Secretin. The Synthesis of a "Wrong" Hexapeptide Amide, Sequence 22–27*

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ABSTRACT: The hexapeptide amide, L-leucyl-L-glutaminyl-L-leucylglycyl-L-leucyl-L-valinamide (V), has been synthesized.

A comparison of this compound with the previously prepared synthetic hexapeptide amide, L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (VI), and with the natural C-terminal tryptic hexapeptide fragment from natural secretin demonstrates that the C-terminal hexapeptide amide does not have the alternative structure V.

The intestinal hormone (porcine) secretin was isolated in pure form by Jorpes and Mutt (1961). The amino acid composition of the hormone (Jorpes *et al.*, 1962), its partial structure (Mutt *et al.*, 1965), and most recently its complete amino acid sequence (Mutt and Jorpes, 1966) have been established. The stepwise synthesis of a tetradecapeptide amide (Bodanszky and Williams, in preparation) and a heptacosapeptide amide having the hormonal activity of secretin (Bodanszky *et al.*, 1966) have been recently communicated from this laboratory. Although the C-terminal sequence 22–27, L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (VI, "right" hexapeptide), was supported by strong evidence (J. E. Jorpes and V. Mutt, 1966, personal communications), an alternative hexapeptide sequence, L-leucyl-L-glutaminyl-L-leucylglycyl-L-leucyl-L-valinamide (V, "wrong" hexapeptide) could not be vigorously excluded. The synthetic "right" hexapeptide VI was indistinguishable on paper chromatograms (Bodanszky and Williams, in preparation; Bodanszky *et al.*, 1966) from the corresponding tryptic fragment from porcine secretin (Mutt *et al.*, 1965); however, this does not prove them to be identical. In order to settle this question, the synthesis of the "wrong" hexapeptide amide was undertaken by the active ester method (Bodanszky, 1955) employing the stepwise strategy (Bodanszky, 1960) from the previously prepared (Bodanszky and Williams, 1966) protected tripeptide, Z-glycyl-L-leucyl-L-valinamide[1] (I).

Treatment of I with hydrobromic acid in acetic acid gave the hydrobromide which was acylated with Z-L-leucine-ONP (Bodanszky and du Vigneaud, 1959) to give Z-L-leucylglycyl-L-leucyl-L-valinamide (II). Removal of the benzyloxycarbonyl group from II gave a hydrobromide which was treated with Z-L-glutamine-ONP (Bodanszky and du Vigneaud, 1959) to afford Z-L-glutaminyl-L-leucylglycyl-L-leucyl-L-valinamide (III). Reaction of Z-L-leucine-ONP with the hydrobromide obtained by decarbobenzoxylation of III gave the protected hexapeptide amide, Z-L-leucyl-L-glutaminyl-L-leucylglycyl-L-leucyl-L-valinamide (IV). The free "wrong" hexapeptide amide (V) was obtained by hydrogenolysis of IV in acetic acid and evaporation of the acetic acid from the frozen state.

The synthetic peptides of both the "right and "wrong" series were then compared (R_F) in two systems (Table I).

TABLE I: Paper Chromatography of the "Right" (R) and "Wrong" (W) Tetra-, Penta-, and Hexapeptide Amides.

Peptide	R_F Values	
	System A[a]	System B[b]
(W) Z-Leu-Gly-Leu-Val-NH_2	0.85[c,d]	0.83
(R) Z-Gln-Gly-Leu-Val-NH_2	0.50	0.53
(W) Z-Gln-Leu-Gly-Leu-Val-NH_2	0.73	0.77
(R) Z-Leu-Gln-Gly-Leu-Val-NH_2	0.64	0.68
(W) Z-Leu-Gln-Leu-Gly-Leu-Val-NH_2	0.77	0.85
(R) Z-Leu-Leu-Gln-Gly-Leu-Val-NH_2	0.79	0.89
(W) Leu-Gln-Leu-Gly-Leu-Val-NH_2	0.83	0.86
(R) Leu-Leu-Gln-Gly-Leu-Val-NH_2	0.83	0.86

[a] 1-Butanol–acetic acid–water (4:1:5) (Partridge, 1948) on Whatman No. 1 paper (descending chromatography). [b] 1-Butanol–pyridine–acetic acid–water (30:20:6:24) (Waley and Watson, 1953) on Whatman No. 42 paper (descending chromatography). [c] The R_F values of the protected peptides were determined after decarbobenzoxylation using HBr–HOAc. [d] The peptides were detected with ninhydrin.

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[1] Abbreviations used: Z, benzyloxycarbonyl; ONP, *p*-nitrophenyl; DMF, dimethylformamide; TEA, triethylamine.

that the "right" and "wrong" hexapeptides areindistinguishable from each other by chromatography.

The two hexapeptides could likewise not be separated electrophoretically (pH 1.9, formic acid–acetic acid buffer). An attempt to differentiate the hexapeptides by partial hydrolysis with hydrochloric acid at room temperature was also unsuccessful; both gave identical patterns of products on paper chromatograms. On the other hand, the volatile ninhydrin-positive components from the pyrolysis (*in vacuo*) of the hexapeptides were different on paper chromatograms, and their nonidentity was proved by partial degradation with a proteolytic enzyme. Samples of the C-terminal hexapeptide tryptic fragment from natural secretin, the synthetic "right" hexapeptide (VI), and the synthetic "wrong" hexapeptide (V) were treated with a microbial proteolytic enzyme (Novo's Bacterial Proteinase) and the products were examined by paper chromatography in two different solvent systems (V. Mutt, 1966, personal communication). The products from the natural fragment and the synthetic "right" hexapeptide were identical and different from the products of the synthetic "wrong" hexapeptide. Thus, these results provide additional evidence for the amino acid sequence 22–27 of procine secretin.

Experimental Section[2]

*Benzyloxycarbonyl-*L*-leucylglycyl-*L*-leucyl-*L*-valinamide* (*II*). The protected tripeptide, benzyloxycarbonylglycyl-L-leucyl-L-valinamide (I, 0.90 g) was suspended in acetic acid (5 ml) and treated with hydrobromic acid in acetic acid (*ca.* 4 N, 5 ml). After 2 hr at room temperature with stirring, ether (80 ml) was added. The precipitated hydrobromide was collected by filtration, washed with ether, and dried *in vacuo* over sodium hydroxide. The hydrobromide was dissolved in DMF (6 ml) and TEA (0.8 ml) was added followed by benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester (1.0 g). After standing overnight at room temperature, the reaction mixture was diluted with ethyl acetate (75 ml) and filtered, and the precipitate was washed with chloroform (25 ml). The product was dried *in vacuo*, 1.1 g, mp *ca.* 190°, Beilstein positive. This material was extracted with hot chloroform (20 ml), filtered, and washed with warm chloroform (10 ml) to give II (0.82 g, mp 226–228°). Repetition of this extraction on a small sample gave the analytical sample, mp 228–230°, $[\alpha]_D^{27}$ −32° (*c* 1, AcOH).

Anal. Calcd for $C_{27}H_{43}N_5O_6$: C, 60.8; H, 8.1; N, 13.1. Found: C, 60.9; H, 8.2; N, 13.2.

*Benzyloxycarbonyl-*L*-glutaminyl-*L*-leucylglycyl-*L*-leucyl-*L*-valinamide* (*III*). The protected tetrapeptide (II, 0.82 g) was suspended in acetic acid (4 ml) and treated with hydrobromic acid in acetic acid (*ca.* 4 N, 4 ml). After 1.5 hr at room temperature with stirring, ether (75 ml) was added. The hydrobromide was collected by filtration, washed with ether, and dried *in vacuo* over sodium hydroxide. The hydrobromide was dissolved in DMF (6 ml) and TEA (0.6 ml) was added followed by benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester (0.75 g). After standing overnight at room temperature, chloroform (60 ml) was added and the precipitate was filtered and washed with ethyl acetate–chloroform (1:1, 30 ml). The product was dried *in vacuo* at 45° to give III (0.86 g, mp 262–263° dec). The analytical sample was prepared by recrystallization from 90% ethanol, mp 267–270° dec, $[\alpha]_D^{27}$ −35° (*c* 1, AcOH).

Anal. Calcd for $C_{32}H_{51}N_7O_8$: C, 58.1; H, 7.8; N, 14.8. Found: C, 58.5; H, 8.1; N, 14.8.

*Benzyloxycarbonyl-*L*-leucyl-*L*-glutaminyl-*L*-leucylglycyl-*L*-leucyl-*L*-valinamide* (*IV*). The protected pentapeptide (III, 0.49 g) was suspended in acetic acid (4 ml) and treated with hydrobromic acid in acetic acid (*ca.* 6 N, 3 ml). After 2 hr at room temperature with stirring, ether (75 ml) was added. The hydrobromide was collected by filtration, washed with ether, and dried *in vacuo* over sodium hydroxide. The hydrobromide was dissolved in DMF (6 ml), and TEA (0.4 ml) was added followed by benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester (0.50 g). After standing overnight at room temperature, the solid mass was disintegrated with chloroform–ethyl acetate (1:1, 30 ml), filtered, and washed with the same mixture (30 ml) to give after drying *in vacuo* IV (0.57 g, mp 272–274° dec). The analytical sample was prepared by recrystallization from 85% ethanol, mp 283–285° dec, $[\alpha]_D^{27}$ −47° (*c* 1.3, AcOH).

Anal. Calcd for $C_{38}H_{62}N_8O_9$: C, 58.9; H, 8.1; N, 14.4. Found: C, 58.7; H, 8.0; N, 14.4.

L-Leucyl-L-glutaminyl-L-leucylglycyl-L-leucyl-L-valinamide (*V*). The protected hexapeptide IV (0.23 g) was dissolved in acetic acid (8 ml) by gentle warming. The solution was cooled to room temperature and stirred in a hydrogen atmosphere in the presence of a 10% palladium-on-charcoal catalyst (0.12 g) for 4.25 hr. Removal of the catalyst by filtration was followed by evaporation of the acetic acid from the frozen state to give V (0.20 g, mp 252–260° dec). For analysis a sample was suspended in ethyl acetate, filtered, washed with 95% ethanol, and dried *in vacuo* at 110° for 2.5 hr. This material contained less than 0.1% acetic acid (titration), mp 253–260° dec, $[\alpha]_D^{27}$ −35° (*c* 1.3, AcOH).

Anal. Calcd for $C_{30}H_{56}N_8O_7$: C, 56.2; H, 8.8; N, 17.4. Found: C, 55.6; H, 8.9; N, 71.1.

After hydrolysis with constant-boiling hydrochloric acid at 110° for 22 hr *in vacuo*, a sample showed the following ratios of amino acids: Glu, 1.0; Gly, 1.0; Val, 1.0; Leu, 2.9; NH_3, 2.0.

Pyrolysis of Hexapeptide Amides V and VI. The samples (*ca.* 7 mg) were heated to 238–239° at 0.03 mm and kept at this temperature for 0.5 hr. The volatile material which deposited along the walls of the sublimation tube was dissolved in 0.5 ml of 90% ethanol and paper chromatographed (system A, Table I).

HEXAPEPTIDE AMIDE V showed two strong spots with ninhydrin, R_F 0.4 and 0.58. (Leucine had the same

[2] Melting points were taken in capillary tubes and are uncorrected.

R_F in this system.) Some yellow ultraviolet-absorbing material was detected near the front.

HEXAPEPTIDE AMIDE VI showed two strong spots with ninhydrin, R_F 0.73 and 0.81. Two slower developing (ninhydrin) spots appeared, R_F 0.58 (trace) and 0.68. Some ultraviolet-absorbing material was detected near the front.

References

Bodanszky, M. (1955), *Nature 175*, 685.
Bodanszky, M. (1960), *Ann. N. Y. Acad. Sci. 88*, 655.
Bodanszky, M., and du Vigneaud, V. (1959), *J. Am. Chem. Soc. 81*, 5688.
Bodanszky, M., Ondetti, M. A., Levine, S. D., Narayanan, V. L., von Saltza, M., Sheehan, J. T., and Williams, N. J. (1966), presented at the 4th International Symposium on the Chemistry of Natural Products, Stockholm, June 28, 1966; *Chem Ind. (London)* (in press).
Jorpes, J. E., and Mutt, V. (1961), *Acta Chem. Scand. 15*, 1790.
Jorpes, J. E., Mutt, V., Magnusson, S., and Steele, B. B. (1962), *Biochem. Biophys. Res. Commun. 9*, 275.
Mutt, V., and Jorpes, J. E. (1966), presented at the 4th International Symposium on the Chemistry of Natural Products, Stockholm, June 28, 1966.
Mutt, V., Magnusson, S., Jorpes, J. E., and Dahl, E. (1965), *Biochemistry 4*, 2358.
Partridge, S. M. (1948), *Biochem. J. 42*, 238.
Waley, S. G., and Watson, G. (1953), *Biochem. J. 55*, 328.

Isolation and Amino Acid Sequences of Glycopeptides Obtained from Bovine Fibrinogen*

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ABSTRACT: After exhaustive pronase digestion of bovine fibrinogen, the resultant glycopeptides were purified and fractionated by gel filtration and ion-exchange chromatography. Three purified fractions were obtained which contained up to 80% of the total carbohydrate of fibrinogen. The amino acid sequences of the three fractions are Asp-Lys, Gly-Glu-Asp-Arg, and Glu-Asp-Arg.

Fibrinogen is a glycoprotein composed of six polypeptide subunits with a total molecular weight of about 340,000. The carbohydrate components comprise about 3% of the total weight of the molecule; the monosaccharide residues include *N*-acetylglucosamine, galactose, mannose, and *N*-acetylneuraminic acid (Davie and Ratnoff, 1965). Our interest in the carbohydrate components of fibrinogen was stimulated initially by suggestions that these constituents might be involved in the fibrinogen–fibrin conversion. For example, several laboratories have reported that about 20% of the carbohydrate content of fibrinogen is released during its conversion to urea-insoluble fibrin (Bagdy and Szara, 1955; Blombäck, 1958; Chandrasekhar *et al.*, 1962; Chandrasekhar and Laki, 1964; Laki, 1951; Szara and Bagdy, 1953), although this has been disputed (Hörmann and Gollwitzer, 1964; Raisys *et al.*, 1966; Rosenberg and Carman, 1964). In addition, it has been reported that oxidation of the carbohydrate components of fibrinogen by periodate results in a loss of clottability by thrombin (Laki and Mester, 1952).

One approach which has been employed by several laboratories in the study of the carbohydrate components of fibrinogen or fibrin has entailed proteolytic digestion followed by purification of the resultant glycopeptide fractions (Cynkin and Haschemeyer, 1964; Haschemeyer and Cynkin, 1964; Lipinski, 1964; Mester *et al.*, 1963a,b, 1965; Mester and Moczar, 1965; Mészáros, 1964). In most of these studies, the preparations were heterogeneous with respect to amino acid composition. In the present study, pronase digestion followed by ion-exchange column chromatography has led to the isolation of three major glycopeptide fractions whose amino acid sequences have been determined.

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