Synthesis and Some Biological Properties of 4-Valine-oxytocin and 1-Deamino-4-valine-oxytocin*

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SUMMARY

4-Valine-oxytocin and 1-deamino-4-valine-oxytocin, analogues of the posterior pituitary hormone containing a valine residue in place of the glutamine residue in position 4, have been synthesized by the p-nitrophenyl ester method of peptide synthesis and tested for pharmacological activity. The 4-valine-oxytocin possesses 140 units per mg of oxytocic activity and 240 units per mg of avian vasodepressor activity. The 1-deamino-4-valine-oxytocin, in which the free amino group on the half-cystine residue in position 1 of 4-valine-oxytocin is replaced by hydrogen, is a highly potent compound, possessing approximately 350 units of oxytocic activity and 800 units of avian vasodepressor activity per mg.

As part of the study of the contribution of the various functional groups of the posterior pituitary hormone, oxytocin, to its biological activities, the carboxamide group of the glutamine residue was replaced with hydrogen through the synthesis of 4-α-aminobutyric acid-oxytocin (4-decarboxamido-oxytocin) (1). This analogue proved to have a fairly high degree of biological activity, possessing, for example, 72 units per mg of oxytocic activity and 108 units per mg of avian vasodepressor activity. Oxytocin, the structure of which is shown in Fig. 1, possesses approximately 500 units per mg of each of these activities (2).

We have now found that the formal substitution of a methyl group for 1 of the hydrogens on the α carbon of the α-aminobutyric acid residue in position 4 of 4-decarboxamido-oxytocin leads to a considerable increase in potency. This new analogue, 4-valine-oxytocin, possesses approximately 140 units per mg of oxytocic activity and 240 units per mg of avian vasodepressor activity. Furthermore, the replacement of the free amino group on the half-cystine residue in position 1 of 4-valine-oxytocin by hydrogen increases the potency to a very high degree. The 1-deamino-4-valine-oxytocin possesses approximately 350 units per mg of avian vasodepressor activity, over 3 times that of 4-valine-oxytocin and 1.5 times that of oxytocin itself. The 1-deamino-4-valine-oxytocin also possesses approximately 350 units per mg of oxytocic activity, more than twice that of 4-valine-oxytocin but about 0.75 that of the hormone. When tested for pressor activity in the rat, both analogues gave no detectable response. The 1-deamino-4-valine-oxytocin was also tested for milk-ejecting and antidiuretic activity. The compound was found to possess 350 units per mg of milk-ejecting activity and 5 units per mg of antidiuretic activity. The methods utilized for the bioassays are referred to under “Experimental Procedure.”

The 4-valine-oxytocin and the 1-deamino-4-valine-oxytocin were prepared by the methods employed in the synthesis of oxytocin (3) and deamino-oxytocin (4), in which the p-nitrophenyl ester method of peptide synthesis (5) was utilized in a stepwise fashion to obtain the desired protected polypeptide intermediates. As described along with the details of the synthetic procedures under “Experimental Procedure,” the purification of 4-valine-oxytocin was accomplished by countercurrent distribution (6) in two different solvent systems, followed by partition chromatography on Sephadex (7). In the case of the deamino analogue, purification was accomplished by countercurrent distribution.

The highly purified analogues gave the expected elementary analyses. When the hydrolyses for the amino acid analyses were carried out for 22 hours, low values for valine and isoleucine were obtained although the ratios of the other amino acids were 1:1. However, after hydrolysis for 42 hours, the molar ratios obtained for isoleucine and valine approached the expected values. It is interesting to note that satisfactory values for isoleucine are obtained when oxytocin is subjected to hydrolysis for 22 hours; thus the results obtained with 4-valine-oxytocin and 1-deamino-4-valine-oxytocin indicate that the peptide linkage between isoleucine and valine in these analogues is difficult to cleave.

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![Fig. 1. Structure of oxytocin. Numbers indicate the positions of the individual amino acid residues.](http://www.jbc.org)
difficulty of hydrolysis of the isoleucylvaline peptide sequence in insulin is discussed by Harfenist (8).

**EXPERIMENTAL PROCEDURE**

**N-Carbobenzoxy-L-valyl-L-asparaginyl-S-benzyl-L-cysteinyll-L-prolyl-L-leucylglycinamide** A solution of 3.6 g of N-carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteinyll-L-prolyl-L-leucylglycinamide (3) in 15 ml of glacial acetic acid was treated with 18 ml of a solution of HBr in glacial acetic acid (32%, w/w). After 1 hour at room temperature the solution was poured into 300 ml of cold, dry ether. The precipitated hydrobromide of the free base was washed by decantation with three 300-ml portions of cold ether. After being dried in a vacuum over KOH and P2O5 overnight, the hydrobromide was dissolved in 120 ml of dry methanol and passed through a column of ion exchange resin, Resyn RGl(OH) (Fisher). The column was washed with 50 ml of dry methanol. The filtrate was slightly basic and, after acidification, gave a negative test for halides. The solid obtained after evaporation of the solvent from the eluate and washings was dried for several hours in a vacuum and then dissolved in 4 ml of dimethylformamide.

To the cold solution, 2.0 g of p-nitrophenyl N-carbobenzoxy-L-valinate (9) were added. The product partially solidified overnight. The addition of 80 ml of ethyl acetate completed the precipitation. The solid was collected and washed with four 80-ml portions of ethyl acetate, and finally with a 100-ml portion of ether. The material was then dried to constant weight over P2O5 in a vacuum at 100°, giving 3.6 g, m.p. 263-264°, [α]D = -46.0° (c, 0.5, in dimethylformamide).

Naphthalene-1,2-dioxide

Calculated: C 60.4, H 6.77, N 11.9
Found: C 60.4, H 6.75, N 11.4

**N-Carbobenzoxy-O-benzyl-L-tyrosyl-L-isoleucyl-L-valyl-L-asparglinyl-S-benzyl-L-cysteinyll-L-prolyl-L-leucylglycinamide** A suspension of 1.0 g of the preceding protected octapeptide in 5 ml of glacial acetic acid was treated with 5 ml of 32% HBr in glacial acetic acid. After 2 hours the hydrobromide was precipitated with ether, repeatedly washed with the same solvent, and then dried and dissolved in 150 ml of dry methanol.

The solution was passed through a column of Resyn RGl(OH) (Fisher). After removal of the solvent the residue, 490 mg, was allowed to react with 3.3 g of p-nitrophenyl N-carbobenzoxy-O-benzyl-L-tyrosinate. After 16 hours, 200 ml of ethyl acetate were added; the precipitate was collected and triturated five times with 100-ml portions of an ethanol-ethyl acetate mixture (9:1). After being dried in a vacuum at 100° for 12 hours, the compound weighed 6.02 g, m.p. 250-251°, [α]D = -78.2° (c, 0.5, in dimethylformamide).

**Calculated:** C 58.2, H 6.34, N 13.6

**Found:** C 57.8, H 6.51, N 13.5

After being dissolved in 15 ml of glacial acetic acid was treated with 5 ml of 32% HBr in glacial acetic acid. After 1 hour at room temperature, 300 ml of dry ether were added; the precipitate was collected and triturated five times with 50-ml portions of a solution of HBr in glacial acetic acid. After 1 hour at room temperature, 300 ml of dry ether were added. The solid which had separated was filtered and carefully washed with cold ether. The hydrobromide of the free heptapeptide was dried overnight in a vacuum over KOH and dissolved in 200 ml of dry methanol. The solution was then passed through a column of Resyn RGl(OH) and washed with an additional 200 ml of dry methanol. After removal of the solvent, the residue was taken up in 20 ml of dimethylformamide and allowed to react with 3.3 g of p-nitrophenyl N-carbobenzoxy-O-benzyl-L-tyrosinate. After 16 hours, 200 ml of ethyl acetate were added; the precipitate was collected and triturated five times with 100-ml portions of an ethanol-ethyl acetate mixture (9:1). After being dried in a vacuum at 100° for 12 hours, the compound weighed 6.02 g, m.p. 250-251°, [α]D = -46.0° (c, 0.5, in dimethylformamide).

Naphthalene-1,2-dioxide

Calculated: C 60.4, H 6.77, N 11.9
Found: C 60.4, H 6.75, N 11.4

**4-Valine-oxytocin**—A sample of the preceding protected nonapeptide, 200 mg, was dissolved in approximately 300 ml of liquid ammonia freshly distilled from sodium. The protected peptide was treated with sodium at the boiling point of the liquid ammonia until a blue color persisted in the solution for about 40 sec. The ammonia solution was subsequently concentrated, and the final 50 ml were removed by lyophilization. The white residue was then dissolved in 200 ml of 0.25% acetic acid. The pH was adjusted to 6.8 with NH4OH, and the solution was oxidized overnight by aeration with CO2-free air followed by titration with 0.011 m aqueous potassium ferricyanide solution. Ferrocyanide and ferricyanide ions were removed by passage of
the oxidized solution through a column of AG8 X4 resin (Calbiochem) in the chloride form. After being concentrated to a volume of 15 ml, the solution was subjected to countercurrent distribution (6) for a total of 750 transfers in the solvent system butanol-benzene-0.5% acetic acid containing 0.1% pyridine (6:1:7). The contents of tubes 75 through 90 were pooled; the solvent was evaporated in a flash evaporator to a volume of approximately 20 ml and the concentrate was lyophilized. The residue weighed 60.5 mg and possessed an avian depressor activity of 245 units per mg. The material from two experiments, 118 mg, was combined, dissolved in 9 ml of lower phase of the solvent system butanol-benzene-0.5% acetic acid containing 0.1% pyridine (6:2:7), and subjected to countercurrent distribution for a total of 450 transfers. A main peak with a partition coefficient (K) of 0.23 was obtained as determined by measurement of Folin-Lowry color values (10). The contents of tubes 75 through 90 were pooled, the solution was concentrated, and the solvent was removed by lyophilization. The material (59 mg) so obtained was subjected to partition chromatography by the method of Yamashiro (7). The sample was dissolved in 1 ml of upper phase of the solvent system butanol-benzene-3.5% acetic acid containing 1.5% pyridine (6:1:7) and applied on a Sephadex G-25 column (2.15 × 111.5 cm) which had been equilibrated with both phases. Elution with the latter phase was performed, and fractions were collected at a flow rate of about 22.5 ml per hour. The Folin-Lowry color values showed a single peak having an Rf (Sephadex) of 0.43. The amount of 4-valine-oxytocin isolated from this peak was 49 mg, [α]25° = -24.6°.

Two days later 100 ml of ethyl acetate were added, and the solid material separated in a gelatinous form. S-benzyl-P-mercaptopropionate, 320 mg, was added. In the course of a few hours the material separated in a gelatinous form. It was dissolved in 4 ml of dimethylformamide, cooled to 0°, and titrated with triethylamine to a pH of 8. Then p-nitrophenyl aspartame was added. The mixture was stirred for 22 hours, the following amino acid ratios were obtained, with the value of glycine taken as 1.0: aspartic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 1.0; valine, 0.95; isoleucine, 1.0; tyrosine, 0.9; ammonia, 2.0. When a sample was hydrolyzed for only 22 hours, the following amino acid ratios were obtained, with glycine taken as 1.0: aspartic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 1.0; valine, 0.8; isoleucine, 0.8; leucine, 1.0; tyrosine, 1.0.

S-Benzyl-β-mercaptopropionyl-L-tyrosyl-L-isoleucyl-L-valyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide—The hydro bromide of the free octapeptide was obtained from 1.0 g of protected octapeptide as already described. After the salt had been dried for several hours in a vacuum over KOH, it was dissolved in 4 ml of dimethylformamide, cooled to 0°, and titrated with triethylamine to a pH of 8. Then p-nitrophenyl S-benzyl-β-mercaptopropionate, 320 mg, was added. In the course of a few hours the material separated in a gelatinous form. Two days later 100 ml of ethyl acetate were added, and the solid material was collected and washed with four 50-ml portions of ethanol ethyl acetate (9:1). Finally, the product was dissolved in dimethylformamide and precipitated from ethanol-ethyl acetate (9:1). After being dried in a vacuum at 100° over P2O5 for 20 hours, the product weighed 730 mg, m.p. 254–256°, [α]25° = -46.0° (c, 0.5, in dimethylformamide).

C17H39N5O6S2
Calculated: C 52.8, H 6.91, N 15.7
Found: C 53.3, H 7.10, N 15.5

A sample was hydrolyzed in 6 N HCl at 110° for 42 hours and then analyzed by the method of Spackman, Stein, and Moore (11) in the 50–50° temperature system on a Beckman/Spinco amino acid analyzer. The following molar ratios were obtained, with glycine taken as 1.0: aspartic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 1.0; valine, 0.95; isoleucine, 0.95; leucine, 1.0; tyrosine, 0.9; ammonia, 2.0. When a sample was hydrolyzed for only 22 hours, the following amino acid ratios were obtained, with glycine taken as 1.0: aspartic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 1.0; valine, 0.8; isoleucine, 0.8; leucine, 1.0; tyrosine, 1.0.

Methods Used for Bioassay—The 4-valine-oxytocin and its 1-deamino analogue were assayed for a number of biological activities known to be possessed by oxytocin, and the units per mg of each activity have been given in the introductory section. Avian depressor assays were performed on conscious chickens as described in the United States Pharmacopeia (15). The milk-ejecting assay was performed according to the method of Cross and Harris (16) as modified by van Dyke, Adamsons, and Engel (17) and by Chan (18). The assay for 1-deamino-4-valine-oxytocin—The debenzylation of the S,S'-dibenzyldiamino-4-valine-oxytocine, 100 mg, was performed with sodium in 100 ml of liquid ammonia freshly distilled from sodium until the blue color persisted for about 2 min. The solution was concentrated, and the last 50 ml of liquid ammonia were lyophilized. The remaining white residue was taken up with 100 ml of water, the pH was adjusted to 6.8, and the resulting clear solution was titrated with 13 ml of 0.01 N potassium ferriyamidc solution (4). This oxidized solution was passed through a column of AG3-X4 resin in the chloride form and concentrated in a flash evaporator at 20° to a volume of 12 ml. The latter solution was then subjected to countercurrent distribution for a total of 300 transfers in the solvent system butanol-benzene-0.5% acetic acid containing 0.1% pyridine (1:3:4). A symmetrical peak with a K value of 0.62 was obtained as determined by measurement of the Folin-Lowry color values. The avian depressor activity was associated with the material of this peak. The contents of tubes 100 through 130 were pooled, concentrated, and lyophilized to yield 29 mg of 1-deamino-4-valine-oxytocin, [α]25° = 107.5° (c, 0.75, in N acetic acid).

For elementary analysis a sample was dried in a vacuum at 100° over P2O5 for 6 hours, and a loss of 5.1% in weight was observed.

C17H39N5O6S2
Calculated: C 53.6, H 6.91, N 14.5
Found: C 53.8, H 6.97, N 14.4

A sample was hydrolyzed in 6 N hydrochloric acid at 110° for 42 hours and then analyzed on a Beckman/Spinco amino acid analyzer. The following molar ratios of amino acids and ammonia were obtained, with the value of glycine taken as 1.0: aspartic acid, 1.0; proline, 1.0; glycine, 1.0; valine, 1.0; isoleucine, 1.0; leucine, 1.0; tyrosine, 0.8; ammonia, 2.0. In addition, cystine (0.3) and the mixed dialkyl of L-cystine and β mercaptopropionic acid (0.4) were present. These two sulfur compounds account for the fall-cystine residue in the analogue (4).
antidiuretic activity was performed on male rats according to the method of Jeffers, Livezey, and Austin (19) as modified by Sawyer (20). All potencies were measured against the U.S.P. posterior pituitary reference standard.

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