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1-Mercaptoacetic Acid-4-β-alanine-oxytocin*

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ABSTRACT: 1-Mercaptoacetic acid-4-β-alanine-oxytocin has been synthesized through the protected polypeptide derivative *S*-benzylmercaptoacetyl-L-tyrosyl-L-isoleucyl-β-alanyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The latter compound was converted to the analog by treatment with sodium in liquid ammonia followed by oxidation of the disulfhydryl intermediate to the cyclic disulfide. The 1-mercaptoacetic acid-4-β-alanine-oxytocin was isolated in highly purified form by means of countercurrent distribution as an amorphous powder which was subsequently crystallized.

Upon bioassay 1-mercaptoacetic acid-4-β-alanine-oxytocin was found to possess less than 0.01 unit/mg of avian vasodepressor activity, and no oxytocic activity could be detected.

It has been reported from this laboratory that 4-glycine-oxytocin, an analog of oxytocin (Figure 1) in which the glutamine residue in position 4 is replaced with a glycine residue, possesses a low but appreciable degree of oxytocic and avian vasodepressor activity, namely, 3 and 5 units/mg, respectively (Drabarek, 1964). The enlargement of the ring of 4-glycine-oxytocin from 20 to 21 members by the formal introduction of a methylene group at position 4 gave 4-β-alanine-oxytocin which was found to possess virtually no oxytocic or avian vasodepressor activity (Manning and du Vigneaud, 1965). This communication presents the synthesis of 1-mercaptoacetic acid-4-β-alanine-oxytocin and the results of bioassays of the compound for the activities under discussion. In this analog, the half-cystine residue in position 1 of 4-β-alanine-oxytocin is replaced by a mercaptoacetic acid residue, a change which is accompanied by a decrease in the size of the disulfide ring from 21 to 20 members. The preparation of this analog was undertaken to see whether restoration of the size of the disulfide ring to 20 members in this manner might possibly be accompanied by restoration of some degree of biological activity. It should be borne in mind that the replacement of the free amino group with hydrogen in a biologically active analog of oxytocin does not decrease oxytocic or avian vasodepressor activity, but in fact in some instances these activities are enhanced.

An important intermediate in the present synthesis was the protected octapeptide, *N*-carbobenzoxy-*O*-benzyl-L-tyrosyl-L-isoleucyl-β-alanyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, which had originally been prepared for the synthesis of 4-β-alanine-oxytocin. The intermediate was treated with hydrogen bromide in acetic acid and the product so obtained was purified by countercurrent distribution. The octapeptide acetate was coupled with *S*-benzylmercaptoacetic acid *p*-nitrophenyl ester to give *S*-benzylmercaptoacetyl-L-tyrosyl-L-isoleucyl-β-alanyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. This protected polypeptide derivative was converted to 1-mercaptoacetic acid-4-β-alanine-oxytocin and isolated in highly purified form by procedures very similar to those described for the preparation of 1-γ-mercaptobutyric acid-oxytocin (Jarvis *et al.*, 1965). The highly purified amorphous analog was crystallized in the same manner in which 1-γ-mercaptobutyric acid-oxytocin was obtained in crystalline form (Jarvis and du Vigneaud, 1964; Jarvis *et al.*, 1965).

The 1-mercaptoacetic acid-4-β-alanine-oxytocin was

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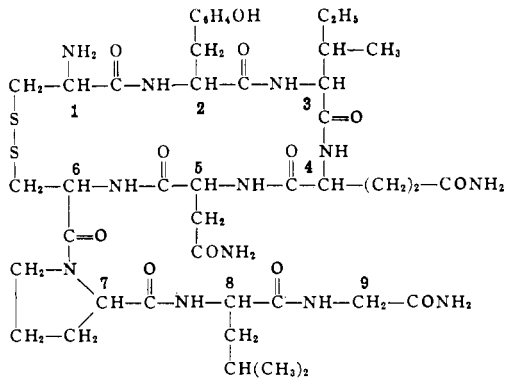

FIGURE 1: Structure of oxytocin with *numbers* indicating the position of the individual amino acid residues.

subjected to bioassays for oxytocic and avian vasodepressor activity against the U.S.P. posterior pituitary reference standard. Avian vasodepressor assays were performed on conscious chickens according to the procedure employed by Munsick *et al.* (1960). Oxytocic assays were performed on uteri from rats in natural estrus according to the method of Holton (1948) with the use of magnesium-free van Dyke–Hastings solution as employed by Munsick (1960). The analog was found to possess less than 0.01 unit/mg of avian vasodepressor activity, and no oxytocic activity could be detected.

Experimental Section[1]

*S-Benzylmercaptoacetyl-*L*-tyrosyl-*L*-isoleucyl-β-alanyl-*L*-asparaginyl-S-benzyl-*L*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* The carbobenzoxy-*O*-benzyl-L-tyrosyl-L-isoleucyl-β-alanyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (2.0 g) was dissolved in 30 ml of warm glacial acetic acid. The solution was cooled and 20 ml of a solution of hydrogen bromide in acetic acid (22%, w/w) was added. After 50 min the yellow solution was poured into 350 ml of cold, dry ether. The precipitate which had formed was collected on a filter, washed with 1000 ml of ether, and dried *in vacuo* over NaOH pellets overnight. The dry solid was dissolved in 500 ml of methanol and portions of resin IRA-410 (OH form) were added until the solution was free from bromide ions. The resin was filtered off and washed with 300 ml of warm methanol. The combined filtrate and washings were evaporated *in vacuo* to give a solid residue (wt 1.43 g).

A portion of the crude product (1.1 g) was dissolved in a mixture of 70 ml of upper phase and 70 ml of lower phase of the solvent system 1-butanol–1-propanol–benzene–water containing 1% acetic acid and 0.15% pyridine (2:1:1:4). The solution was placed in the first seven tubes of a 200-tube countercurrent machine (Craig *et al.*, 1951) and washings were placed in the next three tubes. Distribution was carried out for 220 transfers at 24–26°. Initially a settling time of 5 min was necessary, but this was shortened as the distribution proceeded. A determination of the Folin–Lowry color values (Lowry *et al.*, 1951) on 0.1-ml aliquots of the lower phase of every fifth tube indicated a major peak with a partition coefficient (K) of approximately 0.5 accompanied by two minor peaks having a K value of approximately 0.1 and 1.0. The first 40 tubes were emptied and replenished with fresh solvent system. The distribution was continued, the settling time being 3 min. After 330 transfers a second Folin–Lowry analysis was performed. The major peak (K = 0.5) was well separated from the faster moving minor peak (K = 1.2) and from a very tiny, more slowly moving peak (K = 0.3); it was slightly asymmetrical, indicating the possibility of the presence of small amounts of impurity in the leading edge. The total contents of tubes 98–140 inclusive were pooled and concentrated to about 50 ml in a rotary evaporator at a temperature below 20°. The concentrate was lyophilized to give 785 mg of a fluffy, white powder. A sample of the lyophilized powder was hydrolyzed in 6 N HCl *in vacuo* at 110° for 17 hr and analyzed in the 50–50° system of the Beckman–Spinco amino acid analyzer. The following molar ratios were found, glycine being taken as 1.0: aspartic acid, 1.0; β-alanine, 1.0; proline, 1.0; glycine, 1.0; leucine, 1.0; isoleucine, 1.0; tyrosine, 1.0; *S*-benzylcysteine, 1.0; and ammonia, 1.8. The octapeptide acetate (600 mg) was dissolved in 10 ml of dimethylformamide and 220 mg of *S*-benzylmercaptoacetic acid *p*-nitrophenyl ester (Jarvis and du Vigneaud, 1967) was added to the solution. After the reaction mixture had stood at room temperature for 60 hr, it was diluted with 100 ml of ethyl acetate and kept at 5° for 4 hr. The solid was filtered off, washed with 100 ml of ethyl acetate and 40 ml of ether, and dried *in vacuo* over $CaCl_2$; wt 600 mg, mp 226–230° dec.

The crude product was dissolved, by heating, in a mixture of 7 ml of pyridine and 5 ml of water. The yellow solution was cooled in ice water. To the cooled solution 1.0 ml of 2 N sodium hydroxide solution was added, followed, after 5 min, by 10 ml of 2 N acetic acid. The mixture was left to stand for 60 min at 5° before the solid was filtered off, washed with 150 ml of 2 N acetic acid and 200 ml of water, and dried *in vacuo* over P_2O_5 overnight; wt 500 mg, mp 228–231° dec, $[\alpha]_D^{20}$ −43.8° (*c* 2.2, dimethylformamide).

Anal. Calcd for $C_{54}H_{74}N_{10}O_{11}S_2$ (1103.3): C, 58.8; H, 6.76; N, 12.7. Found: C, 58.5; H, 6.70; N, 12.6.

1-Mercaptoacetic Acid-4-β-alanine-oxytocin. The protected polypeptide intermediate (400 mg) was dissolved in approximately 200 ml of liquid ammonia which had been redistilled from sodium. The solution was stirred magnetically while reduction was carried out by means of a sodium stick. When the blue color had enveloped the solution, the stick was withdrawn. Glacial acetic acid (0.04 ml) was added and the am-

[1] All melting points are capillary melting points and are corrected, unless otherwise stated.

monia was removed by evaporation and lyophilization. The lyophilized powder was dissolved in 250 ml of 0.2% acetic acid. The solution, which gave a strongly positive reaction to alkaline nitroprusside, was adjusted to pH 7 and titrated with a 0.02 N solution of recrystallized potassium ferricyanide (Hope *et al.*, 1962). The solution was poured onto a short column of 12 g of resin AG3X4 (100–200 mesh) in the chloride form, and the column was washed with 80 ml of water. The total effluent, free from ferrocyanide and excess ferricyanide ions, was adjusted to pH 7 with dilute ammonium hydroxide and concentrated to approximately 50 ml in a rotary evaporator at a temperature below 20°.

The concentrate was introduced into the first five tubes of a 200-tube contercurrent distribution machine and distributed in the solvent system 1-butanol–benzene–water containing 0.5% acetic acid and 0.1% pyridine (6:5:11) at 24–26°, the settling time being 55 sec. After 200 transfers a determination of the Folin–Lowry color values indicated one major peak which was symmetrical and had a distribution coefficient (K) of 1.7, and a tiny very slowly moving peak with a K of 0.05. The minor peak, contained in tubes 0–25, and the total contents of tubes 26–90 were removed and the emptied tubes were replenished with fresh, equilibrated solvent system. The distribution was continued by the recycling procedure. After 600 transfers the determination of the Folin–Lowry color values was repeated; one symmetrical peak was revealed in tubes 350–405, with a maximum in tube 377. The distribution curves obtained by plotting the Folin–Lowry color values, and weight determinations for which 1.0-ml aliquots of the lower phase of every fifth tube in the area of the peak were used, agreed very closely with the theoretical curve calculated for the K value 1.7. The total contents of tube 360–395 inclusive were pooled and concentrated to about 80 ml in a rotary evaporator at a temperature below 20°. The concentrate was lyophilized to give 160 mg of a fluffy white powder, $[\alpha]_D^{20}$ −17.6° (c 0.68, 5 N acetic acid).

A sample of the lyophilized product was hydrolyzed in 6 N HCl *in vacuo* for 17 hr at 110° and analyzed in the 50–50° system of the Beckman-Spinco amino acid analyzer. The following molar ratios were obtained, glycine being taken as 1.0: aspartic acid, 1.0; β-alanine, 0.8; proline, 1.0; glycine, 1.0; leucine, 0.9; isoleucine, 0.9; tyrosine, 0.9; half-cystine, 0.5; mixed disulfide of cysteine and mercaptoacetic acid, 0.45; and ammonia, 2.2.

A sample of the analog (100 mg) was treated with 10 ml of 1 N acetic acid solution; the mixture was shaken until almost all of the material had dissolved and then left to stand at room temperature. After several hours, crystals had formed; the crystalline product was filtered off and dried *in vacuo* over P_2O_5, wt 70 mg, mp 262–264° dec (modified Kofler block). Lyophilization of the mother liquor yielded 22 mg of amorphous powder.

Anal. Calcd for $C_{40}H_{60}N_{10}O_{11}S_2$ (921.1): C, 52.2; H, 6.57; N, 15.2. Found: C, 52.2; H, 6.69; N, 15.2.

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