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Design of novel bicyclic analogues derived from a potent oxytocin antagonist‡

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Abstract: Eleven new analogues were synthesized by modification of the potent oxytocin antagonist (OTA) [(S)Pmp^1, D-Trp^2, Pen^6, Arg^8]-Oxytocin, or PA (parent antagonist), in which (S)Pmp = β,β-(3-thiapentamethylene)-β-mercapto-propionic acid. By internal acylation of Lys, Orn, L-1,4-diaminobutyric acid (Dab), L-1,3-diaminopropionic acid (Dap) at position 4 with the *C*-terminal Gly of the peptide tail, we prepared cyclo-(4–9)-[Lys^4, Gly^9]-PA ($pA_2 = 8.77 \pm 0.27$), 1, and cyclo-(4–9)-[Orn^4, Gly^9]-PA ($pA_2 = 8.81 \pm 0.25$), 3, which are equipotent with PA ($pA_2 = 8.68 \pm 0.18$) in the rat uterotonic assay and cyclo-(4–9)-[Dab^4, Gly^9]-PA, 4, cyclo-(4–9)-[Dap^4, Gly^9]-PA, 5, and cyclo-(4–9)-[Pmp^1, Lys^4, Gly^9]-PA, 2, which were weaker OTAs. Neither 1 nor 3 had activity as agonists or antagonists in the antidiuretic assay. In the pressor assay, both analogues 1 and 3, with $pA_2 = 7.05 \pm 0.10$ and $pA_2 = 6.77 \pm 0.12$, respectively, are somewhat weaker antagonists than PA ($pA_2 = 7.47 \pm 0.35$) showing significant gain in specificity. The [desamido^9] PA-ethylenediamine monoamide, 6, and the dimer ([desamido^9]-PA)$_2$ ethylenediamine diamide, 7, had lower potency in the uterotonic assay than PA. Additionally, we synthesized cyclo-(1–5)-[(HN)Pmp^1, Asp^5]-PA, 8, inactive in all tests, which suggests that the intact Asn^5 side chain may be critical in the interaction of the OTAs with the oxytocin (OT) receptor. Similarly, cyclo-(5–9)-[Dap^5, Gly^9]-PA, 9, had very low uterotonic potency. Two derivatives of PA truncated from the *C*-terminus were internally cyclized to Lys^4, giving rise to cyclo-(4–8)-desGly-NH$_2^9$[Lys^4, Arg^8]-PA, 10 ($pA_2 = 8.35 \pm 0.20$), which maintains the high potency of PA and has no activity in the rat antidiuretic assay, and in the rat pressor assay it is about ten times weaker ($pA2 = 6.41 \pm 0.15$) than PA ($pA2 = 7.47 \pm 0.35$), thus showing gains in specificity, and to cyclo-(4–7)-desArg-Gly-NH$_2^{8-9}$[Lys^4, Pro^7)-PA, 11, which has much weaker potency than PA. Synthesis of cyclo-(4–6)-desPro-Arg-Gly-NH$_2^{7-9}$[Lys^4]-PA failed. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: oxytocin; antagonists; bicyclic peptides; premature labor

INTRODUCTION

The synthesis of oxytocin antagonists (OTAs) continues to draw great interest since they would be potential inhibitors of preterm labor and because premature birth still is the major contributory factor to perinatal mortality and morbidity [3–5]. The neurohypophysial hormone (NHH) OT has an uncertain role in the initiation of labor, but seems to have a contributory endocrine and paracrine role in human parturition [6–9]. OT and its sister hormone arginine vasopressin (AVP) act in numerous tissues throughout the human body. Not surprisingly, a number of different NHH receptors have been characterized, including vascular, V_{1a}, pituitary, V_{1b} or V_3, renal, V_2, and uterine OT receptors [10–12]. Both OT and AVP act on each other's receptors with varying potencies. The successful design of highly potent and specific OTAs would be useful tools in the discovery and definition of new OT receptors, as well as in the clarification of the role of OT during normal and preterm labor. Although β-adrenergic agents have been used to arrest preterm labor, e.g. Ritodrine, their use has been deemed unsatisfactory [13].

Considerable effort has been directed in designing specific OTAs [14–19] that would be more specific for OT receptors and devoid of the side effects of nonpeptide

Abbreviations: AcOH, acetic acid; BOP reagent, (benzotriazolyl-1-oxy)-tris(dimethylamino) phosphonium hexafluorophosphate; *n*-BuOH, *n*-butanol; 2-Cl-Z, 2-Chlorobenzyloxycarbonyl; Dab, L-1,4-diaminobutyric acid; Dap, L-1,3-diaminopropionic acid; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; EDAC.HCl, 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride; ESI-MS, electrospray ionization-mass spectrometry; EtOAc, ethyl acetate; (HN)Pmp, β,β-(3-azapentamethylene)-β-mercaptopropionic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; Meb, 4-methylbenzyl; MeCN, acetonitrile; ONp, 4-nitrophenyl ester; OR, optical rotation; OT, oxytocin; OTA/OTAs, oxytocin antagonist/antagonists; PA, [(S)Pmp^1, D-Trp^2, Cys^6, Arg^8]-Oxytocin; PITC, phenylisothiocyanate; Pmp, β,β-pentamethylene-β-mercaptopropionic acid; PTC, phenylthiocarbamyl; Pyr, pyridine; (S)Pmp, β,β-(3-thiapentamethylene)-β-mercaptopropionic acid; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; TLC, thin layer chromatography; Tos, *p*-toluenesulfonyl; Z, benzyloxycarbonyl. Abbreviations used comply with recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* 1989; 264, 688–673), and J. Peptide Sci. 2003: **9**: 1–8.

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‡Preliminary results were presented in part at the 27th European Peptide Symposium in Sorrento, Italy (31 August–6 September 2002), and in the Proceedings of the Symposium [1], and at the 3rd International and 28th European Peptide Symposia in Prague, Czech Republic (5–10 September 2004) and in the Proceedings of the Symposium [2].

mimetics [20]. In recent years, [Mpa^1, D-Tyr(Et)^2, Thr^4, Orn^8]-OT, Atosiban, has been used in more or less high doses to arrest the uterine contractions of preterm labor in clinical studies [21–23], and it is now in clinical use in Europe [24].

In our laboratories, we synthesized a number of NHH derivatives with high potency as OTAs and with decreased action in vasopressin-sensitive organs. Among our earlier prepared analogues, [Pmp^1, D-Trp^2, Phe^3, Ile^4, Arg^8]-Oxytocin [25], in which Pmp is β,β-(pentamethylene)-β-mercaptopropionic acid and $pA_2 = 7.51$ in the uterotonic *in vitro* test, inhibits uterine contractions of an estrus rat in response to exogenous OT and milk let down in the lactating rat, and delays labor contraction in rats. Furthermore, this antagonist inhibits *in vitro* uterine contractions in response to OT in isolated uterine strips obtained from women after cesarean sections [26], and inhibits spontaneous uterine contractions and labor in baboons [27]. Subsequently, [Pmp^1, D-Trp^2, Arg^8]-OT ($pA_2 = 7.77$) [25,28,29], [(S) Pmp^1, D-Trp^2, Cys^6, Arg^8]-OT ($pA_2 = 8.11$), in which (S)Pmp is β,β-(3-thiapentamethylene)-β-mercaptopropionic acid, and more recently [(S) Pmp^1, D-Trp^2, Pen^6, Arg^8]-OT, hereafter PA (or parent antagonist, Figure 1) with $pA_2 = 8.68$ [18], were found to be more potent and more specific, which is very promising for preterm labor inhibitors.

Previous NMR studies have suggested that OTAs appear to be more rigid than OT or its agonists [30]. Some potent bicyclic OTAs were made by bridging Glu and Lys, which were introduced into positions 4 and 8 respectively [31]. In a previous study, we synthesized bicyclic analogues of PA in order to reduce their conformational flexibility. The analogue cyclo-(1–9)-[(HN)Pmp^1, D-Trp^2, Pen^6, Arg^8, Gly^9]-OT had $pA_2 = 8.17 \pm 0.16$ and had gained specificity, since this analogue had no activity in the antidiuretic assay either as an agonist or antagonist. We report here other modes of introducing bicyclic structures

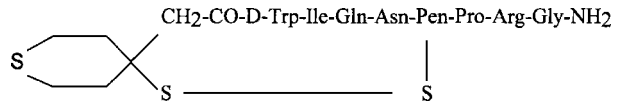


Figure 1 Structure of [β,β-(3-thiapentamethylene-β-mercaptopropionyl^1, D-Trp^2, Pen^6, Arg^8]-oxytocin PA, or parent antagonist.

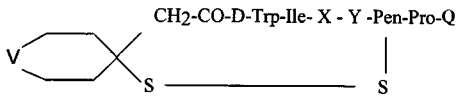
Table 1 List of analogues

Number	Structure
	[(S)Pmp^1, D-Trp^2, Pen^6, Arg^8]OT, PA or parent antagonist
	Cyclo-(1–9)-[(HN)Pmp^1, Gly^9]-PA
1	Cyclo-(4–9)-[Lys^4, Gly^9]-PA
2	Cyclo-(4–9)-[Pmp^1, Lys^4, Gly^9]-PA
3	Cyclo-(4–9)-[Orn^4, Gly^9]-PA
4	Cyclo-(4–9)-[Dab^4, Gly^9]-PA
5	Cyclo-(4–9)-[Dap^4, Gly^9]-PA
6	[desamido^9]-PA ethylenediamine monoamide
7	([desamido^9]-PA)$_2$ ethylenediamine diamide
8	Cyclo-(1–5)-[(HN)Pmp^1, Asp^5]-PA
9	Cyclo-(5–9)-[Dap^5, Gly^9]-PA
10	Cyclo(4–8) desGly-NH$^9_2$[Lys^4, Arg^8]-PA
11	Cyclo(4–7) desArg-GlyNH$^{8-9}_2$[Lys^4, Pro^7]-PA

Table 2 Physicochemical characteristics of oxytocin antagonists

Analogue		MW		TLC[b]					HPLC retention time (min)[d]
Number	Name	Calculated	Found[c]	Yield[a] %	R_fA	R_fB	R_fC	R_fD	
1	Cyclo-(4–9)-[Lys^4, Gly^9]-PA[e]	1155.4	1155.9	7	0.53	0.51	0.47	0.71	11.2
2	Cyclo-(4–9)-[Pmp^1, Lys^4, Gly^9]-PA	1137.4	1137.8	28	0.46	0.51	0.42	0.68	15.6
3	Cyclo-(4–9)-[Orn^4, Gly^9]-PA	1141.4	1141.5	8	0.47	0.49	0.37	0.65	11.6
4	Cyclo-(4–9)-[Dab^4, Gly^9]-PA	1127.4	1127.5	11	0.42	0.49	0.32	0.67	5.8
5	Cyclo-(4–9)-[Dap^4, Gly^9]-PA	1113.4	1113.6	22	0.47	0.49	0.37	0.68	10.0
6	[desamido^9]-PA ethylenediamine monoamide	1215.5	1215.0	27	0.10	0.38	0.07	0.41	5.6
7	([desamido^9]-PA)$_2$ ethylenediamine diamide	2370.8	2370.8	24	0.07	0.40	0.04	0.46	14.0
8	Cyclo-(1–5)-[(HN)Pmp^1, Asp^5]-PA	1138.4	1137.8	32	0.26	0.44	0.19	0.55	4.2
9	Cyclo-(5–9)-[Dap^5, Gly^9]-PA	1127.4	1127.8	15	0.20	0.46	0.12	0.49	3.8
10	Cyclo(4–8) desGly-NH$^9_2$[Lys^4, Arg^8]-PA	1098.4	1098.8	10	0.47	0.56	0.33	0.75	11.4
11	Cyclo(4–7) desArg-GlyNH$^{8-9}_2$[Lys^4, Pro^7]-PA	942.2	942.5	6	0.78	0.82	0.73	0.90	13.8

[a] These yields are based on the milliequivalents of starting Boc-amino acid-resin.
[b] The composition of solvents A–D is given in the 'Experimental' section.
[c] ESI-MS.
[d] The analysis was run isocratically, solvent composition = 45% solvent B, flow rate 1 ml/min, in order to determine the relative hydrophilicities by comparing retention times of analogues. An Alltech alpha Bondapak C18, 300 × 3.9 mm column was used.
[e] PA = [(S)Pmp^1, D-Trp^2, Pen^6, Arg^8]OT.



Analogue	V	X	Y	Q	Second Ring
PA	S	Gln	Asn	Arg-Gly-NH_2	--
1	S	Lys	Asn	Arg-Gly	4-9
2	CH_2	Lys	Asn	Arg-Gly	4-9
3	S	Orn	Asn	Arg-Gly	4-9
4	S	Dab	Asn	Arg-Gly	4-9
5	S	Dap	Asn	Arg-Gly	4-9
6	S	Gln	Asn	Arg-Gly-NH-CH_2-CH_2-NH_2	--
7	S	Gln	Asn	Arg-Gly-NH-CH_2	--
	S	Gln	Asn	Arg-Gly-NH-CH_2	
8	HN	Gln	Asp	Arg-Gly-NH_2	1-5
9	S	Gln	Dap	Arg-Gly	5-9
10	S	Lys	Asn	Arg	4-8
11	S	Lys	Asn	--	4-7

Figure 2 Structure of PA analogues.

to decrease the flexibility of the potential OTAs. We attempted to bridge β,β-(3-azapentamethylene)-β-mercaptopropionic acid (HN)Pmp at positions 1 to Glu at position 4, or to Asp at position 5. In addition, we explored bridging position 4, substituted with different basic amino acids, to Gly at position 9, as well as truncating PA sequences ending in Arg at position 8, in Pro at position 7 and in Pen at position 6. We also included in the study the bridging of L-1,3-diaminopropionic acid (Dap) at position 5 to Gly at position 9. The PA was also bridged at the *C*-terminus by means of ethylene diamine. Considerable time had to be spent determining which reagent would be optimal for each cyclization.

Eleven new analogues were prepared (Table 1) and their structures are summarized in Figure 2. These analogues were tested as OTAs of the OT uterotonic action in the rat bioassay *in vitro* [32] in the presence of magnesium ions. The most potent OTAs were also tested in the rat antidiuretic assay and/or the pressor assay. We report here the physicochemical properties of the new OTAs (Tables 2, 3) and their biological data (Table 4).

EXPERIMENTAL

Synthesis of Protected Peptides

All the protected peptide precursors of the OTAs were first assembled manually by solid phase peptide synthesis (SPPS) [33] using the Boc-strategy [34] with small variations in procedures as we have described [18,19]. The starting resins were Boc-Gly-Resin, Boc-Arg(Tos)-Resin, Boc-Pro-Resin or Boc-Pen(Meb)-Resin (0.5–1.0 mmol of Boc-amino acid/g). These resins were prepared by esterification of a 200–400-mesh chloromethylated polystyrene resin (BioRad) 1% cross-linked, with the cesium salt of the Boc-amino acid [35]. The protected peptide precursor of cyclo-(1–5)-[(HN)Pmp^1, Asp^5]-PA, **8**, was synthesized on a 4-methylbenzhydryl amine-resin [34]. For protection of side-chain functionalities, Boc-Arg(Tos), Boc-Pen(Meb), Boc-Lys(2-Cl-Z), Boc-Orn(Z), Boc-Dab(Z), Boc-Dap(Z), (Boc-N)Pmp, (S)Pmp(Meb) or Pmp(Meb) were chosen. All Boc-amino acids were obtained from Bachem, with the exception of Boc-Pen(Meb). DCHA, which was obtained from Peptides International and was converted to Boc-Pen(Meb) immediately before use. The appropriate Boc-amino acid-resin was lengthened with suitable amino acids until the required coupling cycles led to the desired protected peptide-resin. The ninhydrin test [36] was used to verify completion of coupling at each step. Following the introduction of Boc-D-Trp at position 2, the Boc-groups were removed with 30% trifluoroacetic acid (TFA) in dichloromethane (DCM) containing 1% mercaptoethanol and 5% anisole [25]. (S)Pmp(Meb), Pmp(Meb), and (Boc-N)Pmp(Meb) were coupled where required in threefold excess in dimethylformamide (DMF) solution using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) for activation. There was only one protected peptide amide that was removed from the resin by aminolysis for 3 days with MeOH-ethylene diamine (2 : 1) [37]. The resin was filtered and extracted with hot DMF three times. The filtrate and the DMF extracts were combined and evaporated to dryness under low pressure, and the solid residue obtained was washed with ether. Protected peptides thus isolated usually have show one major component with minor impurities

Table 3 Amino acid analyses of oxytocin antagonists

Analogue		Amino acid ratios								
Number	Name	Asp	Glu	Gly	Arg	Pro	Pen[a]	Ile	Trp[b]	R
1	Cyclo-(4–9)* [Lys^4, Gly^9]-PA	0.98	—	1.00	1.06	1.02	0.31	0.91	0.92	0.90[c]
2	Cyclo-(4–9)-[Pmp^1, Lys^4, Gly^9]-PA	0.94	—	1.00	1.03	0.91	0.30	0.90	0.82	0.91[c]
3	Cyclo-(4–9)-[Orn^4, Gly^9]-PA	1.00	—	1.09	1.07	1.05	0.38	0.92	0.89	1.00[d]
4	Cyclo-(4–9)-[Dab^4, Gly^9]-PA	0.90	—	1.00	1.07	1.06	0.44	0.91	0.81	1.01[e]
5	Cyclo-(4–9)-[Dap^4, Gly^9]-PA	0.99	—	1.00	0.94	0.96	0.33	0.92	0.82	0.98[f]
6	[desamido^9]-PA ethylenediamine monoamide	0.98	0.97	1.00	1.01	1.02	0.28	0.94	0.83	—
7	([desamido^9]-PA)$_2$ ethylenediamine diamide	0.96	0.94	1.03	1.00	0.90	0.30	0.93	0.81	—
8	Cyclo-(1–5)-[(HN)Pmp^1, Asp^5]-PA	0.93	0.94	0.98	1.03	0.97	0.36	0.93	0.80	—
9	Cyclo-(5–9)-[Dap^5, Gly^9]-PA	—	0.92	1.00	0.94	0.95	0.35	0.90	0.83	0.91[f]
10	Cyclo(4–8) desGly-NH_2^9[Lys^4, Arg^8]-PA	0.92	—	—	1.02	1.01	0.29	0.94	0.79	0.95[c]
11	Cyclo(4–7) desArg-GlyNH_2^{8-9}[Lys^4, Pro^7]-PA	0.90	—	—	—	0.92	0.28	0.91	0.80	0.92[c]

[a] Pen-SS-Pen. Values for Pen are low because a derivative of the type (S)Pmp-SS-Pen could form, which cannot be detected.
[b] Tryptophan in peptides was estimated by its UV absorption at 280 nm as we have reported. [18] Values found for Trp suggest that the peptide preparation contains several moles of AcOH, TFA, and/or H_2O.
[c] Lys.
[d] Orn.
[e] Dab.
[f] Dap.

Table 4 Potency of competitive antagonists of oxytocin contractile action in the rat uterus *in vitro* assay

Analogue		Biological activity			
Number	Name	Antioxytocic $pA_2 \pm SEM$	Pressor $pA_2 \pm SEM$	Antidiuretic pA_2	Affinity for human OTR K_i (nM)[a]
	PA	8.68 ± 0.18[b]	7.47 ± 0.35	5.75[b]	n.d[c]
	Cyclo-(1–9)-[(HN)Pmp^1, Gly^9] PA	8.17 ± 0.16	<5.65	0[d]	n.d.
1	Cyclo-(4–9)-[Lys^4, Gly^9]-PA	8.77 ± 0.27	7.05 ± 0.10	n.d.	15.2
2	Cyclo-(4–9)-[Pmp^1, Lys^4, Gly^9]-PA	7.79 ± 0.16	n.d.	n.d.	19.7
3	Cyclo-(4–9)-[Orn^4, Gly^9]-PA	8.81 ± 0.25	6.77 ± 0.12	0[d]	10.9
4	Cyclo-(4–9)-[Dab^4, Gly^9]-PA	7.79 ± 0.36	n.d	n.d.	n.d.
5	Cyclo-(4–9)-[Dap^4, Gly^9]-PA	7.52 ± 0.25	n.d.	n.d.	113
6	[desamido^9]-PA ethylenediamine monoamide	7.73 ± 0.30	n.d.	n.d.	n.d.
7	([desamido^9]-PA)$_2$ ethylenediamine diamide	8.09 ± 0.10	n.d.	n.d.	n.d.
8	Cyclo-(1–5)-[(HN)Pmp^1, Asp^5]-PA	0[e]	0[f]	n.d.	21,800
9	Cyclo-(5–9)-[Dap^5, Gly^9]-PA	7.00 ± 0.29	<5.65	0[d]	346
10	Cyclo(4–8) desGly-NH_2^9[Lys^4, Arg^8]-PA	$8.35 + 0.20$	$6.41 + 0.15$	0[d]	31.7
11	Cyclo(4–7) desArg-GlyNH_2^{8-9}[Lys^4, Pro^7]-PA	7.89 ± 0.20	n.d.	n.d.	40.8

[a] For oxytocin, this value amounted to 1.5 nM.
[b] The parent analogue, PA, was previously reported [18].
[c] n.d. means not determined.
[d] No antidiuretic or diuretic activity is detected up to a dose of 0.2 mg/Kg of the experimental animal, *s.c.*
[e] No antioxytocin activity is detected up to 2 mM concentration.
[f] No pressor or antipressor activity is detected up to a dose of 0.2 mg/Kg of the experimental animal, *i.v.*

on thin layer chromatography (TLC) and were used for removal of blocking groups without further purification.

Analogue Synthesis

The blocking groups on side-chain functionalities of protected peptide amides were removed by reduction with sodium in liquid ammonia [38]. In the case of peptide acids, protected peptide-resin precursors were treated with liquid HF-anisole [39] using an all-Teflon apparatus (Protein Research Foundation, Osaka, Japan), which yielded directly a peptide acid free of blocking groups. In the case of the protected peptide precursor of cyclo-(1–5)-[(HN)Pmp^1, Asp^5]-PA, **8**, synthesized

on a 4-methylbenzhydryl amine-resin, the treatment with liquid HF-anisole led to Asp being free of the benzyl ester blocking group, and simultaneously, the release of the peptide as the glycinamide at the *C*-terminus. In all these cases, the disulfhydryl peptides were converted [40] to the corresponding cyclic disulfide in a very dilute solution by oxidation with a potassium ferricyanide solution [41]. The analogues thus obtained were simultaneously desalted, freed from by-products, and purified by preparative high performance liquid chromatography (HPLC) [18,19,25]. The solvent systems used both for analytical and/or preparative HPLC were (A) 0.05% TFA and (B) MeCN containing 0.05% TFA. Peptide homogeneity was also monitored by analytical HPLC and by TLC (Table 2) on silica gel G precoated Uniplates (0.25 mm, Analtech). After applying a solution of peptide, which contained 20–30 μg, to each plate, the chromatogram was developed for about 10 cm. The solvent systems used (ratios given 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); and (D) *n*-BuOH : AcOH : H_2O : Pyr (5 : 1 : 1 : 1). The peptides were detected with Ehrlich reagent, chlorine, and KI-starch, or chlorine-tolidine [34]. The molecular weight of each peptide was verified by electrospray ionization-mass spectrometry (ESI-MS, Table 2) and the amino acid content of each peptide was also evaluated by amino acid analysis (Table 3). For amino acid analysis, peptides were hydrolyzed with 6N HCl for 24 h at 110 °C. After evaporation under reduced pressure, the residues were derivatized with phenylisothiocyanate (PITC) and analyzed by the Waters Associates picotag method [42] as previously reported [25]. The Trp residue was estimated from the UV absorption of a suitable peptide solution at 280 nm [18].

(Desamido^9)-PA Ethylenediamine Monoamide, 6

(S)Pmp(Meb)-D-Trp-Ile-Gln-Asn-Pen(Meb)-Pro-Arg(Tos) Gly-Resin, obtained by the SPPS method as described above (0.2 mmol), was suspended in MeOH (10 ml) and ethylenediamine (5 ml). After 3 days, the suspension was filtered and washed three times with methanol and three times with hot DMF. All washings were pooled and evaporated to dryness, and the residue was washed with ether yielding 200 mg of product. The protected peptide amide thus obtained (200 mg) was dissolved in anhydrous liquid ammonia (150 ml) freshly distilled from sodium and was treated with sodium until a pale blue color lasted for about 15–30 s. The liquid ammonia reaction was evaporated in a vacuum and the solid residue obtained was dissolved in 20 ml of 50% acetic acid (AcOH). The clear solution was added to water (2 l), the pH was adjusted to 7.0–7.3 with ammonium hydroxide, and the solution was titrated with 0.01N potassium ferricyanide until a permanent yellow color resulted, and then an additional 20% excess of potassium ferricyanide solution was added [41]. After 20 min, the ferrocyanide and ferricyanide salts were removed by addition of an AG1X-2 (Cl-) ion exchange resin (20 g) and stirring for 15 min. The suspension was then added to a chromatographic column containing an additional ion exchange resin (20 g) and was washed two times with 100 ml of water. The combined eluents were acidified with AcOH to pH 5 and then lyophilized. Analysis of the peptide obtained was accomplished by HPLC (analytical μBondapak C_{18} column, 30 × 0.39 cm), monitored at 220 nm, and eluted isocratically with 45% solvent B (see above) at a flow rate of 1 ml/min. For purification by preparative HPLC, a linear gradient was run from 0 to 50% solvent B over 120 min, eluting at a flow rate of 4 ml/min and monitoring the eluent at 254 nm. The purer fractions determined by analytical HPLC were pooled and lyophilized, yielding antagonist PA-ethylenediamine monoamide, **6**, (101 mg) in an estimated purity of 95% or better. The analogue molecular weight was determined by ESI-MS, analogue homogeneity was determined by TLC and by analytical HPLC (Table 2), and amino acid components were determined by amino acid analysis (Table 3).

((Desamido^9)-PA)$_2$ Ethylenediamine Diamide, 7

The starting [desamido^9]-PA ethylenediamine monoamide, **6**, (60 mg, 0.049 mmole), PA acid, [desamido^9]-PA (86 mg, 0.074 mmole), which was previously prepared [19], HOBt (11.3 mg, 0.074 mmol), 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC.HCl) (14 mg, 0.074 mmol) and diisopropylethylamine (DIEA) (0.0166 ml, 0.098 mmol) were dissolved in DMF (3 ml). Additional DIEA (0.033 ml) was added to have a basic solution. After 24 h, the reaction was complete (attempts with DCC or with (benzotriazolyl-1-oxy)-tris(dimethylamino) phosphonium hexafluorophosphate (BOP) reagent gave incomplete reactions). The product was precipitated by adding it to ether, and the precipitate obtained (160 mg) was purified as usual by preparative HPLC as described for analogue **6**. The product obtained was ([desamido^9]-PA)$_2$ ethylenediamine diamide, **7**, (34 mg), homogeneous on TLC and HPLC, with the expected MW and amino acid analysis (Tables 2 and 3).

Cyclo-(4–9)-(Lys^4, Gly^9)PA, 1

(S)Pmp(Meb)-D-Trp-Ile-Lys(2-Cl-Z)-Asn-Pen(Meb)-Pro-Arg(Tos) Gly-Resin (0.25 mmol), obtained by the SPPS method, was suspended in liquid HF (10 ml) containing anisole (1 ml) in an all-Tefflon apparatus as previously described [25]. The suspension was stirred in ice for 1 h and then it was evaporated under reduced pressure. The solid residue was dissolved in 40 ml of 50% AcOH and the clear solution was extracted three times with hexane. The aqueous phase was added to water (2 l) and the pH was adjusted to 7.2 with ammonium hydroxide. This solution was titrated with 0.01N potassium ferricyanide, until a permanent yellow color resulted and then a 20% excess of potassium ferricyanide solution was added [41]. After 20 min, the solution was lyophilized. For purification by preparative HPLC, a linear gradient was run from 0–33% solvent B over 70 min, eluting at a rate of 4 ml/min and monitoring the eluent at 254 nm. The purer fractions determined by analytical HPLC were pooled and lyophilized, yielding the intermediate analogue [Lys^4, Gly^9]-PA (130 mg) in an estimated purity of 95% or better. Analytical data are given in Tables 2 and 3. To a solution of this intermediate, [Lys^4, Gly^9]-PA (130 mg, 0.11 mmol) in DMF (0.65 ml), sodium bicarbonate (93 mg, 0.11 mmol) and BOP reagent (98 mg, 0.22 mmol) were added and the suspension was stirred for 1–3 days at room temperature. When HPLC analysis showed that the product did not increase in concentration, the solvent was evaporated in a vacuum and the residue was dissolved in water and purified by preparative HPLC. Elution with a gradient from 0 to 38% solvent B, flow rate 4 ml/min, for 90 min gave the desired analogue cyclo-(4–9)-[Lys^4, Gly^9]-PA, **1**, (25 mg) (Tables 2 and 3).

BIOASSAYS

All analogues were tested in a rat uterotonic *in vitro* assay using a modified Holton method [32] in Munsick solution [43] containing 0.5 mM Mg^{++}. Rats in induced estrus by an injection of estrogen 48 h before the experiments were used; the height of the single isometric contraction was measured and cumulative dose response curves were constructed in the absence and in the presence of various doses of the analogues. The pA_2 was calculated according to Schild [44]. Pure synthetic oxytocin from Bachem or PolyPeptide Labs was used as standard. Only the most potent or interesting antagonists of the series were also tested in a rat antidiuretic assay performed on conscious rats in a modified Burn's arrangement [45,46] and/or in the rat pressor test performed on phenoxybenzamine-treated male rats [47]. The *in vivo* pA_2 was calculated as the negative logarithm of base 10 of the effective concentration that was calculated from the effective dose divided by the volume of distribution (arbitrarily taken as 67 ml/Kg) and by the molecular weight. Wistar rats were used in all experiments. For details, see Ref. 48.

Binding affinities to the human oxytocin receptor were obtained using membranes of human endothelial kidney (HEK) cells stably expressing the human oxytocin receptor [49] (kindly provided by Dr G. Gimpl from Mainz University) as described previously [50,51], using of tritiated oxytocin from NEN Life Science, Boston, MA, USA. In brief, a crude membrane fraction of the cells was incubated with [^{3}H]-OT (2 nM) and various concentrations of peptides (0.1–10 000 nM) for 30 min at 35 °C. The total volume of the reaction mixture was 0.25 ml. The incubation medium consisted of HEPES (50 mM, pH 7.6), $MnCl_2$ (10 mM) and bovine serum albumin (BSA, 1 mg/ml). The reaction was terminated by quick filtration on a Brandel cell harvester. Oxytocin was used as a control and for the determination of nonspecific binding (1 μM). Binding affinities were expressed as K_i values calculated according to the following expression [52].

$$K_i = IC_{50}/[(c_{3HOT}/K_{dOT}) + 1] \quad (1)$$

where K_{dOT} is taken as 1.8 nM.

RESULTS AND DISCUSSION

A total of 11 analogues were synthesized and evaluated (Table 4). Our previous studies had demonstrated that when PA substituted with basic amino acids at position 4 was acylated with a carbamoyl group, a very high retention of potency as OTAs in the rat uterotonic assay was attained. This suggested that acylation with the carboxyl group of amino acids of the tail sequence could also lead to potent OTAs. This paper aimed at determining the optimal length of the basic amino acid to be acylated with the carboxyl group of *C*-terminal Gly; hence, we prepared analogues **1**, **3–5** using Lys and the progressively shorter Orn, L-1,4-diaminobutyric acid (Dab), and Dap respectively. The finding of their high OTA potency would suggest a more facile fit of the more rigid bicyclic compound with the OT receptor. It was found that cyclo-(4–9)-[Lys^4, Gly^9]-PA ($pA_2 = 8.77 \pm 0.27$), analogue **1**, and cyclo-(4–9)-[Orn^4, Gly^9]-PA ($pA_2 = 8.81 \pm 0.25$), analogue **3**, were equipotent with PA ($pA_2 = 8.68 \pm 0.18$) in the rat uterotonic assay. In order to determine whether substitution with β,β-(pentamethylene)-β-mercaptopropionic acid (Pmp) instead of β,β-(3-thiapentamethylene)-β-mercaptopropionic acid ((S)Pmp) would be advantageous, cyclo-(4–9)-[Pmp^1, Lys^4, Gly^9]-PA ($pA_2 = 7.79 \pm 0.16$), analogue **2**, was also prepared. It was disappointing that the introduction of the Pmp substitution led to such a sharp decrease in potency, although this might suggest an important role for the thia substitution in (S)Pmp to enhance potency. The substitutions with shorter basic amino acids, Dab and Dap, led to weaker analogues. Of additional interest was the finding that neither **1** nor **3** had activity as agonists or antagonists in the antidiuretic assay. In the pressor assay, both analogue **1** ($pA_2 = 7.05$) and analogue **3** ($pA_2 = 6.77 \pm 0.12$) are somewhat weaker antagonists than PA ($pA_2 = 7.47 \pm 0.35$). Hence, for analogues **1** and **3**, the potency has been highly conserved, and significant gains in specificity have been realized.

The [desamido^9]-PA ethylenediamine monoamide, **6**, had lower potency in the uterotonic assay than PA, and so did the diamide dimer, **7**; hence, they did not develop any new insights into structure activity relationships.

During our previous studies of Pmp analogues at position 1 [18], we had found that substitution at position 1 with the highly hydrophilic (HN)Pmp^1 had led to a sharp loss in potency as an OTA. The potency was restored by internal acylation with the glycine carboxylic acid on the peptide tail, which led to bicyclic compounds with high potency as OTAs. It was of interest, however, to determine whether a similar potency increase could be effected by acylation with Glu at position 4 or Asp at position 5. Whereas we could successfully prepare the cyclo-(1–5)-[(HN)Pmp^1, Asp^5]-PA, analogue, **8**, the cyclo(1–4) analogue could not be prepared. Three separate attempts were made to synthesize the cyclo (1–4) analogue by cyclization of [(HN)Pmp^1, Glu^4]-PA. All these attempts gave intractable mixtures and traces of HPLC fractions having a different MW from that expected. Observation of space-filling Dreiding models suggested that significant distortion of the ring portion would have to take place to allow for the Glu side-chain carboxyl group to come into proximity and react with the (HN)Pmp group. Perhaps, this raises the energy of activation high enough that

the formation of the second ring could not take place under the normal mild conditions at room temperature. On the other hand, acylation of the (HN)Pmp group with the Asp side-chain carboxyl led to analogue **8**, which was completely inactive. This seems to corroborate that Asn^5 plays a critical role in the interaction of OT and analogues with the OT receptor.

For preparation of the cyclo (5–9) analogue **9**, [Dap^5, Gly^9]-PA was chosen as the starting material, since Dap is the smallest stable basic amino acid, resembling Asn more and leading to a potent antagonist [2], and there are sufficient degrees of freedom for the tail end Gly of the peptide to reach and react with the amino side group. The uterotonic potency of this analogue is also low, suggesting that it may be important to keep the Asn^5 intact to facilitate interaction with the OT receptor.

We also studied the effect of using truncated peptide acid analogues of PA. We settled for using Lys^4 to provide sufficient length of the side chain, in order to facilitate the reaction with the peptide acid. The truncated derivatives chosen included the octapeptide acid desGly-NH_2^9-[Lys^4, Arg^8]-PA, the heptapeptide acid desArg-Gly-NH_2^{8-9}[Lys^4, Pro^7]-PA, and the hexapeptide acid desPro-Arg-Gly-NH_2^{7-9}[Lys^4]-PA. The cyclo-(4–8)-desGly-NH_2^9[Lys^4, Arg^8]-PA, **10** ($pA_2 = 8.35 \pm 0.20$) maintained the high potency of PA, but it had no activity in the rat antidiuretic assay and was about ten times weaker ($pA_2 = 6.41 \pm 0.15$) than PA ($pA_2 = 7.47 \pm 0.35$) in the rat pressor assay. Hence, **10** has maintained potency but has gained greatly in specificity when compared to PA. The cyclo(4–7) desArg-Gly-NH_2^{8-9}[Lys^4, Pro^7]-PA, **11** (pA_2 7.89 ± 0.20) was much weaker than PA and was not studied further. Three separate attempts were made to synthesize the cyclo(4–6) derivative of the hexapeptide acid desPro-Arg-Gly-NH_2^{7-9}[Lys^4]-PA. All attempts gave intractable mixtures with traces of HPLC fractions with a different MW from that expected.

The data from pharmacological tests on rats were supplemented by determination of the affinities of the analogues to the human oxytocin receptors stably expressed on HEK cells [49] using tritiated oxytocin. The data are given in Table 4. As can be seen, the affinities of the most potent analogues are about 7–10 times lower than that of OT, and the affinities correlate well with the values of the potency in the uterotonic test. Compound **8**, which is not active in the rat biological test, also has an affinity three orders of magnitude lower for the receptors. These findings point to the fact that the analogues should be active not only in the rat but also in the human.

CONCLUSIONS

Our findings lead to the conclusion that bicyclic modifications of some structures including cyclo-(4–9)-[Lys^4, Gly^9]-PA, analogue **1**, cyclo-(4–9)-[Orn^4, Gly^9]-PA, analogue **3**, or cyclo-(4–8)-desGly-NH_2[Lys^4, Arg^8]-PA, analogue **10**, led to highly potent OTAs in the uterotonic assay even if their potency was not higher than that of the monocyclic PA. But they showed increased specificity arising from their decreased activity in the rat antidiuretic or rat pressor assays. We also confirmed that leaving the Asn^5 unsubstituted is crucial, since this residue may be an important component of binding of the analogue to the OT receptor.

On the basis of these results, we are undertaking further SAR studies on our best bicyclic analogues, 1, 3 and 10, with the aim of designing a highly potent and pure antagonist of oxytocin that is free of biological effects on renal and vascular tissues.

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