Synthesis and Some Pharmacological Properties of [4-β-(2-Thienyl)-L-alanine]oxytocin[1]

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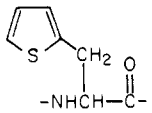
The synthesis and some biological activities of [4-β-(2-thienyl)-L-alanine]oxytocin are reported. This analogue has been studied in an ongoing exploration of the biological effects of introducing amino acid residues with bulky hydrophobic side chains into the second corner position of the β turn present in the conformation of the 20-membered ring portion of oxytocin. The analogue was synthesized in a stepwise manner by solution techniques utilizing ethylcarbamoyl protection for cysteine side chains. The presence of thienylalanine in position 4 evokes a drastic reduction in both affinity and intrinsic activity; the reduction in intrinsic activity was greater than that found for [Leu⁴]oxytocin or [Phe⁴]oxytocin. The analogue possesses 0.51 ± 0.03 unit/mg of rat uterotonic potency and less than 0.05 unit/mg of rat pressor and rat antidiuretic potency and behaves as a competitive inhibitor of the response to oxytocin in the avian vasodepressor assay with a pA_2 value of 7.44 ± 0.19.

In a previous study we investigated the effect on biological activity—with special emphasis on intrinsic uterotonic activity[2]—of introducing an amino acid residue with a bulky hydrophobic side chain into position 4 of oxytocin.[3] This position is one of the corner positions of the two β turns present in the preferred solution conformation of the hormone.[4] It was found that both [Leu⁴]oxytocin and [Phe⁴]oxytocin exhibited a diminished ability to stimulate maximally the isolated rat uterus as compared to oxytocin and that the aromatic phenylalanine side chain in position 4 was more effective in reducing intrinsic activity than was the aliphatic leucine side chain. We have extended these investigations to include [4-β-(2-thienyl)-L-alanine]oxytocin ([Thi⁴]oxytocin), an analogue with an aromatic residue of the following structure in position 4.



The protected nonapeptide was synthesized in a stepwise manner beginning with Pro-Leu-Gly-NH_2.[5] Couplings were achieved with dicyclohexylcarbodiimide (DCC) mediated by 1-hydroxybenzotriazole[6] (HBT), except for the thienylalanine residue which was introduced as the *p*-nitrophenyl ester. For the protection of side-chain sulfhydryl groups of the cysteine residues, the ethylcarbamoyl[7] (Ec) group was employed. The side-chain hydroxyl group of tyrosine was left unprotected. The *tert*-butyloxycarbonyl (Boc) group was used throughout for N^{α} protection. After removal of the NH_2-terminal Boc group, the Ec groups were removed by titrating a methanol solution of the nonapeptide trifluoroacetate salt with equivalent amounts of aqueous 1.0 M NaOH (1 equiv for the acid salt, 1 equiv for the tyrosine hydroxyl, and 1 equiv for each Ec group). Formation of the disulfide bond was accomplished by oxidation with diiodoethane[8] and the product was purified by partition chromatography[9] and gel filtration on Sephadex G-25.

[Thi⁴]oxytocin was tested for some of the biological activities characteristic of neurohypophyseal hormones (rat uterotonic,[10] avian vasodepressor,[11] rat pressor,[12] and rat antidiuretic[13]). However, [Thi⁴]oxytocin behaved as a competitive inhibitor of the vasodepressor response to oxytocin when tested according to the method of Vavrek et al.[14] with a pA_2[15] of 7.44 ± 0.19. When the analogue was given to four rats at doses of 0.1 mg administered in 0.2 mL, no consistent antidiuretic or pressor response was obtained. Nevertheless, the data reveal that these activities of the peptide lie below 0.05 unit/mg. Higher doses could not be tested due to the poor solubility of the analogue in aqueous media. The dose–response behavior of [Thi⁴]-oxytocin, relative to oxytocin, on the isolated rat uterus is shown in Figure 1. The data were obtained by the individual injection method of Chan and Kelly,[16] performed routinely in this laboratory.[3,17] Not only does the analogue exhibit a reduced affinity for the uterotonic receptors reflected by the displacement of its dose–response curve to higher concentrations than oxytocin, but it also has a lower intrinsic activity as shown by its inability to stimulate maximally contractions of the uterus to the same extent as oxytocin. In fact, the maximum response to [Thi⁴]oxytocin was only 64 ± 6% of the maximum achieved with oxytocin. By working in the region of the uterotonic response which was parallel to the USP posterior pituitary reference standard (less than 40% of maximum response), a specific uterotonic potency of 0.51 ± 0.03 unit/mg was determined for [Thi⁴]oxytocin.

In our earlier study it was found that both [Leu⁴]-oxytocin and [Phe⁴]oxytocin exhibited reduced intrinsic uterotonic activities. The maximum of contraction obtained in response to [Leu⁴]oxytocin and [Phe⁴]oxytocin was only 81 ± 4 and 75 ± 4%, respectively, as compared to 100% for oxytocin.[3] It is apparent that the five-membered-ring aromatic thienylalanine residue in position 4 is more effective in reducing intrinsic activity than either the aliphatic leucine residue or the aromatic phenylalanine residue ($p < 0.001$). These observations are in line with our earlier interpretations, based on the biologically active conformation of oxytocin,[18,19] regarding the role of bulky side chains in position 4 of oxytocin in reducing intrinsic uterotonic activity by sterically interfering with the active elements[19] Tyr² and Asn⁵ of neurohypophyseal peptides.

Experimental Section

Details about materials and experimental methods have been described previously.[3] Solvent systems for thin-layer chromatography (TLC) were A, acetone–HOAc (9:1); B, 1-BuOH–HOAc–H_2O (4:1:1); C, 1-BuOH–pyridine–H_2O (20:10:11).

Boc-Cys(Ec)-Pro-Leu-Gly-NH_2 (1). A solution of Boc-Cys(Ec)[20] (4.3 g, 18.2 mmol), HBT (3.5 g, 26 mmol), and DCC (3.7 g, 17.3 mmol) was prepared in 10 mL of DMF cooled to 0 °C. After stirring for 40 min at room temperature, the preactivation mixture was added through a fritted funnel to a solution of Pro-Leu-Gly-NH_2[5] (4.3 g, 14.6 mmol) in DMF (15 mL) with 10 mL of DMF wash. *N*-Methylmorpholine (1.7 mL, 15 mmol) was added 90 min after the preactivation mixture. After 24 h, TLC in system A did not show any residual Pro-Leu-Gly-NH_2. Water (3 mL) was added and the resulting dicyclohexylurea (DCU) precipitate was removed by filtration. The DMF was removed by rotary evaporation and the residue triturated with EtOAc to precipitate a solid. The product was filtered, washed with EtOAc and Et_2O, and dried in vacuo: 6.4 g (78% yield); mp 184–186 °C;

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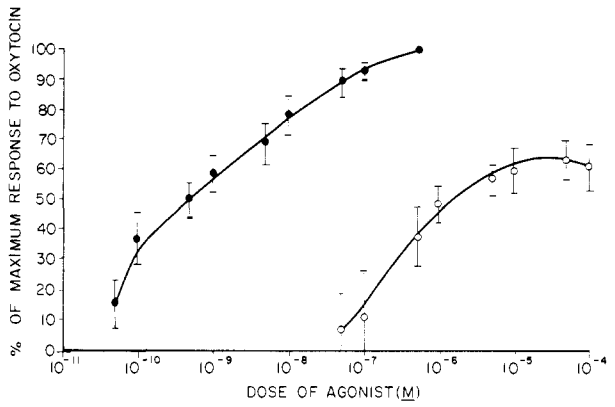

Figure 1. Dose–response curves of oxytocin (●—●) and [Thi⁴]oxytocin (○—○) obtained by the individual injection procedure on the isolated rat uterus in natural estrus according to methods previously described.[3,17] The points along the curves represent the data from eight determinations on six uteri.

$[\alpha]^{25}_D$ −45° (*c* 1, DMF); TLC (A) R_f 0.68. Anal. ($C_{24}H_{42}N_6O_7S$) C, H, N.

Boc-Asn-Cys(Ec)-Pro-Leu-Gly-NH$_2$ (2). A solution of **1** (5.59 g, 10 mmol) in HCl–EtOAc (75 mL, 4 M) was prepared and allowed to stand at room temperature for 30 min. The solvents were removed by rotary evaporation and the resulting crystalline solid was dried in vacuo. The tetrapeptide hydrochloride salt was dissolved in DMF (50 mL) and the pH of the solution adjusted to 7 (Fisher indicator solution) with *N*-methylmorpholine. To the solution was added HBT (3.34 g, 16.5 mmol) and Boc-Asn (2.56 g, 11 mmol). The solution was cooled to 0 °C and DCC (2.27 g, 11 mmol) was added. After 24 h at room temperature, H_2O (100 mL) was added, and the precipitate was filtered and washed with water, 5% $NaHCO_3$, water, 10% HOAc, and water. The product was triturated in boiling EtOH, and after cooling the solid was filtered, washed with EtOH and Et_2O, and dried in vacuo: 4.9 g (75% yield); mp 219.5 °C dec; $[\alpha]^{25}_D$ −66° (*c* 1, DMF); TLC (A) R_f 0.45. Anal. ($C_{28}H_{48}N_8O_9S$) C, H, N.

Boc-Thi-Asn-Cys(Ec)-Pro-Leu-Gly-NH$_2$ (3). A solution of **2** (673 mg, 1.0 mmol) was prepared in 10 mL of CF_3COOH. After 30 min the CF_3COOH was evaporated; the residue was triturated to a solid with Et_2O, filtered, washed with Et_2O, and dried in vacuo. The pentapeptide trifluoroacetate was dissolved in DMF (10 mL) and the pH adjusted to 7.5 with $(i\text{-Pr})_2NEt$ (0.18 mL). Boc-Thi-ONp[20] (432 mg, 1.1 mmol) was added and the progress of the reaction followed by the ninhydrin test.[21] After 24 h, another 0.10 mL of $(i\text{-Pr})_2NEt$ was added and after 48 h the reaction mixture was poured into 75 mL of EtOAc. The resulting precipitate was filtered, washed with EtOAc and Et_2O, and dried in vacuo: 734 mg (85% yield). The product was reprecipitated from HOAc–EtOAc (1:4, v/v) with 89% recovery: 660 mg; mp 218–218.5 °C; $[\alpha]^{25}_D$ −64° (*c* 1.1, DMF); TLC (A) R_f 0.40. Anal. ($C_{35}H_{55}N_9O_{10}S_2$) C, H, N.

Boc-Ile-Thi-Asn-Cys(Ec)-Pro-Leu-Gly-NH$_2$ (4). The Boc group was removed from **3** (600 mg, 0.72 mmol) as described above. The salt was dissolved in DMF (5 mL) and the pH adjusted to 8 with *N*-methylmorpholine. A preactivated solution of Boc-Ile·0.5H_2O (206 mg, 0.86 mmol), HBT (175 mg, 1.3 mmol), and DCC (178 mg, 0.86 mmol) in glyme (3 mL) was added through a fritted funnel. After 60 min another 0.08 mL of *N*-methylmorpholine was added and after 8 h the product was precipitated by the addition of 50 mL of H_2O. The product was filtered, washed with H_2O, EtOH, and Et_2O, and dried in vacuo: 620 mg (91% yield); mp 236–237 °C; $[\alpha]^{25}_D$ −71° (*c* 1, HOAc); TLC (A) R_f 0.48. Anal. ($C_{41}H_{66}N_{10}O_{11}S_2$) C, H, N.

Boc-Tyr-Ile-Thi-Asn-Cys(Ec)-Pro-Leu-Gly-NH$_2$ (5). This compound was prepared from **4** (570 mg, 0.60 mmol) in the same manner as described for the synthesis of **4** using Boc-Tyr (203 mg, 0.72 mmol) and HBT (194 mg, 1.44 mmol) preactivated by DCC (150 mg, 0.72 mmol): 565 mg (85% yield); mp 225–226 °C; $[\alpha]^{25}_D$ −58° (*c* 1, DMF); TLC (B) R_f 0.59. Anal. ($C_{50}H_{75}N_{11}O_{13}S_2 \cdot H_2O$) C, H, N.

Boc-Cys(Ec)-Tyr-Ile-Thi-Asn-Cys(Ec)-Pro-Leu-Gly-NH$_2$ (6). This compound was prepared from **5** (510 mg, 0.46 mmol) in the same manner as described for the synthesis of **4** using Boc-Cys(Ec) (160 mg, 0.55 mmol) and HBT (110 mg, 0.83 mmol) preactivated with DCC (110 mg, 0.55 mmol). The product precipitated from the reaction mixture as it formed and was isolated by diluting the reaction mixture with EtOH, filtering the precipitate, washing with EtOH and Et_2O, and drying in vacuo: 515 mg (87% yield); mp 234 °C dec; $[\alpha]^{25}_D$ −54° (*c* 1, DMF); TLC (B) R_f 0.60. Anal. ($C_{56}H_{85}N_{13}O_{15}S_3 \cdot 2H_2O$) C, H, N.

[4-β-(2-Thienyl)-L-alanine]oxytocin (7). The Boc protection was removed from **6** (100 mg, 77.5 μmol) by treatment with CF_3COOH as described for the preparation of **3**. The residue after removal of the CF_3COOH was lyophilized from glacial HOAc. The lyophilizate was dissolved in 10.0 mL of MeOH and continuously flushed with a stream of N_2 which was saturated with MeOH. To this solution was added 0.31 mL of 1.0 N NaOH and the appearance of sulfhydryl groups was monitored by the Ellman method.[22] After 45 min the rate of sulfhydryl formation had leveled off at 99% of the theoretical concentration and the pH of the solution was 9. The reaction mixture was diluted with 80 mL of MeOH and 100 mL of H_2O, and diiodoethane (23 mg, 78 μmol) dissolved in 6 mL of MeOH was added. The pH of the solution dropped immediately to 6.5 where it was maintained by the dropwise addition of 1 N NaOH. After 5 min the Ellman test was negative for sulfhydryl groups. HOAc (5 mL) was added and the reaction mixture was concentrated to a volume of 1 mL. The product was subjected to partition chromatography in the system 1-BuOH–C_6H_6–HOAc–1.5% aqueous pyridine (10:15:12:18) on a 2.15 × 52 cm column of Sephadex G-25 (block polymerizate, 100–200 mesh). Peptide material was detected by the method of Lowry et al.[23] and the product emerged as a single sharp symmetrical peak with a maximum at R_f 0.25. Fractions comprising the peak area were pooled, H_2O was added, and the product was isolated by evaporation and lyophilization. The product was further purified by gel filtration on a 2.8 × 68 cm column of Sephadex G-25 (200–270 mesh, block polymerizate) in 10% aqueous HOAc. The product emerged as a single, sharp, symmetrical peak at 79% of the column volume. The fractions corresponding to the peak area were pooled and lyophilized: 51 mg (63% yield from the partially protected nonapeptide); $[\alpha]^{25}_D$ −17° (*c* 0.5, 1 M HOAc); TLC (B) R_f 0.36, (C) R_f 0.63. Anal. ($C_{45}H_{64}N_{11}O_{11}S_3 \cdot CH_3CO_2H \cdot 2H_2O$) C, H, N. Amino acid analysis:[24] Cys(O_3H),[25] 2.00; Asp, 1.00; Pro, 1.01; Gly, 1.00; Ile, 0.99; Leu, 1.01; Tyr, 1.00; Thi, 0.87; NH_3, 1.98.

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References and Notes

(1) The symbol Thi stands for the β-(2-thienyl)-L-alanine residue; all other abbreviations follow the IUPAC–IUB Tentative Rules on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1972). Optically active amino acids are of the L configuration.
(2) The term "intrinsic activity" [J. M. van Rossum, *Adv. Drug Res.*, **3**, 189 (1966)] is used according to classical pharmacological concepts and indicates the maximal response of the target organ to the test substance as compared to maximal response to standard.
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Replacement of the Disulfide Bond in Oxytocin by an Amide Group. Synthesis and Some Biological Properties of [*cyclo*-(1-L-Aspartic acid,6-L-α,β-diaminopropionic acid)]oxytocin[1]

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As part of a continuing investigation of the steric and electronic functions of the disulfide group in neurohypophyseal hormones on their biological activity, the synthesis of "oxytocin lactam", [*cyclo*-(1-aspartic acid,6-α,β-diaminopropionic acid)]oxytocin, has been undertaken. The protected nonapeptide was prepared in a stepwise manner by solution techniques; after removal of side-chain protecting groups, formation of the bridging amide bonds was accomplished by oxidation–reduction condensation. The analogue possesses rat uterotonic, avian vasodepressor, and rat antidiuretic potencies of 16 ± 2, 6.6 ± 0.6, and 5.6 ± 3.8 units/mg, respectively.

The disulfide bridge present in the 20-membered ring portion of neurohypophyseal hormones carries a primary responsibility for the determination of the preferred conformation of the peptide backbone[2] and thereby for the topography of the peptides recognized by the various receptors responsive to these hormones. This point is dramatically illustrated by the nearly total lack of biological activity observed for the acyclic analogues, [$Ala^{1,6}$]oxytocin,[3] [$Ala^{1,6}$]arginine-vasopressin,[4] and [$Ser^{1,6}$]oxytocin.[3] The fact that the reactivity of the disulfide is not required for biological activity is amply demonstrated by the significant biological activities possessed by oxytocin analogues in which the 20-membered ring has been preserved, but one sulfur atom replaced by a methylene group[5-11] or both sulfur atoms by an ethylene group.[6,9,11-15] Changes of the dihedral angle of the bridging group exercise such a profound influence on the overall conformational integrity of the molecule that even a minor modification such as substitution of either one or both sulfur atoms by selenium evokes a definite change in the spectrum of biological activities.[16-18] A displacement of the disulfide bond within the four-atom bridge as in [1-mercaptoacetic acid,6-homocysteine]oxytocin[19] also dramatically alters the activities.

In an earlier investigation of the steric and electronic functions of the disulfide group on biological activity, an analogue of deamino-oxytocin, [*cyclo*-(1-β-alanine,6-aspartic acid)]oxytocin, in which the disulfide has been replaced by an amide group was synthesized.[20] As an extension of this work we wish to report the synthesis and some pharmacological properties of "oxytocin lactam", [*cyclo*-(1-aspartic acid,6-α,β-diaminopropionic acid)]-oxytocin, (Figure 1) in which the amide group is in reverse direction of acylation as that in the previously reported "deamino-oxytocin lactam".[20,21]

The synthetic route followed is summarized in Scheme I. In brief, the COOH-terminal tripeptide amide was extended in a stepwise manner using N^{α}-benzoxycarbonyl (Z) protected amino acids coupled with dicyclohexylcarbodiimide (DCC) mediated by 1-hydroxybenzotriazole[22] (HOBt) except for the NH_2-terminal aspartic acid residue which was coupled as the *N*-hydroxysuccinimide active ester. In all cases catalytic hydrogenation was used to remove the Z group. The side chains of the tyrosine and aspartic acid residues were protected by the *tert*-butyl group and the β-amino function of the α,β-diaminopropionic acid residue was protected by the *tert*-butyloxycarbonyl (Boc) group. These groups were removed from the linear nonapeptide by treatment with CF_3COOH and cyclization was accomplished by oxidation–reduction condensation.[23] The analogue was purified by gel filtration and partition chromatography[24] on Sephadex G-25.

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