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

Studies on Polypeptides. XVI. The Preparation of N^{ϵ}-Formyl-L-lysine and its Application to the Synthesis of Peptides[1,2]

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A method for the synthesis of N^{ϵ}-formyl-L-lysine is described which involves acylation of the copper complex of L-lysine with ethyl formate. The direct formylation of L-lysine with a mixture of formic acid and acetic anhydride is shown to afford N^{α}-formyl-L-lysine as the major product. The preparation of a number of peptides containing N^{ϵ}-formyl-L-lysine is given and it is shown that the N^{ϵ}-formyl group survived the treatments which were necessary for synthesis and purification. Syntheses of histidylphenylalanylarginyltryptophylglycyl-N^{ϵ}-formyllysylprolylvaline amide and histidylphenylalanylnitroarginyltryptophylglycyl-N^{ϵ}-formyllysylprolylvaline amide are presented and the *in vitro* melanocyte-expanding activity of these compounds is reported.

The carbobenzoxy[3] and *p*-toluenesulfonyl[4] (tosyl) groups have been employed for protection of the ϵ-amino group of lysine when introducing this amino acid into peptides. Since both these groups have certain disadvantages, as far as their removal from complex peptides is concerned, we have explored the potentiality of the formyl group for this purpose. It appears that this group is readily removed from N^{α}-formylamino acids and peptides by a variety of hydrolytic conditions,[5] and it was to be expected that the ϵ-formamido group of N^{ϵ}-formyl-L-lysine would exhibit a similar behavior.

Although the N^{α}-formyl derivatives of a number of amino acids have been known since Fischer's time,[6] little information is available regarding the formylation of lysine, and the stability and other properties of its N^{α}-, and N^{ϵ}-formyl derivatives.

Wolf, Valiant, Peck and Folkers[7] added acetic anhydride to a solution of N^{ϵ}-carbobenzoxy-L-lysine in formic acid and obtained N^{ϵ}-carbobenzoxy-N^{α}-formyl-L-lysine in the form of an oil. This material was subjected to catalytic hydrogenation to give crystalline N^{α}-formyl-L-lysine.

We have added acetic anhydride to a solution of the formate salt of L-lysine in formic acid and have isolated N^{α}-formyl-L-lysine as the major reaction product. Identification was based on comparison of the material with a sample of N^{α}-formyl-L-lysine which was prepared according to the unequivocal procedure of Wolf, *et al.*[8] These results demonstrate that the direct formylation of lysine in formic acid with acetic anhydride leads to a predominant formation of the N^{α}-formyl derivative.

The copper complex of N^{ϵ}-formyl-L-lysine was obtained when the copper complex of L-lysine[9] was treated with ethyl formate at *p*H 8 to 9. The lower solubility of the chelate derived from the formylated product facilitated purification. The copper complex was decomposed with hydrogen sulfide and N^{ϵ}-formyl-L-lysine was isolated from the metal ion free filtrates in analytically pure form.

N^{ϵ}-Formyl-L-lysine differs from the N^{α}-isomer in melting point, and optical rotation, and by the fact that it develops a blue color at room temperature with the ninhydrin reagent whereas heating is necessary to bring about the color development with the N^{α}-formyl derivative. Both N^{α}- and N^{ϵ}-formyl-L-lysine exhibit essentially the same R_f-value in the Partridge system,[10] but their rate of migration in the 2-butanol–ammonia system[11] is sufficiently different to allow their separation.

Exposure to carbobenzoxy chloride under Schotten–Baumann conditions converted N^{ϵ}-formyl-L-lysine into the crystalline N^{α}-carbobenzoxy derivative which regenerated the starting material on catalytic hydrogenation. N^{ϵ}-Formyl-L-lysine methyl ester hydrochloride was obtained from the N^{α}-carbobenzoxy-N^{ϵ}-formyl-L-lysine methyl ester by catalytic hydrogenation in the presence of formic acid followed by the exchange of the formate by chloride ion. N^{α}-Carbobenzoxy-N^{ϵ}-formyl-L-lysine was readily converted into a mixed anhydride and the latter reacted with N^{ϵ}-formyl-L-lysine methyl ester to give N^{α}-carbobenzoxy-N^{ϵ}-formyl-L-lysyl-N^{ϵ}-formyl-L-lysine methyl ester. The corresponding acylated dipeptide was obtained by saponification of the methyl ester.

The methyl esters of N^{α}-carbobenzoxy-N^{ϵ}-formyl-L-lysine and of N^{α}-carbobenzoxy-N^{ϵ}-formyl-L-lysyl-N^{ϵ}-formyl-L-lysine formed crystalline amides on exposure to methanolic ammonia.

These results demonstrate convincingly that the ϵ-formamido group possesses a degree of stability allowing incorporation of N^{ϵ}-formyl-L-lysine into peptides. It should be noted, however, that care must be exercised when working with peptides containing N^{ϵ}-formyllysine, since the formamido group undergoes hydrolysis when these compounds are exposed to the action of strong acids.

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

(2) The results described in this paper were presented at the Atlantic City Meeting of the American Chemical Society in September, 1959. The amino acid 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 in many instances.

(3) M. Bergmann, L. Zervas and W. F. Ross, *J. Biol. Chem.*, 111, 245 (1935).

(4) R. Roeske, F. H. C. Stewart, R. J. Stedman and V. du Vigneaud, THIS JOURNAL, **78**, 5883 (1956).

(5) J. C. Sheehan and D. D. H. Yang, *ibid.*, **80**, 1154 (1958).

(6) See for example, E. Fischer and O. Warburg, *Ber.*, **38**, 3997 (1905).

(7) D. E. Wolf, J. Valiant, R. L. Peck and K. Folkers, THIS JOURNAL, **74**, 2002 (1952).

(8) We have been unable to duplicate the melting point of 193–193.5° reported by these authors; our product melted at 187–188°.

(9) A. Neuberger and F. Sanger, *Biochem. J.*, **37**, 515 (1943).

(10) S. M. Partridge, *ibid.*, **42**, 238 (1948).

(11) J. F. Roland, Jr., and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954); since the position of the solvent front cannot be determined with this system the R_f's are given in relation to the nearest amino acid on the indicator strip.

In a previous communication[12] we have described a synthesis of the partially blocked tripeptide amide N^{ϵ}-tosyllysylprolylvaline amide, a sequence which corresponds to positions 11 to 13 in the molecule of α-MSH. The N^{ϵ}-formyl analog of this compound has now been prepared by treating a mixed anhydride of N^{α}-carbobenzoxy-N^{ϵ}-formyl-L-lysine with prolylvaline amide[12] followed by decarbobenzoxylation of the resulting blocked tripeptide amide. The ensuing hydrochloride of N^{ϵ}-formyllysylprolylvaline amide formed a single spot on paper and the results of the analytical evaluation of acid hydrolysates and leucine aminopeptidase (LAP) digests provided convincing evidence in favor of the assigned structure. The quantitative recovery of N^{ϵ}-formyllysine from the LAP digest and the absence of free lysine from the chromatograms illustrate the stability of the N^{ϵ}-formyl group to the various treatments which were required in this synthesis.

The preparation of N^{α}-carbobenzoxy-N^{ϵ}-formyllysylprolylvalylglycine in a stepwise manner from the carboxyl end provides another application of N^{ϵ}-formyl-L-lysine to peptide synthesis.

Methyl carbobenzoxyvalylglycinate was prepared by the mixed anhydride procedure and was converted into carbobenzoxyvalylglycine by hydrolysis. Hydrogenation of this acyldipeptide afforded a valylglycine with optical properties which were in remarkable agreement with those given by Fischer who prepared this compound by another route 51 years ago.[13] The dipeptide was also prepared by Sheehan[5] who reported identical optical properties. The methyl ester of valylglycine was coupled with a mixed anhydride of carbobenzoxyproline[4] to give carbobenzoxyprolylvalylglycine methyl ester and, by saponification, carbobenzoxyprolylvalylglycine. This same compound was also obtained when a mixed anhydride of carbobenzoxyproline was added to an aqueous dioxane solution containing the triethylammonium salt of valylglycine. Hydrogenation converted the carbobenzozytripeptide into the free tripeptide which was completely digestible with LAP. The enzymic digest contained valine and glycine in the expected molar ratios; proline was present but was not determined. The tripeptide methyl ester was treated with a mixed anhydride prepared from N^{α}-carbobenzoxy-N^{ϵ}-formyl-L-lysine to give the crystalline methyl ester of N^{α}-carbobenzoxy-N^{ϵ}-formyllysylprolylvalylglycine. Saponification afforded N^{α}-carbobenzoxy-N^{ϵ}-formyllysylprolylvalylglycine which was transformed into N^{ϵ}-formyllysylprolylvalylglycine by catalytic hydrogenation.

The synthesis of the octapeptide amide histidylphenylalanylarginyltryptophylglycyl - N^{ϵ} - formyllysylprolylvaline amide, which was patterned according to the scheme which we had developed for the preparation of histidylphenylalanylarginyltryptophylglycyl - N^{ϵ} - tosyllysylprolylvaline amide,[12] provides an application of N^{ϵ}-formyl-L-lysine to the construction of a complex polypeptide. Carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine[12] was coupled by the use of N,N'-dicyclohexylcarbodiimide[14] with the above described N^{ϵ}-lysylprolylvaline amide and the ensuing crude reaction product (which contained at least three components as judged by paper chromatography) was subjected to exhaustive hydrogenation in order to remove the carbobenzoxy group and to convert the nitroarginine residue into an arginine moiety. The octapeptide was isolated from the crude mixture of reaction products by countercurrent distribution in two different solvent systems. The highly purified product which was isolated as the diacetate tetrahydrate in a yield of 51% (based on the tripeptide amide) formed a single spot on paper and its LAP digest contained the constituent amino acids in the theoretically predicted molar ratios. The absence of free lysine from the chromatograms of the digest attests to the stability of the N^{ϵ}-formyl group to the conditions which were employed in the countercurrent distribution.

In addition to the octapeptide amide containing arginine we have isolated from the hydrogenation mixture the octapeptide analog containing nitroarginine. This material was also completely digestible by LAP, but the digest contained nitroarginine rather than arginine. The availability of this substance for biological testing was of considerable importance since it made possible an assessment of the importance of the presence of a free guanido group for biological activity.

In a previous communication[12] we have recorded the biological activity of a series of peptides which corresponded to sequences which are present in the molecule of α-MSH. One of the biologically active compounds of this series was the octapeptide amide histidylphenylalanylarginyltryptophylglycyl-N^{ϵ}-tosyllysylprolylvaline amide which exhibited an *in vitro* melanocyte-expanding activity of 0.5×10^6 MSH units per gram.[15] The N^{ϵ}-formyl analog of this compound which is described in the present communication possesses sixteen times the activity of the N^{ϵ}-tosyl derivative, *i.e.*, 8×10^6 MSH units per gram.

The finding that the nitroarginine analog of the formyloctapeptide amide possesses biological activity (5.0×10^5 u./g.) casts some doubt on the essentiality of a free guanido group for melanocyte-expanding activity, but additional experimental evidence to substantiate this result must become available before definitive conclusions may be drawn pertaining to this crucial point.

We have been successful in removing the formyl blocker from a number of peptides of N^{ϵ}-formyllysine under conditions which did not result in the fragmentation of peptide bonds. Along these lines we have prepared the octapeptide histidylphenylalanylarginyltryptophylglycyllysylprolylvaline amide. Details of these experiments will be presented at a later date.

Experimental[16]

N^{α}-Formyl-L-lysine. a. By Direct Formylation of L-Lysine.—Acetic anhydride (9.4 ml.) was added slowly with

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

(13) E. Fischer and H. Scheibler, *Ann.*, **363**, 136 (1908).

(14) J. C. Sheehan and G. P. Hess, THIS JOURNAL, **77**, 1067 (1955).

(15) We wish to express our thanks to Drs. A. B. Lerner and M. R. Wright of the Department of Medicine, Yale University School of Medicine, for these assays which were performed according to the method of Shizume, Lerner and Fitzpatrick, *Endocrinol.*, **54**, 553 (1954).

stirring over a period of 10 minutes to a solution of L-lysine formate (1.92 g.), (prepared from the monohydrochloride with Amberlite IR4B in the formate cycle) in 98% formic acid (21 ml.). The temperature was kept at 5–15° during the addition and the reaction mixture was stirred at room temperature for an additional hour. Ice-water (approximately 40 ml.) was added and the solvents were evaporated *in vacuo*. The resulting oil was dissolved in 5 ml. of ethanol, additional ethanol (approximately 30 ml.) was added until the solution became cloudy and the mixture was kept at room temperature for one hour; the resulting crystals were collected and recrystallized from ethanol; yield 1.07 g. (62%), m.p. 187–188° dec. (lit.[7] m.p. 193°), $[\alpha]^{25}D$ −2.2° (*c* 1.1 in satd. sodium bicarbonate), R_f[10] 0.30, R_f[11] his, development of ninhydrin color on paper requires heating for 5 minutes at 70–75°.

Anal. Calcd. for $C_7H_{14}O_3N_2$: C, 48.3; H, 8.1; N, 16.1. Found: C, 48.3; H, 8.2; N, 16.1.

b. **By Hydrogenation of Nε-Carbobenzoxy-Nα-formyl-L-lysine.**—Nε-Carbobenzoxy-Nα-formyl-L-lysine[7] (0.218 g.) was hydrogenated over a palladium catalyst in methanol (8 ml.) containing 10% v./v. of formic acid. The product was isolated in the usual manner and recrystallized from a mixture of ethanol and ether; yield 78 mg. (63%), m.p. 185–186°, mixed m.p. with the material prepared according to a above 185–186°, $[\alpha]^{26}D$ −2.0° (*c* 0.91 in satd. sodium bicarbonate), R_f[10] 0.30.

Nε-Carbobenzoxy-Nα-formyl-L-lysine.—Nε-Carbobenzoxy-L-lysine (0.56 g.) was converted into the formyl derivative essentially in the manner described by Wolf, *et al.*[7] The oily product was dissolved in ethyl acetate and the solution was washed with ice-cold 0.5 *N* hydrochloric acid and water and was dried over sodium sulfate. Evaporation of the solvent gave an oil which was converted into a gelatinous mass by treatment with benzene. The addition of petroleum ether converted the material into a powder. This was dissolved in a small volume of methanol, the solution was filtered and the methanol was evaporated. Treatment of the residue with benzene–petroleum ether gave crystals; yield 0.54 g. (88%), m.p. 74–78°, $[\alpha]^{24}D$ +9.6° (*c* 1.4 in ethanol).

Anal. Calcd. for $C_{15}H_{20}O_5N_2$: C, 58.4; H, 6.5; N, 9.1, Found: C, 58.6; H, 6.6; N, 8.9.

Nε-Formyl-L-lysine.—This entire operation was performed in a 5° cold room. The copper chelate of L-lysine[9] (5.2 g.) was dissolved in ice-cold 1 *N* sodium hydroxide (60 ml.) and methanol (60 ml.) was added. The solution was cooled with an ice-bath, ethyl formate (24 ml.) was added, and the mixture was stirred vigorously for 2 hours with ice-cooling. The *p*H of the mixture was checked periodically and 5 *N* sodium hydroxide was added slowly to maintain the *p*H between 8 and 9 during the entire operation. The organic solvents were removed *in vacuo;* the copper complex of Nε-formyl-L-lysine which precipitated was collected by centrifugation, and was washed with several portions of water. The complex was suspended in water, hydrogen sulfide was passed through the solution and the copper sulfide was removed by filtration with the aid of Norite and Filter-cel. The clear filtrate was concentrated to a small volume *in vacuo* and ethanol was added to bring about crystallization of the product. The crystals were collected, dissolved in a small volume of water and recrystallized by addition of ethanol; yield 1.72 g., m.p. 214–215° dec., $[\alpha]^{26}D$ +4.1° (*c* 0.91 in water), $[\alpha]^{26}D$ +15.5° (*c* 1.4 in satd. sodium bicarbonate), R_f[10] 0.26, R_f[11] pro⁻. Some samples of Nε-formyl-L-lysine were contaminated with small amounts of free lysine. This impurity was readily removed by treatment with Dowex-50 in the ammonia cycle.

Anal. Calcd. for $C_7H_{14}O_3N_2$: C, 48.3; H, 8.1; N, 16.1; N-formyl, 16.6. Found: C, 48.4; H, 8.1; N, 16.6; N-formyl, 16.2.

Nα-Carbobenzoxy-Nε-formyl-L-lysine.—Carbobenzoxy chloride (8.5 g.) was added, during one hour, to a vigorously stirred ice-cold solution of Nε-formyl-L-lysine (6.96 g.) in 1 *N* sodium hydroxide (60 ml.). The mixture was kept at *p*H 8 to 9 by the slow addition of 1 *N* sodium hydroxide (approximately 35 ml. required) and stirring was continued under ice-cooling for 2 hours. The solution was extracted with ether, the ethereal extracts were discarded and the aqueous layer was acidified at 0° with 2 *N* hydrochloric acid to congo red. The organic material was extracted into ethyl acetate, the extracts were washed and dried in the usual manner and the solvent was evaporated. The ensuing sirup soon crystallized. The crystals were washed with ice-cold ethyl acetate and recrystallized from ethyl acetate; yield 8.4 g. (68%), m.p. 94–95°, $[\alpha]^{26}D$ −4.5° (*c* 1.06 in methanol).

Anal. Calcd. for $C_{15}H_{20}O_5N_2$: C, 58.4; H, 6.5; N, 9.1; N-formyl, 9.4. Found: C, 58.7; H, 6.7; N, 9.1; N-formyl, 9.0.

Hydrogenation of a sample of the carbobenzoxy derivative in methanol containing 10% v./v. of formic acid gave Nε-formyl-L-lysine, m.p. 214–215°, no depression of the m.p. when mixed with an authentic sample, $[\alpha]^{25}D$ +4.1° (*c* 0.94 in water), R_f[10] 0.31.

Nε-Formyl-L-lysine Methyl Ester Hydrochloride.—Ethereal diazomethane was added to a solution of Nα-carbobenzoxy-Nε-formyl-L-lysine (1.85 g.) in ice-cold methanol (20 ml.) until a faint yellow color remained. The solution was kept for 5 minutes, a few drops of glacial acetic acid were added and the solvents were removed. The residue was dissolved in methanol containing 10% v./v. of formic acid and was hydrogenated in the usual manner over a palladium catalyst. The catalyst was removed by filtration, the filtrate was evaporated to dryness and the resulting sirup was dissolved in a small volume of water and 1 *N* hydrochloric acid (6 ml.) was added. The solution was lyophilized and the non-crystalline residue dried by evaporation with benzene; yield 1.6 g., R_f[10] 0.50.

Nα-Carbobenzoxy-Nε-formyl-L-lysyl-Nε-formyl-L-lysine Methyl Ester.—A mixed anhydride was prepared in the usual manner from Nα-carbobenzoxy-Nε-formyl-L-lysine (2.68 g.) in ice-cold dioxane (8 ml.) with tri-*n*-butylamine (2.1 ml.) and ethyl chloroformate (0.83 ml.). This solution was added to an ice-cold solution of Nε-formyl-L-lysine methyl ester (prepared from 1.95 g. of the hydrochloride with 1.65 ml. of triethylamine in 5 ml. of chloroform) and the mixture was kept at ice-bath temperature for 30 minutes and at room temperature for one hour. The solvents were removed *in vacuo*, the residue was dissolved in a mixture of ethyl acetate and saturated sodium bicarbonate, the ethyl acetate extract was washed and dried in the usual manner, and the solvents were evaporated. The amorphous residue was purified by precipitation from ethanol with ether, and was dried over phosphorus pentoxide *in vacuo;* yield 1.8 g. (44%), m.p. 117–118°, $[\alpha]^{26}D$ −14.5° (*c* 1.17 in methanol).

Anal. Calcd. for $C_{23}H_{34}O_7N_4$: C, 57.7; H, 7.2; N, 11.7. Found: C, 57.5; H, 7.3; N, 11.5.

Nα-Carbobenzoxy-Nε-formyl-L-lysine Amide.—Nα-Carbobenzoxy-Nε-formyl-L-lysine (1.5 g.) was converted into the methyl ester in the manner described above, and the solvents were removed *in vacuo*. The residue was dissolved in methanol saturated with ammonia (10 ml.) and the mixture was kept at 5° for one week. The crystalline amide was collected and recrystallized from hot methanol to give needles; yield 1.1 g. (73%), m.p. 178–179°, $[\alpha]^{13}D$ +4.0° (*c* 2.28 in dimethylformamide).

Anal. Calcd. for $C_{15}H_{21}N_3O_4$: C, 58.6; H, 6.9; N, 13.7. Found: C, 58.4; H, 7.0; N, 13.4.

Nε-Formyl-L-lysine Amide Formate.—The above carbobenzoxy derivative (0.725 g.) was hydrogenated in the usual manner in methanol containing 10% v./v. of formic acid. The solvent was evaporated, the residue dissolved in water (approximately 5 ml.) and the solution was lyophilized to a thick sirup which crystallized on standing. Crystallization was completed by addition of ethanol. The material was collected and recrystallized from hot ethanol to give

(16) The melting points were determined in capillary tubes and are uncorrected. The organic solvents were freshly distilled. A Craig-type countercurrent machine of 200 tubes having a capacity of 10 ml. of upper and lower phase each (obtained from H. O. Post Scientific Instrument Co., Maspeth 78, N. Y.) was employed. Rotations were determined in a Rudolph precision polarimeter, model 80 with model 200 photoelectric attachment. The statement "washed in the usual manner" implies washing with 0.5 *N* hydrochloric acid followed by saturated sodium bicarbonate and water. Since the Nε-formyl group is labile to acid the acid washings of peptides containing this group were carried out at 0° in the presence of cracked ice which was added directly to the separatory funnels. Unless stated otherwise the solvents were evaporated in a rotatory evaporator at a bath temperature of 40–50°. The analytical procedures used were described in THIS JOURNAL, **80**, 1486 (1958).

hydroscopic needles; yield 0.42 g. (81%), m.p. 120-123°, $[\alpha]^{25}D$ +16.6° (*c* 0.87 in water), R_f^{10} 0.32, R_f^{11} try.

Anal. Calcd. for $C_8H_{17}O_4N_3$: C, 43.8; H, 7.8; N, 19.2. Found: C, 43.7; H, 8.0; N, 19.0.

Nα-Carbobenzoxy-Nε-formyl-L-lysyl-Nε-formyl-L-lysine.—The above methyl ester (0.24 g.) was dissolved in methanol (3 ml.), 1 *N* sodium hydroxide (0.6 ml.) was added and the mixture was kept for 2 hours at room temperature. The bulk of the methanol was removed *in vacuo*, the residue was extracted with ethyl acetate, the aqueous phase was cooled in an ice-bath and was acidified with 2 *N* hydrochloric acid to congo red. The precipitate was collected, washed with several portions of water and dried. For purification the product was precipitated from methanol with ethyl acetate; yield 0.15 g. (65%), m.p. 168-169°, $[\alpha]^{25}D$ −4.5° (*c* 0.75 in methanol).

Anal. Calcd. for $C_{22}H_{32}O_7N_4$: C, 56.9; H, 6.9; N, 12.0. Found: C, 56.5; H, 7.0; N, 12.0.

Nα-Carbobenzoxy-Nε-formyl-L-lysyl-Nε-formyl-L-lysine Amide.—Nα-Carbobenzoxy-Nε-formyl-L-lysyl-Nε-formyl-L-lysine methyl ester (1.2 g.) was dissolved in methanol saturated with ammonia (20 ml.) and the mixture was kept at 5° for one week. The amide which had precipitated was collected and was recrystallized from hot methanol; yield 0.88 g. (76%), m.p. 204-206°, $[\alpha]^{26}D$ −10.5° (*c* 0.89 in dimethylformamide), ninhydrin negative.

Anal. Calcd. for $C_{22}H_{33}O_6N_5$: C, 57.0; H, 7.2; N, 15.1. Found: C, 56.5; H, 7.2; N, 14.8.

Carbobenzoxyvalylglycine Methyl Ester.—A mixed anhydride was prepared in the usual manner from carbobenzoxyvaline (7.55 g.) in ice-cold dioxane (20 ml.) with tri-*n*-butylamine (7.2 ml.) and ethyl chloroformate (2.9 ml.). This solution was added with stirring to a cooled dioxane solution (20 ml.) of glycine methyl ester (prepared from 3.75 g. of the hydrochloride with 5.9 ml. of triethylamine). The mixture was stirred at 0° for 30 minutes and at room temperature for 60 minutes and the solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate, the solution was washed in the usual manner and dried over sodium sulfate. The solvent was evaporated and the residue recrystallized from a mixture of methanol and water, 1:1, yield 7.0 g. (73%), m.p. 160-161°, $[\alpha]^{27}D$ −24.3° (*c* 1.6 in abs. ethanol), $[\alpha]^{24}D$ −30.0° (*c* 1.85 in methanol).

Anal. Calcd. for $C_{16}H_{22}O_5N_2$: C, 59.6; H, 6.9; N, 8.7. Found: C, 59.6; H, 7.0; N, 8.7.

Valylglycine.—The above methyl ester (2.09 g.) was saponified in methanol (40 ml.) and 2 *N* sodium hydroxide (6.5 ml.) and the product was isolated in the usual manner. The oily material was dissolved in hot benzene and precipitated in the form of a gelatinous mass on cooling. The carbobenzoxyvalylglycine was collected and dried; yield 1.8 g. (90%), m.p. 120-125°, $[\alpha]^{30}D$ −21.5° (*c* 1.88 in satd. sodium bicarbonate).

The carbobenzoxy derivative (2.06 g.) was hydrogenated in methanol containing 10% v./v. of glacial acetic acid in the usual manner, and the resulting dipeptide was recrystallized from a mixture of water and ethanol, 1:10, yield 1.1 g. (95%), m.p. 255-260°, $[\alpha]^{30}D$ +93.4° (*c* 1.63 in water); lit. $[\alpha]^{20}D$ +93.6° in water,[13] $[\alpha]^{21}D$ +93.9° in water[5]; R_f^{10} 0.49, R_f^{11} val⁻; amino acid ratios in LAP digest $val_{1.0}$-$gly_{1.0}$.

Carbobenzoxyprolylvalylglycine Methyl Ester.—A mixed anhydride was prepared in the usual manner from carbobenzoxyproline[4] (2.54 g.) in ice-cold dioxane (10 ml.) with tri-*n*-butylamine (2.64 ml.) and ethyl chloroformate (1.05 ml.). This solution was added to a chilled dioxane solution of valylglycine methyl ester (prepared from 2.48 g. of the hydrochloride with triethylamine). The mixture was kept at 0° for 30 minutes and at room temperature for 1 hour and the solvent was evaporated. The residue was dissolved in ethyl acetate, the solution was washed and dried in the usual manner, and was evaporated to dryness *in vacuo*. The residue was dissolved in hot methanol (10 ml.), the solution was cooled, ether (1 ml.) was added and the mixture was placed in a refrigerator. The resulting gelatinous mass was collected, washed with cold methanol and ether and dried; yield 2.85 g. (62%), m.p. 125-127°, $[\alpha]^{24}D$ −89.7° (*c* 1.05 in methanol).

Anal. Calcd. for $C_{21}H_{29}O_6N_3$: C, 60.1; H, 7.0; N, 10.0. Found: C, 59.9; H, 7.1; N, 10.6.

Carbobenzoxyprolylvalylglycine.—a. By coupling of a mixed anhydride of carbobenzoxyproline with the triethylammonium salt of valylglycine: A mixed anhydride was prepared in the usual manner from carbobenzoxyproline[4] (0.50 g.) in dioxane (10 ml.) with tri-*n*-butylamine (0.48 ml.). and ethyl chloroformate (0.19 ml.). This solution was added to an ice-cold solution of valylglycine (0.35 g.) in 70% v./v. aqueous dioxane (10 ml.) and triethylamine (0.28 ml.). The mixture was stirred at ice-bath temperature for 30 minutes and at room temperature for 1 hour, the dioxane was evaporated to dryness and the residue was extracted with ethyl acetate. The extract was washed in the usual manner, dried over sodium sulfate and evaporated to dryness. The resulting oil, which solidified when rubbed with ether, was collected and dried; yield 0.44 g. (54%). A sample for analysis was dissolved in ethyl acetate and precipitated in the form of a gelatinous mass by addition of benzene. The material was dried *in vacuo* at 65° for 12 hours; m.p. 191-193°, $[\alpha]^{24}D$ −91.5° (*c* 1.0 in satd. sodium bicarbonate).

Anal. Calcd. for $C_{20}H_{27}O_6N_3$: C, 59.3; H, 6.7; N, 10.4. Found: C, 59.0; H, 6.8; N, 10.4.

b. By saponification of carbobenzoxyprolylvalylglycine methyl ester: Carbobenzoxyprolylvalylglycine methyl ester (0.42 g.) was dissolved in hot methanol (5 ml.), the solution was cooled at room temperature and 2 *N* sodium hydroxide (1 ml.) was added. The mixture was kept for 2 hours and the acyltripeptide was isolated in the usual manner. For purification the substance was dissolved in ethyl acetate and precipitated by addition of ether; yield 0.33 g. (81%), m.p. 193-195°, mixed m.p. with the material prepared according to method (a) 193-195°, $[\alpha]^{25}D$ −91.2° (*c* 1.0 in satd. sodium bicarbonate).

Prolylvalylglycine.—A sample of the carbobenzoxytripeptide (0.95 g.) was hydrogenated in methanol containing 10% v./v. of glacial acetic acid, and the product was isolated in the usual manner. The compound was dissolved in a small volume of water and was crystallized by addition of absolute ethanol; yield 0.50 g. (74%), m.p. 228-234°, $[\alpha]^{27}D$ −72.0° (*c* 1.40 in water), R_f^{10} 0.53, R_f^{11} met⁺, amino acid ratios in LAP digest $pro_{1.0}val_{1.0}gly_{1.0}$.

Anal. Calcd. for $C_{12}H_{21}O_4N_3 \cdot H_2O$: C, 49.8; H, 8.0; N, 14.5. Found: C, 50.3; H, 8.0; N, 14.9.

Prolylvalylglycine Benzyl Ester Hydrochloride.—The free tripeptide (0.5 g.) was suspended in benzyl alcohol (10 ml.) and the suspension was saturated with dry hydrogen chloride with cooling in an ice-bath. The solution was concentrated to one-half its volume *in vacuo* (0.1 mm.) at a bath temperature of 80° and benzyl alcohol (5 ml.) was added to the residue. The mixture was saturated once more with hydrogen chloride and was partially evaporated. This operation was repeated three times, and then the solution was evaporated to dryness and the residue rubbed with ether to give a white solid which was recrystallized from a mixture of absolute ethanol and ether; yield 0.52 g. (75%), m.p. 183-186°, $[\alpha]^{28}D$ −71.7° (*c* 0.42 in abs. ethanol).

Anal. Calcd. for $C_{19}H_{28}O_4N_3Cl$: N, 10.6; Cl, 8.9. Found: N, 10.5; Cl, 9.2.

Nα-Carbobenzoxy-Nε-formyllysylprolylvaline Amide.—A mixed anhydride, prepared in the usual manner from Nα-carbobenzoxy-Nε-formyl-L-lysine (2.12 g.) in dioxane (15 ml.) with tri-*n*-butylamine (1.68 ml.) and ethyl chloroformate (0.67 ml.) at 10-15°, was added to an ice-cold dioxane solution (10 ml.) containing prolylvaline amide (prepared from 1.74 g. of the hydrochloride with 1.33 ml. of triethylamine). The mixture was kept at 10-15° for 30 minutes and at room temperature for one hour and the solvent was removed *in vacuo*. The sparingly soluble white residue was purified by suspending it three times in ice-cold 0.5*N* hydrochloric acid followed by three similar treatments with 10% ammonium hydroxide. The compound was finally washed with water and dried; yield 1.54 g. (43%), m.p. 217-218°, $[\alpha]^{25}D$ −42.5° (*c* 1.04 in dimethylformamide).

Anal. Calcd. for $C_{25}H_{37}O_6N_5$: C, 59.6; H, 7.4; N, 13.9. Found: C, 59.2; H, 7.5; N, 13.7.

Nε-Formyllysylprolylvaline Amide Hydrochloride.—The carbobenzoxy derivative (1.4 g.) was suspended in methanol containing 10% v./v. of formic acid (15 ml.), palladium catalyst was added and the mixture was shaken in a stream of hydrogen. The material dissolved as the hydrogenation progressed and the operation was interrupted after 2 hours of

shaking. The catalyst was removed by filtration, and the filtrate was evaporated to dryness *in vacuo*. The resulting sirup was dissolved in methanol (10 ml.), 1 *N* hydrochloric acid (2.76 ml.) was added and the solution was evaporated to dryness to give a white solid. This material was dissolved in absolute ethanol (approximately 75 ml.) and the product was precipitated by addition of ether. The hygroscopic solid was collected, washed with ether and dried over phosphorus pentoxide; yield 0.73 g. (61%), $[\alpha]^{25}D$ −54.9° (*c* 0.8 in methanol), R_f^{10} 0.50, R_f^{11} phe$^+$, amino acid ratios in acid hydrolysate $lys_{1.0}val_{0.9}$ (pro present but not determined), amino acid ratios in LAP digest Nε-$formlys_{1.0}$ $val_{1.0}$ (pro present but not determined).

Anal. Calcd. for $C_{17}H_{32}O_4N_5Cl\cdot 1.5\ H_2O$: C, 47.2; H, 8.1; N, 16.2; Cl, 8.2. Found: C, 47.1; H, 8.2; N, 15.8; Cl, 8.2.

Nα-Carbobenzoxy-Nε-formyllysylprolylvalylglycine Methyl Ester.—A mixed anhydride was prepared in the usual manner from Nα-carbobenzoxy-Nε-formyl-L-lysine (2.2 g.) in ice-cold dioxane (8 ml.) with tri-*n*-butylamine (1.7 ml.) and ethyl chloroformate (0.69 ml.). This solution was added to a chilled chloroform solution (approximately 10 ml.) of prolylvalylglycine methyl ester (prepared from 2.29 g. of the hydrochloride[17] with 1.36 ml. of triethylamine in chloroform). The mixture was kept at 0° for 30 minutes and at room temperature for one hour and the solvents were evaporated *in vacuo*. The residue was dissolved in a mixture of ethyl acetate and saturated sodium bicarbonate, the ethyl acetate solution was washed in the usual manner and was dried over sodium sulfate. The peptide derivative precipitated in the form of well formed needles during evaporation of the ethyl acetate. The crystals were collected and recrystallized from methanol; yield 2.5 g. (61%), m.p. 166–167°, $[\alpha]^{24}D$ −98.5° (*c* 0.67 in methanol).

Anal. Calcd. for $C_{28}H_{41}O_8N_5$: C, 58.4; H, 7.2; N, 12.2. Found: C, 58.1; H, 7.2; N, 12.4.

Nα-Carbobenzoxy-Nε-formyllysylprolylvalylglycine.—The above methyl ester (1.0 g.) was dissolved in hot methanol (8 ml.), the solution was cooled at room temperature and 2 *N* sodium hydroxide (1.5 ml.) was added. The mixture was kept at room temperature for 2 hours and the acyl tetrapeptide was isolated in the usual manner and recrystallized from ethyl acetate. The ensuing hygroscopic crystals were dried *in vacuo* over phosphorus pentoxide; yield 0.69 g. (69%), $[\alpha]^{24}D$ −93.4° (*c* 0.46 in satd. sodium bicarbonate).

Anal. Calcd. for $C_{27}H_{39}O_8N_5\cdot H_2O$: C, 55.9; H, 7.1; N, 12.1. Found: C, 55.8; H, 7.1; N, 11.4.

Nε-Formyllysylprolylvalylglycine.—Nα-Carbobenzoxy-Nε-formyllysylprolylvalylglycine methyl ester (1.1 g.) was saponified in the manner described above and the crude product was hydrogenated in the usual manner in methanol containing 10% v./v. of formic acid. The catalyst was removed by filtration, the filtrate was evaporated, the residue was dissolved in water (5 ml.) and the solution was washed with ethyl acetate. The aqueous layer was lyophilized, the residue dissolved in ethanol (approximately 5 ml.) and the product precipitated by addition of ether. For purification the material was twice re-precipitated from methanol with ether and was dried over phosphorus pentoxide *in vacuo;* yield 0.58 g. (67%), $[\alpha]^{25}D$ −90.5° (*c* 0.77 in water), $[\alpha]^{25}D$ −68.0° (*c* 0.53 in methanol), R_f^{10} 0.39, R_f^{11} pro$^+$, amino acid ratios in acid hydrolysate $lys_{1.0}val_{0.9}gly_{1.0}$ (pro present but not determined), amino acids in LAP digest Nε-$formlys_{1.0}val_{0.9}gly_{1.0}$ (pro present but not determined).

Anal. Calcd. for $C_{19}H_{33}O_6N_5\cdot 1.5\ H_2O$: C, 50.2; H, 8.0; N, 15.4. Found: C, 49.9; H, 8.1; N, 15.3.

Histidylphenylalanylarginyltryptophylglycyl-Nε-formyllysylprolylvaline Amide Diacetate Tetrahydrate.—A solution of Nε-formyllysylprolylvaline amide hydrochloride (442 mg.) and triethylamine (0.14 ml.) in methanol (5 ml.) was evaporated to dryness *in vacuo* (bath temp. 35°) and the residue was kept *in vacuo* at room temperature over calcium chloride and phosphorus pentoxide for 30 minutes. A solution of carbobenzoxyhistidylphenylalanylnitroarginyltryptophylglycine[12] (900 mg.) in dimethylformamide (25 ml.) was added followed by N,N′-dicyclohexylcarbodiimide (266 mg.) and the mixture was kept at room temperature for 20 hours when additional N,N′-dicyclohexylcarbodiimide (62 mg.) was added. The mixture was kept at room temperature for an additional 20 hours, a few drops of glacial acetic acid was added and the solvents were removed *in vacuo*. The semisolid residue was triturated with a small volume of ice-cold dimethylformamide, the suspension was filtered, and the N,N′-dicyclohexylurea was washed with a small volume of ice-cold dimethylformamide (urea recovered 188 mg.). The filtrate and washings were evaporated to dryness *in vacuo*, the oily residue was dissolved in 1-butanol equilibrated with 10% acetic acid and the solution was washed with six 8-ml. portions of 10% acetic acid. Evaporation of the butanol gave 1.600 g. of a crude product which was ninhydrin negative and contained three components. This material was hydrogenated in 70% v./v. aqueous acetic acid (30 ml.) over a palladium catalyst for 20 hours, fresh catalyst being added twice during that period. The pink oily hydrogenation products (1.457 g.) which contained three Pauly-positive components with R_f^{10} values of 0.45, 0.52 and 0.78, respectively, were dissolved in 1-butanol saturated with 5% acetic acid containing 0.5% of sodium chloride (50 ml.) and the solution was placed in the first five tubes of a 200-tube countercurrent machine. Spectrophotometric examination at 280 mμ of the lower phases and the effluents after 400 transfers in the solvent system 1-butanol–5% acetic acid containing 0.5% sodium chloride revealed the presence of two major bands, A and B, and a minor pink colored band, C. Band A was located in effluent fractions 3 to 37, band B was present in tubes 17 to 77 (maximum in tube 45), whereas band C (the slowest moving component) occupied tubes 1 to 16 (maximum in tube 5). The effluent fractions containing material corresponding to band A were pooled and the solvent was evaporated. The residue was dissolved in 1-butanol, the solution was washed with five 10-ml. portions of 1% ammonium hydroxide and the butanol was evaporated. Examination of the residue (232 mg.) revealed the presence of a mixture containing a major component with R_f^{10} of 0.78. The material gave a positive reaction with the ninhydrin, Pauly, Sakaguchi and Ehrlich reagents, and its acid hydrolysate contained only those amino acids which were derived from the carbobenzoxypentapeptide. The nature of the material corresponding to band C was not investigated. The tubes containing material corresponding to band B (the desired octapeptide amide) were pooled and the solvents were removed. The residue, which was contaminated with sodium chloride, contained a major component (R_f^{10} 0.52) which reacted positively with the Pauly, Sakaguchi, Ehrlich and ninhydrin reagents and a minor component (R_f^{10} 0.45). A 200-plate distribution of this material in the solvent system 1-butanol–0.25% sodium bicarbonate containing 1.5% of sodium chloride gave a minor band (located in tubes 77–97) and a major component (located in tubes 137–187). The minor component was not investigated. The contents of the tubes containing the material corresponding to the major band were pooled, the solvents were evaporated, and the residue, which was heavily contaminated with inorganic salts, was triturated with four 30-ml. portions of 1-butanol equilibrated with 1% ammonium hydroxide. The butanol extracts were filtered and the filtrates were washed with six 5-ml. portions of 1% ammonium hydroxide (emulsions were broken by centrifugation). The butanol was evaporated, the residue was dissolved in a small volume of 10% acetic acid, the solution was filtered and lyophilized to give a white fluffy material; yield 541 mg. The ammonia washings were combined, evaporated to dryness *in vacuo* and the residue was dissolved in 1-butanol. The butanol was washed with several small portions of 1% ammonium hydroxide and was evaporated to give additional amounts of octapeptide which were combined with the first fraction; total yield 641 mg. (51%), $[\alpha]^{25}D$ −41.2° (*c* 0.7 in 10% acetic acid), single spot with R_f 0.53; positive reaction with the Pauly, Sakaguchi and Ehrlich reagents; completely digestible with LAP, amino acid ratios in digest $his_{1.0}phe_{1.0}arg_{1.0}try_{1.0}$Nε-$formyls_{1.0}val_{1.0}$ (pro present on chromatogram but not determined; biol. activity 8.0×10^6 MSH units per gram).

Anal. Calcd. for $C_{55}H_{80}O_{13}N_{16}\cdot 4H_2O$: C, 53.0, H, 7.1; N, 18.0; try, 16.4. Found: C, 53.1; H, 7.2; N, 18.2; try, 16.0.

Histidylphenylalanylnitroarginyltryptophylglycyl-Nε-formyllysylprolylvaline Amide.—Occasionally, the crude hydrogenation mixture (described above) contained a fourth component. In the initial countercurrent distribution this mate-

(17) Obtained by hydrogenation of the carbobenzoxytripeptide methyl ester in methanol containing hydrogen chloride.

rial travelled at a faster rate than the corresponding octapeptide amide containing arginine (band B).

A representative experiment was carried out as follows: The crude coupling product (707 mg.) was hydrogenated and the hydrogenation products were subjected to a 560-plate distribution in the solvent system 1-butanol-5% acetic acid containing 0.5% sodium chloride. The desired completely reduced octapeptide amide (band B) was located in tubes 20 to 80, the nitroarginine analog occupying tubes 141 to 200. The contents of these tubes were pooled, the solvents were evaporated *in vacuo*, and the residue was desalted by distribution between 1-butanol and 1% ammonium hydroxide in the manner described. The salt-free residue was dissolved in 5% acetic acid and the filtered solution was lyophilized to give a colorless, fluffy solid; yield 108 mg., $[\alpha]^{25}D$ −30.1° (*c* 0.8 in 10% acetic acid), homogeneous on paper (R_f^{10} 0.67); ninhydrin, Pauly and Ehrlich positive, Sakaguchi negative; completely digestible by LAP, amino acid ratios in digest $his_{1.0}phe_{1.0}arg_{1.0}try_{1.0}gly_{1.0}N^{\epsilon}$-$formlys_{1.0}$-$val_{1.0}$ (arg absent, pro present but not determined); biol. activity 5.0×10^5 MSH units per g.

PITTSBURGH, PENNA.

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

Studies on Polypeptides. XVII. The Synthesis of Three Acyltridecapeptide Amides Possessing a High Level of Melanocyte-expanding Activity *in Vitro*[1-3]

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The synthesis of three acyltridecapeptide amides containing of the entire amino acid sequence of the pituitary hormone α-MSH is described. Evidence is presented for the homogeneity and 12-L-configuration of these compounds. All three peptide derivatives were shown to possess a high level of melanocyte-expanding activity *in vitro*. The biological activity of acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N^{ϵ}-formyllysylprolylvaline amide was found to be essentially the same as that reported for natural α-MSH. The relation between structure and biological activity of these α-MSH derivatives is discussed.

Elucidation of the constitution of α-MSH (I) by Harris and Lerner[4] provided the structural foundation for the intrinsic melanocyte-expanding activity of pure corticotropin which was observed by Bell and collaborators.[5] Indeed, the very tridecapeptide sequence of amino acids which constitutes the molecule of α-MSH makes up the N-terminal third of the corticotropin structure. The molecule of α-MSH contains an acetylated serine at the amino end and terminates with a valine amide group in position 13[6]; the amino group of the N-terminal serine in corticotropin is free.

In an attempt to delineate the minimal structural features endowing a peptide with melanocyte-expanding or corticotropic activity we have completed, some time ago,[7,8] the synthesis of two derivatives of α-MSH possessing structures II and III. These blocked tridecapeptide amides which embody within their molecules the entire structure of α-MSH are endowed with *in vitro* melanocyte-expanding activity to a significant degree.

In the present communication we present detailed experimental procedures for the preparation of compounds II and III and record the synthesis of a new derivative of α-MSH (compound IV) exhibiting a level of biological potency approaching that of α-MSH.

In their, by now classical study, on the constitution of the corticotropin molecule, Bell and collaborators[5] established the L-configuration of the constituent amino acids except alanine, aspartic acid, methionine and tryptophan by microbiological procedures. The structure of α-MSH was determined with extremely small amounts of the hormone and information pertaining to the stereochemical nature of its constituent amino acids is not available, but the all-L configuration seems highly probable. Based on the premise that only a molecule possessing the all-L configuration would possess maximal biological activity (an assumption which remains to be verified experimentally) we have employed in our work only those coupling reactions which minimize the chances for racemization. In addition we have purified extensively and have evaluated critically the homogeneity of all subunits prior to their application to the syntheses of the more complex peptides. Such a course of action can be expected to ensure the highest possible degree of purity of the final products.

Studies relating structure to biological activity of complex polypeptides can have fundamental significance only if the compounds to be tested biologically are of a high degree of homogeneity. The biological evaluation of impure samples is likely to provide misleading results.

The general scheme which we employed in the synthesis of the α-MSH derivatives involved the interaction of an acylpentapeptide azide (subunit A) with a partially blocked octapeptide amide (subunit B). Thus, the coupling of the azide of carbobenzoxyseryltyrosylserylmethionylglutamine (subunit A, R = carbobenzoxy, $R^1 = NH_2$) with histidylphenylalanylarginyltryptophylglycyl - N^{ϵ}-tosyllysylprolylvaline amide[9] (subunit B, R^2 =

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(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) Some of the results reported in this paper were presented at the Atlantic City Meeting of the American Chemical Society in September, 1959.

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