Synthesis and Pharmacological Properties of [1-L-Penicillamine,4-L-leucine]oxytocin[1]

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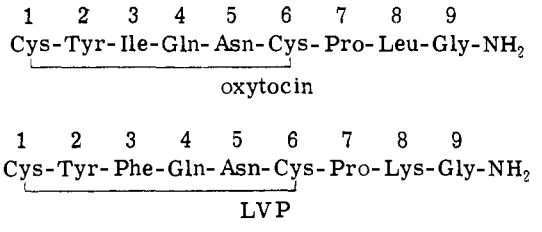
For the synthesis of [1-L-penicillamine,4-L-leucine]oxytocin (2), Z-Tyr(Bzl)-Ile-Leu-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 was treated with anhydrous HBr, and the resulting partially deprotected octapeptide was coupled with Z-penicillamine(Bzl) in a condensation reaction mediated by dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. The protected nonapeptide Z-penicillamine(Bzl)-Tyr-Ile-Leu-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 was treated with Na in NH_3 and the resulting disulfhydryl compound was subjected to oxidative cyclization in H_2O–CH_3OH with ICH_2CH_2I. Purification of 2 was effected by partition chromatography and gel filtration. The analog possesses antioxytocic and antiavian vasodepressor pA_2 values of 6.77 and 7.21, respectively, and has no antipressor or anti-ADH activity. Its biological activity spectrum is qualitatively identical with that of [1-penicillamine]oxytocin. In contrast to the marked natriuretic–diuretic and anti–antidiuretic activity of [Leu^4]oxytocin, 2 exhibits none of these effects on the rat kidney.

Each of the posterior pituitary hormones, oxytocin, 8-lysine-vasopressin (LVP), and 8-arginine-vasopressin, as well as their 1-β-mercaptopropionic acid (β-Mpa^1) analogs, exhibits a number of biological activities. Of these, the oxytocic, avian vasodepressor, and milk-ejecting are most



characteristic of oxytocin and the rat pressor and antidiuretic are exhibited more strongly by the vasopressins. It has been found that certain chemical modifications of the hormone molecules or of their [β-Mpa^1] analogs result in the formation of analogs which reversibly inhibit one or more of the effects of the hormones. For example, a number of analogs have been synthesized with two methyl, ethyl, or other alkyl groups at position 1 of oxytocin,[2] [β-Mpa^1]oxytocin,[2-4] and [β-Mpa^1]-LVP.[5] These analogs antagonize the oxytocic[2-7] and avian vasodepressor (AVD)[2-5] responses to oxytocin and the rat pressor[4,5] responses to LVP, but those tested have no effect on the antidiuretic (ADH) responses to vasopressin.[7,8] Another modification, which confers a more limited range of reversibly inhibitory properties, is the substitution of a leucine residue for that of glutamine at position 4. [4-Leucine]oxytocin,[9] [4-leucine]-LVP,[10] and their [β-Mpa^1] analogs[10,11] have been prepared, and it was found that [Leu^4]oxytocin, but none of the other three compounds, exhibits a marked anti-ADH effect.[10-12] In addition, [Leu^4]-LVP shows weak antioxytocic and anti-AVD effects, and [β-Mpa^1,Leu^4]-LVP is weakly antioxytocic.[10]

To investigate the effect of combining the above-mentioned inhibitory substituents in the same molecules, [1-β-mercapto-β,β-diethylpropionic acid,4-leucine]oxytocin ([β-Mpa(β-Et$_2$)^1,Leu^4]oxytocin) and [1-β-mercapto-β,β-diethylpropionic acid,4-leucine]-LVP ([β-Mpa(β-Et$_2$)^1,-Leu^4]-LVP) were synthesized[8] and found to have lower antioxytocic, anti-AVD, and antipressor potencies than those of [β-Mpa(β-Et$_2$)^1]oxytocin and [β-Mpa(β-Et$_2$)^1]-LVP, respectively. Neither of the new analogs exhibited anti-ADH effects.

It should be noted that because a free amino group is lacking in the 1 position, [β-Mpa(β-Et$_2$)^1,Leu^4]oxytocin is more closely related to [β-Mpa^1,Leu^4]oxytocin, which has very low but definite ADH activity, than to [Leu^4]oxytocin with its unusual anti-ADH properties. In the search for compounds with enhanced anti-ADH activity which might have clinical applications, it was therefore desired to study an analog of [Leu^4]oxytocin which would include an amino group as well as alkyl substituents at position 1. L-Penicillamine (β,β-dimethyl-L-cysteine), which had been used in the synthesis of the first inhibitory analog of the alkyl-substituted type,[2] was chosen for this work. The present paper describes the synthesis and pharmacological properties of [1-L-penicillamine,4-leucine]oxytocin ([Cys(β-Me$_2$)^1,Leu^4]-oxytocin).

Z-Tyr(Bzl)-Ile-Leu-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2[9] was treated with anhydrous HBr[13] and the resulting free amine salt was coupled in dimethylformamide (DMF) in the presence of base with *N*-benzyloxycarbonyl-*S*-benzyl-L-penicillamine[2] [Z-penicillamine(Bzl)-OH] by the dicyclohexylcarbodiimide (DCC)–hydroxybenzotriazole (HBt) preactivation method.[14] The nonapeptide Z-penicillamine(Bzl)-Tyr-Ile-Leu-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 was treated with Na in liquid NH_3,[15] and the resulting disulfhydryl compound was subjected to oxidative cyclization with ICH_2CH_2I[16] in aqueous MeOH. [1-Penicillamine,4-leucine]oxytocin was purified by partition chromatography[17] and gel filtration[18] on Sephadex G-25.

[1-Penicillamine,4-leucine]oxytocin was assayed,[19] and its biological activity spectrum was compared to the spectra of [1-penicillamine]oxytocin and [4-leucine]oxytocin. It was found that [1-penicillamine,4-leucine]oxytocin resembles closely [1-penicillamine]oxytocin. Neither compound has detectable oxytocic, avian vasodepressor, pressor, or antidiuretic activities. Both compounds have an inhibitory effect on the oxytocic and AVD responses to oxytocin but not on the pressor or antidiuretic responses to oxytocin and vasopressin. The antioxytocic potency of [1-penicillamine,4-leucine]oxytocin is slightly less than that of [1-penicillamine]oxytocin, with a pA_2 value of 6.77 ($M = 1.7 \times 10^{-7}$; SD $= 0.5 \times 10^{-7}$; $n = 23$) compared to 6.68 ($\bar{M} = 1.4 \times 10^{-7}$; SD $= 0.4 \times 10^{-7}$; $n = 7$)[3] for the latter. The anti-AVD potency of [1-penicillamine,4-leucine]oxytocin is approximately half that of [1-penicillamine]oxytocin, with a pA_2 value of 7.21 ($\bar{M} = 6.1 \times 10^{-8}$; SD $= 3.4 \times 10^{-8}$; $n = 15$) compared to 7.50 ($\bar{M} = 3.2 \times 10^{-8}$; SD $= 1.0 \times 10^{-8}$; $n = 8$)[3] for [1-penicillamine]oxytocin.

[1-Penicillamine,4-leucine]oxytocin, however, does not

share the characteristics of [4-leucine]oxytocin. [4-Leucine]oxytocin has a marked natriuretic–diuretic activity. It also antagonizes the antidiuretic effect of vasopressin (anti-ADH).[12] Equimolar doses of [1-penicillamine,4-leucine]oxytocin have no effect on the excretion rates of urine and urinary sodium or on the antidiuretic response to vasopressin. The analog appears to have no activity in the kidney.

These results are qualitatively very similar to those obtained with the deamino compound [β-Mpa(β-Et$_2$)^1,Leu^4]-oxytocin in the earlier work[8] mentioned above. Thus, with or without an amino group present (1) the additional substitution of a leucine residue in the 4 position does not enhance the inhibitory power of a 1-position alkyl-substituted analog of oxytocin, and (2) the 1,4-disubstituted analog has none of the natriuretic–diuretic and anti-ADH properties of [4-leucine]oxytocin.

Experimental Section

Precoated plates of silica gel F-254 (0.25 mm, E. Merck) were used for thin-layer chromatography (TCL) of spots containing 10–20-μg samples of compound and were developed with the solvent systems: (A) $CHCl_3$–MeOH–HOAc (10:2:0.5) and (B) BuOH–HOAc–H_2O (4:1:1). Spots were visualized by chlorination followed by KI–starch spray. Optical rotations were measured on a Perkin-Elmer 141 photoelectric polarimeter. Compounds were dried for analysis over P_2O_5 at room temperature for 16–24 hr at 0.005 mmHg. Yields are reported on the basis of the empirical formula weights. Where analyses are indicated only by symbols of the elements, analytical results obtained were within ±0.4% of the theoretical values.

Z-Penicillamine(Bzl)-Tyr-Ile-Leu-Asn-Cys(Bzl)-Pro-Leu-Gly-NH$_2$ (1). Approximately 15 ml of HBr was condensed, after passage through towers of $CaCl_2$ and naphthalene, into a flask containing Z-Tyr(Bzl)-Ile-Leu-Asn-Cys(Bzl)-Pro-Leu-Gly-NH$_2$[9] (108 mg, 0.090 mmol). The suspension was stirred and refluxed for 2 hr, during which time the lyophilized starting material dissolved and a fine white precipitate appeared. The HBr was evaporated at the water aspirator and the residue was washed with Et_2O (two 10-ml portions). This material was dissolved in DMF (0.64 ml) and neutralized with 0.015 ml of *N*-methylmorpholine. Z-Penicillamine(Bzl)-OH (89 mg, 0.24 mmol) was preactivated by treatment with DCC (50 mg, 0.24 mmol) and HBt·0.5H_2O (52 mg, 0.36 mmol) for 1 hr at room temperature in DMF (1.28 ml). Precipitated dicyclohexylurea was centrifuged down, and supernatant amounting to half of the total volume was added to the neutralized solution of octapeptide hydrobromide. The condensation reaction was 90% complete in 2.5 hr as determined by the quantitative Kaiser test.[5,28] After 3.5 hr, EtOAc (~20 ml) was added slowly to the reaction mixture. The resulting suspension was stirred for 1 hr and then centrifuged. The very fine precipitate was washed by centrifugation with 6 and 4 ml of EtOAc and then dissolved in 10 ml of HOAc and lyophilized: 91 mg (0.064 mmol, 71%). This material showed faint traces of impurities on TLC (A, 0.4) but could be used successfully for the preparation of analog. A sample (28 mg) was washed twice by centrifugation from 2 ml of 95% EtOH and recovered in 68% yield: $[\alpha]^{20.5}$D −46.5° (*c* 1, DMF). Anal. ($C_{68}H_{94}N_{11}O_{13}S_2 \cdot C_2H_4O_2 \cdot H_2O$) C, H, N.

[1-Penicillamine,4-leucine]oxytocin (2). A sample of 1 (98 mg, 0.69 mmol) was dissolved in NH_3 (100 ml, freshly distilled from Na) and treated intermittently with a fresh stick of Na enclosed in a 4-mm glass tube until the solution retained a blue coloration for 20 sec. The color was destroyed with NH_4Cl and the solvent was evaporated to a low volume. The remaining 10–15 ml was removed by lyophilization, the flask was opened under N_2, and the residue was dissolved in a MeOH–H_2O mixture (80–90 ml) that had been deaerated and flushed with N_2. A solution of ICH_2CH_2I (22 mg, 0.078 mmol) in MeOH (10 ml) was added, and the disappearance of the SH group was found by the Ellman test[29] to be complete within 5 min. The MeOH was removed by rotary evaporation, the solution was adjusted to pH 3, and concentration was continued. The final 50 ml of solvent was removed by lyophilization. The white residue was dissolved in 3 ml of the upper phase and 2 ml of the lower phase of the solvent system 1-BuOH–benzene–HOAc–H_2O (8:3:2:8) and subjected to partition chromatography on a column (2.2 × 52 cm) of Sephadex G-25 (100–200 mesh) at a flow rate of 17 ml/hr. Fractions of 4.3 ml were collected and tested by the Folin–Lowry procedure.[30] The elution pattern consisted of two relatively large peaks at R_f 0.25 and 0.80 and a small peak at R_f 0.42. The faster running components were isolated from fractions 6–30 and amounted to 22 mg. The material comprising the peak centered at R_f 0.25 was isolated from fractions 31–50 by dilution with H_2O, rotary evaporation, and lyophilization: wt 32 mg. This material was subjected to gel filtration on a column (2.82 × 68 cm) of Sephadex G-25 (200–270 mesh) in 0.2 *N* HOAc and emerged as a single sharp peak at 75% of column volume: wt 25 mg (32% from 1); homogeneous to TLC (B, 0.4); $[\alpha]^{18}$D 37.4° (*c* 1, 1 *N* HOAc). Anal. ($C_{46}H_{73}N_{11}O_{11}S_2 \cdot C_2H_4O_2 \cdot 2H_2O$) C, H, N.

Amino acid analysis[31] was performed on a Beckman 116 analyzer on a 0.9 × 58 cm column of AA-15 resin at 55°, using a single column system employing 0.069 *M* Na citrate buffers at pH 3.28, 4.30, and 6.40 (Na^+ concentrations 0.2, 0.2, and 1.0 *N*, respectively). A sample was hydrolyzed for 41 hr in degassed 6 *N* HCl at 110° and analyzed at a flow rate of 72.7 ml/hr with buffer changes at 90 and 150 min. Peaks representing the mixed disulfide of cysteine and penicillamine and the disulfide form of penicillamine were observed at 129 and 140 min, respectively (compared to Gly, Cys, and Ile at 104, 125, and 144 min, respectively). These peaks were assumed to have color values equal to that of Cys. On this basis the following ratios were obtained: Asp, 1.03; Pro, 0.95; Gly, 0.95; sulfur compounds, 2.07; Ile, 1.01; Leu, 2.12; Tyr, 0.88; NH_3, 2.16. On a sample which was oxidized by the performic acid method of Moore[32] prior to hydrolysis as above, the three sulfur compounds seen in the unoxidized sample were replaced by a single symmetrical peak at the position of cysteic acid (17 min), in a ratio of 1.94 to the average value for all other amino acids.

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[4-Phenylalanine]oxytocin, an Inhibitor of the Antidiuretic Effect of 8-Arginine-vasopressin[1]

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[4-Phenylalanine]oxytocin was prepared from Z-Cys(Bzl)-Tyr(Bzl)-Ile-Phe-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (4) by deprotection with Na in NH_3 followed by cyclization of the resulting disulfhydryl compound with ICH_2CH_2I. The protected peptide 4 was prepared from Boc-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 by the stepwise solution method. Coupling was effected by a modification of the dicyclohexylcarbodiimide–1-hydroxybenzotriazole preactivation method wherein the precipitate of dicyclohexylurea is removed by filtration prior to mixing of the amino and carboxyl components. The analog was found to be an effective inhibitor of the antidiuretic (ADH) response to exogenous arginine-vasopressin. It produced marked diuresis in the anti-ADH assay at approximately the same dose level as does [Leu^4]oxytocin but, in contrast to [Leu^4]oxytocin, showed natriuretic activity only at relatively high dose levels. In addition, [Phe^4]oxytocin exhibited 0.15% of the oxytocic potency of oxytocin, weak antiavian vasodepressor activity ($pA_2 = 6.93$), and no measurable rat pressor activity.

In the course of studies aimed at elucidating the importance of the glutamine residue in position 4 of oxytocin (Figure 1) to the biological activities of this hormone, a series of analogs[2] was synthesized in which lipophilic, aliphatic amino acid residues were substituted in position 4 of oxytocin and deamino-oxytocin. The substantial potencies exhibited by these analogs in the oxytocic, avian vasodepressor (AVD), and milk-ejecting assays demonstrated that considerable variation is possible and that the carboxamide function is not essential for manifestation of these activities. A number of the analogs retained low but definite antidiuretic (ADH) potency as well, but most were inactive in the rat pressor assay.

Unexpectedly, one member of the series was found to possess antihormonal properties. Although [Leu^4]oxytocin possesses 2, 9, and 16%, respectively, of the oxytocic, AVD, and milk-ejecting potencies of oxytocin, it exhibits a weak depressor effect in the rat pressor assay and a strong diuretic effect in the antidiuretic assay.[3] The diuretic response to [Leu^4]oxytocin is due to a strong natriuretic effect as well as to an inhibition of the effect of the antidiuretic hormone 8-arginine-vasopressin.[3] Since [Leu^4]oxytocin can effectively reverse vasopressin-induced free-water reabsorption to free-water excretion but does not counteract the pressor response to vasopressin, it may be called an anti-ADH substance. No other member of this series displayed anti-ADH activity, although the length of the aliphatic side chain was varied from zero to four carbon atoms, and both branched- and straight-chain residues were used. The present synthesis of [Phe^4]oxytocin was undertaken in order to examine the effect of a further increase in lipophilicity and steric bulk in position 4.

The protected nonapeptide precursor to [Phe^4]oxytocin was prepared from Boc-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2[5] by the stepwise solution method, utilizing N^{α}-*tert*-butyloxycarbonyl (Boc) protection of the intermediate peptides and trifluoroacetic acid (TFA) deprotection at each step. Coupling was effected by means of a modification of the dicyclohexylcarbodiimide–1-hydroxybenzotriazole (DCC–HBt) preactivation method of König and Geiger[6] wherein the precipitate of dicyclohexylurea (DCU) is removed by filtration prior to mixing of the amino and carboxyl components. This procedure removes ~95% of the troublesome DCU by-product, and the peptide usually can be freed of

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