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Studies on Polypeptides. VIII. Synthesis of Peptides Related to Corticotropin[1,2]

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A number of peptides and peptide derivatives containing the amino acids glycine, serine, tyrosine, methionine and glutamic acid have been prepared by the azide and mixed anhydride procedures. A modification of the azide procedure was developed, which allows formation of complex acylated peptides in heterogeneous aqueous systems. The application of this method to the synthesis of seryltyrosylserylmethionylglutamic acid, the N-terminal pentapeptide sequence of the corticotropins, is described.

Recent studies[3-6] have led to the elucidation of the structure of swine β-corticotropin, swine corticotropin-A and sheep α-corticotropin.

These hormones were found to be closely similar single-chain peptides composed of 39 amino acid residues derived entirely from known amino acids. The structure elucidation involved digestion of homogeneous hormone preparations with highly purified homospecific proteolytic enzymes, separation of the ensuing split products, and finally the establishment of the structures of the individual peptide fragments. In order to confirm the results of this degradative work, and as model studies for contemplated synthetic experiments, we undertook the preparation of a number of key peptides resulting from the enzymatic breakdown of the corticotropin molecule. This communication describes experiments relating to the five N-terminal positions which are beyond doubt identical in the three corticotropins.

White and Landmann[7] identified among the products of peptic digestion of corticotropin-A the pentapeptide seryltyrosylserylmethionylglutamic acid (I). The demonstration of seryltyrosine as the N-terminus of the hormone placed the pentapeptide at the amino end of its peptide chain. We selected for initial study sequences occurring in this pentapeptide, in particular those containing the amino acids serine, tyrosine, methionine and glutamic acid. Simple peptides of these various amino acids have been described previously, but the sequences occurring in the corticotropin molecule had received little attention prior to this investigation.

The N-terminal dipeptide L-seryl-L-tyrosine was prepared along classical lines by the azide procedure. Carbobenzoxy-L-serine azide[8] was coupled with ethyl L-tyrosinate and the ensuing acylated dipeptide ester saponified. The resulting carbobenzoxy-L-seryl-L-tyrosine afforded L-seryl-L-tyrosine when subjected to hydrogenolysis. The paper chromatographic comparison of the synthetic with the natural peptide (obtained by chymotryptic digestion of corticotropin) demonstrated their identity.[9] Following the initial announcement the preparation of seryl-tyrosine was also recorded by Fischer and Whetstone[10] who employed essentially similar methods for its preparation.

The sequence seryl-methionine, which occupies positions 3 and 4 in the hormone molecule, was obtained readily from carbobenzoxy-L-serine azide and methyl L-methionate followed by saponification and decarbobenzoxylation. Decarbobenzoxylation was effected either catalytically or by reduction with sodium in liquid ammonia; the same dipeptide resulted from both procedures. It is of interest to note that we have subjected a number of carbobenzoxylated peptides of methionine to reduction with sodium in liquid ammonia without incurring significant losses of methionine. The use of an excess of sodium in these reactions must be carefully avoided since Stekol[11] has demonstrated that DL-methionine is converted to DL-homocysteine upon treatment with an excess of sodium in liquid ammonia.

Next we turned our attention toward the preparation of the dipeptide L-methionyl-L-glutamic acid, a sequence occupying positions 4 and 5 in corticotropin. Dekker, *et al.*,[12] have accomplished the synthesis of a number of peptides of methionine by the use of carbobenzoxy-L-methionine azide. We have prepared the ethyl esters of carbobenzoxy-L-methionyl-L-glycine and carbobenzoxy-L-methionyl-L-glutamic acid by the mixed anhydride procedure. Saponification and decarbobenzoxylation converted these derivatives into the corresponding dipeptides.[13]

The dipeptides Ser-Tyr, Ser-Met, Met-Gly and Met-Glu exhibited one single ninhydrin positive spot with R_f values of 0.42, 0.46, 0.50 and 0.54, respectively, on paper in the Partridge system.[14] The chromatographic analysis of their hydrolyzates (6 N hydrochloric acid for 24 hours at 110° in sealed tubes) afforded the expected mixture of amino acids. Considerable destruction of serine was observed in the hydrolyzates of the peptides containing this amino acid.[15]

(1) The authors wish to express their appreciation to Armour and Company and to the U. S. Public Health Service for generous support of this investigation.

(2) A preliminary communication of some of the work reported in this paper has appeared in THIS JOURNAL, **77**, 2914 (1955).

(3) P. H. Bell, *ibid.*, **76**, 5565 (1954).

(4) K. S. Howard, R. G. Shepherd, E. A. Eigner, D. S. Davies and P. H. Bell, *ibid.*, **77**, 3419 (1955).

(5) W. F. White and W. A. Landmann, *ibid.*, **77**, 1711 (1955).

(6) C. H. Li, I. I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris and J. S. Dixon, *Nature*, **176**, 687 (1955).

(7) W. F. White and W. A. Landmann, THIS JOURNAL, **77**, 771 (1955).

(8) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).

(9) W. F. White and W. A. Landmann, THIS JOURNAL, **76**, 4193 (1954).

(10) R. F. Fischer and R. R. Whetstone, *ibid.*, **76**, 5076 (1954).

(11) J. A. Stekol, *J. Biol. Chem.*, **140**, 827 (1941).

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

(13) The optical rotation of L-methionylglycine agreed with that previously reported,[12] but our peptide melted 29° higher.

(14) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(15) We wish to express our appreciation to Mrs. Eleanore T. Schwartz for these determinations.

The successful approach to the synthesis of complex polypeptides such as the pentapeptide I depends, in our opinion, upon the availability of methods allowing the formation of peptide bonds in aqueous media, without racemization under mild conditions. Consequently we have directed our efforts toward the development of such a procedure, based on the azide method of peptide synthesis. In its most commonly used form this method involves the coupling (in anhydrous solvents) of a carbobenzoxyamino acid or peptide azide with an amino acid or peptide ester to give an acylated peptide ester which is converted into the final peptide by saponification and decarbobenzoxylation. This method suffers from serious disadvantages as a generally applicable tool for the preparation of peptides of high molecular weight. Firstly, complex peptide esters and acylated peptide azides (in particular those containing polyfunctional amino acid residues) are only sparingly soluble in most organic solvents. Secondly, many complex carbobenzoxylated peptide esters are racemized by exposure to strong alkali and thus cannot be saponified with retention of stereochemical purity. The latter disadvantage can be eliminated by the use of benzyl esters which are subject to hydrogenolysis. However, the preparation of the benzyl esters of complex peptides is beset with difficulties.

In 1904 Curtius and Wüstenfeld[16] described the preparation of a series of benzoylpolyglycines (up to benzoylhexaglycine). They coupled the azides of benzoylglycine, benzoyldiglycine, benzoyltriglycine and benzoyltetraglycine, respectively, with sodium glycinate or diglycinate. Similar procedures were employed by Curtius and Lambotte[17] for preparing hippuryl-DL-alanine, hippuryl-DL-alanyl-DL-alanine and hippuryl-DL-alanyl-DL-alanyl-DL-alanine. The coupling reactions were carried out in aqueous solutions. This approach seemed to offer great promise as a generally useful tool in peptide synthesis and eliminates the final saponification step. Consequently we have now explored the potentialities of carbobenzoxyamino acid and peptide azides of the L-series as acylating agents for amino acid and peptide salts in aqueous systems. A solution of the azide in a suitable organic solvent (ether or ethyl acetate) was shaken with an aqueous solution containing the triethylammonium salt of an amino acid or peptide. After completion of the reaction, the organic layer was separated from the aqueous phase, the latter was acidified and the resulting carbobenzoxylated peptide isolated in the usual manner. Ion-exchange resins were employed in some instances to separate the desired products from unchanged starting materials. It is apparent from the examples presented in the experimental section that the desired products were obtained (in analytically pure form) in yields which varied from 30 to 65% of the theoretical. Some of the products were converted into the corresponding free peptides by decarbobenzoxylation. Of particular interest was the application of this method to the synthesis of the pentapeptide I, which was accomplished in the following manner. Carbobenzoxy-L-serine azide was coupled with the triethylammonium salt of L-methionyl-L-glutamic acid and the ensuing acylated tripeptide was decarbobenzoxylated by treatment with sodium in liquid ammonia. The product, L-seryl-L-methionyl-L-glutamic acid (again as the triethylammonium salt), then reacted with the azide of carbobenzoxy-L-seryl-L-tyrosine to give carbobenzoxy-L-seryl-L-tyrosyl-L-seryl-L-methionyl-L-glutamic acid which was decarbobenzoxylated to afford the final pentapeptide I. This was purified by repeated precipitation with ethanol from its aqueous solution. It was finally dissolved in warm water and crystallized in the form of small rosettes upon slow cooling of this solution.

The peptide gave a positive ninhydrin reaction and produced a dark purple color with diazotized sulfanilic acid in sodium carbonate solution.

The paper chromatographic comparison of the synthetic product with the natural material in the Partridge[14] and 2-butanol–ammonia systems[18] revealed identical behavior of both compounds. Their R_f value in the Partridge system was 0.51, and both peptides appeared between glutamic acid and lysine in the latter system. Paper chromatography of a 1:1 mixture of the peptides produced one spot only.

The behavior of the synthetic specimen on treatment with carboxypeptidase and aminopeptidase[19] duplicated that observed with the natural material.[7] The four constituent amino acids were liberated in the expected molar ratios by both enzymes. The recovery of serine from an acid hydrolyzate (18 hours at 105° with 6 *N* HCl) was 90% of the theoretical, reflecting the well-known lability of this compound to acid. Excellent recoveries of tyrosine, methionine and glutamic acid were realized.[20]

These results establish the structure and optical purity of the synthetic peptide, and substantiate structure I for the pentapeptide resulting from the peptic digestion of corticotropin-A.

Experimental[21]

Tosyl-L-serine.—To a solution of L-serine (3.0 g.) in water (20 ml.) was added a solution of tosyl chloride (7.2 g.) in ethyl acetate (100 ml.). This mixture was shaken at room temperature for four hours while 2 *N* sodium hydroxide (38 ml.) was added slowly. The organic phase was separated and the aqueous solution cooled in an ice-bath and acidified to congo red with 6 *N* hydrochloric acid. The resulting crystals were collected, washed with ice-water and recrystallized from hot water; yield 5.8 g. (78%); m.p. 235–236°; $[\alpha]^{25}D$ −32.3° (*c* 2.06, in pyridine).

Anal. Calcd. for $C_{10}H_{13}O_5NS$: C, 46.3; H, 5.0; N, 5.4; S, 12.4. Found: C, 46.4; H, 4.8; N, 5.4; S, 12.6.

(16) T. Curtius and R. Wüstenfeld, *J. prakt. Chem.*, [2] **70**, 73 (1904).

(17) T. Curtius and R. Lambotte, *ibid.*, [2] **70**, 109 (1904).

(18) J. F. Roland, Jr., and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954).

(19) D. H. Spackman, E. L. Smith and D. M. Brown, *J. Biol. Chem.*, **212**, 255 (1955).

(20) We wish to express our thanks to Dr. W. F. White of the Armour Laboratories for comparing these properties of the natural and synthetic peptides.

(21) The melting points are uncorrected. Optical rotations were determined in a Rudolph Precision Polarimeter, Model 70, using a 1 dm. tube.

Tosyl-L-serinhydrazide.—Tosyl-L-serine (6.0 g.) was suspended in dry methanol (150 ml.) and the mixture saturated with hydrogen chloride. The solution was evaporated to dryness and the residue was re-esterified in an identical manner. The resulting crystalline tosyl-L-serine methyl ester was dissolved in methanol (35 ml.) and hydrazine hydrate (4 ml.) was added. The mixture was kept in a refrigerator for 15 hours and the crystalline hydrazide which had separated out was collected, washed with ice water, dried and recrystallized from ethanol; yield 4.9 g. (79%); m.p. 179–181°.

Anal. Calcd. for $C_{10}H_{15}O_4N_3S$: N, 15.4. Found: N, 15.4.

Carbobenzoxy-L-methionine.—To an ice-cold solution of L-methionine (4.0 g.) in water (120 ml.) and sodium bicarbonate (6.8 g.) was added in small portions carbobenzoxy chloride (5.0 g.) with vigorous stirring. Stirring was continued for two hours with cooling and the solution was acidified to congo red with 6 *N* hydrochloric acid. The resulting oily precipitate was extracted into ether, the ethereal solution washed with water, and dried over sodium sulfate. Evaporation of the ether solution to a small volume followed by the addition of petroleum ether (b.p. 40–60°) afforded needles which were recrystallized from a mixture of ether and petroleum ether; yield 6.3 g. (83%); m.p. 67–68° (lit.[22] m.p. for carbobenzoxy-D-methionine 69–70°); $[\alpha]^{28}D$ −16.6° (*c* 2.47, in ethanol).

Anal. Calcd. for $C_{13}H_{17}O_4NS$: C, 55.1; H, 6.0. Found: C, 55.1; H, 5.9.

Carbobenzoxy-L-seryl-L-methionine Methyl Ester.—L-Methionine methyl ester hydrochloride (8.0 g.) was dissolved in dry ethanol (30 ml.) and triethylamine (5.5 ml.) was added. The mixture was evaporated to dryness *in vacuo* at a bath temperature of 30° and the residue was extracted with ethyl acetate (40 ml.). To this solution was added at 5° an ethereal solution (100 ml.) of carbobenzoxy-L-serine azide (prepared from 7.6 g. of the hydrazide and 2.1 g. of sodium nitrite in 60 ml. of 1 *N* hydrochloric acid at 0°[8]). The mixture was shaken at 5° for 20 hours and at room temperature for four hours. The solvents were evaporated, the residue was extracted with ethyl acetate and the solution was washed consecutively with 2 *N* hydrochloric acid, saturated sodium bicarbonate and water and was dried over sodium sulfate. Evaporation of the solvent afforded a solid which was recrystallized from ethanol; yield 7.5 g. (65% based on hydrazide); m.p. 101–102°.

Anal. Calcd. for $C_{17}H_{24}O_6N_2S$: C, 53.1; H, 6.3; N, 7.3. Found: C, 52.8; H, 6.3; N, 7.3.

The mother liquors from the above preparation were evaporated to dryness, dissolved in ethanol (30 ml.) and hydrazine hydrate (0.5 ml.) was added. The mixture was kept at room temperature for 12 hours and the ensuing crystalline hydrazide was collected, washed with ethanol and ether and dried. The hydrazide was recrystallized from hot water; yield 1.46 g.; m.p. 215–216°. It was identical in every respect with the hydrazide described below.

Carbobenzoxy-L-seryl-L-methioninhydrazide.—Carbobenzoxy-L-seryl-L-methionine methyl ester (2.5 g.) was dissolved in dry ethanol (25 ml.) and hydrazine hydrate (0.5 ml.) was added to the solution. The mixture was kept at room temperature for 12 hours and the crystalline hydrazide was collected, washed with ethanol and dried. The material was recrystallized from ethanol; yield 1.6 g. (64%); m.p. 215–216°.

Anal. Calcd. for $C_{16}H_{24}O_5N_4S$: C, 50.0; H, 6.3; N, 14.6. Found: C, 49.4; H, 6.4; N, 14.7.

Carbobenzoxy-L-seryl-L-methionine.—Carbobenzoxy-L-seryl-L-methionine methyl ester (3.3 g.) was dissolved in methanol (10 ml.) and 1 *N* sodium hydroxide (9.5 ml.) was added. The solution was kept at room temperature under nitrogen for 30 minutes and water (100 ml.) was added. The mixture was extracted with ethyl acetate, the organic layer was separated and the aqueous phase acidified to congo red with 2 *N* hydrochloric acid. The resulting precipitate was dissolved in ethyl acetate, the solution was washed with water, dried over sodium sulfate and concentrated to dryness *in vacuo*. The substance was recrystallized from ethanol; yield 2.1 g. (67%); m.p. 128–129°; $[\alpha]^{26}D$ −24.2° (*c* 3.2, in saturated sodium bicarbonate).

(22) C. A. Dekker and J. S. Fruton, *J. Biol. Chem.*, **173**, 471 (1948).

Anal. Calcd. for $C_{16}H_{22}O_6N_2S$: C, 51.9; H, 6.0; N, 7.6. Found: C, 52.0; H, 5.9; N, 7.4.

L-Seryl-L-methionine. a. **With Sodium in Liquid Ammonia.**—Carbobenzoxy-L-seryl-L-methionine (1.5 g.) was dissolved in liquid ammonia (approximately 100 ml.) and sodium (0.37 g.) was added with stirring. Dowex 50 (ammonia cycle) (7 g.) was added, the mixture was stirred for 10 minutes and the ammonia was evaporated. The residue was kept over sulfuric acid *in vacuo* to remove the last traces of ammonia and was then suspended in water (40 ml.). The resin was removed by filtration and was re-extracted with several small portions of water. The combined filtrates were acidified to *p*H 5 with glacial acetic acid and evaporated to dryness *in vacuo* at a bath temperature of 30°. The solid residue was recrystallized from aqueous ethanol; yield 0.71 g. (74%); m.p. 215–216°; $[\alpha]^{27}D$ −11.4° (*c* 5.6, in 1 *N* HCl); R_f = 0.46 (Partridge).

Anal. Calcd. for $C_8H_{16}O_4N_2S$: C, 40.7; H, 6.8; N, 11.9. Found: C, 40.0; H, 6.4; N, 11.3.

b. **By Catalytic Hydrogenation.**—Carbobenzoxy-L-seryl-L-methionine (0.25 g.) was hydrogenated for 80 minutes in 90% aqueous acetic acid (20 ml.) with palladium on barium sulfate[23] (2.5 g.). The catalyst was removed by filtration (with the aid of Hyflo filter-cel) and the solvent was removed *in vacuo* at a bath temperature of 45°. The residue was evaporated with two 45-ml. portions of ethanol and dissolved in a small quantity of water and crystallized upon the addition of ethanol; yield 100 mg. (62%); $[\alpha]^{26}D$ −10.4° (*c* 2.2, in 1 *N* HCl); R_f = 0.46 (Partridge).

Carbobenzoxy-L-seryl-L-tyrosine Ethyl Ester.—L-Tyrosine ethyl ester hydrochloride (2.31 g.) was suspended in dioxane (20 ml.) and triethylamine (1.31 ml.) was added. The mixture was shaken at room temperature for 15 minutes, the precipitate of triethylammonium chloride was removed by filtration and the filtrate was cooled in an ice-bath (dioxane solidifies). To this solution was added an ice-cold ethyl acetate solution of carbobenzoxy-L-serine azide (prepared from 1.5 g. of the hydrazide) and the mixture was kept in a refrigerator at 5° for 40 hours. The solvents were removed *in vacuo* and the ensuing oil dissolved in ethyl acetate. The solution was washed consecutively with saturated sodium chloride, 1 *N* hydrochloric acid (saturated with sodium chloride), sodium bicarbonate and finally saturated sodium chloride, and was dried over sodium sulfate. On evaporation a yellow oil was obtained which failed to crystallize; yield 2.1 g. (84% based on hydrazide).

Carbobenzoxy-L-seryl-L-tyrosinhydrazide.—Carbobenzoxy-L-seryl-L-tyrosine methyl ester (4.66 g.) (prepared as described for the ethyl ester) was dissolved in boiling methanol (20 ml.) and hydrazine hydrate (0.9 ml.) was added. The mixture was kept in a refrigerator for 15 hours, and the crystalline hydrazide collected and washed with ice-cold methanol. The compound was recrystallized from dioxane; yield 4.2 g. (89%); m.p. 213–214°.

Anal. Calcd. for $C_{20}H_{24}O_6N_4$: C, 57.7; H, 5.8; N, 13.4. Found: C, 57.2; H, 5.6; N, 13.0.

Carbobenzoxy-L-seryl-L-tyrosine.—Carbobenzoxy-L-seryl-L-tyrosyl ethyl ester (5.3 g.) was dissolved in methanol (20 ml.) and 2 *N* sodium hydroxide (12.5 ml.) was added. The mixture was kept at room temperature for 30 minutes, was then acidified to congo red with 2 *N* hydrochloric acid, and most of the methanol was removed *in vacuo*. The resulting oil soon solidified. The compound was recrystallized from hot water; yield 2.5 g. (50%); m.p. 189–190° (lit.[10] m.p. 187–188°); $[\alpha]^{24}D$ +37.4° (*c* 1.92, in ethanol).

Anal. Calcd. for $C_{20}H_{22}O_7N_2$: C, 59.7; H, 5.5; N, 7.0. Found: C, 60.2; H, 5.8; N, 7.0.

L-Seryl-L-tyrosine.—A sample of the carbobenzoxy derivative (0.48 g.) was hydrogenated over a palladium catalyst in methanol (12 ml.) containing glacial acetic acid (2 ml.). The product was isolated in the usual manner and recrystallized from water to give the monohydrate; yield 0.3 g. (88%); m.p. 254–256° (lit.[10] m.p. 200–260°); $[\alpha]^{24}D$ +38.4° (*c* 1.0, in water); R_f = 0.42 (Partridge).

Anal. Calcd. for $C_{12}H_{16}O_5N_2(H_2O)$: C, 50.3; H, 6.3; N, 9.8; H_2O, 6.2. Found: C, 50.5; H, 6.4; N, 10.0; H_2O, 6.1.

(23) R. Mozingo, S. A. Harris, D. E. Wolf, C. E. Hoffhine, Jr., N. R. Easton and K. Folkers, THIS JOURNAL, **67**, 2092 (1945).

The compound crystallizes from methanol in needles which disintegrate in air to give the half hydrate.

Anal. Calcd. for $C_{12}H_{16}O_5N_2 \cdot 1/2H_2O$: C, 52.0; H, 6.2; N, 10.1; H_2O, 3.3. Found: C, 52.1; H, 6.5; N, 9.6; H_2O, 3.5.

Tosyl-L-seryl-L-tyrosinhydrazide.—Tosyl-L-serinhydrazide (1.25 g.) was dissolved in water (20 ml.) and concentrated hydrochloric acid (1.5 ml.) and the solution was cooled at 0°. Sodium nitrite (0.37 g.) in water (5 ml.) was added and the precipitated solid azide was extracted with ice-cold ethyl acetate. The ethyl acetate extracts were washed with ice-cold sodium bicarbonate and water and were dried over sodium sulfate. This solution, containing the azide of tosyl-L-serine, was then added to a dioxane solution of L-tyrosine methyl ester (prepared from 2.11 g. of the hydrochloride). The mixture was kept at 5° for 20 hours when the solvents were removed *in vacuo*. The ensuing oil was dissolved in ethyl acetate, the solution was washed consecutively with water, 2 *N* hydrochloric acid, sodium bicarbonate and water and was dried over sodium sulfate. The solvent was evaporated, the residue dissolved in hot methanol (9 ml.) and hydrazine hydrate (0.4 ml.) was added. After standing at room temperature for 20 hours the hydrazide was collected and recrystallized from aqueous methanol; yield 1.25 g. (63%); m.p. 200–202°.

Anal. Calcd. for $C_{19}H_{24}O_6N_4S$: N, 12.8. Found: N, 12.9.

Carbobenzoxy-L-methionylglycine Ethyl Ester.—A mixed anhydride was prepared in the usual manner from carbobenzoxy-L-methionine (2.0 g.) in dry dioxane (15 ml.) with tri-*n*-butylamine (1.67 ml.) and ethyl chloroformate (0.76 g.). This solution was added to a dioxane solution of ethyl glycinate (prepared from 1.1 g. of the hydrochloride), and the mixture was kept for 30 minutes at 8–10°, then one hour at room temperature. The solvent was evaporated, and the reaction product isolated in the usual manner, and recrystallized from a mixture of ether and petroleum ether; yield 2.0 g. (77%); m.p. 95–96° (lit.[12] m.p. 93–95°); $[\alpha]^{27}D$ −20.0° (*c* 4.6, in absolute ethanol).

Anal. Calcd. for $C_{17}H_{24}O_5N_2S$: C, 55.4; H, 6.6; N, 7.6. Found: C, 55.7; H, 6.6; N, 7.7.

Carbobenzoxy-L-methionylglycine.—Carbobenzoxy-L-methionylglycine ethyl ester (1.0 g.) was dissolved in methanol (17 ml.) and 1 *N* sodium hydroxide (3.2 ml.) was added. The solution was kept at room temperature for one hour, then cooled in an ice-bath and acidified to congo red with 2 *N* hydrochloric acid. Most of the methanol was evaporated and the resulting crystalline material was collected and recrystallized from hot water; yield 0.87 g. (94%); m.p. 135–136° (lit.[12] m.p. 130–131°); $[\alpha]^{26}D$ −16.0° (*c* 2.2, in absolute ethanol).

Anal. Calcd. for $C_{17}H_{20}O_5N_2S$: C, 53.0; H, 5.9; N, 8.2. Found: C, 52.9; H, 5.9; N, 8.2.

L-Methionylglycine.—A sample of the carbobenzoxy derivative (680 mg.) was dissolved in liquid ammonia and sodium (40 mg.) was added in small pieces with stirring. Dowex 50 (ammonia cycle) (2.5 g.) was added, the ammonia was evaporated and the residue kept over sulfuric acid *in vacuo* at room temperature for 12 hours. The residue was triturated with water (30 ml.), filtered and the filtrate acidified with glacial acetic acid. The solution was evaporated to a sirup at a bath temperature of 50° and the peptide precipitated by the addition of absolute ethanol. The material was recrystallized from aqueous ethanol; yield 292 mg. (71%); m.p. 211–212° with decomposition (lit.[12] m.p. 182–183°); R_f = 0.50 (Partridge); $[\alpha]^{26}D$ +83.0° (*c* 1.9, in H_2O), (lit.[12] rotation $[\alpha]D$ +86.0° in H_2O).

Anal. Calcd. for $C_7H_{14}O_3N_2S$: C, 40.8; H, 6.8. Found: C, 40.7; H, 6.8.

Carbobenzoxy-L-methionyl-L-glutamic Acid Diethyl Ester.—A mixed anhydride was prepared in the usual manner from carbobenzoxy-L-methionine (4.06 g.) in dry dioxane (30 ml.), tri-*n*-butylamine (3.9 ml.) and ethyl chloroformate (1.6 ml.). This solution was added over a period of 30 minutes to a solution of diethyl L-glutamate (prepared from 3.6 g. of the hydrochloride in 30 ml. of dioxane and 2.4 ml. of triethylamine) and the mixture was stirred at 8–10° for one hour. The solvents were evaporated, the residue dissolved in ethyl acetate and the solution washed in the usual manner and dried over sodium sulfate. The solution was concentrated to a small volume and petroleum ether was added. The resulting fine needles were collected and recrystallized from a mixture of ethyl acetate and petroleum ether; yield 5.5 g. (82%); m.p. 95–96°; $[\alpha]^{25}D$ −20.9° (*c* 1.27, in ethanol).

Anal. Calcd. for $C_{22}H_{32}O_7N_2S$: C, 56.4; H, 6.9; N, 6.0. Found: C, 57.1; H, 6.9; N, 5.9.

Carbobenzoxy-L-methionyl-L-glutamic Acid.—Carbobenzoxy-L-methionyl-L-glutamic acid diethyl ester (5.03 g.) was dissolved in methanol (60 ml.) and 2 *N* sodium hydroxide (25.5 ml.) was added. The mixture was kept at room temperature for 45 minutes, was then cooled in an ice-bath and acidified with 2 *N* hydrochloric acid. Most of the methanol was evaporated at a bath temperature of 40° and the resulting crystalline material was collected and recrystallized from a mixture of ethyl acetate and petroleum ether; yield 4.07 g. (92%); m.p. 138–140°; $[\alpha]^{28}D$ −13.5° (*c* 1.0, in ethanol).

Anal. Calcd. for $C_{18}H_{24}O_7N_2S$: C, 52.4; H, 5.9; N, 6.8. Found: C, 52.3; H, 5.8; N, 6.8.

L-Methionyl-L-glutamic Acid.—The carbobenzoxy derivative (3.86 g.) was dissolved in liquid ammonia (25–30 ml.) and sodium (0.86 g.) was added in small pieces with stirring. The ammonia was evaporated and the residue dried over sulfuric acid, and dissolved in 50% acetic acid (25 ml.). The solution was filtered and evaporated to dryness *in vacuo* at a bath temperature of 40°. The residue was evaporated with three portions of methanol and then recrystallized from aqueous ethanol to give shiny plates; yield 2.05 g. (79%); m.p. 205–206°; $[\alpha]^{28}D$ +18.6° (*c* 2.6, in 1 *N* HCl); R_f = 0.54 (Partridge).

Anal. Calcd. for $C_{10}H_{18}O_5N_2S$: C, 43.2; H, 6.5; N, 10.1. Found: C, 43.5; H, 6.7; N, 10.0.

Carbobenzoxyglycyl-L-glutamic Acid.—A solution of L-glutamic acid (1.0 g.) in water (10 ml.) and triethylamine (3 ml.) was shaken for four hours at room temperature with an ethyl acetate solution of carbobenzoxyglycine azide (prepared in the usual manner from 1 g. of the hydrazide). The mixture was acidified with 2 *N* hydrochloric acid to congo red, the ethyl acetate layer was separated and the aqueous phase re-extracted with ethyl acetate. The combined ethyl acetate extracts were washed with water, dried over sodium sulfate and evaporated to dryness. The residue was recrystallized from hot water; yield 1.0 g. (65%); m.p. 160–162° (lit.[24] m.p. 160–162°).

Carbobenzoxy-L-seryl-L-tyrosyl-L-serine Methyl Ester.—Carbobenzoxy-L-seryl-L-tyrosinhydrazide (2.3 g.) was dissolved in a mixture of water (25 ml.), concentrated hydrochloric acid (1.8 ml.) and glacial acetic acid (2.5 ml.) and the solution was cooled in an ice-salt bath. A solution of sodium nitrite (0.42 g.) in water (15 ml.) was added slowly and the oily azide which precipitated was extracted with ice-cold ethyl acetate. The extract was washed consecutively with ice-cold sodium bicarbonate and water and was dried over sodium sulfate. This azide solution was added to an ice-cold ethyl acetate solution of L-serine methyl ester (prepared from 1.72 g. of the hydrochloride with triethylamine (1.6 ml.) in ethyl acetate (70 ml.)). The mixture was kept at 5° for 15 hours, and was then washed with water, 2 *N* hydrochloric acid, sodium bicarbonate and water and was dried over sodium sulfate. The solvent was evaporated *in vacuo* and the residue recrystallized from a mixture of dioxane and petroleum ether (b.p. 60–90°); yield 2.4 g. (86%); m.p. 191–192°; $[\alpha]^{25}D$ −3.9° (*c* 1.77, in pyridine).

Anal. Calcd. for $C_{24}H_{29}O_9N_3$; C, 57.2; H, 5.8; N, 8.3. Found: C, 57.0; H, 6.1; N, 8.2.

Carbobenzoxy-L-seryl-L-tyrosyl-L-serinhydrazide.—The above ester (1.2 g.) was dissolved in methanol (15 ml.) and hydrazine hydrate (0.25 ml.) was added. The solution was kept at room temperature for 24 hours and the hydrazide which had separated in the form of a gel was collected and washed with methanol and ether; it was recrystallized from hot water; yield 1.2 g. (100%); m.p. 210–213°.

Anal. Calcd. for $C_{23}H_{29}O_8N_5$: N, 13.9. Found: N, 13.5.

Carbobenzoxy-L-serylglycyl-L-glutamic Acid.—A solution of glycyl-L-glutamic acid (0.4 g.) in water (10 ml.) and triethylamine (0.82 ml.) was shaken for 18 hours at 5° with an ethyl acetate solution of carbobenzoxy-L-serine azide (prepared in the usual manner from 0.5 g. of the hydrazide). The

(24) K. Hofmann and M. Bergmann, *J. Biol. Chem.*, **134**, 225 (1940).

aqueous phase was separated, Dowex 50 (acetate cycle) (3 g.) was added and the mixture was shaken for 10 minutes. The resin was removed by filtration, was washed with several portions of water, and the combined filtrate and washings were concentrated to a small volume *in vacuo* at a bath temperature of 40°. The solution was placed in a refrigerator where crystallization occurred. The material was recrystallized from water; yield 0.37 g. (44% based on hydrazide); m.p. 106–107°.

Anal. Calcd. for $C_{18}H_{23}O_9N_3$: C, 50.8; H, 5.5; N, 9.9. Found: C, 50.6; H, 5.6; N, 9.6.

Carbobenzoxy-L-seryl-L-methionyl-L-glutamic Acid.—A solution of L-methionyl-L-glutamic acid (0.5 g.) in water (10 ml.) and triethylamine (0.5 ml.) was shaken for 42 hours at 5° with an ethereal solution of carbobenzoxy-L-serine azide (prepared from 1.36 g. of the hydrazide). The ether layer was separated, the aqueous solution re-extracted with ether and Dowex 50 (acetate cycle) (9.0 g.) was added. The mixture was shaken for 10 minutes, the resin was removed by filtration and was washed repeatedly with water. The combined filtrate and washings were evaporated *in vacuo* to a volume of 10 ml., when the reaction product began to separate out. Precipitation was completed by placing the mixture in a refrigerator for 10 hours. The product was collected, washed with a small portion of ice-water and dried over phosphorus pentoxide *in vacuo*. The material was dissolved in hot water and separated on cooling in the form of a gelatinous mass, which was dried at room temperature *in vacuo;* yield 0.42 g. (46%); m.p. 117–121°; $[\alpha]^{25}D$ −22.5° (*c* 1, in ethanol).

Anal. Calcd. for $C_{21}H_{29}O_9N_3S \cdot 1/2H_2O$: C, 49.6; H, 5.9; N, 8.3. Found: C, 49.5; H, 5.5; N, 8.4.

Carbobenzoxy-L-seryl-L-tyrosyl-L-glutamic Acid.—A solution of L-glutamic acid (0.13 g.) in water (7 ml.) and triethylamine (0.23 ml.) was shaken at 5° for 40 hours with an ethereal solution of carbobenzoxy-L-seryl-L-tyrosine azide (prepared from 0.7 g. of the hydrazide). The ethereal phase was removed, the aqueous phase re-extracted with ether and Dowex 50 (acetate cycle) (2.5 g.) was added to the water layer. The mixture was shaken for 10 minutes, was warmed at 60° to dissolve the product which had precipitated, and the resin was removed by filtration. The filtrate was concentrated to a small volume *in vacuo*. The tripeptide derivative which separated in the form of a gelatinous mass was collected and dried *in vacuo* over phosphorus pentoxide; yield 0.18 g. (37%); $[\alpha]^{25}D$ −23.5° (*c* 1, in ethanol).

Anal. Calcd. for $C_{25}H_{29}O_{10}N_3(H_2O)$: C, 54.7; H, 5.7; N, 7.6. Found: C, 54.5; H, 6.0; N, 7.7.

L-Seryl-L-methionyl-L-glutamic Acid.—Carbobenzoxy-L-seryl-L-methionyl-L-glutamic acid (0.40 g.) was dissolved in liquid ammonia (approximately 50 ml.) and sodium (0.12 g.) was added in small pieces with stirring. Dowex 50 (ammonia cycle) (9 g.) was then added, the mixture was stirred for an additional 10 minutes and the ammonia was evaporated. The last traces of ammonia were removed by keeping the residue over sulfuric acid *in vacuo* for 20 hours. Water (30 ml.) was added, the resin was removed by filtration and was washed with additional 20 ml. of water. The combined filtrate and washings were acidified to *p*H 4 with glacial acetic acid and the solution was evaporated to dryness *in vacuo*. The ensuing material was evaporated with three 10-ml. portions of methanol, was suspended in ethanol (10 ml.) containing three drops of glacial acetic acid and the mixture was centrifuged. The supernatant was decanted, the solid residue was washed with three 5-ml. portions of ethanol and was recrystallized from aqueous ethanol. Fine needles were obtained; yield 0.28 g. (86%); $[\alpha]^{25}D$ −26.1° (*c* 1, in water), R_f = 0.48 (Partridge).

Anal. Calcd. for $C_{13}H_{23}O_7N_3S(C_2H_5OH)$: C, 43.8; H, 7.1; N, 10.2. Found: C, 44.5; H, 6.7; N, 10.8.

Carbobenzoxy-L-seryl-L-tyrosyl-L-seryl-L-methionine Methyl Ester.—L-Seryl-L-menthionine (2.6 g.) was suspended in methanol (80 ml.) and the solution saturated with hydrogen chloride with cooling. The solvent was removed *in vacuo* at a bath temperature of 40°, and the residue re-esterified as described. This process was repeated once more. The ester hydrochloride was obtained in the form of an oil; yield 3.2 g. The methyl ester hydrochloride (2.35 g.) was dissolved in methanol (30 ml.) and 0.9 *N* methanolic sodium methoxide (9.1 ml.) was added. The solution was evaporated to dryness and the residue dissolved in a mixture of dioxane (20 ml.) and ethyl acetate (20 ml.). To this solution was added an ethyl acetate solution of carbobenzoxy-L-seryl-L-tyrosine azide (prepared in the manner described above from 2.74 g. of the hydrazide). The mixture was shaken at 5° for 20 hours and was then evaporated to dryness *in vacuo*. The residue was dissolved in ethyl acetate, the solution washed with 1 *N* hydrochloric acid, sodium bicarbonate and water and dried over sodium sulfate. Evaporation of the solvent afforded a solid which was recrystallized from methanol; yield 2.45 g.; decomposes at 190°; $[\alpha]^{27}D$ −39.0° (*c* 0.7, in methanol).

Anal. Calcd. for $C_{39}H_{38}O_{10}N_4S$: C, 54.9; H, 6.0; N, 8.8. Found: C, 55.0; H, 6.3; N, 8.6.

Carbobenzoxy-L-seryl-L-tyrosyl-L-seryl-L-methioninhydrazide.—Carbobenzoxy-L-seryl-L-tyrosyl-L-seryl-L-metlhionine methyl ester (1.64 g.) was dissolved in boiling methanol (20 ml.) and hydrazine hydrate (0.25 ml.) was added. The mixture was kept at room temperature for 17 hours, the resulting gelatinous mass was collected, washed with methanol, and dried *in vacuo* at room temperature. The material was recrystallized from 95% ethanol twice; yield 1.1 g. (67%); decomposes on heating.

Anal. Calcd. for $C_{28}H_{38}O_9N_6S$: C, 53.0; H, 6.0. Found: C, 52.9; H, 5.4.

Carbobenzoxy-L-seryl-L-tyrosyl-L-methionyl-L-glutamic Acid.—A solution of L-methionyl-L-glutamic acid (0.17 g.) in water (8 ml.) and triethylamine (0.17 ml.) was shaken at 5° for 40 hours with an ethereal solution of carbobenzoxy-L-seryl-L-tyrosine azide (prepared as described above from 0.5 g. of the hydrazide). The ether phase was separated, Dowex 50 (acetate cycle) (2.5 g.) was added to the aqueous layer and the mixture was shaken for 10 minutes. The resin was removed by filtration and was washed with several portions of water. The combined filtrate and washings were evaporated to dryness *in vacuo* at a bath temperature of 40°, the residue was dissolved in methanol and water was added. The ensuing gelatinous material was collected and dried over phosphorus pentoxide *in vacuo;* yield 0.13 g. (32%); $[\alpha]^{25}D$ −22.7° (*c* 1, in glacial acetic acid).

Anal. Calcd. for $C_{30}H_{38}O_{11}N_4S$: C, 54.4; H, 5.8; N, 8.5. Found: C, 54.4; H, 6.0; N, 8.2.

L-Seryl-L-tyrosyl-L-methionyl-L-glutamic Acid.—A sample of the carbobenzoxy derivative (0.14 g.) was dissolved in liquid ammonia (20 ml.) and sodium 0.05 g.) was added in small pieces with stirring. Dowex 50 (ammonia cycle) (2 g.) was added and the ammonia was evaporated. The product was isolated as described for the preparation of L-seryl-L-methionyl-L-glutamic acid, and was purified by precipitation from water with ethanol; yield 60 mg. (50%); $[\alpha]^{25}D$ −8.6° (*c* 1, in water).

Anal. Calcd. for $C_{22}H_{32}O_9N_4S(2H_2O)$: C, 46.8; H, 6.4; N, 9.9. Found: C, 46.8; H, 7.0; N, 10.6.

Carbobenzoxy-L-seryl-L-tyrosyl-L-seryl-L-methionyl-L-glutamic Acid.—A solution of L-seryl-L-methionyl-L-glutamic acid (0.27 g.) in water (10 ml.) and triethylamine (0.21 ml.) was shaken at 5° for 48 hours with an ethereal solution of carbobenzoxy-L-seryl-L-tyrosine azide (prepared in the usual manner from 0.92 g. of the hydrazide). The ether layer was separated, the aqueous solution filtered and Dowex 50 (acetate cycle) (4 g.) added to the clear filtrate. The suspension was shaken for 10 minutes. The carbobenzoxypentapeptide precipitated during the treatment with the resin, and was redissolved by the addition of ethanol. The resin was removed by filtration, washed with water and the combined filtrate and washings were concentrated to a small volume *in vacuo* at a bath temperature of 40–50°. The material separated during this operation and precipitation was completed by placing the concentrate in a refrigerator for 12 hours. The product was collected, dissolved in ethanol, precipitated with water and dried over phosphorus pentoxide *in vacuo;* yield 0.26 g. (52%); $[\alpha]^{25}D$ −13.2° (*c* 1, in glacial acetic acid).

Anal. Calcd. for $C_{33}H_{43}O_{13}N_5S$: C, 52.9; H, 5.8; N, 9.3. Found: C, 53.7; H, 6.2; N, 9.1.

L-Seryl-L-tyrosyl-L-seryl-L-methionyl-L-glutamic Acid.—A sample of the carbobenzoxy derivative (0.26 g.) was dissolved in liquid ammonia (30 ml.) and sodium (0.1 g.) was added in small pieces with stirring. Dowex 50 (ammonia cycle) (8 g.) was added and the ammonia was evaporated. The residue was dried over sulfuric acid *in vacuo* and was then extracted with water (20 ml.). The mixture was filtered,

the resin washed with an additional 20 ml. of water and the combined filtrates acidified to *p*H 4 with glacial acetic acid and evaporated to dryness *in vacuo* at a bath temperature of 40–45°. The residue was evaporated with three 10-ml. portions of methanol and suspended in 10 ml. of ethanol containing a few drops of glacial acetic acid. The solids were collected by centrifugation and were re-extracted with three additional 5-ml. portions of ethanol. The residue was dried *in vacuo* over phosphorus pentoxide. The material was purified further by precipitation from water with ethanol. A white granular powder was obtained; yield 0.14 g. (63%); $[\alpha]^{26}D$ −20.6° (*c* 1.95, in 2 *N* HCl); R_f = 0.51 (Partridge). For analysis, the pentapeptide was dissolved in a small quantity of hot water, and separated in the form of small rosettes upon cooling of the solution. It was collected and dried at room temperature *in vacuo* over phosphorus pentoxide.

Anal. Calcd. for $C_{25}H_{37}O_{11}N_8S(H_2O)$: C, 47.4; H, 6.2; N, 11.1. Found: C, 47.0; H, 6.4; N, 11.6.

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[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH, SCHOOL OF MEDICINE]

Studies on Polypeptides. IX. Synthesis of Peptides Containing Basic Amino Acid Residues, Related to Corticotropin and Intermedin

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The sequences histidyl-phenylalanyl-arginine and glutamyl-histidyl-phenylalanyl-arginine, have been shown to occur in the corticotropins and in the melanocyte-stimulating hormone. Peptides corresponding to these sequences have been synthesized and their homogeneity ascertained by paper chromatography and by quantitative determination of the amino acids liberated on acid hydrolysis. Leucine aminopeptidase converted both peptides into an equimolar mixture of the expected amino acids, demonstrating their stereochemical purity. The behavior, on paper chromatography, of the synthetic histidyl-phenylalanyl-arginine was identical with that of a peptide resulting from the enzymatic degradation of corticotropin-A. This provides unequivocal synthetic confirmation for the sequence his-phe-arg in this hormone. The synthesis of a series of compounds related to the two peptides is also described.

In previous communications,[2,3] we have described a synthesis of seryl-tyrosyl-seryl-methionyl-glutamic acid, the N-terminus of swine β-corticotropin, swine corticotropin-A, and sheep β-corticotropin.[4–6] A structural unit, common not only to these corticotropins but also to the melanocyte-stimulating principle of porcine pituitary glands,[7,8] possesses the sequence glutamyl-histidyl-phenylalanyl-arginine. In the present communication, we record a synthesis of this tetrapeptide. Prior to attempting the preparation of the entire peptide, methods were explored for preparing various smaller peptides and peptide derivatives embodying within their structures certain bond types occurring in the over-all sequence. The dipeptide, L-histidyl-L-phenylalanine, was prepared readily by the azide method *via* carbobenzoxy-L-histidyl-L-phenylalanine. Two routes to this substance were explored. In the first, the azide of carbobenzoxy-L-histidine[9] was coupled with the methyl ester of L-phenylalanine and the ensuing carbobenzoxy-L-histidyl-L-phenylalanine methyl ester was subjected to saponification. In the second, the triethylammonium salt of L-phenylalanine was treated with carbobenzoxy-L-histidine azide in a manner recently described.[3] The carbobenzoxy-L-histidyl-L-phenylalanine resulting from both approaches exhibited identical properties, and on catalytic hydrogenation afforded the crystalline free dipeptide. Recrystallization from water gave a product which was homogeneous, as revealed by paper chromatography. Acid hydrolysis converted the dipeptide into an equimolar mixture of histidine and phenylalanine.

The observation that the azide of carbobenzoxy-L-histidine lends itself to coupling in aqueous systems provided a key to a synthesis of L-histidyl-L-phenylalanylnitro-L-arginine. Acid-catalyzed decarbobenzoxylation in glacial acetic acid containing hydrogen bromide converted carbobenzoxy-L-phenylalanylnitro-L-arginine[10] into the hydrobromide of L-phenylalanylnitro-L-arginine, which gave the free peptide when treated with the ion exchanger Amberlite IR-4B in the acetate form. This dipeptide, as its triethylammonium salt, was then coupled with carbobenzoxy-L-histidine azide[9] to give carbobenzoxy-L-histidyl-L-phenylalanylnitro-L-arginine. The carbobenzoxylated tripeptide was sparingly soluble in water, but dissolved readily in dilute ammonia and was precipitated therefrom in crystalline form by glacial acetic acid. This property of the compound provided a convenient method of purification. Hydrogenation in glacial acetic acid in the presence of a palladium catalyst converted the carbobenzoxy derivative into the acetate salt of L-histidyl-L-phenylalanyl-L-arginine. This tripeptide salt was purified by precipitation from its ethanolic solution with ether, and exhibited a single ninhydrin-positive spot on paper in the Partridge[11] and the 2-butanol–ammonia systems.[12] Control strips sprayed with diazotized

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