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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

The Synthesis of the Tetrapeptide Amide S-Benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide

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The synthesis of the tetrapeptide amide, S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, starting from ethyl carbobenzoxy-L-leucylglycinate is described. Ethyl L-leucylglycinate was condensed with carbobenzoxy-L-proline by the mixed anhydride procedure using isovaleryl chloride to give ethyl carbobenzoxy-L-prolyl-L-leucylglycinate. The latter was reduced catalytically and coupled with bis-carbobenzoxy-L-cystinyl bischloride. The resultant ethyl biscarbobenzoxy-L-cystinyl-bis-(L-prolyl-L-leucylglycinate) was hydrolyzed to the corresponding acid which on reduction with sodium in liquid ammonia followed by treatment with benzyl chloride was converted to S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine. The latter was esterified with benzyl alcohol in the presence of hydrogen chloride to the benzyl ester hydrochloride which on treatment with methanolic ammonia afforded S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. Carbobenzoxy-L-propyl-L-leucylglycine and carbobenzoxy-L-prolyl-L-leucylglycinamide were also prepared.

As a result of certain degradative experiments in this Laboratory on oxytocin, the principal milk-ejecting and uterine-contracting hormone of the posterior pituitary gland, the sequence of amino acids in oxytocin was obtained and a structure proposed.[2] In connection with the synthetic approach to this structure,[3] the tetrapeptide amide S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide was desired, and in this paper the synthesis of the latter is described.

The S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide was prepared according to the accompanying series of reactions.

Ethyl carbobenzoxy-L-leucylglycinate was synthesized by the procedure described by Vaughan and Osato.[4] After catalytic removal of the carbobenzoxy group according to the general procedure of Bergmann and Zervas[5] the ethyl L-leucylglycinate (I) was condensed with carbobenzoxy-L-proline also by means of the mixed anhydride technique using isovaleryl chloride. The resultant protected tripeptide, ethyl carbobenzoxy-L-prolyl-L-leucylglycinate (II), was obtained in crystalline form in 92% yield from ethyl carbobenzoxy-L-leucylglycinate.

Ethyl L-prolyl-L-leucylglycinate (IIa) was converted to the tetrapeptide, S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine (IV), *via* the bis-carbobenzoxy-L-cystinyl derivative. Bis-carbobenzoxy-L-cystinyl bischloride, prepared from bis-carbobenzoxy-L-cystine and phosphorus pentachloride,[5,6] condensed smoothly at −40° in ethyl acetate solution with the ethyl L-prolyl-L-leucylglycinate (IIa), which was obtained by catalytic removal of the carbobenzoxy group from II. The protected condensation product, ethyl bis-carbobenzoxy-L-cystinyl-bis-(L-prolyl-L-leucylglycinate) (III), was saponified in aqueous dioxane at +5° using one equivalent of sodium hydroxide to yield the corresponding acid IIIa. The latter was converted to S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine (IV) using the general procedure of Sifferd and du Vigneaud[7] for the removal of carbobenzoxy groups in cystine-containing compounds by the use of sodium in liquid ammonia followed by benzylation[8] of the sulfur of the reduced compound in the same medium. Compounds III, IIIa and IV were obtained in high yields as amorphous products. The crude tetrapeptide IV was readily converted

(1) Appreciation is expressed to the Lederle Laboratories Division, American Cyanamid Company, for a research grant which has aided greatly in this work.

(2) V. du Vigneaud, C. Ressler and S. Trippett, *J. Biol. Chem.*, **205**, 949 (1953).

(3) The preliminary report of the synthesis [V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, THIS JOURNAL, **75**, 4879 (1953)] included some of the data in this paper.

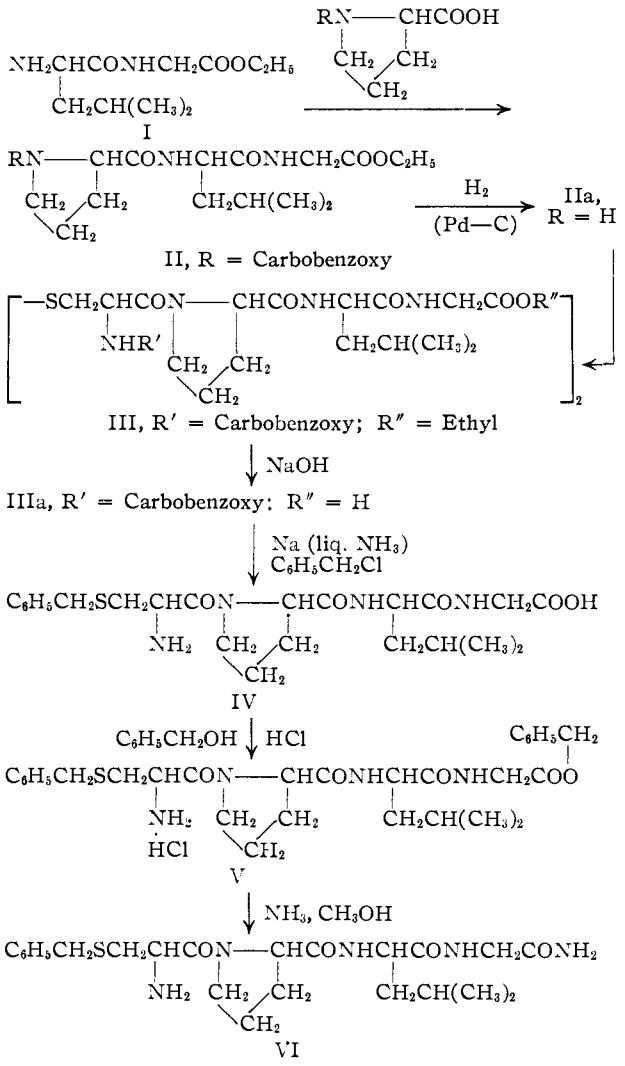
(4) J. R. Vaughan, Jr., and R. L. Osato, *ibid.*, **73**, 5553 (1951).

(5) M. Bergmann and L. Zervas, *Ber.*, **65B**, 1192 (1932).

(6) The biscarbobenzoxycystinyl bischloride was prepared as described by V. du Vigneaud and G. L. Miller in "Biochemical Preparations," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1952, p. 76.

(7) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

(8) V. du Vigneaud, L. F. Audrieth and H. S. Loring, THIS JOURNAL, **52**, 4500 (1930).



using benzyl alcohol and dry hydrogen chloride to the crystalline benzyl ester hydrochloride, benzyl S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinate hydrochloride (V), which was purified through recrystallization and obtained in an over-all yield of 62% from II.

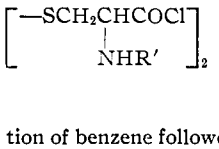
Amination was accomplished by allowing the free base obtained from V to stand in solution in methanolic ammonia for several days at room temperature. This is an extension to a peptide of the method described by Yang and Rising[9] for the preparation of some amino acid amides by direct amination in methanolic ammonia of the corresponding unprotected amino acid esters. S-Benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VI) was isolated in approximately 75% yield from V as a crystalline hydrate.

The preparation of carbobenzoxy-L-prolyl-L-leucylglycine and carbobenzoxy-L-prolyl-L-leucylglycinamide is also described.

Experimental[10]

Carbobenzoxy Amino Acids.—The carbobenzoxy derivatives used as starting materials were prepared in the usual way[5] with the exception that the reaction times were extended up to 1 to 2.5 hours in the larger runs. Carbobenzoxy-L-leucine[11] and carbobenzoxy-L-proline[12] were obtained in 94% yields as oils and bis-carbobenzoxy-L-cystine[5,6] in 85% yield.

Ethyl Carbobenzoxy-L-prolyl-L-leucylglycinate (II).—Ethyl carbobenzoxy-L-leucylglycinate, m.p. 102–103°, was prepared according to Vaughan and Osato[4] by the mixed anhydride procedure using isovaleryl chloride; yield 60–70%. Fifty-gram batches dissolved in 750 ml. of absolute ethanol containing 12.7 ml. of concentrated hydrochloric acid were reduced with hydrogen in the presence of 10 g. of 5% palladium–charcoal catalyst. After the evolution of carbon dioxide had ceased, the catalyst was filtered off and the solvent removed under reduced pressure. The sirupy residue of dipeptide ester hydrochloride was dried by the addition of benzene followed by evaporation under reduced pressure. The residue was then used directly for condensation with carbobenzoxy-L-proline.



A solution of 69.5 g. of carbobenzoxy-L-proline and 28.2 g. of triethylamine in a mixture of 335 ml. of dry toluene and 335 ml. of dry chloroform was cooled to −5° and 33.8 g. of isovaleryl chloride added. After 1.5 hours a cooled solution of ethyl L-leucylglycinate hydrochloride (from 100 g. of ethyl carbobenzoxy-L-leucylglycinate) and 28.2 g. of triethylamine in 700 ml. of chloroform was added and the reaction mixture stored overnight at +5°. The solution was then washed with water and 3% aqueous sodium bicarbonate and concentrated under reduced pressure to a volume of approximately 500 ml. When the solution was diluted with hexane, the white crystalline product separated; wt. 120 g., m.p. 145–146°. One recrystallization from aqueous ethanol gave 115.5 g. (92%) of the carbobenzoxy tripeptide ester, m.p. 148–149°; $[\alpha]^{22.5}D$ −79.8° (*c* 2.5, ethanol).

Anal. Calcd. for $C_{23}H_{33}O_6N_3$: C, 61.7; H, 7.43; N, 9.39. Found: C, 61.8; H, 7.65; N, 9.24.

Carbobenzoxy-L-prolyl-L-leucylglycine.—Two hundred milligrams of II was suspended in 0.5 ml. of acetone and 0.2 ml. of water and 1 equivalent of 1 *N* sodium hydroxide (0.45 ml.) was added. After 25 minutes at room temperature the solution was acidified and a white crystalline solid separated. The product was removed by filtration and after recrystallization from aqueous ethanol melted at 163.5–164°; $[\alpha]^{23.5}D$ −85.2° (*c* 2, 95% ethanol).

Anal. Calcd. for $C_{21}H_{29}O_6N_3$: C, 60.1; H, 6.97; N, 10.0. Found: C, 60.4; H, 7.06; N, 10.2.

Carbobenzoxy-L-prolyl-L-leucylglycinamide.—To 50 ml. of absolute ethanol that had been saturated with dry ammonia at 0° was added 2.0 g. of II and the mixture was allowed to stand at room temperature for 4 days. The solvent was then removed under reduced pressure whereupon the residual sirup crystallized; wt. 1.62 g. (87%), m.p. 159–161°. After recrystallization from either water or nitromethane the compound was obtained as needles melting at 163–163.5°; $[\alpha]^{18.5}D$ −73.3° (*c* 2, 95% ethanol).

Anal. Calcd. for $C_{31}H_{39}O_8N_4$: C, 60.3; H, 7.23; N, 13.4. Found: C, 59.9; H, 7.34; N, 13.4.

Ethyl Bis-carbobenzoxy-L-cystinyl-bis-(L-prolyl-L-leucylglycinate) (III).—Seventeen grams of II was dissolved in 200 ml. of absolute ethanol containing 3.34 ml. of concentrated hydrochloric acid and reduced with hydrogen in the presence of 2.6 g. of 5% palladium–charcoal catalyst. After removal of the catalyst and solvent the reduction product was precipitated as the hydrochloride from 100 ml. of chloroform by the addition of anhydrous ether. The white solid was washed by decantation with ether several times and dissolved in 200 ml. of ethyl acetate. To this solution 6.37 ml. of triethylamine was added in portions and the precipitated triethylamine hydrochloride was removed by filtration. Removal of the ethyl acetate left the ethyl L-prolyl-L-leucylglycinate (IIa) as a viscous sirup which crystallized at ice-box temperature. It was divided into three portions and the coupling with bis-carbobenzoxy-L-cystinyl bischloride was carried out on this scale.

(9) P. S. Yang and M. S. Rising, THIS JOURNAL, **53**, 3183 (1931).

(10) Capillary melting points were determined and are corrected.

(11) M. Bergmann, L. Zervas and J. S. Fruton, *J. Biol. Chem.*, **115**, 593 (1936).

(12) E. Abderhalden and H. Nienburg, *Fermentforschung*, **13**, 573 (1933).

A slurry in 30 ml. of dry ethyl acetate of bis-carbobenzoxy-L-cystinyl bischloride[5,6] (freshly prepared from 4.02 g. of bis-carbobenzoxy-L-cystine) was added to each of two portions of the tripeptide ester IIa in an equal volume of ethyl acetate containing 2.10 ml. of triethylamine under

allowed to stand at +5° for 2–4 hours. The two runs were combined at this point and the precipitated triethylamine hydrochloride was removed by filtration. The ethyl acetate filtrate was washed with 1 *N* hydrochloric acid, 3% aqueous sodium bicarbonate and water and dried over anhydrous magnesium sulfate in the presence of Darco. After filtration the solution was concentrated under reduced pressure in the presence of nitrogen to a volume of approximately 30 ml. and the product was precipitated by the addition of 200 ml. of ether and separated by decantation. Stirring with a fresh portion of cold ether yielded a white amorphous product; wt. 11.9 g. (86% yield from II).

Anal. Calcd. for $C_{52}H_{74}O_{14}N_8S_2$: N, 10.2; S, 5.83. Found: N, 9.82; S, 6.02.

Bis-carbobenzoxy-L-cystinyl-bis-(L-prolyl-L-leucylglycine) (IIIa).—For conversion to the corresponding acid, five 5-g. portions of the carbobenzoxy tetrapeptide ester III were each dissolved in 25 ml. of purified dioxane. Twenty ml. of water was added and the mixture kept in an ice-water bath for 1.5 hours during which time an equivalent amount (9.2 ml.) of 1 *N* sodium hydroxide was added in portions and the mixture shaken frequently. The five runs were combined at this point and acidified with 6 *N* hydrochloric acid. The precipitated viscous gum was separated by decantation of the solution and converted by trituration with 300 ml. of ice-water to a solid which was filtered off and dried *in vacuo*. The crude product was dissolved in approximately 150 ml. of warm ethyl acetate containing several drops of water and precipitated as a white granular solid by the addition of approximately 500 ml. of cold ether; wt. 23.5 g. (99%).

Anal. Calcd. for $C_{48}H_{66}O_{14}N_8S_2$: N, 10.7; S, 6.15. Found: N, 10.2; S, 6.41.

Benzyl S-Benzyl-L-cysteinyl-L-prolyl-L-leucylglycinate Hydrochloride (V).—To a solution of 12.5 g. of IIIa in 800 ml. of liquid ammonia was added approximately 1.9 g. of sodium in portions until a permanent blue color appeared throughout the solution; 3.52 g. of benzyl chloride was then added to the mixture at −30°, when a white precipitate appeared. After 15 minutes an additional 0.5 ml. of benzyl chloride was added. After an additional 30 minutes 3.82 g. of ammonium chloride was added and the resulting clear solution allowed to evaporate; the last traces of ammonia were removed under reduced pressure. The residual white solid was dissolved in 10 ml. of cold water and the solution was extracted with ether and then brought to *p*H 6 by the addition of 6 *N* hydrochloric acid. The resultant viscous oil was extracted several times into a total of 50 ml. of *s*-butyl alcohol previously saturated with water. The extract was concentrated at a pressure of 5 mm. at 35° to a viscous sirup which was converted to a white hygroscopic solid by the addition of 10 ml. of chloroform followed by 200 ml. of cold ether. The S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine (IV) was separated by decantation and dried *in vacuo;* wt. 11.7 g. This product could be further purified by countercurrent distribution in the system 0.05% acetic acid–*s*-butyl alcohol, in which the partition coefficient was 1.07. However, the crude reaction product was converted directly in good yield to the corresponding benzyl ester hydrochloride.

A suspension of 15 g. of IV in 200 ml. of benzyl alcohol was cooled in an ice-water bath and a current of dry hydrogen chloride was introduced over a period of 45 minutes. The mixture was then heated under reduced pressure (15 mm.) in a bath at 80° for 30 minutes. The introduction of hydrogen chloride was then repeated, and after removal of excess hydrogen chloride under reduced pressure the pressure of 2 mm., using a hot water-bath. Addition of 1 l. of hexane to the chilled residue caused the deposition of an oil which crystallized after standing for several hours in the refrigerator. The product was filtered off and recrystallized in approximately 5-g. batches by dissolving the finely divided solid rapidly in 100 ml. of hot, absolute ethanol and adding warm hexane to incipient cloudiness. The benzyl S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinate hydrochloride crystallized as white needles; wt. 13.8 g. (72%), m.p. 193–194° (with slight discoloration); $[\alpha]^{22.5}D$ −73.5° (*c* 1, absolute ethanol). The melting point depended on the rate of heating.

Anal. Calcd. for $C_{30}H_{41}O_5N_4SCl$: C, 59.5; H, 6.83; N, 9.26; S, 5.30; ionic Cl, 5.86. Found: C, 59.4; H, 7.03; N, 9.17; S, 5.36; ionic Cl, 6.01.

S-Benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VI).—Two flasks protected from moisture by drying tubes and each containing a finely divided suspension of 6.25 g. of V in 40 ml. of dry ethyl acetate containing 7 ml. of triethylamine were shaken frequently at +10° for 20 minutes. The precipitated triethylamine hydrochloride was then filtered off and washed with ethyl acetate. The filtrates and washings were combined at this point. Evaporation of the solvent *in vacuo* left a viscous residue which was dissolved in 200 ml. of methanol that had been saturated at 0° with ammonia. The solution was allowed to stand for 2–3 days at room temperature, after which time the solvent was removed under reduced pressure. The residue was repeatedly dissolved in fresh methanol followed by evaporation and this procedure was repeated using benzene. The viscous residue was then dissolved in 10 ml. of acetone. Addition of 200 ml. of dry ether caused the deposition of a semi-solid which was separated by decantation and dissolved in 10 ml. of water. The aqueous solution was extracted with ether, the residual ether removed and the solution cooled. After several hours the white crystalline precipitate which separated was filtered off and washed with ice-water. The product, m.p. 69–71°, was dried *in vacuo* over phosphorus pentoxide; wt. 6.33 g. Concentration of the combined mother liquor and washing yielded a second crop, m.p. 65.5–68.5°; wt. 1.15 g. (after drying). In several experiments the yields ranged from 74 to 82%.

For analysis the compound was recrystallized from water from which it separated as clusters of needles. The air-dried sample, m.p. 69.5–71.5° and $[\alpha]^{22.6}D$ −47.7° (*c* 1, absolute ethanol), analyzed for a hydrate containing 1.5 moles of water.

Anal. Calcd. for $C_{23}H_{35}O_4N_5S\cdot 1^1/_2H_2O$: C, 54.7; H, 7.59; N, 13.9; S, 6.35; amide N, 2.78; H_2O, 5.36. Found: C, 54.9, 54.9; H, 7.41, 7.14; N, 13.9; S, 6.37; amide N, 2.89; H_2O, 5.30, 5.13.

When a small sample was dried over phosphorus pentoxide for 2 hours at room temperature and 0.1 mm., analysis indicated loss of the water of hydration.

Anal. Calcd. for $C_{23}H_{35}O_4N_5S$: C, 57.8; H, 7.39; N, 14.7. Found: C, 58.0; H, 7.48; N, 14.5.

The authors wish to thank Mr. Joseph Albert for carrying out the microanalyses reported herein.

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