# *Syntheses of Peptides Related to the N-Terminal Structure of Corticotropin. II. Synthesis of Gly-Lys-Pro-Val Sequence*

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The sequence, Gly-Lys-Pro-Val is found at positions 10 to 13 in corticotropin and α-MSH[1]. Boissonnas et al.[2,3] synthesized this sequence in the form of tritylglycyl-ε-carbobenzoxylysylprolylvaline methyl ester from the preceding intermediates, tritylglycyl-ε-carbobenzoxylysine (methyl ester) and (carbobenzoxy-)prolylvaline methyl ester, none of which have yet been obtained in any crystalline states.

In connection with our synthetic studies on the *N*-terminal structure of corticotropin we have now obtained this tetrapeptide sequence in another form, formylglycyl-ε-carbobenzoxylysylprolylvaline benzyl ester.

Formylglycine and ε-carbobenzoxylysine methyl ester were coupled in methylene chloride by the use of *N, N'*-dicyclohexylcarbodiimide to give formylglycyl-ε-carbobenzoxylysine methyl ester which was converted by saponification into formylglycyl-ε-carbobenzoxylysine. Both of these dipeptide derivatives were obtained in good yields and in crystalline states. When the coupling was done in dimethylformamide solution the yield of the desired product was less than 30 per cent and in addition a side product, formylglycyl-*N, N'*-dicyclohexylurea was isolated. The mixed acid anhydride procedure also notably lowered the yield and purity of the product.

Carbobenzoxypropoline was condensed with valine benzyl ester by the mixed anhydride method to carbobenzoxyprolylvaline benzyl ester which was easily crystallized. This was then decarbobenzoxylated with hydrogen bromide in glacial acetic acid and the resulting ester hydrobromide was treated with potassium carbonate to the free base.

Coupling of formylglycyl-ε-carbobenzoxylysine with prolylvaline benzyl ester by *N, N'*-dicyclohexylcarbodiimide yielded the tetrapeptide, formylglycyl-ε-carbobenzoxylysylprolylvaline benzyl ester as an amorphous solid. This product was negative to the ninhydrin test and after deformylation with *N*-methanolic hydrochloric acid gave only one spot on the paperchromatogram sprayed with ninhydrin. The complete acid hydrolysate was subjected to the quantitative amino acid analysis and was shown to be composed of glycine, lysine proline and valine in the molar ratio as expected.

## Experimental

All amino acids used are of L-configuration. The melting point was determined in a capillary tube in a sulfuric acid bath and not corrected.

**Formylglycine.**—This was prepared after the usual procedure for DL-formylamino acids of Sheehan and Yang[4]. 3.75 g. of glycine was formylated and the product was recrystallized from water to give 4.20 g. (81.6%), m. p. 149°C with decomposition.

Found: C, 35.12; H, 4.99; N, 13.35. Calcd. for $C_3H_5O_3N$: C, 35.0; H, 4.89; N, 13.6%.

**Formylglycyl-ε-carbobenzoxylysine Methyl Ester.**—To a suspension of 9.10 g. (0.0275 mol.) of ε-carbobenzoxylysine methyl ester hydrochloride[5] in ether was added 23 ml. of ice-cold 50% potassium carbonate. The mixture was shaken and the organic layer separated was dried over sodium sulfate at 0°C. After removal of the solvent in vacuo the oily residue was dissolved in 115 ml. of methylene chloride and 2.58 g. (0.025 mol.) of formylglycine was added. As soon as most of the formylglycine had dissolved, 5.16 g. (0.025 mol.) of *N, N'*-dicyclohexylcarbodiimide in 25 ml. of methylene chloride was quickly added. The mixture was stirred for five hours and allowed to stand overnight at room temperature and refrigerated. The separated dicyclohexylurea was collected by filtration, yielding 5.45 g. (97.2%). The filtrate was cooled at 0°C, washed successively with ice-cold 1 N hydrochloric acid, water, 5% sodium bicarbonate and water and dried over sodium sulfate. Evaporation of the solvent in vacuo gave a clear syrup which was crystallized from methylene chloride-ether. Yield 8.93 g. (94.3 %). This was recrystallized from ethyl acetate-ether to give 8.40 g. (88.7%), m. p. 76~78°C, $[\alpha]_D^{21} = -10.3 \pm 2°$ (*c* 2.901 in methanol).

Found: C, 57.02; H, 6.81; N, 10.89. Calcd. for $C_{18}H_{25}O_6N_3$: C, 57.1; H, 6.60; N, 11.0%.

When dimethylformamide was used as a solvent for this coupling reaction there was obtained, in

1) See the References of the preceding paper of this work. This Bulletin, **34**, 1 (1961).

2) R. A. Boissonnas, St. Guttmann, J. -P. Waller and P. -A. Jaquenoud, *Experientia*, **12**, 446 (1956).

3) R. A. Boissonnas, St. Guttmann, R. L. Huguenin, P. -A. Jaquenoud and Ed. Sandrin, *Helv. Chim. Acta*, **41**, 1867 (1958).

4) J. C. Sheehan and D-D. H. Yang, *J. Am. Chem. Soc.*, **80**, 1154 (1958).

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

addition to the desired peptide (in a 30% yield), a side product, formylglycyl-*N,N'*-dicyclohexylurea, m. p. 164.5~165.5°C.

Found: C, 62.46; H, 8.97; N, 13.69. Calcd. for $C_{16}H_{27}O_3N_3$: C, 62.2; H, 8.81; N, 13.6%.

**Formylglycyl-ε-carbobenzoxylysine.**—To a solution of 3.79 g. (0.01 mol.) of formylglycyl-ε-carbobenzoxylysine methyl ester in 27.5 ml. of dioxane was added 11 ml. of 1 N sodium hydroxide and the mixture was stirred at room temperature for one hour. After removal of a slight amount of insoluble precipitates the solution was diluted with water to about 80 ml., neutralized with 11 ml. of ice-cold 1 N hydrochloric acid and concentrated to about 25 ml. under reduced pressure. The concentrate was stored at 0°C overnight to complete the separation of crystals, 3.47 g. (95.1%). Recrystallization from water gave 3.28 g. (89.8%), m. p. 144~146°C, $[\alpha]_D^{24}=+13.1\pm0.2°$ (*c* 1.358 in methanol).

Found: C, 56.27; H, 6.33; N, 11.56. Calcd. for $C_{17}H_{23}O_6N_3$: C, 56.0; H, 6.35; N, 11.5%.

**Carbobenzoxyproline**[6]. — 4.6 g. (0.04 mol.) of proline was carbobenzoxylated with 8 g. of benzylchloroformate in 30 ml. of 2 N sodium hydroxide at 0°C. When the reaction was over the solution was washed with ether to remove an excess of benzylchloroformate and acidified. The product was taken up into ethyl acetate and the extract was shaken with 5% sodium bicarbonate. The aqueous solution was acidified and reextracted with ethyl acetate. The organic extract was dried over sodium sulfate and concentrated in vacuo to a syrup which was crystallized from carbon tetrachloride-petroleum ether (1 : 2) to give 9.47 g. (95.3%), m. p. 75~78°C. Recrystallization from the same solvent afforded 9.30 g., m. p. 76~78°C, $[\alpha]_D^{29}=-57.7\pm2°$ (*c* 0.975 in glacial acetic acid) (in lit.[6], m. p. 76~77°C, $[\alpha]_D^{20}=-61.7°$ (*c* 5.3 in glacial acetic acid).

Found: C, 62.98; H, 6.05; N, 5.89. Calcd. for $C_{13}H_{15}O_4N$: C, 62.75; H, 6.08; N, 5.63%.

**Valine Benzyl Ester Hydrochloride.**—A mixture of 4 g. of valine and 10 g. of *p*-toluenesulfonic acid monohydrate in 40 ml. of benzyl alcohol was stirred on an oil bath at 100°C (internal temperature). To this solution were added 40 ml. portions of carbon tetrachloride at one hour intervals and distilled out from a side-arm. After 8 hr. the remaining carbon tetrachloride was completely removed under reduced pressure and the crude ester *p*-toluenesulfonate was precipitated with the addition of ether, 10.0 g., m. p. 157~159°C. 10.0 g. of the crystal obtained above was suspended in 150 ml. of ether and mixed with 40 ml. of ice-cold 50% potassium carbonate. The insoluble precipitate was filtered off and the ether layer was dried over sodium sulfate at 0°C. Dry hydrogen chloride gas was bubbled into this ether solution to separate the desired ester hydrochloride, 6.3 g., m. p. 137~139.5°C. Recrystallization from chloroform-ether afforded 6.1 g., m. p. 139~141°C, $[\alpha]_D^{29}=-12.7\pm4°$ (*c* 0.495 in 0.1 N hydrochloric acid).

Found: N, 5.76; Cl, 14.50. Calcd. for $C_{12}H_{18}O_2NCl$: N, 5.75; Cl, 14.60%.

6) A. Berger, J. Kurtz and E. Katchalski, *J. Am. Chem. Soc.*, **76**, 5552 (1954).

**Carbobenzoxyprolylvaline Benzyl Ester.**—To a mixture of 2.49 g. (0.01 mol.) of carbobenzoxyproline and 2.39 ml. (0.01 mol.) of tri-*n*-butylamine in 10 ml. of tetrahydrofuran was added 0.96 ml. (0.01 mol.) of ethylchloroformate at −10°C. After 15 min. a solution of 2.44 g. (0.01 mol.) of valine benzyl ester hydrochloride and 2.39 ml. (0.01 mol.) of tri-*n*-butylamine in 10 ml. of tetrahydrofuran was introduced and the reaction mixture was then stirred at room temperature for 5 hr. and concentrated under reduced pressure. The syrupy residue was dissolved in 35 ml. of ethyl acetate, successively washed with 1 N hydrochloric acid, 15% sodium chloride, 5% sodium bicarbonate, and with 15% sodium chloride, and dried over sodium sulfate. The ethyl acetate solution was concentrated to about 10 ml. in vacuo and to this was added petroleum ether to separate an oily precipitate which was soon crystallized in needles, 4.0 g. (91.3%), m. p. 87~91°C. Recrystallization from the same solvent gave thin needles, m. p. 91~92°C, $[\alpha]_D^{29}=-78.6\pm1°$ (*c* 1.749 in methanol).

Found: C, 68.40; H, 6.96; N, 6.37. Calcd. for $C_{25}H_{30}O_5N_2$: C, 68.4; H, 6.90; N, 6.39%.

**Prolylvaline Benzyl Ester.**—2.63 g. (0.006 mol.) of carbobenzoxyprolylvaline benzyl ester was dissolved in 15 ml. of 21.2% (w/w) hydromromic acid/glacial acetic acid and the mixture was allowed to stand at room temperature for 50 min. at the end of which time about 80 ml. of anhydrous ether was added. The supernatant was decanted and an oily residue was dissolved in 10 ml. of water and washed twice with ether. A small aliquot of this aqueous solution was chromatographed on paper in the system of *n*-butanol-acetic acid-water (4 : 1 : 2) and there were detected two components which gave yellow spots with ninhydrin. The minor component with the lower $R_f$ value is possibly due to prolylvaline, and it may be found that the benzyl ester is cleaved to some extent under the conditions used, but no other side reactions take place.

The aqueous solution of the crude ester hydrobromide was then concentrated to a small volume in vacuo and shaken with 20 ml. of ether and 10 ml. of 50% potassium carbonate at −10°C. The aqueous phase was extracted with additional ether. The combined ether extract was dried over sodium sulfate at 0°C and evaporated in vacuo to an oil, 1.47 g. which was directly used for the following coupling reaction.

**Formylglycyl-ε-carbobenzoxylysylprolylvaline Benzyl Ester.**—The prolylvaline benzyl ester obtained above was dissolved in 30 ml. of methylene chloride and 1.28 g. (0.0035 mol.) of formylglycyl-ε-carbobenzoxylysine was adedd. The mixture was stirred at 0°C to give a homogeneous but somewhat cloudy solution in 15 min. To this solution was then added 0.723 g. (0.0035 mol.) of *N,N'*-dicyclohexylcarbodiimide and the reaction mixture was stirred at room temperature for five hours and allowed to stand overnight at 4°C. Separated dicyclohexylurea was removed by filtration, 0.739 g. (94.3%) and the filtrate was concentrated in vacuo. A syrupy residue was dissolved in ethyl acetate and washed with ice-cold 1 N hydrochloric acid, water, 5% sodium bicarbonate and finally with water and

dried over sodium sulfate. Concentration of the ethyl acetate solution in vacuo gave a foamy residue which was dried over phosphorus pentoxide at room temperature in high vacuum to afford 2.22 g. (97.5%) as colorless powder, $[\alpha]_D^{18} = -62.2 \pm 0.1°$ ($c$ 2.505 in methanol).

Found: C, 62.13; H, 7.43; N, 10.54. Calcd. for $C_{34}H_{45}O_8N_5$: C, 62.7; H, 6.97; N, 10.7%.

This product gave a negative ninhydrin test and after treatment with 1N methanolic hydrochloric acid for 16 hr. only one spot[7] was visible on a paper-chromatogram which was developed in *n*-butanol-acetic acid-water (4 : 1 : 2) and sprayed with ninhydrin.

A sample of the tetrapeptide derivative was hydrolyzed with 6N hydrochloric acid at 100°C for 20 hr. and the hydrolysate was subjected to quantitative amino acid analysis by the method of Levy[8]. It was found that the product had a composition of Gly/Lys/Pro/Val in a molar ratio of 1.0/1.1/0.9/1.1.

7) The color of this spot changed with time from yellow to purple. Such a phenomenon has often been observed in glycylpeptides (K. Narita, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **75**, 487 (1954)).

## Summary

Formylglycyl-ε-carbobenzoxylysylprolylvaline benzyl ester, which has a sequence occurring in corticotropin and α-MSH, has been synthesized.

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8) A. L. Levy, *Nature*, **174**, 126 (1954).