Studies on Serine Peptides. IV. Synthesis of L-Seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic Acid with Strepogenin Activity

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A peptide having strepogenin activity was recently isolated by R. Merrifield and D. Woolley from a partial hydrolysate of beef insulin and its structure was established to be as L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid[1,2].

In a previous paper[3], the author reported that L-seryl-L-histidyl-L-leucine was synthesized from Cbzo-O-Bz-L-seryl-L-histidine and L-leucine methyl ester in good yields by Sheehan's method[4], followed by the elimination of protecting groups with hydrogen bromide in dioxane.

In the present experiment, the pentapeptide was synthesized by the condensation of Cbzo-O-Bz-L-seryl-L-histidine with L-leucyl-L-valyl-L-glutamic acid diethyl ester, followed by the removal of the protecting groups in the same way as above.

During the course of the present work, Woolley and Merrifield reported[5] that the synthesis of this peptide had the same strepogenin activity as that of the native one. The yields in all coupling reactions of these authors' are less than ours.

The synthetic pentapeptide showed strepogenin activity in the biological test using *L. casei**. By addition of zinc acetate to an aqueous solution of the pentapeptide, an insoluble zinc-complex was readily formed.

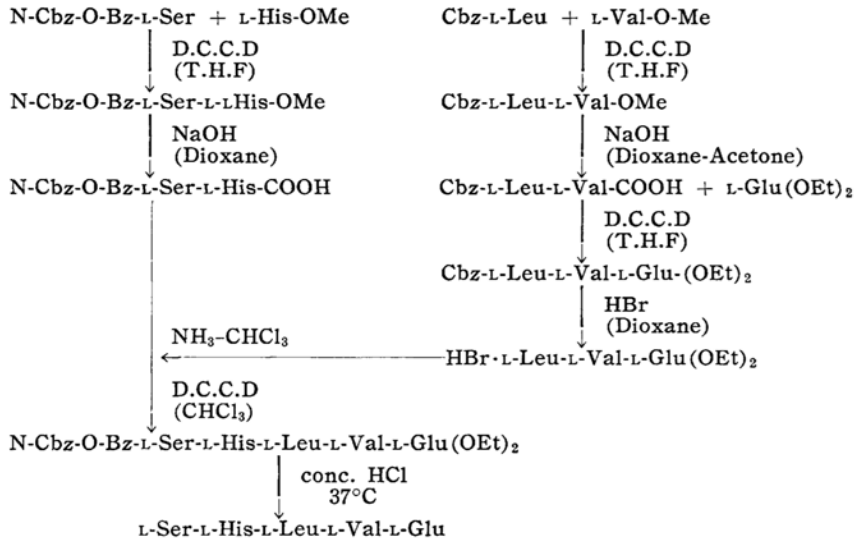
The reaction sequences were the following. Cbzo-L-leucyl-L-valine was prepared by coupling Cbzo-L-leucine with L-valine methyl ester, followed by hydrolysis of Cbzo-peptide methyl ester produced. Then the product was coupled with glutamic acid diethyl ester to give Cbzo-L-leucyl-L-valyl-L-glutamic acid diethyl ester. Then the Cbzo-group was removed by dry hydrogen bromide in dioxane and the tripeptide ester hydrobromide was obtained. Cbzo-O-Bz-L-seryl-L-histidine was prepared by the same method as described in the previous paper[4]. This Cbzo-dipeptide was coupled with the above tripeptide ester in chloroform by means of dicyclohexylcarbodiimide to give Cbzo-O-Bz-L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid diethyl ester in 80% yield. It seems that the good yield of this last stage is due to the high solubility of Cbzo-O-Bz-L-seryl-L-histidine in the organic solvent. The hydrolysis of the ester group by the usual method was unsuccesful, as Woolley pointed out[6], owing to low solubility of this peptide derivative in dioxane or acetone-water system. The desired free pentapeptide was obtained after removal of the protecting groups by treatment of Cbzo-Bz-L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid diethyl ester with concentrated hydrochloric acid at 37°C for 80 minutes.

Experimental

Cbzo-L-leucyl-L-valine.—L-Valine methyl ester hydrochloride (11.5 g.) in 40 cc. of dry tetrahydrofurane was treated with 43 cc. of 1.66 N-ammonia—chloroform. After removal of ammonium chloride, excess of ammonia and chloroform was evaporated in vacuo at 40°C, and the residue was mixed with dicyclohexylcarbodiimide (14.17 g.) and Cbzo-L-leucine (18.25 g.) in 20 cc. of tetrahydrofurane at room temperature. Immediately after mixing, dicyclohexylurea deposited in the above reaction mixture. After the product being kept overnight at room temperature, acetic acid (1 cc.) was added to decompose the unchanged dicyclohexylcarbodiimide. The mixture was allowed to stand at room temperature for an hour and the precipitate produced was filtered off. Then, tetrahydrofurane was replaced by ethyl acetate and the solution was washed with 0.1 N hydrochloric acid, 1% sodium bicarbonate and water successively, dried over anhydrous sodium sulfate and concentrated in vacuo. The syrupy product was saponified with N-sodium hydroxide in dioxane-acetone (about 14 : 1 v/v) solution and Cbzo-L-leucyl-L-valine was extracted twice with ethyl acetate. The extract was dried over anhydrous sodium sulfate and then concentrated in vacuo. Addition of petroleum ether

1) R. B. Merrifield and D. W. Woolley, *Arch. Biochem. Biophys.*, **56**, 265 (1955).
2) R. B. Merrifield and D. W. Woolley, *J. Am. Chem. Soc.*, **78**, 358 (1956).
3) K. Okawa, Part III This Bulletin, **30**, 976 (1957).
4) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
5) R. B. Merrifield and D. W. Woolley, ibid., **78**, 4646 (1956).
* L. casei; (Bioassay).
6) R. B. Merrifield and D. W. Woolley, ibid., **78**, 4646 (1956).

TABLE I



T.H.F; Tetrahydrofurane D.C.C.D; Dicyclohexylcarbodiimide

gave 15 g. (65.2%) of white crystals. The product was recrystallized from ethyl acetate-petroleum ether, yield 13.5 g. (59%), m.p. 107—108°C. $[\alpha]_D^{13} = -18.52$ (10 mg./cc. EtOH)[7].

Anal. Found: C, 62.88; H, 7.38; N, 7.81. Calcd. for $C_{19}H_{23}O_5N_2$: C, 62.60; H, 7.77; N, 7.68%.

Cbzo-L-leucyl-L-valyl-L-glutamic Acid Diethyl Ester.—L-Glutamic acid diethyl ester from L-glutamic acid diethyl ester hydrochloride (4.83 g.) was coupled with Cbzo-L-leucyl-L-valine (6.69 g.) in 20 cc. of tetrahydrofurane by the same procedure as described above. Dicyclohexylurea was precipitated more slowly in this case than in the above. After standing overnight, Cbzo-L-leucyl-L-valyl-L-glutamic acid diethyl ester was precipitated together with dicyclohexylurea since it was difficultly soluble in tetrahydrofurane. Then Cbzo-tripeptide ester was extracted with hot ethyl acetate. After evaporation of ethyl acetate, the precipitate was recrystallized from hot ethylacetate. The yield was 6.5 g. (68%), m. p. 178—179°C. $[\alpha]_D^{13} = -41.1$ (15 mg./cc. acetic acid).

Anal. Found: C, 61.16; H, 7.39; N, 7.85. Calcd. for $C_{28}H_{43}O_8N_3$: C, 61.18; H, 7.70; N, 7.65%.

L-Leucyl-L-valyl-L-glutamic Acid Diethyl Ester-HBr.—Cbzo-tripeptide ester was suspended in dry dioxane (10 g.) into which dry hydrogen bromide was passed through until saturation was completed. After an hour, absolute ether (150 cc.) was added to the reaction mixture and the ether solution was concentrated in vacuo. The residual syrup was dissolved in absolute ethanol and the solvent was distilled off in vacuo. After the white residue was recrystallized from absolute ethanol and absolute ether, 1.7 g. of the hydrobromide was obtained in 90% yield. m. p. 220°C. $[\alpha]_D^{13} = -30.46$ (20 mg./cc. ethanol).

Anal. Found: C, 48.20; H, 7.48; N, 8.70. Calcd. for $C_{20}H_{38}O_6N_3Br$: C, 48.38; H, 7.66; N, 8.46%.

Cbzo-O-Bz-L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic Acid Diethyl Ester.—To the solution of Cbzo-O-Bz-L-seryl-L-histidine (1.0 g.) in 25 cc. of dioxane, was added 50 cc. of the dioxane solution of the tripeptide ester which was prepared from the hydrobromide (1.2 g.) using the chloroform-ammonia solution, and dicyclohexylcarbodiimide (0.5 g.). After the solution was left overnight at room temperature, acetic acid (0.2 cc.) was added to the mixture, which was allowed to stand for an hour. The reaction mixture was concentrated in vacuo, the residual precipitate was dissolved in chloroform and the urea derivative produced was filtered off. The filtrate was washed with dilute acid, but the aqueous layer was not separated from the organic layer. After chloroform was distilled off, the precipitate was filtered and washed with aq. solution of sodium bicarbonate. The precipitate was recrystallized from hot ethanol; 1.5 g. of crystals was obtained in 82.3% yield. m. p. 192—3°C. $[\alpha]_D^{13} = -34.5$ (10 mg./cc. acetic acid).

Anal. Found: C, 59.91; H, 6.94; N, 11.25. Calcd. for $C_{44}H_{61}O_{11}N_7$: 61.11; H, 7.06; N. 11.34%.

L-Seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic Acid.—The above Cbzo-peptide ester (0.9 g.) was treated with 50 cc. of 12 N hydrochloric acid at 37°C for 80 min. After thirty min. Cbzo-peptide ester was almost dissolved and slight turbidity appeared. At the end of the reaction period, the solution was dried over sodium hydroxide in a vaccum desiccator. The residual solid was dissolved in 10 cc. of water and the insoluble product was filtered off. The filtrate was treated with triethylamine and brought to pH 6.8. After

7) E. L. Smith, D. H. Spackman and W. J. Polglase, *J. Biol. Chem.*, **199**, 801 (1952).

concentration in vacuo, the residual product was washed with ethanol. The crude pentapeptide (0.4 g.) was obtained, in 70%. The product was dissolved in a small amount of water and 5 times as great an amount of alcohol was added; the mixture was allowed to stand in an ice-box until crystallization was completed. Recrystallization was repeated four times in the same way. Paper chromatography of this synthetic pentapeptide in butanol-acetic acid-water (4 : 1 : 1) gave one spot with R_f 0.53. Paper chromatography of an acid hydrolysate of the synthetic peptide gave approximately equimolecular amounts of five spots corresponding to serine, histidine, valine, leucine and glutamic acid. m. p. 233—5°C. $[\alpha]_D^{13} = -52.3$ (10 mg./cc. water).

Anal. Found: C, 50.88; H, 7.02; N, 16.50. Calcd. for $C_{25}H_{41}O_9N_7$: C, 51.45; H, 7.03; N, 16.80%.

Biological Activity. — The synthetic pentapeptide was shown to have strepogenin activity three times as great as that of the casein hydrolysate. With regard to both the peptides, L-seryl-L-histidine and L-seryl-L-histidyl-L-leucine, activity was detected.

Zinc Complex of Pentapeptide.—A zinc complex was readily formed in an aqueous solution of the pentapeptide by adding equimolecular zinc acetate. The analytical value of this product varied according to the reaction condition, a inly concentration of the solution. One of these data suggests the under signed formula.

Anal. Found: N, 12.78; Zn, 0.81. Calcd. for $C_{29}H_{47}O_{11}N_7Zn$: N, 12.78; Zn, 0.85%.

Summary

L-Seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid having strepogenin activity has been synthesized from O-Bz-L-serine as a starting material in a good yield by Sheehan's dicyclohexylcarbodiimide method.

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