94 g.) was dissolved in methanol (20 ml.) and 1 *N* sodium hydroxide (6.0 ml.) was added. The solution was kept at room temperature for 30 min., 1 *N* hydrochloric acid (6.0 ml.) was added, and the solvents were removed *in vacuo*. The residue was recrystallized from water; yield 1.65 g. (92%); m.p. 210–212°; R_f^1 0.79; R_f^2 0.53; single ninhydrin-negative, Pauly- and chlorine-positive spot; $[\alpha]^{25}D$ −12.5° (*c* 2.46, dichloroacetic acid).

Anal. Calcd. for $C_{18}H_{22}O_5N_4$: C, 57.7; H, 5.9; N, 15.0. Found: C, 57.6; H, 5.9; N, 15.1.

Histidyl-α-amino-n-butyric Acid (XX, R = But·OH). The above benzyloxycarbonyl derivative (1.18 g.) was hydrogenated in the usual manner over palladium in methanol–water (2:1, v./v.) (40 ml.) containing acetic acid (0.65 ml.). The catalyst was removed by filtration through "filter cel" and the filtrate was evaporated *in vacuo*. The residue was evaporated twice with ethanol (approximately 15 ml. each) and dissolved in boiling water (20 ml.), and boiling ethanol (80 ml.) was added. Needles formed on cooling; yield 0.66 g. (88%); m.p. 210–212°; $[\alpha]^{28}D$ −25.1° (*c* 2.19, 10% acetic acid); R_f^1 0.18; R_f^3 1.6 × His; completely digestible by LAP with formation of His and But.

Anal. Calcd. for $C_{10}H_{16}O_3N_4$: C, 50.0; H, 6.7; N, 23.3. Found: C, 49.7; H, 6.9; N, 23.3.

Benzyloxycarbonyl-γ-benzylglutamylarginylglutamine. *p*-Nitrophenyl benzyloxycarbonyl-γ-benzylglutamate[24] (6.60 g.) in dioxane (42 ml.) was added to a solution of arginylglutamine monoacetate hemihydrate (2.49 g.) in water (14.0 ml.) and methanol (100 ml.) and the mixture was kept at room temperature for 48 hr. The solvents were removed *in vacuo*, the residue was dissolved in 30% (v./v.) aqueous acetic acid (70 ml.), and the solution was washed with four 30-ml. portions of ether which were re-extracted with two 30-ml. portions of 30% (v./v.) aqueous acetic acid. The combined aqueous layers were concentrated and the resulting residue was dissolved in 1-butanol (60 ml.). This solution was extracted with six 30-ml. portions of 10% (v./v.) aqueous acetic acid which were washed in countercurrent fashion with two 60-ml. portions of 1-butanol. The combined organic layers were concentrated to approximately 10 ml. and ether (300 ml.) was added. The resulting amorphous solid was collected by filtration and dried over P_2O_5; yield 2.62 g. (69%); m.p. 196–197° dec.; $[\alpha]^{27}D$ −9.11° (*c* 0.65, glacial acetic acid); single Sakaguchi- and chlorine-positive, ninhydrin-negative spot with R_f^1 0.71; R_f^2 0.86; R_f^3 4.0 × His. A sample for analysis was recrystallized from boiling water to give needles; m.p. 198–199° dec.

Anal. Calcd. for $C_{31}H_{41}O_9N_7$: C, 56.8; H, 6.3; N, 14.9; O, 22.0. Found: C, 56.8; H, 6.4; N, 15.2; O, 21.7.

Glutamylarginylglutamine. The above partially protected tripeptide (2.15 g.) was hydrogenated for 3 hr. in the usual manner over palladium in methanol (150 ml.), water (80 ml.), and glacial acetic acid (0.56 ml.). The catalyst was removed by filtration, the filtrate was concentrated to an oil *in vacuo*, and water (5 ml.) was added. The solution was lyophilized to give a white powder; yield 1.29 g. (91%); $[\alpha]^{26}D$ −20.3° (*c* 0.67, 0.5 *M* sodium bicarbonate); single ninhydrin-, chlorine-, and Sakaguchi-positive spot with R_f^3 0.39 × His.

Anal. Calcd. for $C_{16}H_{29}O_7N_7$: C, 44.5; H, 6.8; N, 22.7; O, 26.0. Found: C, 44.7; H, 7.1; N, 22.5; O, 26.0.

Benzyloxycarbonylphenylalanylglutamylarginylglutamine. Succinimido benzyloxycarbonylphenylalaninate[7b] (3.56 g.) in tetrahydrofuran (16.0 ml.) was added to a solution of glutamylarginylglutamine (3.50 g.) in water (8.15 ml.) containing triethylamine (3.35 ml.) and the mixture was stirred vigorously for 15 min. when a clear solution resulted. The solution was stirred for an additional 24 hr. at room temperature when the solvents were removed *in vacuo*. The resulting residue was dissolved in 30% (v./v.) aqueous acetic acid (100 ml.) and extracted with four 50-ml. portions of ether which were re-extracted with three 100-ml. portions of 30% (v./v.) aqueous acetic acid. The combined aqueous layers were evaporated *in vacuo* and the resulting residue was dissolved in 1-butanol (50 ml.). The butanol solution was extracted with six 50-ml. portions of 10% (v./v.) aqueous acetic acid which were washed with three 50-ml. portions of 1-butanol. The combined organic layers were concentrated to approximately 30 ml. and ether (200 ml.) was added. The resulting amorphous solid was collected by filtration, washed well with ether, and dried over P_2O_5; yield 3.86 g. (67%); m.p. 190–194° dec.; $[\alpha]^{27}D$ −14.1° (*c* 0.60, dichloroacetic acid); single chlorine- and Sakaguchi-positive, ninhydrin-negative spot with R_f^1 0.80, R_f^2 0.49; R_f^3 3.61 × His. A sample for analysis was recrystallized from methanol–ether; m.p. 200–202° dec.

Anal. Calcd. for $C_{33}H_{44}O_{10}N_8$: C, 55.6; H, 6.2; N, 15.7; O, 22.5. Found: C, 55.4; H, 6.5; N, 15.6; O, 22.1.

Phenylalanylglutamylarginylglutamine (II). The above benzyloxycarbonyltetrapeptide (3.0 g.) was hydrogenated for 2.5 hr. in the usual manner over palladium in methanol (150 ml.), water (100 ml.), and glacial acetic acid (0.69 ml.). The catalyst was removed by filtration, the filtrate was concentrated to approximately 8 ml., and ethanol (40 ml.) was added slowly. The product was collected after 24 hr., washed with ethanol, and dried over P_2O_5; needles; yield 2.08 g. (86%); m.p. 170–175°; $[\alpha]^{25}D$ −11.8° (*c* 0.74, 10% acetic acid); single ninhydrin-, chlorine-, and Sakaguchi-positive spot with R_f^3 1.1 × His; amino acid ratios in acid hydrolysate $phe_{0.93}glu_{2.04}arg_{1.01}NH_{3\,0.94}$; amino acid ratios in 24 hr. LAP digest $phe_{1.03}glu_{1.38}$-arg + $orn_{0.90}gluta_{0.70}$ (81%); amide nitrogen 0.83 μmole/μmole.

Anal. Calcd. for $C_{25}H_{38}O_8N_8$: C, 51.9; H, 6.6; N, 19.4; O, 22.1. Found: C, 52.1; H, 6.8; N, 19.2; O, 22.0.

t-Butyloxycarbonylphenylalanyl-γ-t-butylglutamyl-nitroarginylglutamine "Carbobenzoxyhydrazide" Hydrate (XVIII). *(a) By the Standard Azide Procedure.* Sodium nitrite (194 mg.) in water (1 ml.) was added to an ice-cold solution of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamic acid hydrazide (1.39 g.) in 90%

(24) F. Marchiori, R. Rocchi, and E. Scoffone, *Ric. Sci. Rend. Sez.* A2 [6] 647 (1962).

aqueous tetrahydrofuran (10 ml.) and 1 *N* hydrochloric acid (6.0 ml.). After 2 min. a 10% (v./v.) solution of triethylamine in DMF (4.46 ml.) was added. This solution containing *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamic acid azide (XV) was added to a DMF solution (5 ml.) containing nitroarginylglutamine "carbobenzoxyhydrazide" hydrate (XIV, 1.60 g.) and this was followed by enough ice-cold DMF to give a homogeneous solution. After stirring for 24 hr. at 4° a second portion of the azide (XV) (prepared from 464 mg. of the hydrazide) was added and stirring at 4° was continued for 24 hr. The solvents were removed *in vacuo*, the residue was dissolved in ethanol (50 ml.), and ice-cold 2% aqueous acetic acid (300 ml.) was added. The precipitate was collected, washed with water, and dried. The material was recrystallized from ethanol; yield 1.85 g. (63%); m.p. 199–200.5; $[\alpha]^{23}D$ −18.4° (*c* 1.51, DMF); R_f^1 0.91; single ninhydrin-negative, chlorine- and ultraviolet-positive spot; amino acid ratios in acid hydrolysate arg + $orn_{1.06}glu_{2.02}phe_{0.94}$.

Anal. Calcd. for $C_{42}H_{61}O_{13}N_{11}\cdot H_2O$: C, 53.3; H, 6.7; N, 16.3; O, 23.7. Found: C, 53.2; H, 6.8; N, 16.1; O, 24.0.

(*b*) *By the Rudinger Modification.*[15] *t*-Butyl nitrite (3.62 ml.) was added to a stirred solution cooled to −30° of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamic acid hydrazide (13.9 g.) in DMF (100 ml.) containing 19.8 ml. of 6.1 *N* hydrogen chloride in dioxane. The solution, after stirring at −25° for 15 min., was cooled to −60° and triethylamine (16.7 ml.) was added. To this mixture, containing the azide (XV), was added a solution of nitroarginylglutamine "carbobenzoxyhydrazide" hydrate (XIV, 15.39 g.) in DMF (50 ml.). After stirring for 1 hr. at −20° the solution was stirred at 4° for 20 hr. A second portion of azide, prepared from 3.49 g. of the hydrazide, was added and after stirring a further 24 hr. at 4° and 12 hr. at room temperature the solvents were removed *in vacuo*. The residue was dissolved in ethanol and the product was precipitated by addition of ice-cold water. Recrystallization from boiling ethanol gave 25.1 g. (89%); m.p. 203–205°; $[\alpha]^{28}D$ −18.3° (*c* 2.00, DMF); chromatographically pure.

t-Butyloxycarbonylphenylalanyl-γ-t-butylglutamylarginylglutamine Hydrazide Diacetate Trihydrate (I). The "carbobenzoxyhydrazide" (XVIII, 0.473 g.) was hydrogenated in the usual manner over palladium in methanol–acetic acid–water (2:1:1) (20 ml.). The catalyst was removed by filtration through "filter cel" and the filtrate was evaporated to dryness *in vacuo*. The resulting oil was lyophilized from 5% acetic acid to give a colorless hydroscopic solid; yield 436 mg. (95%); $[\alpha]^{28}D$ −33.8° (*c* 0.62, water); R_f^1 0.84; R_f^2 0.80; trace impurity with R_f^2 0.95; amino acid ratios in acid hydrolysate $arg_{0.96}glu_{2.00}phe_{1.04}NH_{3.0.98}$.

Anal. Calcd. for $C_{34}H_{56}O_9N_{10}\cdot 2CH_3COOH\cdot 3H_2O$: C, 49.6; H, 7.4; N, 15.2; O, 27.8. Found: C, 49.7; H, 7.6; N, 15.3; O, 27.2.

t-Butyloxycarbonylphenylalanyl-γ-t-butylglutamylnitroarginylglutamic Acid "α-Carbobenzoxyhydrazide." Sodium nitrite (291 mg.) in water (2 ml.) was added to an ice-cold solution of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamic acid hydrazide (2.09 g.) in 95% aqueous tetrahydrofuran (20 ml.) and 1 *N* hydrochloric acid (9 ml.). After 5 min. triethylamine (0.67 ml.) was added and this solution containing the azide of γ-*t*-butyloxycarbonylphenylalanylglutamic acid (XV) was added to a DMF solution (15 ml.) containing nitroarginylglutamic acid "α-carbobenzoxyhydrazide" monoacetate (2.4 g.) and triethylamine (0.65 ml.). After stirring for 24 hr. at 4° a second portion of the azide (prepared from 522 mg. of the hydrazide) was added and stirring continued for 24 hr. at 4°. The solvent was removed *in vacuo* and the residue was distributed between ethyl acetate (50 ml.) and 0.2 *N* ammonium hydroxide (50 ml.). The aqueous phase was washed with three 50-ml. portions of 0.2 *N* ammonium hydroxide. The combined aqueous phases were cooled at 0°, acidified (to approximately pH 3.0) with acetic acid, and extracted with six 50-ml. portions of 1-butanol (equilibrated with 5% acetic acid). The combined butanol phases were evaporated to dryness *in vacuo*, the residue was dissolved in dioxane (20 ml.), and the solution was lyophilized; yield 2.2 g. (55%); $[\alpha]^{28}D$ −22.4° (*c* 1.0, methanol); R_f^1 0.92; R_f^2 0.95; R_f^{VII} 0.81; amino acid ratios in acid hydrolysate arg + $orn_{1.06}glu_{1.92}phe_{1.00}$.

Anal. Calcd. for $C_{42}H_{60}O_{14}N_{10}$: C, 54.3; H, 6.5; N, 15.1; O, 24.1. Found: C, 54.4; H, 6.8; N, 15.1; O, 24.3.

t-Butyloxycarbonylphenylalanyl-γ-t-butylglutamylarginylglutamic Acid α-Hydrazide Diacetate. t-Butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylnitroarginylglutamic acid "α-carbobenzoxyhydrazide" (2 g.) was hydrogenated in the usual manner over palladium in methanol–acetic acid–water (2:1:1) (100 ml.). The catalyst was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The resulting oil was dissolved in 90% aqueous dioxane (20 ml.) and the solution was lyophilized; yield 1.8 g. (96%); R_f^1 0.92; R_f^2 0.94.

Anal. Calcd. for $C_{34}H_{56}O_{10}N_9\cdot 2CH_3COOH$: C, 52.4; H, 7.4; N, 14.5; O, 25.7. Found: C, 52.2; H, 7.6; N, 14.4; O, 25.5.

t-Butyloxycarbonylphenylalanyl-γ-t-butylglutamylarginylglutaminylhistidine Amide Acetate Trihydrate (XXI, R = NH₂). *t*-Butyl nitrite (0.25 ml.) was added to a stirred solution cooled at −30° of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamine hydrazide diacetate trihydrate (I, 1.8 g.) in DMF (20 ml.) containing 1.32 ml. of 6.1 *N* hydrogen chloride in dioxane. The mixture was stirred at −20° for 20 min., and then neutralized with triethylamine (1.1 ml.). To this solution of the azide (XIX) histidine amide (XX, R = NH_2) dihydrochloride[25] (910 mg.) in 50% aqueous DMF (8 ml.) and triethylamine (1.1 ml.) were added. After stirring for 30 min. at −20° and 75 hr. at 4° the solvents were removed *in vacuo*. The residue was dissolved in 20% acetic acid (100 ml.) and the solution was extracted with six 100-ml. portions of 1-butanol (equilibrated with 20% acetic acid). The butanol phases were washed with ten 50-ml. portions of 20% acetic acid (equilibrated with 1-butanol) and then evaporated to dryness. The residue was dissolved in 20% aqueous acetic acid (100 ml.) and the solution was extracted again with 1-butanol as described above. All the acetic acid phases were combined and evaporated to dryness. The residue was dissolved in water

(25) N. C. Davis, *J. Biol. Chem.*, 223, 935 (1956).

(100 ml.) and the solution was extracted with six 80-ml. portions of 1-butanol (equilibrated with water). The butanol phases were washed with ten 50-ml. portions of water (equilibrated with 1-butanol) and then evaporated to dryness. The residue was lyophilized twice from water; yield 690 mg. (36%); $[\alpha]^{27}D$ −26.5° (*c* 0.34, 10% acetic acid); single Pauly-, Sakaguchi-, and chlorine-positive spot; R_f^1 0.59; R_f^2 0.80; amino acid ratios in acid hydrolysate $phe_{1.09}glu_{1.94}arg_{1.00}his_{1.00}NH_{3\ 2.27}$.

Anal. Calcd. for $C_{40}H_{62}O_{10}N_{12}\cdot CH_3COOH\cdot 3H_2O$: C, 51.2; H, 7.4; N, 17.1; O, 24.4. Found: C, 50.9; H, 7.1; N, 16.9; O, 24.6.

Phenylalanylglutamylarginylglutaminylhistidine Amide Monoacetate Tetrahydrate (III). The above protected pentapeptide (XXI, R = NH_2) (700 mg.) was dissolved in anhydrous trifluoroacetic acid (10 ml.) and the solution was kept at room temperature for 15 min. The trifluoroacetate of the free pentapeptide was precipitated by addition of dry, ice-cold ether (500 ml.). After standing at −10° for 30 min. the precipitate was collected and dissolved in water (200 ml.). Amberlite IRA-400 (acetate cycle) (50 ml. settled in water) was added to the solution and the mixture stirred for 45 min. at room temperature. The resin was removed by filtration and washed with two 100-ml. portions of 2% acetic acid, and the combined filtrate and washings were evaporated to dryness. The residue dissolved in water (500 ml.) was applied to a CMC column (3 × 30 cm.) which was eluted with 500 ml. each of the following pH 6.9 ammonium acetate buffers: 0.005, 0.01, 0.025, 0.05, 0.075, and 0.1 *M*. Individual fractions (20 ml. each) were collected at a flow rate of approximately 3 ml./min. The desired peptide was located in the 0.1 *M* eluates by the Pauly reaction. These eluates were pooled and the solvent was evaporated. The residue was lyophilized to constant weight from small volumes of water yielding 510 mg. (85%) of a colorless powder; $[\alpha]^{29}D$ −19.7° (*c* 0.41, 10% acetic acid); single ninhydrin-, chlorine-, Pauly-, and Sakaguchi-positive spot with R_f^3 1.1 × His; amino acid ratios in acid hydrolysate $phe_{1.00}glu_{2.14}arg_{0.94}his_{0.92}NH_{3\ 1.95}$; amino acid ratios in LAP digest $phe_{1.06}glu_{2.04}arg + orn_{0.94}$-$his_{0.91}$ (86%); amide nitrogen 2.28 μmoles/μmole.

Anal. Calcd. for $C_{31}H_{46}O_8N_{12}\cdot CH_3COOH\cdot 4H_2O$: C, 46.8; H, 6.9; N, 19.8; O, 26.5. Found: C, 46.4; H, 6.9; N, 20.2; O, 26.5.

t-Butyloxycarbonylphenylalanyl-γ-t-butylglutamylarginylglutaminylhistidylmethionine Monoacetate Tetrahydrate (XXI, R = Met·OH). (*a*) *By the Standard Azide Procedure.* Sodium nitrite (20.1 mg.) in water (0.5 ml.) was added to an ice-cold solution of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamine hydrazide diacetate trihydrate (I, 277 mg.) in water (3.0 ml.) and 1 *N* hydrochloric acid (0.87 ml.) and the solution was kept at 0° for 2 min. The azide XIX was "salted out" by addition of saturated sodium chloride (5.0 ml.) and solid sodium chloride, and was collected by filtration. It was washed with saturated sodium chloride and was dried at 0° over potassium hydroxide pellets for 1 hr. This azide was dissolved in ice-cold DMF (6.0 ml.), the solution was added to a stirred, ice-cold solution of histidylmethionine (XX, R = Met·OH) (86 mg.) in 50% aqueous DMF (1 ml.) containing 10% (v./v.) triethylamine in DMF (0.84 ml.), and the mixture was stirred at 4° for 24 hr. Additional azide (prepared from 92 mg. of the hydrazide) in DMF (3.0 ml.), and 10% (v./v.) triethylamine in DMF (0.14 ml.) were added, and the mixture was stirred for 24 hr. at 4° and for 12 hr. at room temperature. The DMF was removed *in vacuo* and the residue was distributed between 1-butanol (equilibrated with 2% acetic acid) (50 ml.) and 2% acetic acid (equilibrated with 1-butanol) (40 ml.). The aqueous phase was extracted in countercurrent fashion with six 50-ml. portions of 1-butanol (equilibrated with 2% acetic acid) and the organic phases were washed with ten 40-ml. portions of 2% acetic acid (equilibrated with 1-butanol). The combined butanol phases were evaporated to dryness *in vacuo*, and the residue was lyophilized to give an amorphous powder (376 mg.) which was shown by paper chromatography to contain three components. This material was dissolved in water (500 ml.) and the solution was added to a CMC column (3 × 25 cm.) which was successively eluted with the following solvents: water (500 ml.); pH 6.9 ammonium acetate buffers 0.005 (500 ml.) and 0.01 *M* (1250 ml.). Individual fractions, 15 ml. each, were collected at a flow rate of approximately 2 ml./min. The desired peptide was located in the 0.01 *M* ammonium acetate eluates by the use of the Pauly reaction. These fractions were pooled and evaporated to a small volume *in vacuo*, and the residue was lyophilized to constant weight from small volumes of water; colorless, fluffy solid; yield 144 mg. (42%); $[\alpha]^{28}D$ −35.1° (*c* 0.26, 10% acetic acid); R_f^1 0.71; R_f^2 0.76; contaminated with the sulfoxide R_f^1 0.50 and R_f^2 0.53; both components ninhydrin negative, Pauly, Sakaguchi, methionine, and chlorine positive; amino acid ratios in acid hydrolysate $phe_{1.02}glu_{2.02}$-$arg_{1.00}his_{1.00}met_{1.00}NH_{3\ 0.96}$.

Anal. Calcd. for $C_{45}H_{70}O_{12}N_{12}S\cdot CH_3COOH\cdot 4H_2O$: C, 49.7; H, 7.3; N, 14.8; O, 25.4. Found: C, 49.9; H, 7.3; N, 14.8; O, 25.8.

(*b*) *By the Rudinger Modification.*[15] *t*-Butyl nitrite (0.25 ml.) was added to a stirred solution cooled at −20° of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamine hydrazide diacetate trihydrate (1, 1.84 g.) in DMF (20 ml.) containing 1.32 ml. of 6.1 *N* hydrogen chloride in dioxane. The mixture, after stirring at −20° for 15 min., was cooled at −60° and triethylamine (1.11 ml.) was added. To this solution containing the azide XIX was added histidylmethionine (XX, R = Met·OH) (0.573 g.) in DMF (3 ml.), water (7 ml.), and triethylamine (0.28 ml.). After stirring for 30 min. at −20°, 48 hr. at 4°, and 12 hr. at room temperature the solvents were evaporated. The product was isolated in the manner described under a, above; yield 934 mg. (41%); chromatographically indistinguishable from the previous preparation.

Phenylalanylglutamylarginylglutaminylhistidylmethionine Monoacetate Trihydrate (IV). The protected hexapeptide (XXI, R = Met·OH) (144 mg.) was dissolved in anhydrous trifluoroacetic acid (2.0 ml.) and the solution was kept at room temperature for 10 min. The trifluoroacetic acid was removed *in vacuo* at room temperature, the residue was dissolved in water (40 ml.), Amberlite IRA-400 (acetate cycle) (15 ml. settled in water) was added, and the mixture was

stirred for 10 min. The resin was removed by filtration and washed with 5% acetic acid (50 ml.), and the combined filtrate and washings were concentrated to a small volume and lyophilized. The residue was dissolved in 1% (v./v.) freshly distilled aqueous thioglycolic acid (10 ml.) and the solution was kept at 48° for 24 hr. Water (10 ml.) was then added followed by Amberlite IRA-400 (acetate cycle) (10 ml. settled in water) and the mixture was stirred for 30 min. The resin was removed by filtration and washed with 2% acetic acid (15 ml.), and filtrate and washings were evaporated. The residue was dissolved in water (200 ml.) and the solution was added to a CMC column (1.5 × 15 cm.) which was eluted with the following solvents: water (100 ml.); pH 6.9 ammonium acetate buffers 0.005 (150 ml.), 0.01 (200 ml.), and 0.025 *M* (400 ml.). Individual fractions (10 ml. each) were collected at a flow rate of approximately 2 ml./min. The desired peptide was located in the 0.025 *M* eluates by the Pauly reagent. These fractions were pooled, concentrated to a small volume *in vacuo*, and lyophilized to a constant weight from small volumes of water; colorless, fluffy powder; yield 111 mg. (90%); $[\alpha]^{30}D$ −32.7° (*c* 0.41, 10% acetic acid); single ninhydrin-, Pauly-, Sakaguchi-, and methionine-positive spot on paper chromatograms; $R_f{}^3$ 1.5 × His; and on paper electrophoresis at pH 1.9, 3.5, 6.5, and 8.0. Some samples were slightly contaminated with the sulfoxide; $R_f{}^3$ 0.73 × His; amino acid ratios in acid hydrolysate $phe_{1.00}glu_{2.07}arg_{0.98}his_{0.97}met_{1.00}NH_{3\ 1.36}$; amino acid ratios in LAP digest $phe_{1.08}glu_{1.30}arg_{1.10}$-$gluta_{0.55}his_{0.99}met_{0.83}$ (71%); amide nitrogen 0.98 μmole/μmole.

Anal. Calcd for $C_{36}H_{54}O_{10}N_{12}S\cdot CH_3COOH\cdot 3H_2O$: C, 47.5; H, 6.7; N, 17.5; O, 25.0. Found: C, 47.3; H, 6.7; N, 17.2; O, 24.2.

Phenylalanylglutamylarginylglutaminylhistidyl-α-n-aminobutyric Acid Monoacetate Tetrahydrate (*V*). *t*-Butyl nitrite (0.26 ml.) was added to a stirred solution cooled at −20° of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamine hydrazide diacetate trihydrate (I, 1.91 g.) in DMF (20 ml.) containing 1.41 ml. of 6.1 *N* hydrogen chloride in dioxane. The mixture after stirring at −20° for 15 min. was cooled at −60° and triethylamine (1.18 ml.) was added. To this solution containing the azide (XIX) was added histidyl-α-amino-*n*-butyric acid (XX, R = But·OH) (0.503 g.) in DMF (3 ml.), water (7 ml.), and triethylamine (0.29 ml.). After stirring for 30 min. at −20°, 48 hr. at 4°, and 12 hr. at room temperature the solvents were evaporated. The protected peptide (XXI, R = But·OH) was isolated essentially in the manner described for the preparation of XXI (R = Met·OH). Two purification attempts on CMC yielded a material (869 mg.; $R_f{}^1$ 0.67; $R_f{}^2$ 0.79; ninhydrin negative, Pauly, Sakaguchi, and chlorine positive) which was still contaminated with a material having $R_f{}^1$ 0.84 which was ninhydrin and Pauly negative but Sakaguchi and chlorine positive. This impure protected hexapeptide (XXI, R = But·OH) was dissolved in anhydrous trifluoroacetic acid (6.0 ml.) and the solution was kept at room temperature for 10 min. The trifluoroacetic acid was removed *in vacuo* at room temperature and the residue was lyophilized from water. This material was dissolved in water (50 ml.) and treated with Amberlite IRA-400 (acetate cycle) (30 ml. settled in water) and the suspension stirred for 10 min. The resin was removed by filtration and washed with 2% acetic acid (50 ml.) and the combined filtrate and washings were concentrated to a small volume and lyophilized. The residue was dissolved in water (1000 ml.) and the solution added to a CMC column (3 × 30 cm.) which was eluted with the following solvents: water (500 ml.); pH 6.9 ammonium acetate buffers 0.005 (500 ml.), 0.010 (1000 ml.), and 0.025 *M* (2000 ml.). Individual fractions (15 ml. each) were collected at a flow rate of approximately 3 ml./min. The desired peptide was located in the 0.025 *M* eluates by the Pauly reaction. These fractions were pooled, concentrated to a small volume *in vacuo*, and lyophilized to constant weight from small volumes of water; colorless, fluffy powder; 537 mg. (27%); $[\alpha]^{28}D$ −31.0° (*c* 0.50, 10% acetic acid); single ninhydrin-, Pauly-, and Sakaguchi-positive spot, $R_f{}^3$ 1.25 × His; homogeneous on paper electrophoresis at pH 1.9, 3.5, 6.5, and 8.0; amino acid ratios in acid hydrolysate $phe_{1.02}glu_{2.02}arg_{0.95}his_{0.95}but_{1.08}NH_{3\ 1.35}$; amino acid ratios in LAP digest $phe_{1.06}glu_{1.37}arg$ + $orn_{1.01}gluta_{0.48}$-$his_{0.95}but_{0.98}$ (82%); amide nitrogen 0.94 μmole/μmole.

Anal. Calcd. for $C_{35}H_{52}O_{10}N_{12}\cdot CH_3COOH\cdot 4H_2O$: C, 47.6; H, 6.9; N, 18.0; O, 27.4. Found: C, 47.4; H, 6.9; N, 18.2; O, 27.1.

Phenylalanylglutamylarginylglutamylhistidylmethionine Monoacetate Dihydrate. *t*-Butyl nitrite (0.25 ml.) was added to a stirred solution cooled at −20° of *t*-butyloxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamic acid α-hydrazide diacetate (1.80 g.) in DMF (20 ml.) containing 1.32 ml. of 6.1 *N* hydrogen chloride in dioxane. The mixture after stirring at −20° for 20 min. was cooled at −30° and triethylamine (1.42 ml.) was added. To this solution containing the azide was added histidylmethionine (XX, R = Met·OH) (0.573 g.) in DMF (3 ml.), water (7 ml.), and triethylamine (0.28 ml.). After stirring for 30 min. at −20°, 24 hr. at 4°, and 12 hr. at room temperature the solvents were removed *in vacuo*. The residue was distributed between 1-butanol and 2% acetic acid in the usual manner and the combined butanol phases were evaporated *in vacuo*. The residue was dissolved in 90% aqueous dioxane (20 ml.) and the solution was lyophilized to give an amorphous powder. This material dissolved in dioxane (20 ml.) and water (600 ml.) was applied to a CMC column (3 × 30 cm.) which was eluted with water (750 ml.) and pH 6.9 0.005 *M* ammonium acetate buffer (1500 ml.). The desired peptide was located (Pauly reaction) in the ammonium acetate eluates from which it was isolated in the usual manner. Chromatography on CMC was repeated since the protected peptide ($R_f{}^V$ 0.61) plus sulfoxide ($R_f{}^V$ 0.81) Pauly and Sakaguchi positive was still contaminated by Pauly-negative, Sakaguchi-positive materials with $R_f{}^V$ 0.50 and 0.72. Material from the second CMC column (600 mg.) was dissolved in anhydrous trifluoroacetic acid (3.0 ml.) and the solution was kept at room temperature for 20 min. The trifluoroacetic acid was removed *in vacuo*, and the residue dissolved in 15 ml. of water was applied to an Amberlite IRA-400 (acetate cycle) column (0.9 × 15 cm.). The column was eluted with 2% acetic acid

(100 ml.) and the eluate was evaporated to a small volume *in vacuo* and lyophilized; yield 560 mg. The crude hexapeptide dissolved in water (200 ml.) was applied to a DEAE-cellulose column (15 × 20 cm.) which was eluted with the following solvents: water (200 ml.) and pH 6.9 ammonium acetate buffer 0.001 *M* (300 ml.). Individual fractions of 10 ml. each were collected at a flow rate of 2.5 ml./min. The desired peptide was located in the 0.001 *M* ammonium acetate eluates from which it was isolated in the usual manner. Rechromatography on DEAE-cellulose gave a homogeneous material; yield 380 mg. (18%); $[\alpha]^{27}D$ −13.3° (*c* 0.29, 10% acetic acid); R_f[3] 1.6 × His; ninhydrin-, Pauly-, Sakaguchi-, and methionine-positive spot slightly contaminated with the sulfoxide; R_f[3] 1.1 × His; amino acid ratios in acid hydrolysate $phe_{1.08}glu_{2.02}arg_{1.02}his_{1.00}met_{0.85}$; amino acid ratios in LAP digest $phe_{1.18}glu_{1.48}arg_{1.00}his_{0.93}met_{0.93}$.

Anal. Calcd. for $C_{36}H_{53}O_{11}N_{11}S \cdot CH_3COOH \cdot 2H_2O$: C, 48.3; H, 6.6; N, 16.3; O, 25.4. Found: C, 48.4; H, 7.1; N, 16.1; O, 25.5.

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Studies on Polypeptides. XXXI. Synthetic Peptides Related to the N-Terminus of Bovine Pancreatic Ribonuclease (Positions 12–20)[1,4]

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Syntheses are described of peptides related to positions 12–20 in the amino acid sequence of bovine pancreatic ribonuclease A. In particular, methods are given for preparation of the d-sulfoxides of histidylmethionylaspartylserine and histidylmethionylaspartylserylserylthreonylserylalanylalanine. Evidence is presented for homogeneity of these compounds, one of which served as an intermediate in a synthesis of S-peptide.

In previous communications[3,5] we described syntheses of the protected peptide hydrazides II and III and related compounds whose amino acid sequences correspond to positions 1–7 and 8–11, respectively, of the primary structure of bovine pancreatic ribonuclease A and hence of S-peptide[6] (I). The present study relates synthetic routes to the *d*-sulfoxides of the tetrapeptide histidylmethionylaspartylserine (IV) and the nonapeptide histidylmethionylaspartylserylserylthreonylserylalanylalanine (V).

Potts, *et al.*,[7] digested S-peptide[6] with carboxypeptidase A and observed liberation of 2 moles each of alanine and serine and 1 mole of threonine per mole of peptide. The crude digestion mixture was assayed for its ability to reconstitute active ribonuclease in combination with S-protein. Full activity was restored when an aliquot of the carboxypeptidase digest, corresponding to 1 mole of S-peptide, was added per mole of S-protein. From this result the authors concluded that a peptide corresponding to the N-terminal pentadecapeptide of S-peptide (positions 1–15) was fully as active as S-peptide in restoring enzymic activity with S-protein. Since the pentadecapeptide was neither isolated nor purified it was of importance to produce pure synthetic samples of this compound to verify these conclusions. Histidylmethionylaspartylserine *d*-sulfoxide (IV), a necessary intermediate for a synthesis of this pentadecapeptide, was prepared for this reason.

The nonapeptide *d*-sulfoxide (V) which corresponds to positions 12–20 of the amino acid sequence of pancreatic ribonuclease A provided one of three subunits essential for construction of the entire S-peptide molecule. The previously described compounds (II) and (III) served as the other subunits in this scheme.

In addition to our own studies along these lines Marchiori, *et al.*,[8] have recently reported the preparation of ethyl benzyloxycarbonylserylthreonylserylalanylalaninate, which they obtained by a stepwise process starting with ethyl alanylalaninate.

The section of the S-peptide molecule which is the subject of this study contains the sequence aspartylserine which has attracted considerable attention in view of its occurrence in a number of esteratic and proteolytic enzymes.[9] Peptide derivatives containing

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

(3) See K. Hofmann, W. Haas, M. J. Smithers, R. D. Wells, Y. Wolman, N. Yanaihara, and G. Zanetti, *J. Am. Chem. Soc.*, **87**, 620 (1965), for paper XXX in this series.

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