The Effect of Decreasing the Size of the Ring Present in Deamino-oxytocin by One Methylene Group on Its Biological Properties

THE SYNTHESIS OF 1-MERCAPTOACETIC ACID-OXYTOCIN*

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SUMMARY

1-Mercaptoacetic acid-oxytocin, an analogue of deamino-oxytocin in which the size of the disulfide ring is decreased from 20 to 19 members, has been synthesized and isolated in highly purified form by means of countercurrent distribution. The analogue possessed 4 units per mg of avian vasodepressor activity, 25 units per mg of oxytocic activity, 50 units per mg of milk-ejecting activity, 0.1 unit per mg of antidiuretic activity, and 0.01 unit per mg of pressor activity.

The mixed disulfide of L-cysteine and mercaptoacetic acid has been synthesized, and its chromatographic behavior in the Beckman/Spinco amino acid analyzer has been shown to be the same as that of the mixed disulfide produced by acid hydrolysis of 1-mercaptoacetic acid-oxytocin.

Deamino-oxytocin is a crystalline analogue of oxytocin (Fig. 1) in which the free amino group of the half-cystine residue in position 1 has been replaced by a hydrogen atom. Compared with the natural hormone, deamino-oxytocin possesses a considerably greater degree of avian vasodepressor, oxytocic, and antidiuretic activities and a somewhat greater degree of milk-ejecting activity, whereas the pressor activity was found to be decreased (1-6). In these pharmacological assays equivalent doses of the two compounds produce patterns of response which are virtually identical. Recently the synthesis of 1-γ-mercaptoprobutyric acid-oxytocin was described (7). In this analogue the β-mercaptohexanoic acid residue of deamino-oxytocin is replaced by a γ-mercaptohexanoic acid residue and so the 20-membered disulfide ring of deamino-oxytocin is expanded to a 21-membered ring by the formal introduction of a methylene group at position 1. This structural change was found to have brought about an almost complete loss of oxytocic and avian vasodepressor activity. We therefore wished to study the effect of decreasing the size of the disulfide ring of deamino-oxytocin to 19 members. This was accomplished by the formal removal of a methylene group at position 1 through the synthesis of 1-mercaptoacetic acid-oxytocin, which is described in this communication.

The required synthetic intermediate for this analogue was the polypeptide derivative S-benzylmercaptoacetyl-L-tyrosyl-L-iso-leucyl-L-glutaminyl-L-asparaginyl-L-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, which was prepared by application of the p-nitrophenyl ester method of peptide synthesis (8) in the manner described in the stepwise synthesis of oxytocin (9) and deamino-oxytocin (2, 3). The final protected intermediate was reduced with sodium in liquid ammonia according to the method of Sifferd and du Vigneaud (10) as employed in the synthesis of oxytocin (11, 12) to remove the benzyl groups, and the disulfhydryl compound so produced was oxidized to the disulfide form by an aqueous solution of potassium ferricyanide (2, 3). After removal of ferrocyanide and excess ferricyanide ions by means of an ion exchange resin, the analogue was purified by countercurrent distribution (13).

Biological assays were performed according to a four-point assay design with the United States Pharmacopeia posterior pituitary reference powder as standard. Avian vasodepressor assays were performed on conscious chickens according to the procedure used by Munsick, Sawyer, and van Dyke (14). Oxytocic assays were performed on isolated uteri from rats in natural estrus according to the method of Holton (15), as modified by van Dyke and Munsick (16), with the use of magnesium-free van Dyke-Hastings solution as the bathing fluid. Milk-ejecting activity was measured on anesthetized rabbits by the method of Cross and Harris (17), as modified by van Dyke, Adamsons, and Engel (18), and by Chan (19). Rat pressor assays were carried out on anesthetized male rats as described in the United States Pharmacopeia (20). Assays for antidiuretic activity were performed on anesthetized male rats according to the method of Jeffers, Livezey, and Austin (21), as modified by Sawyer (22). This analogue was found to possess 4 units per mg of avian vasodepressor activity, 25 units per mg of oxytocic activity, and 20 units per mg of milk-ejecting activity. The antidiuretic potency was found to be...
approximately 0.1 unit per mg and the pressor potency approxi-
mately 0.01 unit per mg. The corresponding activities of oxy-
tocin are approximately 500, 500, 400, 3, and 3 units per mg, re-
spectively (4). This severe diminution of biological activity may
be a reflection of an altered relationship of the constituent amino
acid side chains to each other and to the disulfide bond due to the
decrease in the size of the ring, thereby affecting the action of the
compound at the receptor sites in the various tissues.

It may be pointed out that avian vasopressor activity, ex-
hibited in low degree by mercaptoacetic acid-oxytocin, cannot be
detected in 1 γ mercaptobutyric acid o xo tocin. Further, the
oxytocic potency of the former is appreciably greater than that of
the latter. Thus, it is evident that the decrease in the size of the
ring of deamino-oxtocin by the deletion of a methylene group in
position 1 has a less drastic effect upon biological activity than an
increase in the size of the ring by the addition of a methylene
group in the same position.

EXPERIMENTAL PROCEDURE

S-Benzylmercaptoacetic Acid p-Nitrophenyl Ester—Sodium (24
g) and mercaptoacetic acid (25 g) were added alternately in small
amounts to 1000 ml of liquid ammonia during 3½ hours, the reac-
tion mixture being stirred mechanically. The long reaction time
required was due to the insolubility of mercaptoacetic acid in
liquid ammonia. Ammonium chloride was added to discharge
the blue color and then benzyl chloride (38 ml) was added. Stir-
ring was continued for a further 3 hours before the ammonia was
allowed to evaporate. The crude product was dissolved in water
(500 ml) and the aqueous solution was extracted with ether (100
ml), acidified to Congo red with concentrated HCl, and left to
stand at 5° overnight. A colorless crystalline solid precipitated
and was filtered off, washed with water and dried in a vacuum
over P2O5; weight, 43.2 g; m.p. 59-60°. Holmberg (23) re-
ported m.p. 61-63°. The material was used without further
purification.

S-Benzylmercaptoacetic acid (36.4 g) and p-nitrophenol (30.8
g) were dissolved in ethyl acetate (250 ml) and the solution was
cooled in an ice-water bath. Molten N,N'-dicyclohexylcarbodi-
imide (41 g) was added and the mixture was left to stand over-
night. N,N'-Dicyclohexylurea was filtered off and washed with
ethyl acetate (100 ml). The filtrate was evaporated to an orange
powder was dissolved in 150 ml of 0.1% acetic acid. The solu-
tion, which gave a positive reaction to alkaline nitroprusside, was
filtered off, washed with 2

CH(\(\text{CH}_2\))

and was dried in a vacuum over CaCl2; weight, 200 mg.

The crude product was dissolved in pyridine (2.5 ml) and 2
acetic acid (2.5 ml). The hot solution was diluted with 2
acetic acid (25 ml) and dried in a vacuum over CaCl2; weight, 180 mg;
m.p. 239-242° (with decomposition); [α]D = -42.1° (c, 0.97 in
dimethylformamide).

C15H33N2O4S2
Calculated: C 58.0, H 6.69, N 13.3
Found: C 58.2, H 6.61, N 13.4

I-Mercaptoacetic Acid-oxytocin—The protected polypeptide
intermediate (218 mg) was dissolved in liquid ammonia (200 ml)
which had been redistilled from sodium. The solution was stirred
magnetically while reduction was carried out by means of a so-
dium stick. When the blue color had persisted for 10 sec, the
stick was withdrawn and the ammonia was removed by evapora-
tion and lyophilization at the water pump. The lyophilized
powder was dissolved in 150 ml of 0.1% acetic acid. The solu-
tion, which gave a positive reaction to alkaline nitroprusside, was
adjusted to pH 7 with dilute ammonium hydroxide and titrated
with a 0.02 N solution of recrystallized potassium ferricyanide,
15 ml being consumed. The solution was passed through a short
column containing 5 g of resin AG-3-X4 (Bio-Rad) in the chloride
form, and the column was washed well with water. The total
effluent, free from ferricyanide and ferricyanide ions, was con-
centrated to approximately 60 ml in a rotary evaporator at a
temperature below 20°.

The concentrate was introduced into the first six tubes of a
200-tube countercurrent distribution machine, washings being placed
in the next three tubes. Distribution was carried out in the
solvent system 1-butanol-benzene-water containing 0.5% acetic acid
and 0.1% pyridine (6:5:11) at 24-26° with a settling
time of 60 sec. A determination of the Folin-Lowry (see Refer-
cence 22) color values with the use of 0.2-ml aliquots of the lower
phase of every fifth tube after 194 and 440 transfers indicated a
major peak with a partition coefficient (K) of approximately 0.4
accompanied by two very small peaks, one having a K of approxi-
mately 0.9, the other approximately 0.01. The very slowly mov-
ing small peak, contained in tubes 0 to 20, and the contents of
tubes 21 to 90 were removed and the emptied tubes were replen-
ished with fresh equilibrated solvent system. The distribution

\[
\begin{align*}
\text{Oxytocin with numbers indicating the positions of the component amino acid residues.}
\end{align*}
\]
was continued by the recycling procedure. After 700 transfers the Folin-Lowry color values indicated one major peak which appeared in tubes 170 to 223 with a maximum in tube 198, and was completely separated from a very small peak which was contained in tubes 280 to 320. The distribution curves obtained by plotting the Folin-Lowry color values, and weight determinations for which 1.0 ml aliquots of lower phase from every fifth tube in the area of the peak were used, agreed closely with the theoretical curve calculated for the \( K \) value 0.4. The total contents of tubes 177 to 217 were pooled and concentrated to about 70 ml in a rotary evaporator at a temperature below 90°. The concentrate was lyophilized to give 75 mg of a fluffy white powder; [\( \alpha \)]
\[^{25}\text{D}\] = 47.0° (c, 0.66 in 1 N acetic acid). When other solvent systems were used for the purification of the analogue, a similar pattern of distribution was obtained. Thus in the system 1-butanol-benzene-0.5% acetic acid (3:2:5) the main peak had a \( K \) value of 0.55, in 1-butanol-benzene-ethanol-water (6:5:2:10) \( K \) value was 0.8, and in 1-butanol-ethanol-0.65% acetic acid (4:1:5) \( K \) value was 3.6.

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\text{C}_9\text{H}_6\text{N}_4\text{O}_6\text{S}_2
\]

Calculated: C 51.6, H 6.49, N 15.8

Found: C 51.7, H 6.61, N 15.6

A sample of the lyophilized product was hydrolyzed in 6 N HCl in a vacuum at 100° for 17 hours and analyzed (25) in the 50° system of the Beckman/Spinco amino acid analyzer. The following molar ratios were obtained, glycine being taken as 1.0; aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 1.0; leucine, 1.0; isoleucine, 1.0; tyrosine, 0.9; cystine 0.65; mixed disulfide of cysteine and mercaptoacetic acid, 0.4; and ammonia, 3.0.

A sample of the product (5 mg) was dissolved in 0.5 ml of potassium acetate-potassium chloride buffer (0.15 m, pH 4.8) and the molecular weight was determined by short column equilibrium centrifugation (26). The molecular weight was found to be 968; the calculated value for the monomer is 978.

Mixed Disulfide of L-Cysteine and Mercaptoacetic Acid—A solution of perbenzoic acid in benzene was prepared by the method of Braun (27) as modified by Kolthoff, Lee, and Mairs (28). By iodometric titration the concentration of perbenzoic acid was found to be 33.6 mg per ml.

Twice recrystallized dithiodiglycolic acid (13.7 g) was suspended in chloroform (100 ml) in a 500 ml three-necked flask which was fitted with a \( \text{CaCl}_2 \) tube. The suspension was stirred and cooled in ice-water throughout the experiment. A solution of perbenzoic acid in benzene (272 ml) was added dropwise to the suspension over a period of 3 hours from a separatory funnel which was also fitted with a \( \text{CaCl}_2 \) tube. Stirring was continued for 3½ hours after the addition had been completed. The colorless precipitate was filtered off, washed with anhydrous ether (15 ml), and left to stand overnight at 5°. The precipitate which formed was filtered off; weight, 1.0 g. Four recrystallizations from aqueous acetone were carried out in order to obtain material which was homogeneous when chromatographed on Whatman No. 1 paper in 1-butanol-acetic acid-water (2:1:1) and in the Beckman/Spinco amino acid analyzer. The product weighed 600 mg; m.p. 169-171° (with decomposition); [\( \alpha \)]
\[^{20}\text{D}\] = -112.0° (c, 0.36 in 1 N HCl). Schöbel and Gräfl (29) reported m.p. 168-169° (with decomposition); [\( \alpha \)]
\[^{20}\text{D}\] = 116.1° (c, 0.31 in 1 N HCl).

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\text{C}_9\text{H}_6\text{NO}_3\text{S}_2
\]

Calculated: C 28.4, H 4.29, N 6.63, S 30.4

Found: C 28.6, H 4.37, N 6.69, S 30.6

Acknowledgments—The authors wish to thank the following members of this department: Mr. Joseph Albert for the elementary microanalyses, Mr. Roger Sebbane for the amino acid analyses, Dr. Esther Breslow for the molecular weight determination, and Dr. W. Y. Chan for the biological assays which were carried out under his direction.

REFERENCES

The Effect of Decreasing the Size of the Ring Present in Deamino-oxytocin by One Methylene Group on Its Biological Properties: THE SYNTHESIS OF 1-MERCAPTOACETIC ACID-OXYTOCIN
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