# Systematic Substitution of an Oxytocin Antagonist with D-Amino Acids: Unexpected High Antagonistic Potency of the D-Cys[6]-Substituted Analogue†

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We report twelve analogues (1-12) of [Pmp[1],D-Trp[2],Arg[8]]oxytocin, PA (parent antagonist), (Pmp = β,β-pentamethylene-β-mercaptopropionic acid), which is a potent antagonist ($pA_2 = 7.77$) of the uterotonic effect of oxytocin (OT) in rats. The analogues were designed by replacement of each optically active amino acid residue at positions 3-8 in PA with a D-amino acid. Analogues 1-8, featuring D-amino acids in the ring portion, were weaker antagonists than PA or were inactive. Unexpectedly, replacement with D-Cys[6] gave analogue **9**, $pA_2 = 8.29$, which is more than 3 times as potent as PA, and replacement with D-Pen[6] gave analogue **10**, $pA_2 = 7.98$, also more potent than PA. Replacement with D-Pro[7] and D-Arg[8] gave analogues **11** and **12**, which are approximately equipotent or somewhat more potent than PA. These data suggest that neither the orientation of the tail sequence with respect to the plane of the ring portion of an antagonist nor the configuration of individual amino acids in the tail sequence may be critical for preservation of antagonism to the uterotonic action of OT. In the antidiuretic assay, analogues **9** and **12** were very weak partial agonists and had estimated $pA_2 = <6.3$ and $<5.6$, respectively. Analogue **9** constitutes an interesting lead for the future design of OT antagonists with different molecular requirements than those featuring L-Cys[6] as a substituent.

## Introduction

The design of potent, reversible antagonists of OT is of current interest because they would be useful clinically to inhibit preterm labor. One of these analogues[1] is in advanced clinical trials. Studies have attempted to enhance specificity of action of OT antagonists by enhancing their uterotonic potency and limiting their undesirable antidiuretic properties.[2,3]

We have recently designed [Pmp[1],D-Trp[2],Phe[3],Ile[4],Arg[8]]-OT, which inhibits (a) in vivo uterine contractions in the rat and disrupts the progress of labor, (b) the in vitro contractile response to exogenous OT of human myometrial tissue obtained by cesarean section at term, (c) the OT-induced uterine contractions in the pregnant baboon, and (d) the spontaneous uterine contractions during the last third of pregnancy in the baboon.[4] Further design led to [Pmp[1],D-Trp[2],Arg[8]]OT (PA), which has even stronger potency on the rat uterus and lower antidiuretic potency,[4] and inhibits spontaneous uterine contractions in the pregnant baboon.[5] We designed analogues of PA substituted with D-amino acids at positions 3-8 in attempts to enhance antagonistic potency and/or decrease breakdown by tissue peptidases. These analogues were tested as antagonists of OT in a uterotonic assay,[6] and the most potent ones were also tested in a rat antidiuretic assay.[6]

**Peptide Synthesis.** Suitably protected peptides were assembled manually by the solid phase (SP) method.[7] The modified synthetic procedure and HPLC purification synthetic are analogous to the ones previously described,[8] and analogue purity was assessed by HPLC, TLC (Table I), and amino acid analysis.

**Bioassays.** All analogues were tested as antagonists of OT uterotonic action in the presence of magnesium salts as we previously described[6] (Table II). The $pA_2$, or negative logarithm of the molar concentration of an antagonist that reduces the response to an agonist by one-half, was determined for each antagonist by the method of Schild[9] in at least four separate assays. Analogues **9** and **12** were also tested in a rat antidiuretic assay performed in water-loaded rats under ethanol anesthesia, as previously described.[6] An in vivo $pA_2$ was estimated by dividing the effective dose by the estimated volume of distribution of 67 mL/kg.

**Structure-Activity Relationships.** Analogues 1-8, substituted with D-amino acids in the ring portion, had either weaker potency than PA as uterotonic antagonists of OT or were inactive.

Unexpectedly, replacement of Cys[6] with D-Cys[6] led to analogue 9, with $pA_2 = 8.29$, which is more than 3 times as potent as PA. Replacement with D-Pen[6] led to antagonist **10**, with $pA_2 = 7.98$, which is still somewhat more potent than PA. Substitutions with D-Cys[6] have been reported for AVP antagonists, most of them causing an approximate lowering of the affinity for renal AVP receptors.[10] Additionally, [D-Cys[6]]OT was practically inactive in the oxytocic assay.[11]

Replacement with D-Pro[7] and D-Arg[8] gave analogues **11** and **12**, with $pA_2$ values of 7.85 and 7.91, respectively. Thus, substitutions with D-amino acids in the tail portion of the molecule maintain the potency of the antagonist, and may be of help in enhancing duration of action of antagonists by inhibiting breakdown by peptidases.

† The abbreviations used conform with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* 1989, *264*, 688-673). Abbreviations used include: aIle, alloisoleucine; Pen, penicillamine; Cit, citrulline; OT, oxytocin; AVP, [arginine[8]]-vasopressin; PA (for parent antagonist), [Pmp[1],D-Trp[2],Arg[8]]oxytocin; Pmp, β,β-pentamethylene-β-mercaptopropionic acid; MBHA, 4-methylbenzhydrylamine; Pyr, pyridine; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; UV, ultraviolet.

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Table I. Physicochemical Characteristics of OT Antagonists (OTAs)

| analogue[a] | OTA no. | MW | % yield[b] | OR[c] (deg) | TLC, $R_f$[d] A | B | C | D | HPLC retention time (min)[e] |
|---|---|---|---|---|---|---|---|---|---|
| [D-Ile$^3$]PA | 1 | 1126 | 17 | −2 | 0.37 | 0.36 | 0.24 | 0.61 | 7.0 |
| [D-alle$^3$]PA | 2 | 1126 | 13 | +6 | 0.30 | 0.43 | 0.21 | 0.56 | 6.6 |
| [D-Gln$^4$]PA | 3 | 1126 | 11 | −106 | 0.30 | 0.20 | 0.19 | 0.46 | 5.2 |
| [D-Thr$^4$]PA | 4 | 1099 | 27 | −6 | 0.36 | 0.36 | 0.25 | 0.70 | 2.6 |
| [D-Asn$^4$]PA | 5 | 1112 | 31 | −97 | 0.30 | 0.21 | 0.20 | 0.57 | 5.2 |
| [D-Glu$^4$]PA | 6 | 1127 | 27 | −100 | 0.35 | 0.30 | 0.27 | 0.50 | 5.2 |
| [D-Cit$^4$]PA | 7 | 1157 | 32 | −67 | 0.25 | 0.23 | 0.21 | 0.54 | 5.4 |
| [D-Asn$^5$]PA | 8 | 1126 | 20 | −37 | 0.27 | 0.18 | 0.18 | 0.54 | 5.2 |
| [D-Cys$^6$]PA | 9 | 1126 | 24 | −63 | 0.31 | 0.43 | 0.25 | 0.54 | 9.6 |
| [D-Pen$^6$]PA | 10 | 1154 | 10 | −69 | 0.50 | 0.50 | 0.40 | 0.68 | 22.1 |
| [D-Pro$^7$]PA | 11 | 1126 | 28 | −47 | 0.38 | 0.44 | 0.25 | 0.62 | 8.0 |
| [D-Arg$^8$]PA | 12 | 1126 | 30 | −78 | 0.25 | 0.37 | 0.14 | 0.49 | 5.4 |

[a] The structures of the analogues (1–12) is given as derivatives of PA = [Pmp$^1$,D-Trp$^2$,Arg$^8$]OT. [b] These yields are based on the milliequivalents of starting Boc-amino acid-resin, or MBHA resin. [c] OR = Optical rotation. OR was determined as $[\alpha]^{27}$D, in degrees (c 1, 1 N AcOH). [d] The composition of solvents A–D is given in the Experimental Section. [e] Solvent composition = 50% solvent B; flow rate = 1.8 mL/min. All compounds were 95–98% pure, as shown by the HPLC pattern.

Table II. $pA_2$ of Competitive Antagonists of OT Contractile Action in the Rat Uterus and $pA_2$ of Competitive Antagonism to ADH Antidiuretic Action in the Rat

| analogue[c] | analogue no. | $pA_2$ OT[a] | $pA_2$ ADH[b] |
|---|---|---|---|
| PA | | 7.77 ± 0.03 | <5.9 |
| [D-Ile$^3$]PA | 1 | 5.23 ± 0.31 | |
| [D-alle$^3$]PA | 2 | 5.11 ± 0.11 | |
| [D-Gln$^4$]PA | 3 | 5.24 ± 0.27 | |
| [D-Thr$^4$]PA | 4 | 4.95 ± 0.08 | |
| [D-Asn$^4$]PA | 5 | 5.41 + 0.06 | |
| [D-Glu$^4$]PA | 6 | 0 | |
| [D-Cit$^4$]PA | 7 | 5.51 + 0.03 | |
| [D-Asn$^5$]PA | 8 | 0 | |
| [D-Cys$^6$]PA | 9 | 8.29 ± 0.23 | <6.3 |
| [D-Pen$^6$]PA | 10 | 7.98 ± 0.15 | |
| [D-Pro$^7$]PA | 11 | 7.85 ± 0.17 | |
| [D-Arg$^8$]PA | 12 | 7.91 ± 0.06 | <5.6 |

[a] Rat oxytocic assay in vitro in the presence of $Mg^{2+}$. [b] Rat antidiuretic assay. $pA_2$ for a compound with agonist and antagonist properties were only approximated. [c] This analogue, [Pmp$^1$,D-Trp$^2$,Arg$^8$]OT or PA, was previously reported.[4]

In earlier studies it was ascertained that the ring structure of oxytocin may be the smallest structure that preserves measurable oxytocic activity.[12] This suggested that the tail portion may be regarded as having the role of enhancing affinity of binding to the OT receptor.[2,13,14] Our findings suggest that Cys$^6$ is not one of the essential components of the OT ring required for recognition by the OT receptor, since both L- or D-Cys substituents lead to good antagonist potency. In addition, the configuration of the D-Cys$^6$ residue changes the direction in which the tail peptide will be oriented with respect to the 20-membered ring by about 120°. This makes it likely that the tail portion of the antagonist, whether bound to the same amino acids or to alternate ones on the receptor, would impart what may be a different but still inactive conformation to the receptor–antagonist complex. We built space-filling models of PA and [D-Cys$^6$]PA with a $\beta$-turn involving D-Trp$^2$-Ile$^3$-Gln$^4$-Asn$^5$ with an intramolecular H-bond between the Asn$^5$ peptide amide proton and the D-Trp$^2$ carbonyl oxygen, patterned on the OT conformation in DMSO as proposed by Urry and Walter[15] and later modified by Brewster et al.[16] Observation of the model, with the ring conformation held unchanged, suggests that introduction of D- or L-Cys may facilitate the folding of the tail segment above or below the plane of the ring. [D-Cys$^6$]PA is an interesting new lead, and could give rise to a generation of OT antagonists probably having significantly different binding requirements than for the L-Cys$^6$ type of analogue. Recently, a bicyclic OT antagonist with L-Cys$^6$ has been reported, with $pA_2 = 8.74$,[17] perhaps possessing one of the possible conformations favorable to bind to the receptor but not to transmit the hormonal message. We have undertaken the synthesis of bicyclic analogues of OT in which L- and D-cysteine will be featured to evaluate the usefulnes of this type of conformational constraints.

The most potent antagonists, **9** and **12**, are weak partial agonists in the antidiuretic assay, with analogue **9** having a $pA_2$ of less than 6.3, whereas analogue **12** has a $pA_2$ of less than 5.6.

**Conclusions.** We have shown that the potency of [Pmp$^1$,D-Trp$^2$,Arg$^8$]OT, PA, a reversible antagonist in the OT uterotonic assay with a $pA_2$ value of 7.77, is unexpectedly maintained or somewhat increased by substitutions with the corresponding D-amino acid at positions 7 or 8. Surprisingly, the potency was somewhat increased by the replacement of Cys$^6$ with D-Pen$^6$, but was markedly increased by substitution with D-Cys$^6$. Hence, Cys$^6$ may not be one of the essential components of the OT ring required for recognition by the OT receptor. In addition, the D-Cys$^6$ residue changes the direction of the tail sequence with respect to the plane of the tocin 20-membered ring, so the tail could now be bound to alternate amino acid sequences on the receptor, enhancing binding affinity and imparting a different, but still inactive, conformation to the receptor. [D-Cys$^6$]PA could give rise to a generation of OT antagonists having significantly different binding requirements than for the L-Cys$^6$ type of analogue. Antidiuretic studies on the most potent antagonists showed that they are partial agonists and have very weak antidiuretic activity.

## Experimental Section

Analogues were synthesized by the manual solid-phase method, using a special vessel and a mechanical shaker. The 4-methylbenzhydrylamine resin was purchased from Bachem and the chloromethylated resin and ion-exchange resins were supplied by Bio-Rad. Boc-amino acid derivatives were purchased from Bachem Inc. or Advanced Chem Tech. Other reagents were of analytical grade and were purchased from Aldrich Chemical Co., Chemical Dynamics, or Pierce Chemical Co. When peptides were treated with liquid HF, they were handled in an all-Teflon apparatus (Protein Research Foundation, Osaka, Japan). Preparative HPLC was performed on a Gilson autopreparative HPLC System 71, and the purity of analogues was monitored by analytical HPLC with a Millipore apparatus by methods pre-

viously reported.[4] The solvents used for syntheses or for chromatography were HPLC grade (Fisher Scientific). For TLC, silica gel G precoated plates (0.25 mm, Uniplates, Analtech) were employed. The solvent systems used (ratios given volume by volume) were (A) *n*-BuOH–AcOH–$H_2O$ (4:1:1); (B) *n*-BuOH–AcOH:$H_2O$ (4:1:5, upper phase); (C) *n*-BuOH–AcOH–$H_2O$ (5:1:1); (D) *n*-BuOH–AcOH–$H_2O$–Pyr (5:1:1:1). Peptides were visualized with Ehrlich reagent and/or chlorine–tolidine color reactions.[7] For amino acid analyses of analogues, we used the Waters Associates Picotag method,[18] as we have previously described.[4] The optical rotations of peptides were determined using a Rudolph polarimeter (precision of ±0.01°).

**Solid-Phase Synthesis of Protected Peptides.** All analogues were synthesized using the SP method in a manner analogous to the synthesis of OT antagonists previously reported.[4] All peptides were removed from the resin by ammonolysis and were freed from blocking groups with HF, except for peptide **6**, which was assembled on the MBHA resin and was obtained by treating the peptide resin directly with HF. Analysis of the solid residue obtained was accomplished by HPLC on an analytical μBondapak $C_{18}$ column (30 × 0.39 cm), with monitoring of the effluent at 220 nm with a UV detector and isocratic elution with 60% solvent B (solvent A, 0.05% TFA; solvent B 60% MeCN–40% of 0.05% TFA). Preparative purification of the peptide by HPLC was accomplished with a Dyanamax-60A column using conditions analogous to those previously reported.[19] Analogue purity was established by TLC, HPLC (Table I), and amino acid analysis, which showed the expected amino acid molar ratios to be within 10% of the expected values.

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