# Synthesis and Some Pharmacological Properties of [4-Threonine,7-sarcosine]oxytocin, a Peptide with High Oxytocic Potency, and of [4-Threonine,7-*N*-methylalanine]oxytocin

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Two analogues of oxytocin, [Thr$^4$,Sar$^7$]- and [Thr$^4$,MeAla$^7$]oxytocin, were synthesized and their pharmacological properties investigated. [Thr$^4$,Sar$^7$]oxytocin was found to exhibit high biological activity (uterotonic activity of 1174 ± 104 and milk ejection activity of 731 ± 57 units/mg) and high selectivity for oxytocin-like relative to vasopressin-like activities (antidiuretic activity of 0.037 ± 0.012 unit/mg, undetectable pressor activity). [Thr$^4$,MeAla$^7$]oxytocin was characterized by markedly lower biological activities. In both analogues, the additivity of the effects of the residues in positions 4 and 7 of oxytocin on the biological activity of the analogues was ascertained.

Recently we have reported the synthesis of oxytocin and arginine-vasopressin analogues with interesting selective biological properties.[1] These analogues contained the sarcosine or *N*-methylalanine residues in place of the proline residue in position 7 of the oxytocin and vasopressin compounds. The replacement of proline by sarcosine yielded an analogue of high oxytocic activity. At the same time, [Sar$^7$]oxytocin proved to be highly selective, its vasopressin-like activities being suppressed. On the other hand, incorporation of the *N*-methylalanine residue in position 7 of oxytocin resulted in a considerable loss of oxytocic activity. Nevertheless, this analogue retained a degree of selectivity.

The 7-substituted analogues of arginine-vasopressin also displayed high selectivity, their antidiuretic potency being quite high, but their pressor activity being quite low.

These results prompted us to synthesize oxytocin analogues in which, in addition to incorporation of the sarcosine and *N*-methylalanine residues in position 7, the glutamine residue was replaced by threonine. It has been well established that incorporation of threonine in place of glutamine in position 4 of oxytocin affords highly active analogues.[2-4] Taking as an example [Thr$^4$,Gly$^7$]oxytocin, Manning has found additivity of the effects of the 4- and 7-residues on biological activity. This compound shows the highest selectivity yet encountered among oxytocin analogues.[5]

## Results and Discussion

[Thr$^4$,Sar$^7$]- and [Thr$^4$,MeAla$^7$]oxytocins were synthesized on a solid (resin) support.[1,6,7] The protected peptides were removed from the resin by ammonolysis.[7] They were then reacted with sodium metal in liquid ammonia,[8] and the disulfhydryl derivatives formed were cyclized with the aid of $K_3Fe(CN)_6$.[9] The oxytocin analogues were purified by gel filtration on Sephadex G-15.[10] The overall yields of the chromatographically homogeneous [Thr$^4$,Sar$^7$]-oxytocin and [Thr$^4$,MeAla$^7$]oxytocin were 55 and 39%, respectively.

In Table I are summarized the results of the bioassays of the analogues. For comparison, the activities of oxytocin itself and its [Thr$^4$], [Sar$^7$], and [MeAla$^7$] derivatives are shown as well. [Thr$^4$,Sar$^7$]oxytocin exhibits exceptionally high oxytocic activity. Its uterotonic activity is more than twice as high as that of the parent hormone and [Sar$^7$]-oxytocin. There is also a considerable increase in the milk-ejection activity.

On the other hand, the pressor activity of this analogue is below the limits of detection, while its antidiuretic activity is lower than that of oxytocin by two orders of magnitude. It is thus one of the most selective oxytocin analogues known.[11] The oxytocin/antidiuretic (O/A) ratio is 3170, while the oxytocic/pressor (O/P) ratio may approach infinity (since pressor activity is below the limits of detection in our assay system) (Table I).

The uterotonic activity increases markedly when the bioassay is carried out in the presence of $Mg^{2+}$ ion. A similar enhancement of the activity in the presence of $Mg^{2+}$ ion was observed previously with other highly selective oxytocin analogues, e.g., those containing a glycine residue in position 7.[5]

[Thr$^4$,MeAla$^7$]oxytocin shows markedly lower biological activity (Table I). However, in this case also, the incorporation of a threonine residue in position 4, in addition to the 7-*N*-methylalanine residue, potentiated the activity compared to that of [MeAla$^7$]oxytocin.[1] This provides support for a principle of additivity of effects of individual amino acid residues in positions 4 and 7 of oxytocin.[5,11] The markedly lower activity of the analogues carrying the *N*-methylalanine residue in position 7 compared with those carrying the sarcosine in this position might possibly be explained in terms of conformational changes due to the effect of the interaction of the methyl groups occupying the neighboring N and $C^\alpha$ atoms in the *N*-methylalanine residue. The change of angle $\Phi$ in the *N*-methylalanine residue may not allow for suitable accommodation of the C-terminal peptide relative to the cyclic fragment of the oxytocin molecule, thus hindering the formation of the analogue–receptor complex.

## Experimental Section

The procedure of solid-phase peptide synthesis conformed to that published.[1] Triethylamine was distilled from ninhydrin. Other solvents and reagents were of analytical grade. Thin-layer chromatography (TLC) was carried out on silica gel plates (Merck),

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Table I. Biological Activity of Oxytocin and Its Analogues in Rats

| peptide | biol act. ± SEM, U/mg | | | | | | |
|---|---|---|---|---|---|---|---|
| | uterotonic[a] | | milk[b] ejection | vasopressor[c] | antidiuretic[d] | O/P[e] | O/A[e] |
| | no $Mg^{2+}$ | $Mg^{2+}$ | | | | | |
| [Thr[4],Sar[7]]oxytocin | 1173 ± 104 | 1561 ± 57 | 731 ± 57 | <0.5 | 0.037 ± 0.012 | >2346 | 31 700 |
| [Thr[4],MeAla[7]]oxytocin | 115 ± 12 | 219 ± 21 | 243 ± 12 | <0.6 | 0.047 ± 0.014 | >192 | 2 450 |
| oxytocin[f] | 520 ± 12 | 486 ± 15 | 474 ± 16 | 4.3 ± 0.12 | 4.0 ± 0.8 | 120 | 130 |
| [Thr[4]]oxytocin[g] | 923 ± 95 | 719 ± 83 | 543 ± 62 | 0.43 ± 0.01 | 1.8 ± 0.3 | 2146 | 510 |
| [Sar[7]]oxytocin[h] | 459 ± 41 | 609 ± 14 | 564 ± 27 | <0.35 | 0.18 ± 0.04 | >1311 | 2 550 |
| [MeAla[7]]oxytocin[h] | 62 ± 6 | 184 ± 21 | 402 ± 30 | <0.35 | 0.12 ± 0.03 | >177 | 516 |

[a] In vitro assay, ref 14. [b] Reference 15. [c] Reference 16. [d] Reference 17. [e] In vitro uterine potencies (without $Mg^{2+}$) were used in calculating O/P and O/A ratios. [f] Reference 11. [g] Reference 2. [h] Reference 1.

and the products were detected by ninhydrin or iodine vapor. The following solvent systems were used: BAW, 1-butanol–acetic acid–water (4:1:5, v/v, upper phase); BAWP, 1-butanol–acetic acid–water–pyridine (15:3:3:10, v/v). Melting points are uncorrected. Optical rotations were determined with a Hilger–Watts polarimeter with an accuracy of 0.01°. Samples for analytical purposes were dried over $P_2O_5$ in vacuo for 24 h. Analytical results, determined on a Carlo Erba Model 1106 analyzer and indicated by the elemental symbols, were ±0.4% of theoretical values. For amino acid analysis,[12] peptides (ca. 0.5 mg) were hydrolyzed with constant-boiling hydrochloric acid (400 μL) containing phenol (20 μL) in evacuated and sealed ampules for 18 h at 110 °C. The analyses were performed with a Beckman automatic amino acid analyzer Model 121. Sarcosine and *N*-methylalanine ratios were not calculated considering the difficulties in detection of *N*-methyl amino acids.[13] The presence of sarcosine or *N*-methylalanine in the structure of the synthesized peptides was determined by TLC of hydrolysates (silica gel; two dimension solvent systems: (I) 1-butanol–acetic acid–water, 4:1:5, v/v, upper phase; (II) phenol–water, 3:1, v/v; ninhydrin detection). Molecular ions of pure analogues were determined by mass spectrometry (field desorption) with a Varian MAT 711 instrument.

**Z-Cys(Bzl)-Tyr(Bzl)-Ile-Thr(Bzl)-Asn-Cys(Bzl)-Sar-Leu-Gly-$NH_2$ (I).** Boc-Gly-resin (1.0 g, 0.51 mmol of Gly) was subjected to eight cycles of deprotection, neutralization, and coupling to yield the protected 9-peptide-resin (1.632 g, 96%). The protected 9-peptide-resin was ammonolyzed, and the product was extracted into warm DMF (60 °C). The product was precipitated with boiling water and left overnight at room temperature. The peptide was collected by filtration, washed with water, and dried in vacuo over $P_2O_5$. The product was further purified by dissolving it in DMF and reprecipitating it with boiling EtOH: yield 632 mg (86% based on substitution of Gly); mp 248–250 °C dec; $[\alpha]^{22}_D$ −30.1° (*c* 1.03, DMF), TLC $R_f$ 0.65 (BAW), 0.91 (BAWP). Anal. ($C_{76}H_{95}N_{11}O_{14}S_2$) C, H, N. Amino acid analysis: Cys(Bzl), 1.92; Tyr, 0.95; Ile, 1.02; Thr, 0.97; Asp, 1.00; Leu, 1.03; Gly, 1.00; $NH_3$, 2.1.

**Z-Cys(Bzl)-Tyr(Bzl)-Ile-Thr(Bzl)-Asn-Cys(Bzl)-MeAla-Leu-Gly-$NH_2$ (II).** This peptide was prepared from Boc-Gly-resin (1.0 g, 0.51 mmol), ammonolyzed, and isolated in the same manner as described for I: yield 690 mg (93%); mp 234–236 °C; $[\alpha]^{22}_D$ −38.5° (c 0.88, DMF), TLC $R_f$ 0.64 (BAW), 0.86 (BAWP). Anal. ($C_{77}H_{97}N_{11}O_{14}S_2$) C, H, N. Amino acid analysis: Cys(Bzl), 1.86; Tyr, 0.92; Ile, 1.00; Thr, 0.95; Asp, 0.98; Leu, 1.01; Gly, 1.00; $NH_3$, 1.95.

**[4-Threonine,7-sarcosine]oxytocin (TSOT, III).** The protected 9-peptide I (168 mg, 0.116 mmol) was dissolved in 400 mL of ammonia freshly distilled from sodium and treated at the boiling point with sodium from a stick of the metal contained in a small-bore glass tube until a light blue color persisted in the solution for 20 s. The color was discharged by the dropwise addition of dry glacial AcOH. The solution was evaporated, and the residue was taken up in 0.2% AcOH (700 mL). The pH was adjusted to 7.5 with 2 N $NH_4OH$, and 0.01 M $K_3Fe(CN)_6$ was added with stirring until a yellow color persisted for 30 min. The solution was stirred for an additional 10 min with AG-3-X4 resin (chloride form, 10 g), and the suspension was filtered through a bed of the resin (70 g damp weight). The resin was washed with 0.2% AcOH (100 mL), and the combined filtrate and washings were lyophilized. The material was desalted on a Sephadex G-15 column (100 × 2.5 cm), eluting with aqueous AcOH (50%) with a flow rate of 4 mL/h. The fractions corresponding to the major peak were pooled, diluted with water, and lyophilized. The resulting powder (76 mg) dissolved in 0.2 N AcOH was subjected to gel filtration on a Sephadex G-15 column (120 × 1.2 cm) in 0.2 N AcOH with a flow rate of 2.5 mL/h. [Thr[4],Sar[7]]oxytocin (62 mg, 55%) was obtained from the fractions comprising the single symmetrical peak by lyophilization: $[\alpha]^{22}_D$ +28.9° (c 0.47, 1 N AcOH); TLC $R_f$ 0.38 (BAW), 0.57 (BAWP). $M_r$ calcd, 954.2; found, 954. Anal. ($C_{40}H_{63}N_{11}O_{12}S_2 \cdot CH_3CO_2H \cdot H_2O$) C, H, N. Amino acid analysis: $^1/_2$-$Cys_2$, 1.96; Tyr, 0.98; Ile, 1.02; Thr, 0.97; Asp, 1.00; Leu, 1.03, Gly, 1.00; $NH_3$, 2.05.

**[4-Threonine,7-*N*-methylalanine]oxytocin (TMAOT, IV).** The protected 9-peptide derivative II (173 mg, 0.118 mmol) was reduced, reoxidized, and purified as described for III to give the [Thr[4],MeAla[7]] analogue (53 mg, 39%); $[\alpha]^{22}_D$ +15.6° (c 0.27, 1 N AcOH); TLC $R_f$ 0.40 (BAW), 0.61 (BAWP). $M_r$ calcd, 968.2; found, 968. Anal. ($C_{41}H_{65}N_{11}O_{12}S_2 \cdot CH_3COOH \cdot H_2O$) C, H, N. Amino acid analysis: $^1/_2$-$Cys_2$, 1.94; Tyr, 0.96; Ile, 1.00, Thr, 0.98; Asp, 1.03; Leu, 1.02, Gly, 1.00; $NH_3$, 1.92.

**Bioassay Methods.** Pressor assays were performed as described by Dekanski.[15] Antidiuretic assays were performed on water-loaded, ethanol-anesthetized rats.[16] In vitro uterine assays (with and without 0.5 mM $Mg^{2+}$) were carried out by the method of Munsick.[17] Milk-ejection assays were performed according to the procedure of Bisset et al.[18]

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