ture fluid. After a final hour at room temperature the thick mass was diluted to 150–200 ml. with water and acidified to congo red with concentrated HCl. The gelatinous suspension was stirred for one-half hour to ensure complete reaction with the acid and the product was then filtered off, washed thoroughly with water and dried. When dry, it was triturated with ether and then purified in a manner identical to that already described for purification of the tosyl tripeptide. The yield of purified, crystalline product, m.p. 223–225°, was 65–70%; $[\alpha]^{21}D$ −26° (*c* 0.1, 0.5 *N* $KHCO_3$).

Anal. Calcd. for $C_{26}H_{31}O_8N_5$: C, 57.7; H, 5.77; N, 12.9. Found: C, 57.6; H, 5.95; N, 12.6.

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The Synthesis of L-Cysteinyl-L-tyrosyl-L-isoleucine

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In a previous report[3] the synthesis of L-cysteinyl-L-tyrosine and L-tyrosyl-L-cysteine, the corresponding cysteic acid analogs and the derived dibromotyrosine compounds were described. Subsequent to this report evidence was submitted that the sequence of amino acids in oxytocin, the principal milk-ejecting and uterine-contracting hormone of the posterior pituitary, was cysteine-tyrosine-isoleucine-etc.[4,5] As already communicated,[6] the key intermediate for the synthesis of oxytocin was N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl-S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide. In the preparation of the latter the dipeptide N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine was condensed with the heptapeptide, L-isoleucyl - L - glutaminyl - L - asparaginyl - S-benzyl - L - cysteinyl - L - prolylleucylglycinamide. At an early stage in the problem, it appeared reasonable to build the desired nonapeptide from the carbobenzoxybenzyl-cysteinyl end. The synthesis of the simple tripeptide was pursued in order to have available an alternate intermediate for the total synthesis as well as to have a model compound with which results of the original degradative studies on natural oxytocin might be explained.

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Experimental[7]

L-Isoleucine Ethyl Ether.—The epimeric mixture of L-isoleucine and D-alloisoleucine was converted to the N-isobutyryl derivative, m.p. 175.5–176°, and resolved by way of the anilide in a manner essentially identical with Doherty and Popenoe.[8] From 201 g. of the N-isobutyryl derivative there was obtained 108.5 g. of N-isobutyryl-L-isoleucine anilide, m.p. 223–223.5°, $[\alpha]^{20}D$ −71° (*c* 3 in glacial acetic acid). Hydrolysis with 6 *N* hydrochloric acid gave 38 g. (85%) of L-isoleucine from 92.5 g. of the anilide. The isolated L-isoleucine has a $[\alpha]^{22}D$ 40.4° (*c* 5 in 6 *N* HCl).

By the procedure described by Smith[9] for the preparation of L-isoleucine methyl ester hydrochloride, 20 g. (0.152 mole) of isoleucine was converted to the isoleucine ethyl ester hydrochloride; this compound could not be satisfactorily crystallized. Instead, the oily residue from the evaporation of the reaction mixture was taken up in water, treated with an excess of an aqueous solution of potassium carbonate, and the ethyl ester extracted with two 100-ml. portions of ethyl ether. The washed and dried solution of the ethyl ester was filtered and treated with an ether solution of an excess of *p*-toluenesulfonic acid monohydrate. The solid product which was obtained amounted to 48 g. (96%), m.p. 161–162°. Recrystallization of a portion of this from ethyl acetate gave a solid, m.p. 161–162°, $[\alpha]^{21}D$ 10.67° (*c* 3 in distilled water). Calcd. for $C_{15}H_{25}O_5NS$: N, 4.23; S, 9.66. Found: N, 4.14; S, 9.55.

A separate preparation of isoleucine ethyl ester was distilled to yield the pure free ethyl ester, b.p. 75–76° (10 mm.), $n^{20}D$ 1.4328, d^{20} 0.9316. Calcd. for $C_8H_{17}O_2N$: N, 8.80; *MR*, 44.36. Found: N, 8.53; *MR*, 44.40.

L-Isoleucine Benzyl Ester.—Seven and one-half grams (0.05 mole) of L-isoleucine was converted to 15.6 g. (78%) of the isoleucine benzyl ester toluenesulfonic acid salt in essentially the manner described by Miller and Waelsch.[10] Recrystallization from ethyl acetate–ether mixture gave the compound, m.p. 143–145°, $[\alpha]^{21}D$ 10.83 (*c* 3 in dioxane). Calcd. for $C_{20}H_{27}O_5NS$: N, 3.56; S, 8.15. Found: N, 3.46; S, 8.04.

A portion of the salt was treated as in the case of the ethyl ester and the pure undistilled isoleucine benzyl ester was dried under vacuum. The quantitative yield of the free benzyl ester was found to have the following constants: $n^{20}D$ 1.5028, d^{20} 1.0253. Calcd. for $C_{13}H_{19}O_2N$: N, 6.34; *MR*, 62.85. Found: N, 6.07; *MR*, 63.77.

Both the free isoleucine benzyl and ethyl esters appeared to be unstable when stored over any length of time at room temperature, there being formed an ether-insoluble precipitate even after a week. It was therefore found to be advantageous to store each of these esters as their *p*-toluenesulfonic acid salts. The free esters were liberated from the salts just prior to their use in coupling reactions by dissolving in water, treating with a slight excess of potassium carbonate and extracting the resulting mixture with ethyl ether or ethyl acetate.

S-Benzyl-N-carbobenzoxycysteinyltyrosine Hydrazide.—The oily residue from a preparation of S-benzyl-N-carbobenzoxycysteinyltyrosine ethyl ester[3,11] amounting to 20 g. (0.04 mole) was let stand for 48 hr. in 300 ml. of absolute ethanol containing 8 g. of hydrazine hydrate. The resulting solid was removed by filtration, washed with three 25-ml. portions of hot ethanol, and recrystallized from boiling ethanol. There was obtained a total of 15.3 g. (77%) pure hydrazide, m.p. 207.5–209°. Calcd. for $C_{27}H_{30}O_5N_4S$: N, 10.72; S, 6.13. Found: N, 10.46; S, 6.06.

S-Benzyl-N-carbobenzoxycysteinyltyrosyl Azide.—In a typical run, 15.66 g. (0.03 mole) of the pure hydrazide was dissolved in a solution of 24 ml. of 3 *N* hydrochloric acid in 150 ml. of glacial acetic acid; the solution was chilled to 0° and a solution of 2.2 g. of sodium nitrate in 20 ml. of distilled water was added all at once; after remaining for ten minutes at 0°, the mixture was diluted with 500 ml. of water. The mixture was then extracted with five 150-ml. portions of chloroform; the chloroform extracts were combined, washed with water and then with a solution of sodium bicarbonate to a neutral reaction; at this point it was necessary to add 100 ml. of ethyl acetate to keep the azide in solution. The dried solution of the azide was then used as soon as possible in the subsequent preparations.

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(3) C. W. Roberts and V. du Vigneaud, *J. Biol. Chem.*, **204**, 871 (1953).

(4) C. Ressler, S. Trippett and V. du Vigneaud, *ibid.*, **304**, 861 (1953).

(5) V. du Vigneaud, C. Ressler and S. Trippett, *ibid.*, **205**, 949 (1953).

(6) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. C. Katsoyannis and S. Gordon, THIS JOURNAL, **75**, 4879 (1953); **76**, 3115 (1954).

(7) All the melting points reported herein are corrected, capillary melting points. Appreciation is expressed to Mr. Joseph Albert for the microanalytical results.

(8) D. G. Doherty and E. A. Popenoe, Jr., *J. Biol. Chem.*, **189**, 447 (1951).

(9) E. L. Smith, D. H. Spackman and W. J. Polglase, *ibid.*, **199**, 801 (1952).

(10) H. K. Miller and H. Waelsch, THIS JOURNAL, **74**, 1092 (1952).

(11) C. R. Harrington and R. V. Pitt Rivers, *Biochem. J.*, **38**, 417 (1944).

S-Benzyl-N-carbobenzoxycysteinyltyrosyl Anilide.—From an aliquot of the solution theoretically containing 0.002 mole of azide and 0.188 g. (0.002 mole) of redistilled aniline, there was obtained, after standing overnight at room temperature, a crude solid product. This was recrystallized from ethyl acetate to give 0.7 g. (60%) of a crystalline solid, m.p. 186.5–188°. Calcd. for $C_{32}H_{33}O_5N_3S$: N, 7.20; S, 5.49. Found: N, 7.42; S, 5.55.

S-Benzyl-N-carbobenzoxycysteinyltyrosylisoleucine Ethyl Ester.—An ethyl acetate solution of S-benzyl-N-carbobenzoxycysteinyltyrosyl azide from 14.5 g. (0.028 mole) of the hydrazide was mixed with an ethyl ether solution of isoleucine ethyl ester from 9.93 g. (0.03 mole) of isoleucine ethyl ester toluenesulfonic acid salt. After standing for 48 hr. the reaction mixture was evaporated to dryness and triturated with ether and filtered; there was obtained 16.7 g. (93%) of crude condensation product. The crude product was extracted with three 100-ml. portions of ethyl ether and refiltered. The yield of almost white ether-insoluble crystalline solid was 13 g., m.p. 142–143°; a portion on recrystallization from aqueous ethanol had m.p. 142–143°. Calcd. for $C_{35}H_{43}O_7N_3S$: C, 64.69; H, 6.67; N, 6.48; S, 4.93. Found: C, 64.23; H, 6.69; N, 6.47; S, 4.97.

S-Benzyl-N-carbobenzoxycysteinyltyrosylisoleucine by Hydrolysis of the Ethyl Ester.—Repeated attempts to prepare the free acid from the ethyl ester by hydrolysis under the following conditions were unsatisfactory; hydrolysis in aqueous dioxane with 1 to 5 *N* sodium hydroxide, hydrolysis in aqueous acetone or ethanol using similar alkali concentrations and varying lengths of time and temperatures from 0 to 25°. From the various attempts there was obtained a small amount of material which on recrystallization from aqueous ethanol gave a product, m.p. 161–163°; 300 mg. obtained from 5 g. of ethyl ester. Calcd. for $C_{33}H_{39}O_7N_3S$: N, 6.76; S, 5.16. Found: N, 6.78; S, 5.23.

Attempted Preparations of S-Benzyl-N-carbobenzoxycysteinyltyrosine Benzyl Ester.—An aliquot of the azide solution containing 0.002 mole was treated with 0.004 mole of redistilled benzyl alcohol; the mixture was let stand for 12 days at room temperature. At the end of this time the reaction mixture was evaporated to dryness and the residue extracted with five 50-ml. portions of boiling ether. The insoluble residue was dried and amounted to 2.5 g.; on recrystallization from acetic acid and then from boiling ethanol there was obtained 2.0 g. of solid, m.p. 186–187°. On further recrystallization the m.p. remained constant at 188–189° and showed no depression with an authentic sample of S-benzyl-N-carbobenzoxycysteinyltyrosine.[3]

In a subsequent run the identical quantities were used and the reaction mixture was maintained at 30° for 60 hr. The resulting solid was filtered and recrystallized from aqueous acetic acid; in this instance 0.40 g. of compound, m.p. 212–213°, was obtained. On analysis it appeared that S-benzyl-N-carbobenzoxycysteinyltyrosyl amide had been formed. Calcd. for $C_{27}H_{29}O_5N_3S$: N, 8.28; S, 6.32. Found: N, 8.31; S, 6.27. In several other instances of the preparation of the dipeptide amide and subsequent coupling this same compound was encountered.

S-Benzyl-N-carbobenzoxycysteinyltyrosylisoleucine Benzyl Ester.—Two preparations were made under slightly different conditions. The first utilized a stock solution of the dipeptide amide and treatment of this with a mixture of equivalent amounts of isoleucine benzyl ester, toluenesulfonic acid salt, and triethylamine in ethyl acetate. From 0.0025 mole of the azide there was obtained after 16 hr. at room temperature a mixture of the desired tripeptide benzyl ester and the free acid derived therefrom. The crude mixture, nearly a quantitative coupling, gave a fraction, m.p. 150–151°, which on recrystallization gave a product with a m.p. 161–163° in about 30% yield. The second fraction obtained by leaching the first fraction with ether and evaporating the solvent gave a product which on recrystallization from ether–hexane had a m.p. 120–122°.

The second preparation involved coupling the azide in a chloroform–ethyl acetate mixture from 15.66 g. (0.03 mole) of the dipeptide hydrazide with the isoleucine benzyl ester obtained in ether solution from 12 g. of the toluenesulfonic acid salt by liberation using potassium carbonate solution. The mixed solutions were evaporated under reduced pressure; the residual chloroform solution was heated for 2 hr. at 45–50° on a water-bath. After standing an additional 20 hr. at 30° the resulting mixture was freed of chloroform by vacuum evaporation. The residual solid was dissolved in a total of 100 ml. of anhydrous acetone and filtered; the filtrate was treated with a total of 800 ml. of hexane and let stand for 24 hr. at 0°. The resulting crystalline solid was removed by filtration and dried. There was obtained 16.5 g., m.p. 132–134°; after two recrystallizations from acetone–hexane a product, m.p. 139.5–141°, was obtained; analysis of this material indicated that a mixture of the benzyl ester and free acid had been obtained. The free acid appeared to have little solubility in potassium bicarbonate solution. The crude mixture was used directly in the preparation of the peptide, cysteinyltyrosylisoleucine.

Cysteinyltyrosylisoleucine.—Three and one-half grams (0.005 mole based on pure benzyl ester) of the S-benzyl-N-carbobenzoxycysteinyltyrosylisoleucine benzyl ester and acid mixture was dissolved in 50 ml. of anhydrous liquid ammonia; to the mixture there was added, with vigorous stirring, sufficient metallic sodium to give a permanent blue color. The excess sodium was destroyed by chilling the reaction mixture to −70° and adding an excess of glacial acetic acid. Cautious evaporation of the liquid ammonia and subsequent complete removal under vacuum led to a solid residue; this was dissolved in a minimum of water and filtered rapidly. The filtrate was made acid with sulfuric acid and treated with an excess of Hopkin's reagent. The mercury salt was washed free of salts and acid and then suspended in 50 ml. of distilled water. Complete saturation of the mixture with hydrogen sulfide and removal of the mercuric sulfide by centrifugation and repetition of the procedure gave a total of 250 ml. of aqueous filtrate containing the tripeptide. Evaporation of the solution to 10 ml. and adjustment to *p*H 5.5 gave a slowly formed crystalline solid. This was removed by filtration and washed with 5-ml. portions of ice-water. The resultant product amounted to 900 mg. (50%), m.p. 172–175°, $[\alpha]^{21}D$ 8.5° (*c* 2 in 5% acetic acid). Calcd. for $C_{18}H_{27}O_5NS$: N, 10.57; S, 8.07. Found: N, 10.78; S, 8.21. Hydrolysis of this solid in 6 *N* HCl and ascending paper chromatograms in 75% phenol, using ninhydrin reagent to develop the spots, indicated the presence of cystine, tyrosine and isoleucine.

S-Benzylcysteinyltyrosylisoleucine.—Three and one-half grams (0.005 mole) of the mixed S-benzyl-N-carbobenzoxycysteinyltyrosylisoleucine acid and benzyl ester was dissolved in 100 ml. of anhydrous liquid ammonia; to the mixture was added a slight excess of metallic sodium to a permanent blue color at the boiling point of the liquid ammonia with vigorous stirring. The excess sodium was destroyed by ammonium chloride and 0.63 g. (0.005 mole) of benzyl chloride added during ten minutes.[12] The reaction mixture was let stand at reflux temperature for 1 hr.; after removal of all the liquid ammonia and after an hour under vacuum the residue was taken up in 25 ml. of water, filtered quickly and brought to a *p*H 6 with glacial acetic acid. The crystalline product which separated was removed by filtration and washed with ice-water. The compound was soluble in alkali, strong acid, hot water, ethanol and acetone. The isolated solid was recrystallized from aqueous ethanol to give 2.2 g., m.p. 175.5–177°. Calcd. for $C_{25}H_{33}O_5N_3S$: C, 61.58; H, 6.82; N, 8.28; S, 6.58. Found: C, 61.73; H, 6.92; N, 8.28; S, 6.52.

S-Benzyl-N-carbobenzoxycysteinyltyrosylisoleucine.—The S-benzylcysteinyltyrosylisoleucine prepared above, 1.45 g. (0.003 mole), was treated with 0.48 g. of carbobenzoxy chloride in a solution of 0.636 g. (0.006 mole) of sodium carbonate.[9] The mixture was shaken at 25° for one hour when a semi-solid oil separated. An excess of 3 *N* hydrochloric acid was added and the mixture shaken for one-half hour to ensure neutralization. The water was decanted and the solid triturated in water and then in hexane and filtered. A yield of 1.65 g. (88%) was obtained, m.p. 156–161°. A portion recrystallized from ethanol gave a solid, m.p. 161–163°, identical with the compound prepared by hydrolysis of the ethyl ester. Hydrolysis of a portion of the analytical sample in 6 *N* hydrochloric acid and ascending paper chromatograms in 75% phenol, using ninhydrin reagent to develop the spots showed the presence of some cystine, tyrosine and the overlapping spots of S-benzylcysteine and isoleucine.

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(12) V. du Vigneaud, L. F. Audrieth and H. S. Loring, THIS JOURNAL, **72**, 4500 (1950).