filtration, and the filtrate was assayed for isoleucine content using *L. arabinosus* which responds to both isoleucine and alloisoleucine and *S. faecalis* under conditions such that only isoleucine and not alloisoleucine will promote growth.[10] That the 2-amino-3-methyl-4-pentenoic acid herein reported is approximately 50% DL-dehydroisoleucine and 50% DL-allodehydroisoleucine is apparent from the biological data.

N-Acetyl Derivatives of 2-Amino-3-methyl-4-pentenoic Acid.[20]—A 5 g. sample of 2-amino-3-methyl-4-pentenoic acid indicated above was added to a mixture of 9 ml. of acetic anhydride in 57 ml. of glacial acetic acid and heated to its boiling point for about 12 minutes. The resulting solution was reduced in volume *in vacuo* to yield a yellow oil which was taken up in 7 ml. of water and cooled in the refrigerator. There was recovered 1.2 g. of product, m.p. 115°, which was fractionally recrystallized three times to give 600 mg. of material, m.p. 129–130°.

Anal. Calcd. for $C_8H_{13}NO_3$: N, 8.19. Found: N, 8.35.

Repeated attempts to obtain another crystalline derivative from the mother liquors resulting from the first batch of crystals above were unsuccessful. Alkaline hydrolysis of the final product, m.p. 129–130°, produced a derivative with comparable inhibitory activities to those of the original material. Further, after hydrogenation, the reduced material upon microbial analysis was indicated to be about an equimolar mixture of the two forms, isoleucine and alloisoleucine, indicating that no appreciable resolution of the diastereoisomers had been accomplished.

(20) This procedure was patterned after that of Greenstein, *et al.*,[12] for the separation of isoleucine and alloisoleucine.

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE, PITTSBURGH 13, PENNA.]

Studies on Polypeptides. XIX. Improved Synthetic Routes to Histidylphenylalanylarginyltryptophylglycine, a Key Intermediate in the Synthesis of ACTH Peptides[1-3]

BY KLAUS HOFMANN AND SAUL LANDE

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Improved procedures are described for the synthesis of the 4-L form of the pentapeptide histidylphenylalanylarginyltryptophylglycine which served as a key intermediate for the construction of the adrenocorticotropically active portion of the ACTH molecule.

In a previous communication[4] we have described the preparation of the 4-L form of the pentapeptide histidylphenylalanylarginyltryptophylglycine (I), a key intermediate in the synthesis of analogs of α-MSH[5] and of the adrenocorticotropically active portion of the ACTH molecule.[3] A different route to this same peptide was reported in a preliminary communication by Schwyzer and Li,[6] but experimental details pertaining to their synthesis are unavailable.

In our original preparation of I[4] we employed N,N′-dicyclohexylcarbodiimide (DCC)[7] to couple carbobenzoxyhistidylphenylalanylnitroarginine (II) with benzyl tryptophylglycinate (III) and subjected the ensuing crude benzyl ester (IV) to exhaustive hydrogenation. Evaluation of the stereochemical homogeneity of the hydrogenation product with leucine aminopeptidase (LAP) and trypsin pointed to marked racemization of the arginine moiety. The crude carbobenzoxyhistidylphenylalanylarginyltryptophylglycine benzyl ester IV was then subjected to fractional crystallization from ethanol and the most sparingly soluble material, obtained in a yield of 19%, proved to be the desired 4-L form of the protected pentapeptide benzyl ester. This material on hydrogenation afforded the 4-L form of the pentapeptide I which was completely digestible by LAP. Hydrogenation of the material from mother liquors gave a pentapeptide whose acid hydrolysate contained the expected amino acids in the correct molar ratios but which was only partially digestible by LAP and trypsin. This material must have contained a mixture of L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine and of L-histidyl-L-phenylalanyl-D-arginyl-L-tryptophylglycine. Since the possibility existed that the incomplete digestibility of the pentapeptide I from mother liquors could have been the result of an inhibition of the enzymes by impurities in the peptide, we have now employed a strictly chemical approach to confirm the results which were obtained by the use of enzymes. Samples of the mother liquor pentapeptide were hydrolyzed with acid, and arginine was isolated from the hydrolysate by the flavianate procedure.[8] The resulting arginine monohydrochloride exhibited an optical rotation of ±1° in water and thus was unquestionably of the DL-variety. A sample of L-arginine monohydrochloride ($[\alpha]_D$ +12.1°) that was subjected to the same isolation procedure exhibited an optical rotation of +11.2° under identical experimental conditions.

These results provided unequivocal support for the conclusions which were reached by the sole use of enzymatic techniques.

Since the 4-L pentapeptide I served as a key intermediate for further synthetic work and since our previously developed route afforded this compound in low yield, it became of importance to develop

(1) The authors wish to express their appreciation to the U. S. Public Health Service, the National Science Foundation, the American Cancer Society and Armour and Co. for generous support of this investigation.

(2) Most of the peptides and peptide derivatives mentioned in this communication are of the L-configuration. In the interest of space conservation we have eliminated for each individual amino acid residue the customary L-designation where the configuration is clearly L.

(3) See *J. Am. Chem. Soc.*, **83**, 487 (1961), for paper XVIII in this series, erroneously numbered XIII.

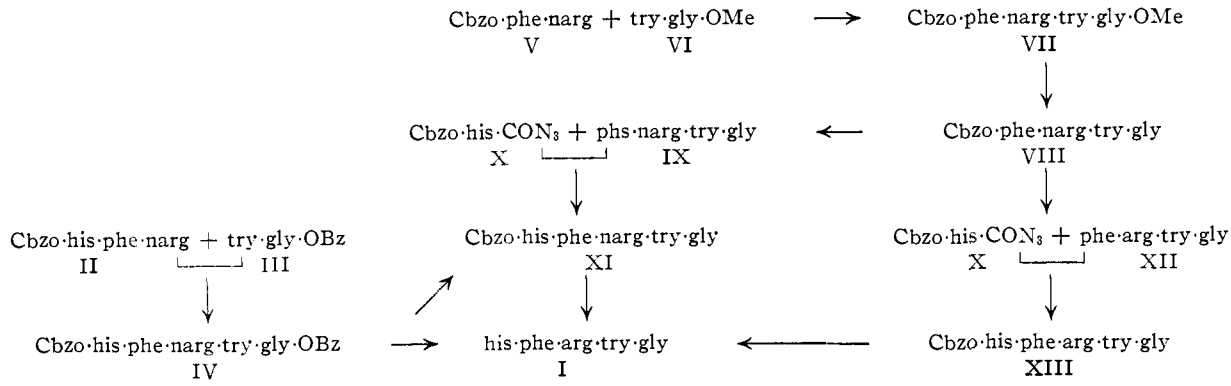
(4) K. Hofmann, M. E. Woolner, G. Spühler and E. T. Schwartz, *J. Am. Chem. Soc.*, **80**, 1486 (1958).

(5) K. Hofmann, H. Yajima and E. T. Schwartz, *ibid.*, **82**, 3732 (1960).

(6) R. Schwyzer and C. H. Li, *Nature*, **182**, 1669 (1958).

(7) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

(8) G. J. Cox, *J. Biol. Chem.*, **78**, 475 (1928).



improved procedures allowing its preparation on a larger scale. This goal has now been accomplished by two related routes in which carbobenzoxyphenylalanylnitroarginyltryptophylglycine served as a common intermediate. This protected tetrapeptide resulted from saponification of the methyl ester VII obtained by treating a mixed anhydride[9] of carbobenzoxyphenylalanylnitroarginine (V) with methyl tryptophylglycinate (VI). Since conversion of V into a mixed anhydride could have given rise to racemization of the C-terminal nitroarginine residue, the carbobenzoxytetrapeptide VIII was converted into XII by catalytic hydrogenation and the stereochemical homogeneity of this tetrapeptide was ascertained by enzymatic procedures. The observation that the paper chromatographically homogeneous compound was completely digestible by LAP with formation of the constituent amino acids in the correct molar ratios with an average amino acid recovery of 96% left little doubt regarding the assigned 4-L configuration. Reaction with the azide of N^{α}-carbobenzoxyhistidine (X) converted the tetrapeptide XII into carbobenzoxyhistidylphenylalanylarginyltryptophylglycine (XIII) which was readily transformed into I by catalytic hydrogenation. The properties of the ensuing paper chromatographically homogeneous pentapeptide agreed closely with those reported previously for the same compound prepared by another route. The yield of pentapeptide based on tryptophylglycine methyl ester was 24%.

Exposure to hydrogen bromide in glacial acetic acid[10] converted the protected peptide VIII into the dihydrobromide of IX in practically quantitative yield.[11] The free tetrapeptide IX was obtained from the dihydrobromide in analytically pure form by treatment with Amberlite IR-4B in the acetate cycle followed by countercurrent distribution. Reaction of the triethylammonium salt of IX with N^{α}-carbobenzoxyhistidine azide[12] afforded N^{α}-carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine (XI), which we had prepared previously by saponification of the benzyl ester IV.[13] The properties of the material obtained by the alternate route agreed closely with those reported previously. Hydrogenation of XI gave I. The over-all yield of I by this route was 29% based on the tryptophylglycine methyl ester. Both protected pentapeptides XI and XIII have been employed as intermediates for construction of more complex peptides since the C-terminal glycine precludes racemization during activation of the carboxyl group. This principle, first employed in our synthesis of analogs of the melanocyte-expanding hormone α-MSH,[5,14] recently was adapted to a synthesis of the adrenocorticotropically active portion of ACTH.[3]

Experimental[15]

Methyl Tryptophylglycinate Hydrochloride Half Hydrate (VI).—Carbobenzoxytryptophylglycine methyl ester[4] (8 g.) was hydrogenated in the usual manner over palladium in aqueous methanol (120 ml.) containing acetic acid (8 ml.) and water (6 ml.). The catalyst was removed by filtration and the clear solution was evaporated to dryness *in vacuo* at 30°. The resulting oil was dissolved in ice-cold 1 *N* hydrochloric acid (20.5 ml.), the solution was filtered and lyophilized and the residue dried over potassium hydroxide pellets *in vacuo;* yield 5.7 g. (91%), $[\alpha]^{28}D$ +48.6° (*c* 1.1 in 1 *N* hydrochloric acid), R_f^1 0.7, single ninhydrin and Ehrlich positive spot.

Anal. Calcd. for $C_{14}H_{18}O_3N_3Cl \cdot 0.5H_2O$ C, 52.4; H, 6.0; N, 13.1; Cl, 11.1. Found: C, 52.7; H, 6.3; N, 13.3; Cl, 11.6.

The corresponding diketopiperazine was obtained when the oily acetate salt of the dipeptide ester was stored under

(9) J. R. Vaughan, Jr., and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).

(10) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

(11) Although the nitroguanidine group fails to accept a proton when nitroarginine is esterified with methanolic hydrogen chloride [H. O. Van Orden and E. L. Smith, *J. Biol. Chem.*, **208**, 751 (1954); K. Hofmann, W. D. Peckham and A. Rheiner, *J. Am. Chem. Soc.*, **78**, 238 (1956)], protonization may ensue when peptides containing nitroarginine are exposed to hydrogen bromide in glacial acetic acid. In addition to the example mentioned above, we have observed the formation of the dihydrobromide when decarbobenzoxylating carbobenzoxynitroarginylprolylvaline methyl ester (*Anal.* Calcd. for $C_{17}H_{33}O_6N_7Br_2$: Br, 27.0. Found: Br, 24.0). Elemental analysis of these dihydrobromides gave low bromine values.

(12) (a) R. W. Holley and E. Sondheimer, *J. Am. Chem. Soc.*, **76**, 1326 (1954); (b) K. Hofmann, H. Kappeler, A. E. Furlenmeier, M. E. Woolner, E. T. Schwartz and T. A. Thompson, *ibid.*, **79**, 1641 (1957).

(13) K. Hofmann, T. A. Thompson, M. E. Woolner, G. Spühler, H. Yajima, J. D. Cipera and E. T. Schwartz, *ibid.*, **82**, 3721 (1960).

(14) (a) K. Hofmann, M. E. Woolner, H. Yajima, G. Spühler, T. A. Thompson and E. T. Schwartz, *ibid.*, **80**, 6458 (1958); (b) K. Hofmann, H. Yajima and E. T. Schwartz, *Biochim. Biophys. Acta*, **36**, 252 (1959).

(15) The organic solvents were freshly distilled. The melting points are uncorrected. Rotations were determined in a Rudolph precision polarimeter model 80 with model 200 photoelectric attachment. The amino acid composition of the acid and LAP hydrolysates was routinely determined with a Beckman–Spinco amino acid analyzer, model 120, according to the method of S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958). Solvents were evaporated *in vacuo* in a rotary evaporator at a bath temperature of 40–50°. R_f^1 values refer to the Partridge system (S. M. Partridge, *Biochem. J.*, **42**, 238 (1948)); R_f^2 values refer to the 2-butanol–ammonia system (J. F. Roland and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954)) and are expressed as a multiple of the distance traveled by phenylalanine under identical conditions.

ether; m.p. 292–303°, $[\alpha]^{28}D$ +38.2° (*c* 0.5 in dimethylformamide), R_f^1 0.75, R_f^2 1.9; single ninhydrin negative, Ehrlich positive spot.

Anal. Calcd. for $C_{13}H_{13}O_2N_3$: C, 64.2; H, 5.4; N, 17.3. Found: C, 64.3; H, 5.4; N, 17.1.

Carbobenzoxyphenylalanylnitroarginyltryptophylglycine Methyl Ester Monohydrate (VII).—Isobutyl chloroformate (3.3 ml.) was added to a cold solution (−8 to −10°) of carbobenzoxyphenylalanylnitroarginine[11] (12.0 g.) in tetrahydrofuran (80 ml.) containing triethylamine (3.4 ml.) and the mixture was stirred at −10° for 15 minutes under anhydrous conditions. This solution was added at −10° to a tetrahydrofuran solution (50 ml.) containing methyl tryptophylglycinate (prepared from 7.5 g. of the hydrochloride with 5.7 ml. of tri-*n*-butylamine). The reaction mixture was stirred for 10 minutes at −10°, was allowed to reach room temperature and was kept at that temperature for 1 hour with stirring. The solvents were evaporated, the resultant oil was dissolved in ethyl acetate and the solution was washed and dried in the usual manner. The product which precipitated during evaporation of the solvent was collected, washed with ethyl acetate and dried; yield 12.0 g. (67%), m.p. 145–148° with sintering at 125°, $[\alpha]^{26}D$ −18.8° (*c* 1.0 in dimethylformamide), R_f^2 1.9; sharp single spot, Ehrlich positive, ninhydrin negative.

Anal. Calcd. for $C_{37}H_{43}O_9N_9 \cdot H_2O$: C, 57.3; H, 5.8; N, 16.3. Found: C, 57.5; H, 5.8; N, 16.0.

Carbobenzoxyphenylalanylnitroarginyltryptophylglycine Dihydrate (VIII).—The above methyl ester (8.0 g.) was dissolved in methanol (80 ml.) and 2 *N* sodium hydroxide (8 ml.) was added. The solution was kept at room temperature for 1 hour, was acidified to congo red with 2*N* hydrochloric acid and was cooled in an ice-bath. The precipitate was collected, washed with ice-cold methanol and water and dried; yield 6.4 g. (80%), m.p. 190–193°, $[\alpha]^{25}D$ −18.0° (*c* 1.2 in dimethylformamide), R_f^1 0.9, R_f^2 1.7; sharp ninhydrin negative, Ehrlich positive spot.

Anal. Calcd. for $C_{36}H_{41}O_9N_9 \cdot 2H_2O$: C, 55.5; H, 5.8; N, 16.2. Found: C, 55.9; H, 5.8; N, 16.1.

Phenylalanylnitroarginyltryptophylglycine (IX). Dihydrobromide Hydrate.—A 3.7*N* glacial acetic acid solution of hydrogen bromide (12 ml.) was added to a suspension of carbobenzoxyphenylalanylnitroarginyltryptophylglycine (3.0 g.) in glacial acetic acid (36 ml.). The mixture was kept at room temperature for 1 hour under nitrogen with periodical gentle swirling and the reaction product was precipitated from the ensuing clear solution by addition of ether. The supernatant was removed by decantation, the solid residue was washed extensively by trituration with ether and was dried over potassium hydroxide pellets *in vacuo;* hygroscopic amorphous solid, yield 3.0 g. (97%), R_f^1 0.7, R_f^2 1.1, ninhydrin and Ehrlich positive spot. Trace of a ninhydrin negative, Ehrlich positive impurity with R_f^1 0.90 was present on the chromatograms.

Anal. Calcd. for $C_{28}H_{37}O_7N_9Br_2 \cdot H_2O$: C, 42.6; H, 5.0; N, 16.0; Br, 20.2. Found: C, 43.1; H, 5.2; N, 15.2; Br, 18.5.[11]

Monohydrate.—For conversion into the analytically pure free peptide a sample of the dihydrobromide (1.0 g.) was dissolved in 50% aqueous methanol and Amberlite 1R-4B in the acetate cycle was added in small portions with stirring until the supernatant was free of bromide ions. The resin was removed by filtration, the filtrate was concentrated to a small volume and the rest of the solvent was removed by lyophilization. The residue was placed into the first three tubes of a countercurrent machine and was subjected to a 100-plate distribution in the solvent system 1-butanol–10% acetic acid. Absorbancy of the lower phases at 280 mμ served to locate the desired peptide in tubes 39–54. The contents of these tubes were pooled, the bulk of the solvents removed and the residue lyophilized and dried over potassium hydroxide pellets; yield 450 mg. (60%), $[\alpha]^{28}D$ −18.6° (*c* 1.0 in dimethylformamide), R_f^1 0.6, R_f^2 1.1, single sharp ninhydrin and Ehrlich positive spot.

Anal. Calcd. for $C_{28}H_{35}O_7N_9 \cdot H_2O$: C, 52.8; H, 6.0; N, 19.8. Found: C, 52.3; H, 6.1; N, 20.4.

Phenylalanylarginyltryptophylglycine Acetate Monohydrate (XII).—Carbobenzoxyphenylalanylnitroarginyltryptophylglycine (VIII) (6.0 g.) was suspended in acetic acid (100 ml.) containing 40% methanol and 10% of water and the mixture was shaken in presence of a palladium catalyst in an atmosphere of hydrogen for 18 hours. Fresh catalyst was added after 8 hours of hydrogenation. The catalyst was separated from the clear solution which was evaporated to dryness. The ensuing pink oil was dissolved in methanol, the peptide was precipitated by addition of ether and was dried over potassium hydroxide pellets *in vacuo;* yield 4.8 g. (90%), $[\alpha]^{28}D$ −4.1° (*c* 1.0 in 1*N* hydrochloric acid), R_f^1 0.6, R_f^2 1.0; single ninhydrin, Ehrlich and Sakaguchi positive spot; amino acid ratios in acid hydrolysate $phe_{1.01}arg_{0.99}gly_{1.00}$ (96%)[16]; amino acid ratios in LAP digest $phe_{1.00}arg_{0.97}try_{1.00}gly_{1.00}$ (86%).[16]

Anal. Calcd. for $C_{30}H_{40}O_7N_8 \cdot H_2O$: C, 56.1; H, 6.6; N, 17.4. Found: C, 55.8; H, 7.1; N, 17.1.

Nα-Carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine One and One-half Hydrate (XI).—An ice-cold ethyl acetate solution (approximately 75 ml.) containing the azide of Nα-carbobenzoxyhistidine (prepared from 1.2 g. of the hydrazide)[12] was added to an ice-cold solution of phenylalanylnitroarginyltryptophylglycine dihydrobromide (2.7 g.) and triethylamine (1.2 ml.) in dimethylformamide (25 ml.). The mixture was kept at 4° for 48 hours when an ethyl acetate solution containing additional azide (prepared from 0.6 g. of the hydrazide) was added. The mixture was kept at 4° for an additional 48 hours, the product precipitated by addition of water was collected by centrifugation, was washed with water, dried *in vacuo* over phosphorus pentoxide and recrystallized from a mixture of dimethylformamide and water, 1:3 v./v.; yield 2.4 g. (73%), m.p. 246–249°, $[\alpha]^{26}D$ −29.2° (*c* 1.2 in dimethylformamide) [lit.[13] m.p. 241–242°, $[\alpha]D$ −29.6° in dimethylformamide], R_f^2 1.6; single ninhydrin negative, Pauly and Ehrlich positive spot.

Anal. Calcd. for $C_{42}H_{48}O_{10}N_{12} \cdot 1.5H_2O$: C, 55.6; H, 5.7; N, 18.5. Found: C, 55.5; H, 5.8; N, 18.6.

Nα-Carbobenzoxyhistidylphenylalanylarginyltryptophylglycine (XIII) Dihydrate.—An ice-cold ethyl acetate solution (approximately 75 ml.) containing Nα-carbobenzoxyhistidine azide (prepared from 2.7 g. of the hydrazide)[12] was added to an ice-cold solution of phenylalanylarginyltryptophylglycine (4.4 g.) and triethylamine (1.1 ml.) in dimethylformamide (35 ml.). The mixture was kept at 4° for 48 hours when an ethyl acetate solution containing additional azide (prepared from 1.3 g. of the hydrazide) was added. The mixture was kept for an additional 48 hours. The product, precipitated by addition of water (approximately 350 ml.), was collected by centrifugation, washed with water (equilibrated with ethyl acetate) and dried *in vacuo* over phosphorus pentoxide; yield 4.0 g. (62%), m.p. 212–217° dec., $[\alpha]^{24}D$ −17.2° (*c* 1.0 in dimethylformamide).

Anal. Calcd. for $C_{42}H_{49}O_8N_{11} \cdot 2H_2O$: C, 57.8; H, 6.1; N, 17.7. Found: C, 57.4; H, 6.3; N, 17.3.

Dihydrochloride Dihydrate.—Nα-Carbobenzoxyhistidylphenylalanylarginyltryptophylglycine (XIII) (200 mg.) was suspended in methanol (1.5 ml.) and 1 *N* hydrochloric acid (0.46 ml.) was added. The product was precipitated by addition of ether, was washed exhaustively with the same solvent and dried; yield 200 mg. (91%), $[\alpha]^{28}D$ −22.8° (*c* 1.0 in dimethylformamide); single ninhydrin negative, Pauly, Ehrlich and Sakaguchi positive spot; R_f^1 0.7, R_f^2 1.8.

Anal. Calcd. for $C_{42}H_{51}O_8N_{11}Cl_2 \cdot 2H_2O$: Cl, 7.8. Found: Cl, 7.3.

Histidylphenylalanylarginyltryptophylglycine Monoacetate Dihydrate (I). a. From Nα-Carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine (XI).—The above-mentioned carbobenzoxy derivative (200 mg.) was suspended in 90% v./v. aqueous acetic acid (20 ml.) and the suspension was shaken with hydrogen in presence of a palladium catalyst for 18 hours. Fresh catalyst was added after 8 hours of hydrogenation. The catalyst was removed from the clear solution which was evaporated to dryness and the resulting oil was dissolved in water. The solution was decolorized with Norit-A, the charcoal-free filtrate was lyophilized and the residue dried over potassium hydroxide pellets *in vacuo;* yield 140 mg. (77%), $[\alpha]^{26}D$ −11.8° (*c* 0.9 in 1*N* hydrochloric acid) (lit.[4] $[\alpha]D$ −10.0° in 1*N* hydrochloric acid); single ninhydrin, Pauly, Ehrlich and Sakaguchi positive spot; R_f^1 0.48, R_f^2 0.76; amino acid ratios in acid hydrolysate $his_{0.99}phe_{1.04}arg_{0.91}gly_{1.06}$ (92%); amino acid ratios in LAP digest $his_{1.09}phe_{1.02}arg_{0.99}try_{0.96}gly_{1.01}$ (88%).

(16) Average recovery of amino acids.

b. From Nα-Carbobenzoxyhistidylphenylalanylarginyltryptophylglycine (XIII).—The carbobenzoxy derivative (200 mg.) was suspended in 90% v./v. aqueous acetic acid and hydrogenated over a palladium catalyst in the usual manner until the evolution of carbon dioxide ceased. The product was isolated in the manner described under (a) above; yield 162 mg. (82%), $[\alpha]^{28}D$ −12.0° (*c* 1.0 in 1*N* hydrochloric acid); single ninhydrin, Pauly, Ehrlich and Sakaguchi positive spot; R_f^1 0.50, R_f^2 0.74; amino acid ratios in acid hydrolysate $his_{0.97}phe_{1.03}arg_{0.98}gly_{1.05}$ (95%); amino acid ratios in LAP digest $his_{0.97}phe_{1.00}arg_{0.98}try_{1.00}gly_{1.03}$ (90%).

Anal. Calcd. for $C_{36}H_{47}O_8N_{11}\cdot 2H_2O$: C, 54.2; H, 6.4; N, 19.3. Found: C, 53.7; H, 6.6; N, 19.8.

Isolation of DL-Arginine from Racemized Histidylphenylalanylarginyltryptophylglycine.—Crude pentapeptide I (1 g.) [obtained by hydrogenating mother liquor material from preparation of 4-L (IV)[4]] was refluxed for 36 hours with 6*N* hydrochloric acid (110 ml.). The hydrolysate was decolorized with Norit-A, the filtrate evaporated to dryness, the residue freed from excess of hydrochloric acid by repeated evaporation with water and the resultant oil dissolved in water (2 ml.). Flavianic acid (1 g.) was added, the mixture was kept at 4° for 5 hours with occasional shaking. The precipitate was collected by centrifugation, was dissolved in hot 5% ammonium hydroxide and precipitated by addition of 1*N* hydrochloric acid. This process was repeated three times and the final product was washed with ethanol and dried. The flavianate was dissolved in concentrated hydrochloric acid (3 ml.) and the mixture was kept at room temperature for 1 hour and at 0° for 30 minutes and then filtered. The flavianic acid precipitate was washed with several small portions of ice-cold concentrated hydrochloric acid and the combined filtrate and washings were evaporated to dryness. The residue was dissolved in ethanol (2 ml.), the solution was filtered after standing at 4° for 18 hours and the filtrate was decolorized with Norit-A and evaporated. The residue was dissolved in methanol and crystallization of the arginine monohydrochloride effected by addition of aniline. The arginine monohydrochloride was recrystallized from ethanol; m.p. 201–203°, $[\alpha]^{24}D$ ±1° (*c* 2.0 in water); paper chromatographically homogeneous in Partridge and 2-butanol–ammonia systems[15]; R_f's identical with those of arginine. L-Arginine monohydrochloride, m.p. 216–218°, $[\alpha]^{24}D$ +12.6° (*c* 2.0 in water), subjected to the isolation procedure exhibited the properties: m.p. 215–216°, $[\alpha]^{24}D$ +11.2° (*c* 2.0 in water).

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[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE, PITTSBURGH 13, PENNA.]

Studies on Polypeptides. XX. Synthesis and Corticotropic Activity of a Peptide Amide Corresponding to the N-Terminal Tridecapeptide Sequence of the Corticotropins[1-4]

BY KLAUS HOFMANN AND HARUAKI YAJIMA

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Exposure of carbobenzoxyseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N$^{\epsilon}$-tosyllysylprolylvaline amide to sodium in liquid ammonia resulted in marked destruction of the peptide chain. A systematic study pertaining to stability to 0.5 *N* hydrochloric acid at 100° of certain peptide derivatives corresponding to the α-MSH sequence demonstrated: (a) that the peptide chain of the α-MSH molecule undergoes some hydrolysis under these conditions; (b) that the N-terminal acetyl group and the glutamine amide function are removed from N-acetylseryltyrosylserylmethionylglutamine with formation of seryltyrosylserylmethionylglutamic acid; (c) that histidylphenylalanylarginyltryptophylglycyl-N$^{\epsilon}$-formyllysylprolylvaline amide affords histidylphenylalanylarginyltryptophylglycyllyslylprolylvaline amide; (d) that acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine is converted into stereochemically homogeneous seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycine; (e) that acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N$^{\epsilon}$-formyllysylprolylvaline amide is converted to seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvaline amide, which was homogeneous stereochemically, exhibited pronounced *in vitro* melanocyte-expanding activity (1.9×10^9 MSH units/g.) and brought about *in vivo* adrenal ascorbic acid depletion and plasma corticosterone elevation in the rat-(activity level <0.1 I.U./mg.).

Peptic digestion of the 39 amino acid residue β-corticotropin molecule affords, among other products, an N-terminal octacosapeptide fragment which is reported to retain the full ascorbic acid depleting activity of the intact hormone. From the results of partial hydrolysis with acid it was inferred that the four C-terminal amino acids may be removed from this fragment with formation of a tetracosapeptide without altering significantly the biological potency.[5]

The smallest fragment of the corticotropin molecule possessing *in vivo* corticotropic activity remains to be established. Indeed, Steelman and Guillemin[6] were able to elicit *in vitro* and *in vivo* corticotropic activity with purified samples of α-MSH, the porcine melanocyte expanding hormone (I) which contains the same N-terminal tridecapeptide sequence as do the corticotropins.[7] The availability for biological studies of the synthetic tridecapeptide amide (VI) which corresponds to the N-terminal third of the amino acid sequence of the corticotropin molecule and which is closely related to α-MSH (I) is of interest in this connection.

(1) The authors wish to express their appreciation to the U. S. Public Health Service, the National Science Foundation, the American Cancer Society, and Armour and Co. for generous support of this investigation.

(2) The peptides and peptide derivatives mentioned in this communication are of the L-configuration. In the interest of space conservation we have eliminated the customary L-designation for individual amino acid residues.

(3) See *J. Am. Chem. Soc.*, **83**, 2286 (1961), for paper XIX in this series.

(4) A preliminary report of some of the results presented in this paper was given at the Brookhaven Conference on Protein Structure and Function on June 8, 1960; K. Hofmann, *Brookhaven Symp. in Biol.*, **13**, 184 (1960).

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