many of the usual recrystallization procedures, would be lost leaving the more stable monoacetylamino product.

(3) There was no evidence of acetylation of hydroxyl groups, when present, under the conditions of the current study. The hydroxyl-bearing pyrimidines were all alkali soluble after acetylation, and were precipitated without any apparent change upon the addition of acid.

Experimental

Standard Acetylation Conditions.—A mixture of 0.02 mole of the aminopyrimidine and 25 cc. of acetic anhydride was refluxed for two hours in a metal-bath at 160°. After cooling, the insoluble product was collected by filtration. This crude material was suspended in 50 cc. of water and the mixture was brought to *p*H 8–9 with dilute ammonium hydroxide solution. The insoluble product was again collected by filtration, was washed with much cold water and was purified by recrystallization as described in Table I for the individual compounds. Yields of acetylamino compounds were all close to 100%.

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Synthesis of L-Phenylalanyl-L-glutaminyl-L-asparagine[1]

BY EDWIN A. POPENOE AND VINCENT DU VIGNEAUD

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The structure CyS.Tyr.Phe.Glu(NH_2).Asp(NH_2).CyS.Pro.Lys.Gly(NH_2) has recently been proposed[2,3] for lysine–vasopressin, the pressor-antidiuretic hormone which has been found to occur in extracts of hog posterior pituitary glands.[4] As an intermediate in a projected synthesis of an octapeptide amide with this structure, carbobenzoxy-L-phenylalanyl-L-glutaminyl-L-asparagine was desired. The preparation of L-glutaminyl-L-asparagine[5] and tosyl-L-isoleucyl-L-glutaminyl-L-asparagine[6] used in the synthesis of oxytocin[7] have recently been reported. The tosyl tripeptide was prepared by treatment of the magnesium salt of L-glutaminyl-L-asparagine in aqueous solution with tosyl-L-isoleucyl chloride in the presence of excess magnesium oxide. The present paper reports an analogous preparation of tosyl-L-phenylalanyl-L-glutaminyl-L-asparagine and the conversion of this compound to the desired carbobenzoxy derivative. The preparation of the free tripeptide is also described.

Experimental[8]

Tosyl-L-phenylalanyl Chloride.—To 5.4 g. of tosyl-L-phenylalanine[9] suspended in 75 ml. of anhydrous ether at 0° there was added 3.90 g. of phosphorus pentachloride. The mixture was shaken for 10 minutes at 0°, then for 10 minutes at room temperature and finally stored at 0° for 1 hour. The crystalline product was filtered off, washed on the funnel quickly with a little ether and then with ice-water and dried for 2 hours in a vacuum desiccator under the vacuum provided by a good oil pump. The yield was 5.07 g. (88%) of a product which melted with decomposition at 128–129°.

Anal. Calcd. for $C_{16}H_{16}O_3NSCl$: Cl, 10.5. Found: Cl, 10.6.

Tosyl-L-phenylalanyl chloride is considerably more stable than carbobenzoxy-L-phenylalanyl chloride. A sample stored over phosphorus pentoxide at room temperature for 5 months showed no change in melting point. This stability is advantageous for reactions of the type used here.

Tosyl-L-phenylalanyl-L-glutaminyl-L-asparagine.—A mixture of 3.44 g. of L-glutaminyl-L-asparagine,[5] 0.82 g. of magnesium oxide and 6 ml. of water was shaken on a machine for 20 minutes. The mixture was then chilled in an ice-bath and 4.46 g. of tosyl-L-phenylalanyl chloride was added in small portions during 1 hour. Stirring was best accomplished by hand. As the reaction proceeded the mixture became so thick that it was necessary to add 5 ml. of water after about half of the acid chloride had been added. After all of the acid chloride had been added, 15 ml. of water was added and the mixture was allowed to come to room temperature during the next half-hour. The gelatinous product which was obtained by acidification of the mixture to congo red with concentrated HCl, was filtered off after 1 hour, washed thoroughly with water and dried. To remove any tosyl-L-phenylalanine the solid was ground in a mortar with 15 ml. of ethyl acetate, filtered and dried; wt. 5.78 g. (78%).

For purification the product was stirred with an excess of $NaHCO_3$ in 100 ml. of water and treated with Darco to remove some turbidity. Sufficient water was then added to make 200–300 ml. per g. of substance. The product which separated slowly after acidification was amorphous, but was usually crystalline after the second or third similar reprecipitation. The yield of purified product, m.p. 193–195°, was usually about 60%; $[\alpha]^{21}D$ −26.0° (*c* 1.95, 0.5 *N*, $KHCO_3$).

Anal. Calcd. for $C_{25}H_{31}O_8N_5S$: C, 53.5; H, 5.56; N, 12.5. Found: C, 53.3; H, 5.68; N, 12.4.

L-Phenylalanyl-L-glutaminyl-L-asparagine.—The tosyl group was removed from the tosyl tripeptide with sodium and liquid ammonia according to the method of du Vigneaud and Behrens.[10] Four grams of the tosyl tripeptide was dissolved in 300 ml. of liquid ammonia and reduced at the boiling point by the addition of metallic sodium until a persistent blue color was produced. Approximately 1.4 g. of sodium was required. Three grams of ammonium chloride was added and then the ammonia was allowed to evaporate. The last traces of ammonia were removed on the water pump. The solid residue was dissolved in 50 ml. of water at 0°, the solution treated quickly with Darco, filtered, washed with ether and the *p*H adjusted to 6 by the cautious addition of concentrated HCl. The crystalline product which separated was filtered off after a few hours at 0°, washed with cold water and dried; wt. 2.15 g. (75%). This product was pure enough for use in the next step. For purification it was dissolved in a slight excess of 0.15 *N* HCl, filtered if necessary and reprecipitated by the addition of dilute NH_4OH to *p*H 6–7. The compound melted with decomposition between 226 and 230° when inserted at 215° into a bath, the temperature of which was rising 2° per minute; $[\alpha]^{21}D$ 0.1° (*c* 4.9, 0.15 *N* HCl).

Anal. Calcd. for $C_{18}H_{25}O_6N_5$: C, 53.1; H, 6.19; N, 17.2. Found: C, 53.0; H, 6.35; N, 17.1.

Carbobenzoxy-L-phenylalanyl-L-glutaminyl-L-asparagine.—A mixture of 2.0 g. of L-phenylalanyl-L-glutaminyl-L-asparagine, 0.25 g. of magnesium oxide and 15 ml. of water was shaken for 10 minutes. The mixture was cooled in ice and 1.23 g. of carbobenzoxy chloride was added in 4 portions. The flask was kept in ice and shaken vigorously between additions. Water was added occasionally to keep the mix-

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

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(5) J. M. Swan and V. du Vigneaud, *ibid.*, **76**, 3110 (1954).

(6) P. G. Katsoyannis and V. du Vigneaud, *ibid.*, **76**, 3113 (1954).

(7) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954).

(8) Capillary melting points were determined for all compounds and are corrected.

(9) M. p. = 163–165°. E. Fischer and W. Lipschitz [*Ber.*, **48**, 360 (1915)] found m.p. 164–165° (cor.).

(10) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).

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.

Acknowledgment.—Grateful appreciation is expressed for guidance and enthusiasm generously given by Dr. Vincent du Vigneaud.

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

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