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Pharmacological Effects of Introducing a Double Bond into a Binding Site of Oxytocin. Analogues with L-3,4-Dehydroproline in Position 7[1]

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The side chain of the proline residue in position 7 of oxytocin has been proposed as a binding site of the hormone for the uterotonic receptor. This is the first in a series of studies in which the possibility is explored that amino acid residues located at such sites and bearing unsaturated side chains may contribute more strongly to binding than neutral, aliphatic side chains. To test this hypothesis [7-(L-3,4-dehydroproline)]oxytocin, [1-β-mecaptopropionic acid,7-(L-3,4-dehydroproline)]oxytocin, and [1-L-α-hydroxy-β-mercaptopropionic acid,7-(L-3,4-dehydroproline)]oxytocin were prepared by the solid-phase technique of peptide synthesis. Some of the pharmacological properties of the analogues were determined, and the following specific activities, respectively, were found: rat uterotonic, 1071 ± 59, 1066 ± 95, 880 ± 180; avian vasodepressor, 548 ± 10, 1008 ± 42, 1295 ± 62; rat antidiuretic 5.9 ± 0.2, 23.3 ± 1.1, 76.7 ± 2.3. All analogues possess a lower rat pressor activity than oxytocin. Compared to oxytocin, [7-(L-3,4-dehydroproline)]oxytocin exhibits a parallel displacement of the cumulative uterotonic log dose vs. response curve toward lower concentration (pD_2 = 9.26 vs. 8.63) but elicits the same maximum response. These data would seem to support the hypothesis that the introduction of unsaturation into a binding element of a peptide hormone can enhance the affinity of the hormone for some of its receptors and thereby its selectivity.

A comparison of the primary structure of oxytocin and of the other eight characterized neurohypophyseal nonapeptides found in nature reveals that mutations during evolution have occurred only at positions 3, 4, and 8.[2] The conformation of oxytocin (Figure 1) proposed by Urry and Walter[3] places these residues and the residue in position 7, which is proline in all of the naturally occurring peptides, at the four corner positions of the two β turns in the hormone. Side chains of residues located at corner positions are exposed and possess maximal structural freedom. Therefore, at that time in 1971[4] conformational considerations suggested that modifications at these four positions could yield hormone analogues in which one or more of the biological activities of oxytocin were highly accentuated in terms of potency relative to other activities characteristic of the hormone. Since that time, more specific assignments to individual amino acid side chains of oxytocin have been possible with regard to their roles in the interaction of the hormone with its uterine receptor. It has been proposed that side chains of Ile^3 and Pro^7 are "binding elements" (the specific atoms of a binding site responsible for binding; for details see ref 5) which are involved in the recognition and binding of the hormone by the uterotonic receptor and that the side chain of Leu^8 and the hydrocarbon portion of the side chain of Gln^4 also contribute to binding.[5] In addition to the topological arrangement of the binding elements at the corner positions of the two β turns, which makes them most visible to the receptor, they share a common chemical nature in their lypophilicity.

This is the first of a series of studies in which the possibility is explored that residues bearing unsaturated side chains with their deformable electron clouds and with their ability to undergo π–π interactions may contribute more strongly to binding (provided steric fit at the receptor can be achieved) than neutral, aliphatic side chains. Among aliphatic side chains, a high molecular weight group (again, provided no steric problems are encountered) with a substantial degree of van der Waals binding forces may be more favorable for binding than a low-molecular-weight

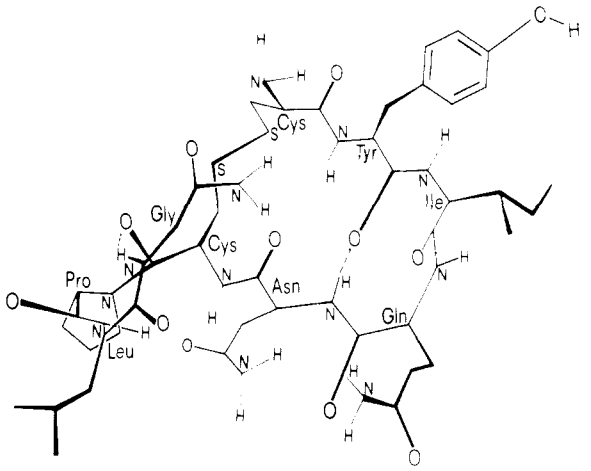

Figure 1. Proposed conformation of oxytocin in dimethyl sulfoxide.

group such as that of alanine or glycine. If this argument is correct, the introduction of a double bond into an aliphatic side chain, which has been identified as a binding site, may increase the affinity of the resultant analogue.

In peptide hormones with multiple binding elements, such as oxytocin, it should not be surprising to find that different receptors, which mediate the various biological responses of the hormone, exhibit different sensitivities to chemical modifications in the binding elements. Such a selectivity appears also to apply to position 7. For example, analogues of oxytocin with glycine in position 7 were found to possess high uterotonic potencies while exhibiting negligible antidiuretic and pressor potencies.[6-10] Some analogues even act to depress the rat blood pressure under the conditions of the assay.[6,10] [7-(Thiazolidine-4-carboxylic acid)]oxytocin is even twice as potent on the rat uterine horn as oxytocin, but it still has a pressor potency about the same as that of the natural principle.[11] [7-Glycine]lysine-vasopressin possesses very low antidiuretic and pressor potencies.[12]

In a concerted effort to test the hypothesis of unsaturation on potency, and to obtain analogues with a high specific uterotonic activity, [7-(L-3,4-dehydroproline)]-oxytocin ([Δ^3-Pro^7]oxytocin) was synthesized and some of its pharmacological activities were studied. Since the formal substitution of the NH_2-terminal amino group by a hydrogen atom[13] or a hydroxyl group[14] can yield analogues with enhanced uterotonic potency, [1-β-mercaptopropionic acid,7-(L-3,4-dehydroproline)]oxytocin (deamino-[Δ^3-Pro^7]oxytocin) and [1-L-α-hydroxy-β-mercaptopropionic acid,7-(L-3,4-dehydroproline)]oxytocin (hydroxy[Δ^3-Pro^7]oxytocin) were also synthesized and studied.

Results

The required partially protected peptide intermediates were prepared by the solid-phase technique[15] (for a recent review see ref 16). In general, the *tert*-butyloxycarbonyl group[17] was used for the temporary protection of the N^{α} group and was removed by treatment with 50% trifluoroacetic acid (TFA) in CH_2Cl_2.[18] Coupling was affected by dicyclohexylcarbodiimide (DCC)[19] or DCC mediated with 1-hydroxybenzotriazole (HBT)[20] except when indicated. The completeness of each coupling reaction was monitored by the ninhydrin test[21] or the fluorescamine test.[22] Repeat couplings were performed when necessary.

For the precursor of [Δ^3-Pro^7]oxytocin the benzyhydrylamine resin[23] served as a support to which Boc-Gly-OH was attached using DCC. Any unsubstituted benzhydrylamine groups were subsequently benzoylated.[24] The acetamidomethyl group (Acm)[25] was used for the protection of the sulfhydryl group of cysteine and the 2,6-dichlorobenzyl group[26] was used for the protection of the tyrosine hydroxyl moiety. In this instance, Boc-Asn-OH and Boc-Gln-OH were introduced by the oxidation–reduction technique using triphenylphosphine and 2,2-dipyridyl disulfide.[27] After each coupling or repeated coupling, chain termination by fluorescamine[28] was carried out except after the coupling of Boc-Cys(Acm)-OH to the Δ^3-Pro residue since fluorescamine reacts with imino acids to yield a derivative with a free carboxyl function. The completed nonapeptide was removed from the resin by treatment with liquid, anhydrous HF[29] which removed all protecting groups but the Acm moiety. Partial purification of the bis(*S*-Acm)-protected nonapeptide was achieved by gel filtration[30] on Sephadex G-10. The Acm groups were removed using mercuric acetate at pH 4 and the disulfide bond was formed by oxidation with $K_3Fe(CN)_6$.[31] The crude [Δ^3-Pro^7]oxytocin was purified by countercurrent distribution[32] and partition chromatography on Sephadex G-25.[33]

For the precursors of deamino- and hydroxy[Δ^3-Pro^7]oxytocin, a chloromethylated polystyrene copolymer (1%)–divinylbenzene resin was used as a support to which Boc-Gly-OH was esterified using the cesium salt method of Gisin.[34] The benzyl group was used for the protection of the sulfhydryl groups and for the tyrosine hydroxyl group. The acetyl group served to protect the hydroxyl function of the α-hydroxy-β-mercaptopropionic acid residue.[14] Boc-Asn-OH and Boc-Gln-OH were also incorporated using DCC–HBT. The completed peptide was removed from the resin by ammonolysis in methanol,[35] which also removed the acetyl group in the case of the hydroxy analogue. The remaining protecting groups were removed by treatment with sodium in anhydrous liquid NH_3[36] and the disulfide bond was formed by oxidation with diiodoethane.[37] The crude products were purified by gel filtration and partition chromatography on Sephadex.

Some of the biological activities of the three analogues were investigated by methods previously described[38-46] and the results are shown in Table I. Cumulative log dose vs. response curves were obtained on the rat uterus in vitro by the method of van Rossum.[51] Compared to oxytocin, [Δ^3-Pro^7]oxytocin exhibits a parallel displacement of the log dose vs. response curve toward lower concentration. For [Δ-Pro^7]oxytocin the value of the negative decimal logarithm of the concentration which produces 50% of the maximum effect (pD_2, see ref 51) is 9.26 as compared to oxytocin with a value of 8.63. [Δ^3-Pro^7]oxytocin elicits the same maximum response as oxytocin. Difficulties were encountered with the Δ^3-Pro^7 analogues in the rat pressor assay. The response to an injection of any [Δ^3-Pro^7] analogue was typified by an initial small decrease in blood pressure (1–5 mmHg lasting ~30 s) followed by an increase lasting 5–10 min. After an initial injection all responses to further injections of USP Posterior Pituitary Reference Standard or analogue were attenuated. A period of 3–4 h was required before reproducible, unattenuated responses to the standard were obtained. Even using experimental conditions designed to circumvent this phenomenon, it is clear that the dose–response relationships for [Δ^3-Pro^7]oxytocin and vasopressin are not parallel (Figure 2) and no quantification of the pressor potency was possible. Qualitatively all three [Δ^3-Pro^7]oxytocin analogues behaved in the same manner in the pressor assay.

Discussion

The introduction of a double bond between the carbon atoms in positions 3 and 4 of the side chain of the proline

Table I. Comparison of Biological Activity of Oxytocin, Deamino-oxytocin, and Hydroxy-oxytocin with the Corresponding Analogues Containing L-3,4-Dehydroproline in Position 7[a]

Peptide	Uterotonic (rat)	Vasodepressor (fowl)	Antidiuretic (rat)	Pressor (rat)
[Δ^3-Pro^7]oxytocin	1071 ± 59	548 ± 10	5.9 ± 0.2	n.d.[b]
Deamino[Δ^3-Pro^7]oxytocin	1066 ± 95	1008 ± 42	23.3 ± 1.1	n.d.[b]
Hydroxy[Δ^3-Pro^7]oxytocin	880 ± 180[c]	1295 ± 62	76.7 ± 2.3	n.d.[b]
Oxytocin	546 ± 18[d]	507 ± 15[e]	2.7 ± 0.2[e]	3.1 ± 0.1[e]
Deamino-oxytocin	803 ± 36[f]	975 ± 24[f]	19[f]	1.44 ± 0.06[f]
Hydroxy-oxytocin	1542 ± 18[g]	1778 ± 25[g]	40.3 ± 2.4[g]	32.7 ± 0.4[g]
	1275 ± 51[h]		16.6 ± 1.3[h]	14.7 ± 0.3[h]
	1607 ± 20[i]			32[i]

[a] Results are expressed in USP units/mg ± SEM. Activities of peptides reported for the first time are based on the anhydrous weight. [b] Potency values were not obtained. See text for discussion. [c] The large SEM was caused by assay difficulties. Longer periods between injections and more extensive washing of the tissue were required to obtain reproducible results. Even with these precautions individual four-point determinations were encountered which gave values for the potency as low as 640 and as high as 1640 units/mg. The results reported are derived from 27 four-point determinations on 16 uterine horns. [d] See ref 47. [e] See ref 48. [f] See ref 13. [g] See ref 10. [h] See ref 49. [i] See ref 14. [j] See ref 50.

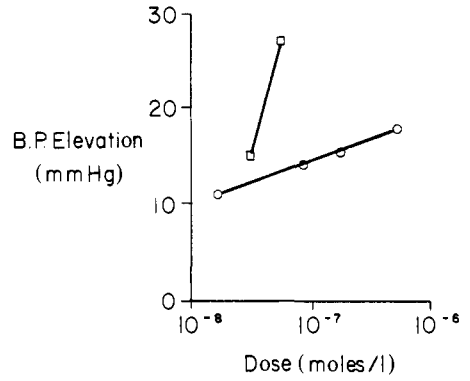

Figure 2. Log dose vs. response relationship in the rat pressor assay of [Δ^3-Pro^7]oxytocin (○—○) and arginine-vasopressin (□—□) with a specific activity of 400 units/mg. An animal was first given 2.2×10^{-8} and 4.5×10^{-8} M doses of vasopressin to test for reproducibility of the pressor responses. Then a single dose (1.6×10^{-8}; 8.4×10^{-8}, 1.8×10^{-7}, or 5.2×10^{-7} M) of [Δ^3-Pro^7]oxytocin was injected.

residues of oxytocin has resulted in an analogue ([Δ^3-Pro^7]oxytocin) which possesses twice the uterotonic potency of oxytocin. The results from the log dose vs. response curve indicate that the analogue possesses a higher affinity but the same intrinsic activity as oxytocin. This seems to support the idea that introducing a deformable electron cloud into a binding element should increase the affinity of the analogue for the receptor. It is also possible that the introduction of the double bond, which would be expected to restrict the rapid interconversion of the pyrrolidine ring of the proline residue between various ring-puckered forms,[52] changes the overall conformation in such a manner as to provide a topography which is more favorable for receptor interactions. The enhanced uterotonic potency of [Δ^3-Pro^7]oxytocin is apparently not due to any change in its overall lipophilicity as the analogue and oxytocin have almost identical R_f's on partition and thin-layer chromatography. As previously observed,[10] the effect of modifications in binding elements when combined with changes at positions other than binding elements may not be additive. The activities found for the disubstituted analogues, deamino[Δ^3-Pro^7]oxytocin and hydroxy[Δ^3-Pro^7]oxytocin, when compared to the three corresponding singly substituted analogues, reinforce this view.

The antidiuretic potency of [Δ^3-Pro^7]oxytocin is also about twice that of oxytocin, indicating perhaps that both the uterotonic receptor and the antidiuretic receptor are able to bind the Δ^3-Pro^7 analogue better than the corresponding Pro peptide. This does not, however, appear to be the case for the vascular receptors, which seem to be more sensitive to changes at position 7 than do the uterotonic and antidiuretic receptors.

Experimental Section

All melting points were determined in open capillary tubes and are reported uncorrected. Thin-layer chromatography was performed on precoated plates of silica gel G-60 F-254 (E. Merck). Compounds were applied in loads up to 50 μg and the chromatograms were developed for 10–15 cm in the following solvent systems: A, 2-BuOH–HCO_2H–H_2O (150:27:23); B, 1-BuOH–HOAc–H_2O (4:1:1); C, 1-BuOH–pyridine–H_2O (20:10:11); D, 1-BuOH–pyridine–HOAc–H_2O (15:10:3:6). Visualization was performed with chlorine–tolidine reagent.[53] Amino acid analyses[54] were performed on a Durrum Model 500 amino acid analyzer following hydrolysis in degassed 6 N HCl at 110 °C for 22 h. All analyses were performed on a 1.75 mm × 50 cm column of Durrum DC 4A resin at a flow rate of 8.3 ml/h. A standard run used sodium citrate buffers of pH 3.25, 4.25 (0.2 M Na^+), and 7.90 (1.1 M Na^+) with buffer changes at 30 and 42 min after sample injection. The column temperature was 50 °C at the beginning of the run and changed to 65 °C after 35 min. Under these conditions 3,4-dehydroproline[55] eluted with a retention time of 13.7 min after injection, preceded by Asp at 12.8 min and followed by Glu at 19.0 min. Where elemental analyses are indicated only by the symbols for the elements, analytical results obtained were within ±0.4% of the theoretical values.

Cumulative dose–response curves were obtained by the technique of van Rossum[51] with doses being increased geometrically according to the $^1/_3$ log procedure until a maximum response was reached. The female rats were chosen and the same bathing fluid was used as described for the measurement of the specific oxytocic activity.[38,39] Isometric contractions were recorded with a Grass polygraph in connection with a Grass force-displacement transducer (FTO3C, springs removed). Alternate determinations for oxytocin and analogue were performed on the same uterine horn allowing contractions to subside to baseline tension (1 g) between determinations. Measurements were performed in a 10-mL tissue bath with the temperature maintained constant at 30 °C. At least three animals (six horns) were used to obtain each curve.

tert-**Butyloxycarbonylglycylbenzhydrylamine-resin.** The resin hydrochloride (10 g) (Beckman, total N_2 by microanalysis 0.57 mequiv/g) was neutralized with 25% Et_3N in CH_2Cl_2 (100 mL × 2) and suspended in CH_2Cl_2 (150 mL) containing Boc-Gly-OH (1.97 g, 2.5 equiv). DCC (2.32 g, 2.5 equiv) was added and the suspension was shaken for 2 h. After washing, the resin was suspended in CH_2Cl_2–pyridine (105:3.5) and benzoyl chloride was added at 0 °C. After 10 min the resin was filtered, washed with CH_2Cl_2, and dried. A sample was treated with TFA, neutralized with Et_3N, and analyzed for N_2 by microanalysis. The N_2 content of the resin was found to be 0.87 mequiv/g as compared to 0.57 mequiv/g at the start which indicated a level of substitution for Boc-Gly-OH of 0.3 mequiv/g.

[7-(L-3,4-Dehydroproline)]oxytocin. A 4-g portion of the above resin was used with the following procedure which comprised a complete sequence of deprotection, neutralization, and

coupling unless otherwise indicated: CH_2Cl_2 wash (80 mL × 3); 50% TFA in CH_2Cl_2 (80 mL) for 15 min (×2); CH_2Cl_2 (×3); 10% NEt_3 in CH_2Cl_2 (80 mL), CH_2Cl_2 (×3); CH_3OH (×3); CH_2Cl_2 (×3); *tert*-butyloxycarbonylamino acid (2.5 equiv); DCC (2.5 equiv); and HBT (2.5 equiv) in CH_2Cl_2 (80 mL), 3 h; CH_2Cl_2 (×3); DMF (×3); CH_3OH (×3); CH_2Cl_2 (×3); fluorescamine termination, which involved neutralization followed by treatment with fluorescamine (1 equiv) in CH_2Cl_2. Samples (10–15 mg) were taken for the Kaiser ninhydrin or fluorescamine tests. Repeat couplings (at Gln, Ile, Tyr, and Cys[1]) were performed prior to the fluorescamine termination using the same conditions and quantities given, but with the omission of the TFA treatment. In the cases of Asn and Gln, coupling was affected using 2.5 equiv of *tert*-butyloxycarbonylamino acid, 2.5 equiv of 2,2′-dipyridyl disulfide, and 2.5 equiv of triphenylphosphine in CH_2Cl_2 for 3 h. With the completion of the final step of the synthesis, the peptide resin was dried in vacuo to yield 5.3 g of material (87% of the theoretical weight gain uncorrected for samples withdrawn for analysis). To remove the completed peptide from the resin, the dry peptide resin (5.3 g) was stirred in liquid anhydrous HF (50 mL) containing anisole (6.5 mL) at 0 °C for 1 h. The HF was evaporated and the residue washed five times with EtOAc (40 mL) and then extracted five times with 0.2 N HOAc (40 mL). The aqueous solution was extracted again three times with EtOAc (40 mL) and lyophilized to yield 1.65 g of crude partially protected nonapeptide. The lyophilizate was partially purified by gel filtration on a 4.8 × 100 cm column of Sephadex G-10 in 0.2 N HOAc. The main peak was rechromatographed to give 716 mg (51% yield based on Gly content of the resin).

The bis(*S*-Acm) nonapeptide (190 mg, 0.16 mmol) was dissolved in H_2O under an argon atmosphere, and mercuric acetate (209 mg, 4 equiv) was added while the pH was adjusted to 4 with 10% HOAc. The progress of the reaction was followed by TLC using solvent system A. After 3.5 h H_2S was bubbled through the solution for 10 min followed by N_2 (oxygen free) for 1 h. The solution was filtered and diluted to 1 L with H_2O under an argon atmosphere. The pH was adjusted to 6.8 with 2 M NH_4OH and the solution was titrated with 0.02 M $K_3Fe(CN)_6$ until a permanent yellow color was observed. The pH was maintained at 6.8 with 2 M NH_4OH during the addition of $K_3Fe(CN)_6$ and then adjusted to 2.5 with 10% HOAc after which AG2-X8 (100–200 mesh, Cl^- form) was added to remove the excess ferri- and ferrocyanide ions. The resin was filtered off and the volume of filtrate reduced by rotary evaporation. The above process was repeated with another equal size batch and both products were combined and subjected to countercurrent distribution (616 transfers) in the system 1-BuOH–1-PrOH–0.05% HOAc (2:1:3). Peptide material was detected by monitoring the absorbancy of the lower phase at 278 nm and the major peak (K = 0.4, fractions 165–195) was isolated by lyophilization: 87 mg (27% yield from the nonapeptide). A 75-mg portion of the above material was further purified by partition chromatography on a 2.15 × 108 cm column of Sephadex G-25 (100–200 mesh, block polymerizate) previously equilibrated with both phases of the solvent system 1-BuOH–3.5% HOAc containing 1.5% pyridine (1:1). The column was eluted with upper phase at 12 mL/h and collected in fractions averaging 6.9 mL. The peptide material, which was detected by the method of Lowry et al.,[56] eluted as a sharp peak with a maximum at R_f 0.21, well resolved from several minor impurities. The fractions comprising the peak area (62–71) were pooled with 2 vol of H_2O, the organic phase was removed by rotary evaporation, and the aqueous phase was lyophilized: 32 mg (43% recovery); $[\alpha]^{23}D$ −80° (*c* 0.5, 1 N HOAc). Anal. ($C_{43}H_{64}N_{12}O_{12}S_2 \cdot CH_3CO_2H \cdot 3H_2O$) C, H, N. The product gave single spots on TLC in systems B (R_f 0.32) and C (R_f 0.50). Amino acid analysis gave the following molar ratios: Asp, 1.0; Δ^3-Pro, 1.0; Glu, 1.0; Gly, 1.0; $^1/_2$Cys, 2.0; Ile, 1.0; Leu, 1.0; Tyr, 0.81; NH_3, 3.0.

β-Mpr(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Δ³-Pro-Leu-Gly-NH_2. Polystyrene copolymer (1%)–divinylbenzene resin (4.9 g) to which Boc-Gly-OH had been esterified to the extent of 0.51 mequiv/g[34] was used with cycles of deprotection, neutralization, and coupling previously published.[57] In this procedure 2.5 equiv of *tert*-butyloxycarbonylamino acid and DCC were used during the coupling step with 5 equiv of HBT and 1 equiv of *i*-Pr_2NEt was added after the first hour. The Boc-Δ^3-Pro-OH[58] was successfully incorporated using only 1.5 equiv of *tert*-butyloxycarbonylamino acid and DCC with 3 equiv of HBT. Repeat couplings, when performed, used 0.5 equiv of *tert*-butyloxycarbonylamino acid and DCC with 1 equiv of HBT. Repeat couplings were required for the incorporation of the Asn, Gln, Ile, and Tyr residues. After each coupling or repeat coupling the ninhydrin test[21] indicated a degree of completeness in excess of the sensitivity of the test and no further termination step was performed. After the incorporation of the Tyr residue, a 50% aliquot of the octapeptide resin was elongated with *S*-benzyl-β-mercaptopropionic acid [β-Mpr(Bzl)-OH][31] in the usual manner. A repeat coupling was required for completion. The dried resin showed a weight gain of 1.3 g (80% of the theoretical amount not corrected for samples withdrawn for testing).

The protected peptide was removed from the resin by ammonolytic cleavage.[35] The NH_3 and MeOH were removed by evaporation in vacuo and the cleaved peptide was extracted with dimethylformamide (DMF) at 60 °C and dimethyl sulfoxide (Me_2SO). The volume of extract was reduced to 25 mL by rotary evaporation and the product was precipitated with H_2O (100 mL). The precipitate was filtered, washed with H_2O, EtOH, and Et_2O, and dried in vacuo: 1.30 g (69% based on the Gly content of the resin); $[\alpha]^{24}D$ −75° (*c* 1, DMF); mp 253–254 °C. Anal. ($C_{64}H_{83}N_{11}O_{12}S_2 \cdot H_2O$) C, H, N. The TLC of the product in system B gave a major spot (R_f 0.58) with a trace impurity (R_f 0.66).

[1-β-Mercaptopropionic acid,7-(L-3,4-dehydroproline)]oxytocin (Deamino[Δ³-Pro⁷]oxytocin). The protected peptide above (250 mg, 0.20 mmol) was dissolved in liquid NH_3 (75 mL; freshly distilled from Na) and reduced with Na at the boiling point of NH_3.[36] After 30 s the slight excess of Na was discharged with HOAc and the NH_3 was removed by evaporation under a stream of N_2. The residue was dissolved in N_2 flushed MeOH–H_2O (1:1, 400 mL) and diiodoethane (58 mg, 0.2 mmol) dissolved in 10 mL of MeOH was added. The disappearance of the sulfhydryl groups was followed by the Ellman test.[59] After 5 min HOAc (5 mL) was added and the volume of the reaction mixture was reduced to 1–3 mL by rotary evaporation. The crude product was subjected to partition chromatography in the solvent system 1-BuOH–C_6H_6–3.5% HOAc containing 1.5% pyridine (1:1:2) on a 2.82 × 68 cm column of Sephadex G-25 (100–200 mesh, block polymerizate). The product emerged as a sharp peak with a maximum at R_f 0.15, well resolved from several minor impurities. The fractions (7.1 mL each) comprising the peak area (84–107) were pooled, 2 vol of H_2O was added, the organic phase was removed by evaporation, and the aqueous phase lyophilized: 103 mg (52% yield from the protected peptide). An 83-mg portion was dissolved in 5 mL of 20% HOAc and subjected to gel filtration on a 2.82 × 68 cm column of Sephadex G-25 (200–270 mesh, block polymerizate). The column was eluted with 0.2 N HOAc at 35 mL/h and the peptide material was detected by monitoring the absorbancy of the eluate at 280 nm. The product emerged as a single sharp peak with a maximum at 80% of the column volume. Fractions (4.9 mL each) comprising the peak area (63–75) were pooled and lyophilized: 77 mg (93% recovery); $[\alpha]^{26}D$ −156° (*c* 0.6, 1 N HOAc). Anal. ($C_{43}H_{63}N_{11}O_{12}S_2 \cdot 2H_2O$) C, H, N. The analogue gave a single spot on TLC in systems B (R_f 0.37) and C (R_f 0.60) and amino acid analysis gave the following molar ratios: Asp, 1.0; Δ^3-Pro, 1.0; Glu, 1.0; Gly, 1.0; $^1/_2$Cys, 0.42; mixed disulfide of β-Mpr-Cys, 0.53; Ile, 1.0; Leu, 1.0; Tyr, 0.99; NH_3, 2.9. The mixed disulfide eluted with a retention time of 36.5 min using the standard conditions described above.

Hmp(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Δ³-Pro-Leu-Gly-NH_2. To the remaining 50% aliquot of protected octapeptide resin was coupled *S*-benzyl-L-α-acetoxy-β-mercaptopropionic acid [Ac-Hmp(Bzl)-OH][14] (1.5 equiv) with DCC (1.5 equiv) and HBT (3.0 equiv) with *i*-Pr_2NEt (1.0 equiv) added after 1 h. After 5 h the ninhydrin test indicated incomplete coupling. The reaction mixture was drained from the resin but saved. The resin was washed as usual in preparation for a repeat coupling and the original reaction mixture was reintroduced with another 1.5 equiv of DCC added. After 4 h the ninhydrin test indicated complete reaction. The observed weight gain on the resin was 1.5 g (86% uncorrected for samples withdrawn for analysis). The completed protected peptide was removed from the resin by ammonolytic cleavage (which also removed the acetyl group from Hmp), extracted with DMF and Me_2SO, precipitated, washed, and dried in vacuo as described above: 1.32 g (69% yield based on the Gly

content of the resin); mp 247–250 °C dec; $[\alpha]^{22}D$ −82° (c 1, DMF). Anal. ($C_{64}H_{83}N_{11}O_{13}S_2 \cdot 2H_2O$) C, H, N. The product gave a single spot on TLC in system D (R_f 0.70), but in system B showed a major component (R_f 0.61) with two trace impurities.

[1-(L-α-Hydroxy-β-mercaptopropionic acid),7-(L-3,4-dehydroproline)]oxytocin (Hydroxy[Δ^3-Pro^7]oxytocin). The protected peptide above (128 mg, 0.10 mmol) was reduced with Na in liquid NH_3 and the disulfide bond formed by oxidation with diiodoethane in the manner previously described. The crude product was taken up in 50% HOAc and subjected to gel filtration chromatography on a 2.15 × 116 cm column of Sephadex G-15. The product emerged as a major and minor peak with maxima at 42 and 35% of the column volume, respectively. Fractions (3.1 mL each) corresponding to the peak areas (61–74 and 48–58, respectively) were pooled, rotary evaporated to a small volume, diluted with glacial HOAc, and lyophilized: yield 66.3 and 31.5 mg, respectively. TLC in system B showed the larger peak, which eluted in the usual place where oxytocin appears, to consist of a major component (R_f 0.37) with several small impurities. The entire 66.3-mg sample was subjected to partition chromatography in the solvent system 1-BuOH–C_6H_6–3.5% HOAc containing 1.5% pyridine (3:2:5) on a 2.20 × 52 cm column of Sephadex G-25 (100–200 mesh, block polymerizate). The product emerged as a sharp symmetrical peak with a maximum at R_f 0.31 well resolved from several minor impurities. Fractions (3.8 mL each) comprising the peak area (56–76) were pooled with 2 vol of H_2O, the organic phase was removed by rotary evaporation, and the aqueous phase was lyophilized: 49.1 mg (49% yield from the protected peptide); $[\alpha]^{22}D$ −122° (c 0.5, 1 N HOAc). Anal. ($C_{43}H_{63}N_{11}O_{13}S_2 \cdot 2.5H_2O$) C, H, N. The product gave a single spot on TLC in system B (R_f 0.37). Using the standard conditions for amino acid analysis described above, a broad peak, presumed to be the mixed disulfide of Hmp-Cys, elutes with a retention time of 14.6 min overlapping the Δ^3-Pro peak. To resolve this difficulty it was necessary to lower the pH of the first buffer to 2.95 and the temperature to 32 °C. Under these conditions Δ^3-Pro emerges at 21.8 min followed by Asp at 24 min. Cysteine was determined following performic acid oxidation[60] and hydrolysis at 110 °C for 16 h in 6 N HCl as cysteic acid which emerges at 4.8 min using standard conditions. The following molar ratios were found: Cys(O_3H), 1.0; Δ^3-Pro, 1.0; Asp, 1.0; Glu, 1.0; Gly, 1.0; Ile, 1.0; Leu, 1.0; Tyr, 0.94; NH_3, 3.0. The peak presumed to be mixed disulfide of Hmp-Cys and the Δ^3-Pro peak were absent in the performic acid oxidized sample.

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References and Notes

(1) The symbols Δ^3-Pro, β-Mpr, and Hmp are used to indicate L-3,4-dehydroproline, β-mercaptopropionic acid (deamino), and α-hydroxy-β-mercaptopropionic acid (hydroxy) residues, respectively. 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.
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Polyamine Biosynthesis in Rat Prostate. Substrate and Inhibitor Properties of 7-Deaza Analogues of Decarboxylated *S*-Adenosylmethionine and 5′-Methylthioadenosine

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The 7-deaza analogue of 5′-methylthioadenosine, a nucleoside end product in polyamine biosynthesis, has been synthesized. This analogue has been shown to competitively inhibit the hydrolytic cleavage of the purine–ribose bond in methylthioadenosine with $K_i \simeq K_m$. In addition, the 7-deaza analogue of decarboxylated *S*-adenosylmethionine, a cofactor in the biosynthesis of both spermidine and spermine, has been synthesized. This analogue has been shown to act as a substrate in the reaction catalyzed by spermidine synthase, in which severe substrate inhibition by both the normal nucleoside substrate and the 7-deaza analogue is observed. These results are discussed in terms of possible end product regulation of polyamine biosynthesis and the possible substitution of the nucleoside antibiotic, tubercidin, for adenosine in reactions involving *S*-adenosylmethionine and its metabolites.

The role of *S*-adenosylmethionine (SAM, **4a**), and its various metabolites, in a myriad of cellular events has been extensively investigated in recent years[1] (Scheme I). As part of a program aimed at the regulation of SAM-dependent methylation, we have described the synthesis and in vitro activity of several analogues of *S*-adenosylhomocysteine (SAH, **5a**), a potent product inhibitor of most SAM-dependent methylases.[2-5] One of these compounds, *S*-tubercidinylhomocysteine (**5b**), the 7-deaza analogue of SAH, proved to be an extremely potent inhibitor of several SAM-dependent methylases.[4] Since our work[2-5] and others[1] have shown that only minor structural variations in the basic SAH structure can be made and still maintain inhibitory activity, we decided to study the 7-deaza derivatives of other SAM metabolites. Of particular interest is the possibility of regulating other biochemical reactions involving SAM metabolites, such as the biosynthesis of polyamines.[6] Previous work on synthetic inhibitors of these reactions has been directed at analogues of ornithine[7-12] and spermidine.[13,14] In addition, we have studied the action of *N*-(5′-phosphopyridoxyl)ornithine, an analogue of the reaction intermediate, on the enzyme ornithine decarboxylase.[15] However, no work has been done on analogues of the nucleoside substrates or products involved in polyamine biosynthesis.

Our rationale for synthesizing **5b**[4] and several other SAH analogues[2,3] was to incorporate a stable base–ribose bond into the molecule. Cleavage of the base–ribose bond is the major pathway of SAH metabolism in bacteria,[16] but recent studies in rat liver[17] and in stimulated rat lymphocytes[18] indicate that cleavage of the 5′-thioether to give homocysteine (**6**) and the nucleoside, **1**, is the major hydrolytic pathway in mammalian cells. However, in the case of polyamine biosynthesis, the nucleoside end product, 5′-methylthioadenosine (**9a**), is readily cleaved to adenine (**11a**) and 5-methylthioribose (**12**) by a phosphorylase (E.C. 2.4.2.) isolated from rat ventral prostate.[19] Thus an analogue of **9a** in which a stable base–ribose bond is incorporated should be resistant to the action of phosphorylases and should inhibit the hydrolysis of **9a** in vitro and, hopefully, in vivo. In this paper, we describe the synthesis of 5′-methylthiotubercidin (**9b**) and show that it is completely resistant to the action of the methylthioadenosine cleaving enzyme isolated from rat ventral prostate. As a result, **9b** competitively inhibits the enzyme-catalyzed cleavage of **9a**. Based on these findings, we have investigated the possibility that **9b** might be formed, and therefore accumulate, via the utilization of decarboxylated *S*-tubercidinylmethionine (**7b**) in the spermidine synthase (E.C. 2.5.1.16.) reaction.

Chemistry. 5′-Methylthioadenosine used in most previous biochemical studies has been obtained by acid hydrolysis of SAM.[19,20] The latter sulfonium compound is itself available only in limited amounts at considerable expense, so that a more economical route of synthesis for **9** was desired. Recent work by Kigugawa and colleagues[21,22] makes possible the synthesis of 5′-alkyl-(aryl-) thioadenosines in two steps from adenosine (eq 1). We have used this new procedure to prepare both [$^{14}CH_3S$]-**9a** and [$^{14}CH_2S$]-**9b**, in addition to the nonlabeled compounds.

Similarly, decarboxylated SAM (**7a**) used in biochemical studies has usually been obtained by enzymatic decarboxylation of SAM,[23,24] although **7a** has been synthesized by Jamieson[25] by coupling 5′-*O*-tosyl-2′,3′-isopropylideneadenosine and 3-(benzylthio)-1-propylamine in Na–NH_3. With a view toward synthesis of the 7-deaza analogue **7b**, we investigated alternate routes to decarboxylated SAM which did not use the intermediate 5′-*O*-tosyl derivative, since we had previously found 5′-*O*-tosyl-2′,3′-isopropylidenetubercidin to be quite unstable.[4] Considering the success achieved using the method of Kigugawa et al.[21,22] to prepare **9**, we sought unsuccessfully to prepare 3-phthalimidopropane-1-thiol[26] for use as an