Synthesis and Some Pharmacological Properties of 6-L-Penicillamine-oxytocin[1]

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6-L-Penicillamine-oxytocin has been synthesized from the protected nonapeptide intermediate, N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-penicillaminyl-L-prolyl-L-leucylglycinamide, by reduction with sodium in liquid ammonia and subsequent oxidation of the disulfhydryl compound with potassium ferricyanide. The protected nonapeptide intermediate was synthesized from L-prolyl-L-leucylglycinamide by use of the stepwise *p*-nitrophenyl ester method. In contrast to 1-L-penicillamine-oxytocin, 6-L-penicillamine-oxytocin does not show an inhibitory effect on the oxytocic activity of oxytocin. 6-L-Penicillamine-oxytocin possesses approximately 2.5 units/mg of oxytocic activity. On the other hand, 6-L-penicillamine-oxytocin exhibits a somewhat greater inhibitory effect on the avian vasodepressor activity of oxytocin than that shown by 1-L-penicillamine-oxytocin.

It has been shown that the replacement of the half-cystine residue at position 1 of oxytocin (Figure 1) by an L-penicillamine residue causes total loss of oxytocic and avian vasodepressor activity and that this analog (1-L-penicillamine-oxytocin) is a highly potent inhibitor of the oxytocic activity of oxytocin on the rat uterus both *in vitro* and *in vivo*.[3,4] 1-L-Penicillamine-oxytocin also exhibits a slight inhibitory effect on the avian vasodepressor activity of oxytocin. It then became of interest to determine the effect of the replacement of the half-cystine residue at position 6 of oxytocin by an L-penicillamine residue on the oxytocic and avian vasodepressor activities of the hormone. The present paper reports the synthesis of 6-L-penicillamine-oxytocin (I), in which the two hydrogens attached to the β-carbon of the half-cystine residue at position 6 of oxytocin are replaced by two methyl groups.

For the synthesis of 6-L-penicillamine-oxytocin the protected nonapeptide N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-penicillaminyl-L-prolyl-L-leucylglycinamide (II) was prepared by the use of the stepwise *p*-nitrophenyl ester method as employed in the synthesis of oxytocin.[5] L-Prolyl-L-leucylglycinamide[6] served as the starting material for the series of reactions, in which the first step involved the coupling of this tripeptide with *p*-nitrophenyl N-carbobenzoxy-S-benzyl-L-penicillaminate.[3] All of the intermediate peptides containing the S-benzyl-L-penicillamine residue were found to be more soluble in organic solvents than the corresponding peptides containing the S-benzyl-L-cysteine residue. The protected nonapeptide (II) was reduced with sodium in liquid ammonia to remove the protecting groups by the method of Sifferd and du Vigneaud[7] as used in the original synthesis of oxytocin.[8] The disulfhydryl compound was oxidized at pH 6.8–7.0 with potassium ferricyanide[9] to the cyclic octapeptide amide (I), and the crude material was purified by countercurrent distribution[10] in the solvent system 1-butanol–pyridine–benzene–0.1% aqueous acetic acid (6:1:2:9) (K = 1.0) and also by partition chromatography on Sephadex G-25[11] in the solvent system 3.5% aqueous acetic acid (containing 1.5% pyridine)–1-butanol (1:1) (R_f 0.32). The material was then subjected to gel filtration on Sephadex G-25 in 0.2 N acetic acid, and 6-L-penicillamine-oxytocin (I) emerged in a peak at the same position as oxytocin.

The 6-L-penicillamine-oxytocin was assayed for avian vasodepressor[12] and rat oxytocic activites.[13] In contrast to 1-L-penicillamine-oxytocin, the 6-L-penicillamine-oxytocin did not show any inhibition of the oxytocic activity of oxytocin and in fact the 6-L-penicillamine-oxytocin possessed 2.7 + 0.5 units/mg of oxytocic activity. On the other hand, the 6 L-penicillamine-oxytocin exhibited a somewhat greater inhibitory effect on the avian vasodepressor activity of oxytocin than that shown by the 1-L-penicillamine-oxytocin.[3] Thus we have observed somewhat different effects on the oxytocic and avian vasodepressor activities of oxytocin, depending upon whether the hydrogens on the β-carbon of the half-cystine residue at position 1 or position 6 are replaced by methyl groups.

Experimental Section[14]

N-Carbobenzoxy-S-benzyl-L-penicillaminyl-L-prolyl-L-leucylglycinamide.—L-Prolyl-L-leucylglycinamide[6] (4.2 g) was dissolved in 15 ml of dimethylformamide (DMF) and *p*-nitrophenyl N-carbobenzoxy-S-benzyl-L-penicillaminate[3] (8 g) was added. After the solution was stirred for 3 days at room temperature, 100 ml of ethyl acetate was added, and the solution was washed ten

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(12) Avian vasodepressor assays were performed on conscious chickens according to the procedure employed by R. A. Munsick, W. H. Sawyer, and H. B. van Dyke, *Endocrinology*, **66**, 860 (1960).

(13) Oxytocic assays were performed according to the method of P. Holton, *Brit. J. Pharmacol.*, **3**, 328 (1948), on uteri from rats in natural estrus with the use of Mg-free van Dyke–Hastings solution as employed by R. A. Munsick, *Endocrinology*, **66**, 451 (1960).

(14) Capillary melting points were determined and are corrected.

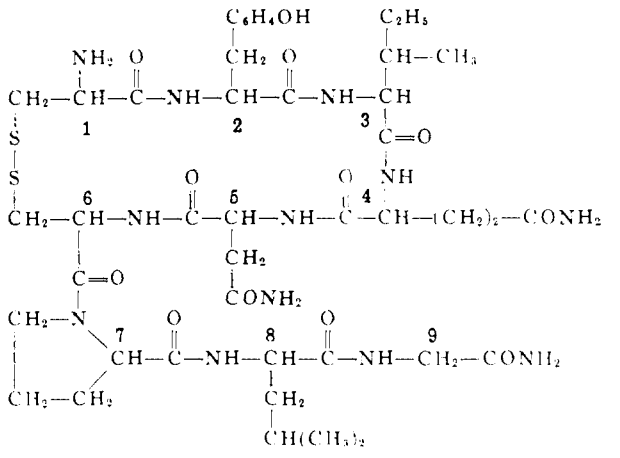

Figure 1.—Structure of oxytocin, with numbers indicating the position of the individual amino acid residues.

times with H_2O saturated with Et_3N and twice with 20% H_2SO_4. The organic phase was then dried ($MgSO_4$) and evaporated to dryness. Crystals (7.6 g) were obtained from the residue on addition of ethyl acetate–hexane. The compound was recrystallized from ethyl acetate–hexane; $[\alpha]^{20}D$ −36.8° (c 1, DMF), mp 85–89°.

Anal. Calcd for $C_{33}H_{45}N_5O_6S$: C, 62.0; H, 7.09; N, 11.0. Found: C, 61.9; H, 7.14; N, 10.9.

N-Carbobenzoxy-L-asparaginyl-S-benzyl-L-penicillaminyl-L-prolyl-L-leucylglycinamide.—The protected tetrapeptide (7.3 g) was dissolved in 15 ml of glacial acetic acid, and 5 *N* HBr in glacial acetic acid (30 ml) was added. The solution was kept for 1.5 hr at room temperature. The product was precipitated by addition of ether, filtered off, washed three times with ether, and dried over KOH. The tetrapeptide was dissolved in 100 ml of methanol and passed through a column containing an ion-exchange resin in the hydroxyl form [Rexyn RG1(OH)]. The methanol was removed by evaporation, and the residue (5.3 g) was dissolved in 25 ml of DMF and allowed to react with 4.5 g of *p*-nitrophenyl N-carbobenzoxy-L-asparaginate.[5] After 4 days at room temperature, 100 ml of ethyl acetate was added. The solution was washed eight times with H_2O saturated with Et_3N and twice with 20% H_2SO_4. The organic phase was dried ($MgSO_4$) and evaporated *in vacuo;* 6.4 g, mp 101–105°, $[\alpha]^{20}D$ −45.3° (c 1, DMF).

Anal. Calcd for $C_{37}H_{51}N_7O_8S$: C, 58.9; H, 6.82; N, 13.0. Found: C, 58.6; H, 6.91; N, 12.9.

N-Carbobenzoxy-L-glutaminyl-L-asparaginyl-S-benzyl-L-penicillaminyl-L-prolyl-L-leucylglycinamide.—The protected pentapeptide (6.2 g) in 50 ml of acetic acid was treated with 5 *N* HBr in acetic acid (50 ml). After 2 hr at room temperature, 400 ml of ether was added. The cooled suspension was filtered, and the precipitate was washed three times with ether. The white solid in 150 ml of methanol was passed through a column containing Rexyn RG1(OH). The methanol was removed by evaporation, and the residue (4.7 g) was dissolved in 12 ml of DMF and treated with 3.3 g of *p*-nitrophenyl N-carbobenzoxy-L-glutaminate.[5] After the solution was stirred for 4 days at room temperature, the product was precipitated by addition of ether and ethyl acetate. The supernatant liquid was removed by decantation, and the residual oil was dissolved in DMF and precipitated with ethyl acetate and ether. The semisolid precipitate in 70 ml of ethanol was passed through a column containing Rexyn RG1(OH). The filtrate was evaporated: 4.9 g, mp 113–116°, $[\alpha]^{20}D$ −42.7° (c 1, DMF).

Anal. Calcd for $C_{42}H_{58}N_9O_{10}S$: C, 57.2; H, 6.74; N, 14.3. Found: C, 56.9; H, 6.76; N, 14.0.

N-Carbobenzoxy-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-penicillaminyl-L-prolyl-L-leucylglycinamide.—The protected hexapeptide (4.7 g) was dissolved in 40 ml of glacial acetic acid, and 5 *N* HBr in acetic acid (50 ml) was added. The solution was kept for 2 hr at room temperature. Ether was added and the precipitate was collected, washed with ether, dissolved in 100 ml of methanol, and passed through a column containing Rexyn RG1(OH). The filtrate was evaporated to dryness, and the residue (3.5 g) was dissolved in 6 ml of DMF. After 1.9 g of *p*-nitrophenyl N-carbobenzoxy-L-isoleucinate[5] was added, the solution was stirred for 2 days at room temperature. The product was precipitated by addition of 100 ml of ethyl acetate and then treated with three 100-ml portions of ethyl acetate to yield 3.4 g.

The protected heptapeptide (3.2 g) was dissolved in 20 ml of glacial acetic acid, and 6 *N* HBr in acetic acid (20 ml) was added. The solution was kept for 2 hr at room temperature, and the product was precipitated by addition of ether, filtered off, washed with ether, dissolved in methanol, and passed through a column containing Rexyn RG1(OH). The filtrate was evaporated to dryness, and the residue (2.6 g) was dissolved in 7 ml of DMF. *p*-Nitrophenyl N-carbobenzoxy-O-benzyl-L-tyrosinate[5] (1.7 g) was added, and the solution was stirred for 4 days at room temperature. The product was precipitated by addition of 100 ml of ethyl acetate, filtered, and treated with two 100-ml portions of ethyl acetate to yield 3.1 g. The compound was precipitated from DMF–ethyl acetate–ethanol and from tetrahydrofuran–water; mp 201–206°, $[\alpha]^{20}D$ −35.8° (c 1, DMF).

Anal. Calcd for $C_{61}H_{78}N_{10}O_{13}S$: C, 61.6; H, 6.86; N, 12.3. Found: C, 61.2; H, 6.86; N, 12.2.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-penicillaminyl-L-prolyl-L-leucylglycinamide.—The protected octapeptide (1.2 g) was suspended in 20 ml of trifluoroethanol, and HBr was bubbled through for 30 min. The reaction mixture was left for another 30 min at room temperature. The solvent was evaporated, and the residue was washed three times with ether, dissolved in 40 ml of methanol, and passed through a column containing the ion-exchange resin (IRA-410) in the hydroxyl form. The filtrate was evaporated to dryness and dissolved in 5 ml of DMF. *p*-Nitrophenyl N-carbobenzoxy-S-benzyl-L-cysteinate[5,15] (0.43 g) was added, and the solution was stirred for 4 days at room temperature. The reaction product was precipitated by addition of 100 ml of ethyl acetate, collected, precipitated from 20 ml of ethanol by addition of ethyl acetate, and finally washed with ethyl acetate to yield 0.95 g. A sample was precipitated from tetrahydrofuran–water; mp 191–195°, $[\alpha]^{20}D$ −45° (c 1, DMF).

Anal. Calcd for $C_{67}H_{84}N_{12}O_{13}S_2$: C, 59.5; H, 6.71; N, 12.4. Found: C, 59.5; H, 6.74; N, 12.1.

6-L-Penicillamine-oxytocin.—The protected nonapeptide (0.3 g) was dissolved in 60 ml of anhydrous liquid NH_3, and Na was added until the blue color lasted for 10 sec. The NH_3 was removed by evaporation and lyophilization. The white residue was dissolved in 300 ml of deaerated 0.1% acetic acid, the pH was adjusted to 6.8–7.0, and the theoretical amount of 0.01 *N* potassium ferricyanide (44 ml) was added. The yellow solution was deionized by passage through a column containing the ion-exchange resin (AG 3-X4) in the chloride form. The filtrate was concentrated and placed in the first five tubes of a countercurrent distribution machine and subjected to 200 transfers in the solvent system 1-butanol–pyridine–benzene–0.1% aqueous acetic acid (6:1:2:9). The main peak, as determined by the Folin–Lowry color values,[16] had a partition coefficient (K) of 1.0. The contents of the tubes representing the main peak together with a small side peak were concentrated and lyophilized. For further purification this material (143 mg) was subjected to partition chromatography on Sephadex G-25 in the solvent system 3.5% aqueous acetic acid (containing 1.5% pyridine)–1-butanol (1:1). The compound emerged in a large peak with an R_f of 0.32, well separated from a small faster moving peak. The material (72 mg) obtained from the tubes representing the main peak was subjected to gel filtration on Sephadex G-25 in 0.2 *N* acetic acid and found to emerge in two separate peaks. The main peak appeared at the position of oxytocin, and the other peak was in the region where dimeric compounds are expected to be eluted. The main peak represented 27 mg of 6-L-penicillamine-oxytocin, $[\alpha]^{20}D$ 0° (c 0.5, 1 *N* acetic acid). On paper chromatography (Whatman No. 1) in the solvent system 1-butanol–acetic acid–water (4:1:5, descending) the compound showed only one spot (developed with Pauly reagent). Electrophoresis performed on Whatman No. 1 paper at 4° in pyridine–acetate buffer of pH

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5.6 at 300 v for 22 hr also showed only one spot (developed with Panly reagent). For analysis a sample was dried over P_2O_5 at 100° *in vacuo;* a loss in weight of approximately 10% was observed.

Anal. Calcd for $C_{45}H_{70}N_{12}O_{12}S_2$: C, 52.2; H, 6.82; N, 16.2. Found: C, 51.9; H, 6.87; N, 15.9.

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The Synthesis, Stereochemistry, and Biology of 16-Hetero and 17-Oxa-D-homo Steroids[1]

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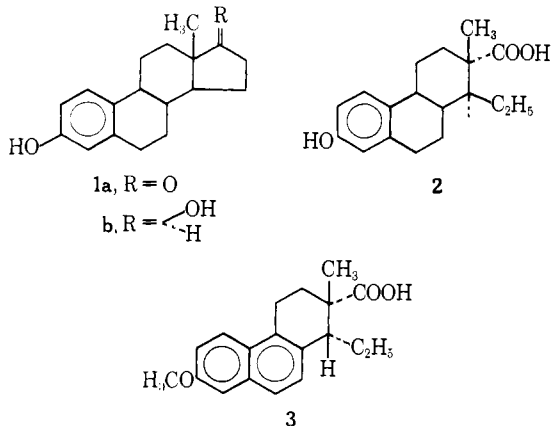
The synthesis, stereochemistry, and biological activities of 16-oxa, 16-aza-, and 17-oxa-D-homo steroids and related seco steroids derived from estrone 3-methyl ether, (±)-13-ethyl-3-methoxygona-1,3,5(10)-trien-17-one, and 14-isoequilenin are presented.

Stamler, Marmiston, Oliver, and others[2] have presented evidence that estrogens play a significant role in human female resistance toward atherosclerosis by virtue of their ability to alter serum lipid concentrations. However, the effect of estrogens on secondary sex characteristics is an obvious deterrent to any therapeutical value they may have in man. At our laboratories, there has been a consistent effort to obtain a substance which might mimic estrone (**1a**) or 17β-estradiol (**1b**) in its ability to alter blood fat patterns in animals without affecting the reproductive organs. A program which began with the investigation of new ring-D seco steroids related to some estrogenolic acids,[3-5] an example of which is doisynolic acid (**8**), led to the synthesis of 16-oxaestra-1,3,5(10)-triene-3,17-diol 3-methyl ether (**14b**), a substance which, in the rat, has significant effects on blood lipids and which is devoid of estrogenic effects at screening levels.[6] The synthesis and stereochemistry of **14b** and related seco and hetero steroids are described presently.



Synthesis and Stereochemistry.—The synthesis began with the ozonolysis of the enol acetate **4a** followed by hydrolysis to the aldehyde acid **5a** (Scheme I). The next synthetic step, the internal enol esterification between the reactive alkyl aldehyde and carboxyl groups, was without precedent and required study. Typical conditions[7] which have been used for the conversion of γ- and δ-ketocarboxylic acids to enol lactones or aldehydes to enol acetates gave only polymer or a low yield of the acetoxy lactone **11a**. Treatment of **5a** in methanol with *p*-toluenesulfonic acid led to the methoxy lactone **11b**.[8] However, rapid, azeotropic distillation of water from a dilute solution of **5a** in toluene containing *p*-toluenesulfonic acid gave a good yield of the enol lactone **6a**. Ozonolysis of the enol lactone **6a** followed by hydrolysis yielded **7a**. Reduction of the aldehyde acids **5a** and **7a** with sodium borohydride followed by acidification produced the six-membered ring lactone **9a** and the hydroxy acid **8a**, respectively. Azeotropic distillation of water from a toluene solution of **8a** containing a catalytic amount of *p*-toluenesulfonic acid yielded the lactone **9b**. Cleavage of the methyl ethers **9a** and **9b** with potassium hydroxide in ethanol[9] at 200° followed by treatment with strong acid gave the phenolic derivatives 17-oxa-D-homoestrone (**9d**) and 16-oxaestrone (**9e**), respectively. The lactones **9a** and **9b**, when reduced with lithium aluminum hydride, yielded the diols **12a** and **12b**, respectively, and, when reduced with diisobutylaluminum hydride[10] in toluene at −60°, yielded the hemiacetals **14a** and **14b**, respectively (Scheme II). When each was dissolved in methanol containing strong acid, a corresponding mixture of methyl ethers was obtained which was

(1) Presented in part at the 2nd International Congress on Hormonal Steroids, Milan, Italy, May 1966; J. S. Baran, *Excerpta Med.*, **111**, 387 (1966).

(2) For leading references see J. Stamler in "Atherosclerosis and Its Origin," M. Sandler and G. H. Bourne, Ed., Academic Press Inc., New York, N. Y., 1963, p 231.

(3) The related estrogenolic acid, 7-methylbisdehydrodoisynolic acid[4] (**3**), is a potent estrogen in the mouse and a weak estrogen in man.[5] It was reasoned that steroids related to **2** and **3** might still have pronounced effects on the lipid metabolism in man without estrogenic effects especially if they affected the lipid metabolism of animals with little or no effects on the reproductive organs.

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(7) See preparation of **6a** in the Experimental Section.

(8) The assignment of configuration to the C-16 hydrogen is based on its nmr spectrum. The half-line width for the C-16 hydrogen is about 5 cps which would be associated with a coupling of an equatorial C-16 hydrogen atom with the C-15 hydrogen atoms; see N. S. Bhacca and D. H. Williams, "Applications of NMR Spectroscopy in Organic Chemistry," Holden-Day, Inc., San Francisco, Calif., 1964, p 51.

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