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4-β-Alanine-oxytocin: An Oxytocin Analog Containing a Twenty-one-membered Disulfide Ring*

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ABSTRACT: 4-β-Alanine-oxytocin, an analog of oxytocin containing a β-alanine residue in place of the glutamine residue in the 4 position in the hormone, has been synthesized and tested for various pharmacological activities in comparison with 4-glycine-oxytocin. Both of these analogs lack the carboxamide-containing side chain attached to the ring in oxytocin at position 4, but the β-alanine-oxytocin contains a ring larger by one methylene unit than the 20-membered ring present in 4-glycine-oxytocin and oxytocin. This increase in ring size resulted in a marked decrease in oxytocic, avian depressor, and milk-ejecting activities.

The synthesis of oxytocin (du Vigneaud *et al.*, 1953, 1954), the principal oxytocic and milk-ejecting hormone of the posterior pituitary gland, has provided a means of studying the relationship of chemical structure to biological activity in this octapeptide hormone, the structure of which is shown in Figure 1. Efforts to elucidate this relationship in this and other laboratories during the past decade have centered on the total synthesis of numerous analogs of the hormone, incorporating various modifications of its structure, and the comparison of the pharmacological properties of these synthetic analogs with those of oxytocin. Along with some other aspects of the general problem, the studies in our laboratory have been focused particularly on the importance of the presence of the individual chemical functional groups, the specificity of the 20-membered disulfide ring, and the relation of the stereostructure of the seven optically active amino acid residues to the pharmacological manifestations of oxytocin.

Recently Drabarek (1964) in this laboratory synthesized three analogs of oxytocin in which the tyrosine, isoleucine, and glutamine residues in positions 2, 3, and 4 were replaced, respectively, by a glycine residue. The only one of these compounds that showed pharmacological activity was 4-glycine-oxytocin, which possessed 5.5 units/mg of oxytocic activity, 17 units/mg of milk-ejecting activity, and 2.8 units/mg of avian depressor activity. Oxytocin possesses approximately 500 units/mg of oxytocic and avian depressor activities, and approximately 400 units/mg of milk-ejecting activity (Chan and du Vigneaud, 1962). In 4-glycine-oxytocin the $-CH_2CH_2CONH_2$ portion of the glutamine residue at the 4 position in oxytocin is replaced by hydrogen, thus eliminating the carboxamide-containing side chain attached to the ring at this position. Even though the activities of this analog were low, we decided to determine whether the activity of the 4-glycine-oxytocin would be further diminished by increasing the ring size from 20 to 21 members through the insertion of a methylene unit at the 4 position. The present paper describes the synthesis and study of such an analog of oxytocin, namely 4-β-alanine-oxytocin. By replacing the glutamine residue in the 4 position of oxytocin by a β-alanine residue the ring size is increased by one methylene unit from 20 to 21 members. This analog also lacks the side chain at position 4 containing the carboxamide group and, as in 4-glycine-oxytocin, no asymmetric carbon is present at this position.

The key intermediate for the synthesis of 4-β-alanine-oxytocin was the protected nonapeptide *S*-benzyl-*N*-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-isoleucyl-β-alanyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. This protected nonapeptide intermediate was prepared by the stepwise *p*-nitrophenyl ester procedure used previously in this laboratory for the synthesis of oxytocin (Bodanszky and du Vigneaud, 1959). Carbobenzoxy-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide served as starting material. After removal of the carbobenzoxy group, the

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free base was coupled with *p*-nitrophenyl carbobenzoxy-β-alaninate to give the protected hexapeptide. The chain was then lengthened by the stepwise *p*-nitrophenyl ester method to give the required protected nonapeptide. Conversion of the latter compound to 4-β-alanine-oxytocin was effected by reduction with sodium in liquid ammonia (Sifferd and du Vigneaud, 1935; du Vigneaud *et al.*, 1953, 1954) and aeration of the reduced material in aqueous solution at *p*H 6.8. Oxidation of the dithiol was completed by the addition of potassium ferricyanide (Hope *et al.*, 1962). The product was purified by countercurrent distribution (600 transfers) in the system butanol–propanol–0.5% acetic acid containing 0.1% pyridine (6:1:8) at 4°. One major component was found, which was isolated by concentration and lyophilization. Paper chromatography of the purified material indicated it to be homogeneous. Elemental analysis and analysis for amino acids and ammonia gave the expected results for 4-β-alanine-oxytocin. Assay of the 4-β-alanine-oxytocin against the USP Standard Reference Powder by the methods used for oxytocin and its analogs in this laboratory (Chan and du Vigneaud, 1962) showed it to possess approximately 0.4 unit/mg of avian depressor activity, 0.12 unit/mg of rat oxytocic activity, 0.5 unit/mg of rabbit milk-ejecting activity, 0.03 unit/mg of rat pressor activity, and negligible rat antidiuretic activity. No inhibitory activity was observed under normal bioassay procedures.

It is also of interest to compare, from the pharmacological standpoint, 4-β-alanine-oxytocin with the analog studied by Guttmann and Boissonnas (1963), in which α-L-alanine is present in the 4 position. The 4-alanine-oxytocin, in contrast to the 4-β-alanine-oxytocin, possesses a substantial degree of avian depressor, oxytocic, and milk-ejecting activities.

Experimental[1]

p-Nitrophenylcarbobenzoxy-β-alaninate. Carbobenzoxy-β-alanine (22.3 g) (Sifferd and du Vigneaud, 1935) was dissolved in 300 ml of warm ethyl acetate. *p*-Nitrophenol (16.6 g) was added, the resulting light yellow solution was cooled to 0°, and 21.5 g of dicyclohexylcarbodiimide was added. The reaction mixture was stirred at 0° for 0.5 hour and then at room temperature for 1 hour. The *N,N'*-dicyclohexylurea was filtered off and washed with 100 ml of ethyl acetate. The filtrate and washings were combined and ethyl acetate was removed *in vacuo*. The resulting crystalline residue was triturated successively with two 50-ml portions of 95% ethanol and four 50-ml portions of ether; wt 23.6 g, mp 91–93°. A portion (3.0 g) of this material was recrystallized twice from warm ethanol; wt 2.6 g, mp 92–94°.

Anal. Calcd for $C_{17}H_{16}O_6N_2$: C, 59.3; H, 4.68; N, 8.1. Found: C, 59.4; H, 4.76; N, 8.1.

[1] All the melting points are capillary melting points and are corrected.

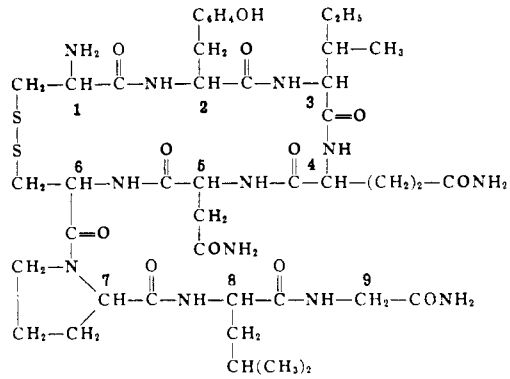

FIGURE 1: Oxytocin.

*Carbobenzoxy-β-alanyl-*L*-asparaginyl-S-benzyl-*L*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* Finely powdered carbobenzoxy-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (10 g) (Bodanszky and du Vigneaud, 1959) was dissolved in 30 ml of acetic acid, hydrogen bromide in acetic acid (25% w/w, 35 ml) was added, and the solution was stirred at room temperature for 2.25 hours. The solution was poured into 400 ml of cold, dry ether and the precipitated pentapeptide hydrobromide was filtered off, washed with three 150-ml portions of ether, and dried for 16 hours *in vacuo* over P_2O_5 and NaOH. The dry solid was dissolved in 150 ml of dry methanol, and Amberlite IRA-410 was added with stirring until the solution was free of bromide ion. The resin was filtered off and washed with methanol. The methanol was removed from the combined filtrate and washings *in vacuo*. The dry residue was dissolved in 30 ml of dimethylformamide and 5.5 g of *p*-nitrophenyl carbobenzoxy-β-alaninate was added. The resulting solution was allowed to stand at room temperature. After 1 day a sizable precipitate was present. After 6 days 200 ml of ethyl acetate was added and the precipitate was collected and triturated with four 50-ml portions of ethyl acetate; wt 9.3 g, mp 227–229°. A 1.0-g portion of this material was purified for analysis by trituration with 30 ml of 95% ethanol; wt 0.95 g, mp 228–229°, $[\alpha]_D^{16}$ −77.6° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{38}H_{52}O_9N_8S$: C, 57.3; H, 6.53; N, 14.1. Found: C, 57.5; H, 6.65; N, 14.3.

*Carbobenzoxy-*L*-isoleucyl-β-alanyl-*L*-asparaginyl-S-benzyl-*L*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* The protected hexapeptide (5.0 g) was dissolved in 30 ml of acetic acid and hydrogen bromide in acetic acid (25% w/w, 28 ml) was added. After 2 hours at room temperature the solution was poured into 300 ml of cold dry ether. The precipitated hydrobromide was collected, washed with 400 ml of ether, and dried for 12 hours *in vacuo* over P_2O_5 and NaOH. The dry white solid was dissolved in 150 ml of dry methanol and converted to the free base by treatment with Amberlite IRA-410 as described in the preceding section. The amorphous residue obtained after evaporation of the

methanol was dissolved in 30 ml of dimethylformamide and 3.0 g of *p*-nitrophenyl carbobenzoxy-L-isoleucinate (Bodanszky and du Vigneaud, 1959) was added. The clear solution was allowed to remain at room temperature and after 10 minutes the product began to separate as a gel. After 3 days 75 ml of ethyl acetate was added, and the precipitate was collected and triturated successively with two 50-ml portions of ethyl acetate, two 50-ml portions of 95% ethanol, and three 50-ml portions of ether; wt 5.0 g, mp 231–232°, $[\alpha]_D^{16}$ −67° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{44}H_{63}O_{10}N_9S$: C, 58.1; H, 6.93; N, 13.9. Found: C, 58.0; H, 7.03; N, 13.9.

*O-Benzyl-N-carbobenzoxy-*L*-tyrosyl-*L*-isoleucyl-β-alanyl-*L*-asparaginyl-S-benzyl-*L*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* A solution of 4.0 g of the protected heptapeptide in 40 ml of acetic acid was treated with hydrogen bromide in acetic acid (32% w/w, 30 ml) and the resulting solution was stirred at room temperature for 2 hours and then slowly poured into 400 ml of cold dry ether. The precipitated hydrobromide was collected, washed with 800 ml of dry ether, and dried for 14 hours *in vacuo* over P_2O_5 and NaOH. The heptapeptide was liberated from the dry hydrobromide by the procedure already described. The amorphous solid remaining after removal of the methanol was dissolved in 40 ml of dimethylformamide. *p*-Nitrophenyl *O*-benzyl-*N*-carbobenzoxy-L-tyrosinate (2.6 g) (Bodanszky and du Vigneaud, 1959) was added and the resulting solution was allowed to stand at room temperature for 2 days. Ethyl acetate (200 ml) was added, and the precipitate was collected and triturated successively with three 50-ml portions of ethyl acetate, 50 ml of absolute ethanol, and two 50-ml portions of ether; wt 3.5 g, mp 235–237°, $[\alpha]_D^{16}$ −60.0° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{60}H_{78}O_{12}N_{10}S$: C, 61.9; H, 6.71; N, 12.0. Found: C, 62.0; H, 7.01; N, 12.5.

*S-Benzyl-N-carbobenzoxy-*L*-cysteinyl-*L*-tyrosyl-*L*-isoleucyl-β-alanyl-*L*-asparaginyl-S-benzyl-*L*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* The protected octapeptide (1.0 g) was dissolved in 20 ml of dry glacial acetic acid and hydrogen bromide in acetic acid (32% w/w, 8 ml) was added. The mixture was stirred at room temperature for 2 hours. The clear solution was poured into 400 ml of cold dry ether and the precipitated hydrobromide was collected, washed with ether, and dried for 14 hours *in vacuo* over P_2O_5 and NaOH. The free octapeptide was liberated from the dry hydrobromide by treatment with Amberlite IRA-410 according to the procedure already described. The solid residue obtained upon removal of the methanol was dried *in vacuo* over P_2O_5 and dissolved in 15 ml of dimethylformamide. *p*-Nitrophenyl *S*-benzyl-*N*-carbobenzoxy-L-cysteinate (0.50 g) (Bodanszky and du Vigneaud, 1959) was added and the resulting clear solution was stirred at room temperature for 3 days. Ethyl acetate (200 ml) was added and the precipitated solid was collected and washed with 150 ml of ethyl acetate. Further purification was effected by trituration of the solid successively with two 50-ml portions of ethyl acetate, three 50-ml portions of 95% ethanol, and three 50-ml portions of ether; wt 0.85 g, mp 230.5–232°, $[\alpha]_D^{20.5}$ −52.5° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{63}H_{83}O_{13}N_{11}S_2$: C, 59.8; H, 6.56; N, 12.2.

Found: C, 59.9; H, 6.62; N, 12.0.

4-β-Alanine-oxytocin. The protected nonapeptide (200 mg) was dissolved in 300 ml of anhydrous liquid ammonia which was at its boiling point. Sodium was added from a small-bore glass tube until a faint blue color enveloped the solution for a 90-second period. Glacial acetic acid (3 drops) was added to remove the color and the ammonia was evaporated *in vacuo*, the last 50 ml being removed from the frozen state. The fluffy residue was dissolved in 200 ml of 0.1% acetic acid, and after adjustment of the *p*H to 6.8 the solution was aerated with CO_2-free air for 3 hours. The oxidation was completed by the addition of potassium ferricyanide (0.011 M, 0.6 ml) (Hope *et al.*, 1962). The ferrocyanide and ferricyanide ions were removed from the solution by passage over a column of AG3X4 (Bio-Rad) resin in the chloride form. The combined eluate and washings contained a total of approximately 12 units of avian depressor activity. Reduction of a second 200-mg portion of the protected nonapeptide under the same conditions also afforded an aqueous solution containing approximately 12 units of avian depressor activity. Reduction of a 100-mg portion of the protected nonapeptide gave an aqueous solution containing approximately 9 units of avian depressor activity. The solutions from these three preparations were pooled and concentrated to a volume of about 30 ml in a rotary evaporator at a bath temperature lower than 15°. This solution was placed in the first 15 tubes of a 6-ml 400-tube countercurrent distribution machine and subjected to a total of 600 transfers in the solvent system butanol–propanol–0.5% acetic acid containing 0.1% pyridine (6:1:8) at 4°. After 400 transfers, analysis by the Folin-Lowry color reaction (Lowry *et al.*, 1951) indicated that the material had separated into a major peak with a *K* value of 0.81 and two much smaller more slowly moving peaks with respective *K* values of 0.17 and 0.25. After 600 transfers the distribution pattern remained the same, although the *K* value of the major peak had decreased slightly to 0.77. This curve was in good agreement with the calculated curve for material with a *K* value of 0.77. The material from the slow-moving peaks did not possess avian depressor activity. The contents of tubes 240–295 from the central portion of the major peak were combined, concentrated to a small volume (150 ml) in a rotary evaporator, and lyophilized to obtain approximately 175 mg of a white, fluffy powder, $[\alpha]_D^{20.5}$ +120° (*c* 0.25, 1 N acetic acid). For elemental analysis a sample was dried at 100° over P_2O_5 *in vacuo* for 5 hours.

Anal. Calcd for $C_{41}H_{63}O_{11}N_{11}S_2$: C, 51.8; H, 6.64; N, 16.2. Found: C, 51.4; H, 6.71; N, 16.0.

A portion of the product was hydrolyzed in 6 N

hydrochloric acid at 110° for 17 hours and the amino acid content of the hydrolysate was determined (Moore *et al.*, 1958; Spackman *et al.*, 1958). The following molar ratios of amino acids and ammonia were obtained (with the value of glycine taken as 1.0): aspartic acid, 1.0; β-alanine, 0.9; proline, 1.0; glycine, 1.0; cystine, 1.0; leucine, 1.0; isoleucine, 1.0; tyrosine, 0.9; and ammonia, 2.0. A portion of the 4-β-alanine-oxytocin was applied to strips of Whatman No. 1 paper and subjected to descending chromatography in the solvent system butanol–acetic acid–water (4:1:5) for a period of 10 hours. When the papers were sprayed with platinum reagent (Toennies and Kolb, 1951) and with a modified ninhydrin spray (Moffat and Lytle, 1959), the product was found to have traveled as a single spot (R_F 0.59). The R_F of oxytocin in this system is 0.56 (Cash *et al.*, 1962).

Discussion

It can be clearly seen that the characteristic pharmacological activities of oxytocin possessed by 4-glycine-oxytocin were almost completely eliminated when an additional methylene unit was introduced into the ring at the 4 position. It may be recalled that several other analogs of oxytocin possessing intramolecular disulfide rings larger than oxytocin have been synthesized and subjected to pharmacological study. Thus 4-isoglutamine-oxytocin (Ressler and du Vigneaud, 1957), an analog in which isoglutamine replaces glutamine in the 4 position giving a 22-membered ring, was found to be completely devoid of oxytocic and avian depressor activities. 5-Isoasparagine-oxytocin (Lutz *et al.*, 1959), in which isoasparagine replaces asparagine in the 5 position giving rise to a 21-membered ring, was also inactive. An analog of oxytocin in which the disulfide ring was enlarged to 23 members has also been synthesized. In this case, the insertion of an additional tyrosine residue between the tyrosine and isoleucine residues of oxytocin resulted in the disappearance of the pharmacological properties characteristic of oxytocin (Guttmann *et al.*, 1957). 1-(Hemi-homocystine)-oxytocin (Jarvis *et al.*, 1961), an analog of oxytocin in which the hemicystine residue of the hormone that bears the free amino group is replaced by a hemi-homocystine residue, thereby forming a 21-membered disulfide ring, did not exhibit avian depressor or rat pressor activity. It did possess a very slight but definite oxytocic activity (0.75 unit/mg). However, the pharmacological data on these analogs do not offer unequivocal evidence that the loss of biological activity is due to an increase in ring size since the shift in the position of some of the functional groups may also play an important role. For instance, the carboxamide group at position 4, which in oxytocin is separated from the ring by two methylene units, is attached directly to the ring in 4-isoglutamine-oxytocin, the isomeric octapeptide. In 5-isoasparagine-oxytocin, the carboxamide group at position 5 is also attached directly to the ring, whereas in oxytocin it is separated from the ring by one methylene unit. That the proximity of the carboxamide group to the ring may be of significance to the biological activity of oxytocin is indicated by the almost complete lack of activity observed for 5-glutamine-oxytocin (Boissonnas *et al.*, 1956; Berde *et al.*, 1957) in which the only change in structure is in the carboxamide group in the 5 position, which is one methylene unit more remote from the ring than in oxytocin. Finally, it may be noted that in 1-(hemi-homocystine)-oxytocin the free amino group is one methylene unit more remote from the disulfide bond than in oxytocin.

The decrease in activities resulting from the increase in ring size upon going from 4-glycine-oxytocin to 4-β-alanine-oxytocin recalls the very recent findings in this laboratory (Jarvis and du Vigneaud, 1964) involving structural changes in a highly active analog of oxytocin, deamino-oxytocin (1-β-mercaptopropionic acid–oxytocin) (du Vigneaud *et al.*, 1960; Hope *et al.*, 1962). By replacing the β-mercaptopropionic acid residue in the 1 position by the homologous γ-mercaptobutyric acid residue, an analog containing a 21-membered ring, 1-γ-mercaptobutyric acid–oxytocin, was obtained. This analog was found to be almost devoid of avian depressor and oxytocic activities. 4-β-Alanine-oxytocin and 1-γ-mercaptobutyric acid–oxytocin differ, respectively, from 4-glycine-oxytocin and deamino-oxytocin only in having an additional methylene group in the ring at the position specified in each case. The loss in activity observed upon going from 4-glycine-oxytocin to 4-β-alanine-oxytocin is much less than that encountered with the higher homolog of deamino-oxytocin, since the 4-glycine-oxytocin was not itself highly active. Nevertheless, it is of considerable interest that the enlargement of the ring at this 4 position in an analog lacking the side chain also causes an almost complete loss of biological activity. The alterations in pharmacological properties may be due to changes in the relationship of the side chains of neighboring amino acid residues to one another and to the disulfide bond, such changes having been induced by the changes in ring size.

From all the work so far reported it appears that a change in the size of the ring produces a change in some vital aspect of the conformation of the molecule, affecting its biological action.

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Preparation of Crystalline Thyroxine-binding Prealbumin from Human Plasma*

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ABSTRACT: A procedure is described for the large-scale preparation of highly purified thyroxine-binding prealbumin from human plasma. The protein is obtained in 10% yield and 150-fold purification from the starting material, Cohn fraction IV-6. The procedure employs the sequential application of ammonium sulfate fractionation, batch adsorption to DEAE-cellulose, DEAE-column chromatography, and preparative electrophoresis in Pevikon C-870. The protein can then be crystallized from ammonium sulfate solution.

In human plasma, L-thyroxine (T_4)[1] is transported in association with several proteins. The majority of data (cf. reviews by Robbins and Rall, 1960; Ingbar, 1960; Tata, 1964) suggests that two plasma proteins play a predominant role in T_4 binding. These are an α-globulin, termed the T_4-binding globulin (TBG), and a prealbumin, the T_4-binding prealbumin (TBPA), which has an electrophoretic mobility 30% greater than albumin when subjected to Tiselius electrophoresis at *p*H 8.6. Albumin and perhaps other proteins appear to serve as secondary carriers of T_4 in plasma.

The electrophoretic migration of TBPA in starch gel at *p*H 8.6 is the same as that of the most rapidly migrating protein of normal plasma (band I of Poulik and Smithies, 1958), and the two proteins are thought to be identical (Blumberg *et al.*, 1961; Squef *et al.*, 1963; Oppenheimer *et al.*, 1965). A more slowly migrating prealbumin in starch gel electrophoresis has been identified as an acidic α_1-glycoprotein (Poulik and Smithies, 1958) and apparently does not bind thyroxine.

A large-scale preparation of purified TBPA has not

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[1] Abbreviations used in this work: T_4, L-thyroxine; TBG, T_4-binding globulin; TBPA, T_4-binding prealbumin.