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Analogues of a Potent Oxytocin Antagonist with Truncated *c*-Terminus or Shorter Amino Acid Side Chain of the Basic Amino Acid at Position 8‡

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Abstract: Twelve analogues were synthesized, their structure derived from modifications of [(S)Pmp^1, D-Trp^2, Pen^6, Arg^8]oxytocin, PA, in which (S)Pmp = β,β-(3-thiapentamethylene-β-mercaptopropionic acid). PA is a potent antagonist of the uterotonic effect of oxytocin in the rat (uterotonic test *in vitro*, $pA_2 = 8.86$) and in the baboon. Truncated analogues of PA from the *C*-terminus were systematically prepared ending in either the free acid or the amide, i.e. PA$^{1-9}$ acid, PA$^{1-8}$ acid, PA$^{1-7}$ acid, PA$^{1-6}$ acid, PA$^{1-8}$ amide, PA$^{1-7}$ amide and PA$^{1-6}$ amide. PA$^{1-8}$ amide was roughly as potent as PA in the rat uterotonic assay *in vitro*, and the shorter amides were only somewhat weaker antagonists. All four acid analogues were weaker antagonists than PA but still maintained rather high antagonistic potency. These findings suggest that, if these truncated acids form as metabolites *in vivo*, they may contribute to the overall biological effect of PA and their contribution should be taken into account. Furthermore, using these analogues, the radioimmunoassay measurements of PA may be standardized, as they may cross react with PA antibodies and interfere with the determination. In addition, five analogues were made by substituting Arg^8 of PA with Lys^8, Orn^8, Dab^8, Dap^8 and Cit^8. All of these analogues maintained high potency as OTAs in the uterotonic assay, although their activity was only about 1.5–3 times lower than PA. The most potent analogue in the uterotonic assay, [Dap^8]PA, $pA_2 = 8.53$, had weak pressor activity ($pA_2 = 6.90$) and no antidiuretic effect. The pressor activity was lower for all tested acids, and for PA$^{1-6}$ acid it was even below the detection limit. Additionally, PA$^{1-9}$ acid, PA$^{1-7}$ acid and PA$^{1-6}$ acid showed no antidiuretic activity. Hence, the PA$^{1-6}$ acid is a potent OTA with $pA_2 = 8.27$ and no measurable effect in the pressor or antidiuretic tests and thus it is a pure oxytocin antagonist. This fact makes it an attractive candidate for further studies on inhibition of OT biological effects and on preterm labour.

Keywords: oxytocin; antagonists; truncated analogues; metabolites

Abbreviations: AcOH, acetic acid; *n*-BuOH, *n*-butanol; 2-Cl-Z, 2-chlorobenzyloxycarbonyl; Dab, L-1,4-diaminobutyric acid; Dap, L-1,3-diaminopropionic acid; DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DCM, dichloromethane; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MBHA, 4-methylbenzhydrylamine; Meb, 4-methylbenzyl; MeCN, acetonitrile; MeOH, methanol; ONp, 4-nitrophenyl ester; OR, optical rotation; OT, oxytocin; OTA/OTAs, oxytocin antagonist/antagonists; PA, [(S)Pmp^1, D-Trp^2, Pen^6, Arg^8]oxytocin; 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).

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INTRODUCTION

Premature birth is the major factor contributing to perinatal mortality and morbidity [1–3] and has generated continued interest in the design of inhibitors of preterm labour. The posterior pituitary hormone oxytocin (OT) may have an endocrine and paracrine role in human parturition, although it has an uncertain role in the initiation of labour [4–7]. Oxytocin antagonists (OTAs) of high potency and high specificity might be useful tools to define the possible role of OT in parturition as well as for the prevention of preterm birth. In this regard, the use in the USA of a β-adrenergic mimetic, such as Ritodrine, as a tocolytic agent is viewed as unsatisfactory [8], but more recently an OT antagonist, Atosiban, has been successfully used in Europe in high doses to inhibit the uterine contractions of premature labour [9–11].

Non-peptide mimetics of OT have been designed [12] but their development seems to have been abandoned probably due to side effects. Instead, studies have focused on designing specific OT analogues with antagonistic properties [13–19] with the expectation that they would be more specific for OT receptors than non-peptide mimetics. The early antagonists of the neurohypophysial hormones oxytocin and [Arg^8]vasopressin (AVP) introduced β-mercapto-propionic acid (Mpa) in position 1 in addition to other substitutions, which led to [Mpa^1, D-Tyr(Et)^2, Thr^4, Orn^8]OT, Atosiban [9]. Atosiban was sufficiently potent to inhibit preterm labour in high doses in clinical studies and is now in clinical use (under the name Tractocile) in Europe [20]. Other analogues featuring β,β-dialkyl-β-mercaptopropionic acid in position 1 were synthesized and shown to have higher antagonistic potencies [21]. Among these analogues, the substitution at position 1 with β,β-pentamethylene-β-mercaptopropionic acid (Pmp), led to the most potent OTAs [22] and Pmp is currently the preferred substituent at position 1 to obtain the highest antagonistic potency.

In our laboratories [Pmp^1, D-Trp^2, Phe^3, Ile^4, Arg^8]oxytocin (*in vitro* $pA_2 = 7.51$) [23] was synthesized, which antagonizes uterine contractions in the nonpregnant rat in response to exogenous OT, inhibits milk letdown in the lactating rat, and disrupts the progress of labour in the pregnant rat [24].

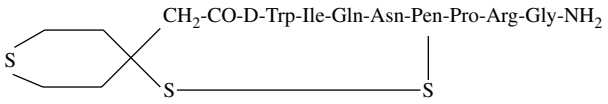


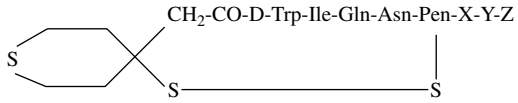
Figure 1 Structure of [β,β-(3-thiapentamethylene-β-mercaptopropionyl^1, D-Trp^2, Pen^6, Arg^8]oxytocin PA, or parent antagonist.

In addition, this OTA inhibits uterine contractions in response to OT in human myometrial tissue strips obtained by caesarean section at term [24]. Furthermore, this OTA was found to inhibit spontaneous contractions and labour in the baboon [25]. Later, [Pmp^1, D-Trp^2, Arg^8]OT was found to be more potent (*in vitro* $pA_2 = 7.77$), and more specific [23]. Subsequently, [(S)Pmp^1, D-Trp^2, Pen^6, Arg^8]OT (PA or parent antagonist, see Figure 1) was designed with *in vitro* $pA_2 = 8.86$, which is an attractive candidate for further studies as a preterm labour inhibitor [19]. These studies confirmed as well that numerous substitutions of the *C*-terminal tail of OTAs are well tolerated and lead to retention and often increase in OTA potency [26,27].

For our studies, it became desirable to prepare sequences of PA truncated at the *C*-terminus. These truncated analogues might be useful tools to facilitate HPLC identification and analysis of metabolic products, to enable preparation of radioactive ligands labelled at the *C*-terminus [28] for pharmacokinetic studies, as well as to help in assessing the specificity of a radioimmunoassay.

To this effect PA$^{1-9}$ acid and its truncated sequences PA$^{1-8}$ acid, PA$^{1-7}$ acid and PA$^{1-6}$ acid and amides of the latter three compounds were synthesized. Also prepared were analogues of PA in which Arg^8 was substituted with Lys or amino acids with shorter or modified side chains, including Orn, Dab, Dap or Cit, in order to determine the effect on biological activity of grading the degree of proximity of the side chain basic group to the peptide backbone.

Altogether, 12 new OTAs were prepared (Figure 2, Table 1) and tested as OTAs in the rat uterotonic assay *in vitro* [29] in the presence of 0.5 mM magnesium ions. Some of the most potent ones were also tested for vasopressin-like activities in the rat antidiuretic assay and/or the pressor assay. The physicochemical properties and amino acid analyses of the new OTAs (Tables 2 and 3) and their biological data (Table 4) are reported.



Analogue	X	Y	Z
PA	Pro	Arg	Gly-NH_2
1	Pro	Arg	Gly-OH
2	Pro	Arg-NH_2	-----
3	Pro	Arg-OH	-----
4	Pro-NH_2	-----	-----
5	Pro-OH	-----	-----
6	NH_2	-----	-----
7	OH	-----	-----
8	Pro	Lys	Gly-NH_2
9	Pro	Orn	Gly-NH_2
10	Pro	Dab	Gly-NH_2
11	Pro	Dap	Gly-NH_2
12	Pro	Cit	Gly-NH_2

Figure 2 Structure of PA analogues.

EXPERIMENTAL SECTION

Synthesis of Protected Peptides

The protected peptide precursors of the OTAs were assembled manually by the solid phase peptide synthesis (SPPS) method of Merrifield [30] employing the Boc-amino acid strategy [31] as previously modified [23]. Boc-Gly-resin (0.7–1.0 mmol of Boc-Gly/g) was prepared on a 200–400 mesh chloromethylated polystyrene resin (BioRad), 1% cross-linked with divinylbenzene, by esterification with the caesium salt of Boc-Gly [32]. Other starting resins, prepared by the same method but using the appropriate Boc-amino acid, included: Boc-Arg(Tos)-resin, Boc-Pro-resin and Boc-Pen(Meb)-resin (0.5–1.0 mmol of Boc-amino acid/g). The protected peptide precursor of PA^{1-6} amide was assembled on a 4-methylbenzhydryl amine (MBHA) resin [31]. For protection of side chain functionalities, Boc-Arg(Tos), Boc-Pen(Meb), Boc-Lys(2-Cl-Z), Boc-Orn(Z), Boc-Dab(Z) and Boc-Dap(Z) were used. Boc-amino acids were supplied by Bachem, except for Boc-Pen(Meb). DCHA, which was supplied by Peptides International and converted to Boc-Pen(Meb) prior to use. Each Boc-amino acid-resin was processed manually through the required coupling cycles. Completion of coupling was evaluated by the ninhydrin Kaiser test, which usually gave a negative response [33]. After unprotected Boc-D-Trp was introduced at position 2, Boc-groups were removed from the Boc-peptide-resin with 30% TFA in DCM containing 1% mercaptoethanol and 5% anisole [27]. (S)Pmp(Meb) was incorporated at position 1 of the peptide using a 3-fold excess in DMF solution by activation with DCC and HOBt. The protected peptides amides precursors of analogue amides **2**, **4**, and **8**–**12** were removed from their respective protective peptide benzyl esters on standard polystyrene resins by

Table 1 List of Analogues

No.	Structure	Abbreviation
	Cyclo[S-S-(S)Pmp^1, Pen^6] (S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro-Arg-Gly-NH_2, or [(S)Pmp^1, D-Trp^2, Pen^6, Arg^8] OT	PA
1	Cyclo[S-S-(S)Pmp^1, Pen^6](S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro-Arg-Gly	PA^{1-9} acid
2	Cyclo[S-S-(S)Pmp^1, Pen^6](S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro-Arg-NH_2	PA^{1-8} amide
3	Cyclo[S-S-(S)Pmp^1, Pen^6](S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro-Arg	PA^{1-8} acid
4	Cyclo[S-S-(S)Pmp^1, Pen^6](S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro-NH_2	PA^{1-7} amide
5	Cyclo[S-S-(S)Pmp^1, Pen^6](S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro	PA^{1-7} acid
6	Cyclo[S-S-(S)Pmp^1, Pen^6](S)Pmp-D-Trp-Ile-Gln-Asn-Pen-NH_2	PA^{1-6} amide
7	Cyclo[S-S-(S)Pmp^1, Pen^6](S)Pmp-D-Trp-Ile-Gln-Asn-Pen	PA^{1-6} acid
8	Cyclo[S-S-(S)Pmp^1, Pen^6](S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro-Lys-Gly-NH_2	[Lys^8]PA
9	Cyclo[S-S-(S)Pmp^1, Pen^6](S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro-Orn-Gly-NH_2	[Orn^8]PA
10	Cyclo[S-S-(S)Pmp^1, Pen^6](S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro-Dab-Gly-NH_2	[Dab^8]PA
11	Cyclo[S-S-(S)Pmp^1, Pen^6](S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro-Dap-Gly-NH_2	[Dap^8]PA
12	Cyclo[S-S-(S)Pmp^1, Pen^6](S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro-Cit-Gly-NH_2	[Cit^8]PA

ammonolysis for 3 days with MeOH saturated with ammonia [34]. The resin was filtered and extracted with hot DMF three times. The methanol filtrate and the DMF extracts were combined and evaporated to dryness, and the solid residue obtained was dissolved in DMF and the protected peptide amide was precipitated by addition to ether : petroleum ether (1 : 2 ratio), yielding 0.4–1.0 g of products. Because TLC analysis of these protected peptides usually showed one major component with minor impurities, they were used directly without purification for removal of blocking groups.

Removal of peptide blocking groups and cyclization. Removal of blocking groups of protected peptide amide precursors of analogue amides **2**, **4** and **8**–**12** was performed by treatment with sodium in liquid ammonia [35]. In the case of PA^{1-6} amide, **6**, assembled as a protected peptide bound to the amino group of MBHA resin, or for analogue acids **1**, **3**, **5** and **7**, assembled as benzyl esters on standard polystyrene resins, free peptides were obtained by treatment with liquid HF-anisole [36] using an all-Teflon apparatus (Protein Research Foundation, Osaka, Japan). In this way, the peptides were simultaneously removed from the resin and freed from blocking groups. The disulfhydryl peptides were oxidized in very dilute solution [37] to the cyclic disulfides by treatment with potassium ferricyanide [38].

Purification and analysis of analogues. The cyclic peptides thus obtained were freed from small by-products and salts by gel filtration on Sephadex G-15 [39], monitoring the eluate at 254 nm. This was followed by purification by preparative HPLC [19,27]. The solvent systems used both for analytical and/or preparative HPLC were: (A) 0.05% TFA; (B) MeCN containing 0.05% TFA or (C) 60% MeCN containing 0.05% TFA. For purification of analogues, preparative HPLC was conducted as previously described [19,27]. An isocratic concentration X for solvent B was determined by analytical HPLC, which would lead to an analytical elution time of 20–30 min. The preparative run was performed with a gradient between 0–X% solvent B for a period of time of 1 to several hours, suitably chosen to lead to the separation of the impurities from the desired peptide. Peptide purity was also assessed by analytical HPLC and by TLC (Table 2) on silica gel G pre-coated Uniplates (0.25 mm, Analtech). After applying 20–30 μg to each plate, the chromatogram was developed for about 10 cm. The solvent systems used (ratios

Table 2 Physicochemical Characteristics of Oxytocin Antagonists

Analogue		ESMS[a]	Yield[b] %	OR[c] deg.	TLC[d]				HPLC retention
No.	Name				R_fA	R_fB	R_fC	R_fD	time (min)[e]
	PA[f]			−39	0.24	0.42	0.11	0.58	5.8
1	PA^{1-9} acid	1173.8	20	−56	0.38	0.44	0.19	0.45	7.2
2	PA^{1-8} amide	1115.8	47	−21	0.33	0.46	0.18	0.69	6.2
3	PA^{1-8} acid	1116.6	35	−42	0.42	0.49	0.29	0.50	8.6
4	PA^{1-7} amide	959.1	12	26	0.65	0.59	0.57	0.77	12.2
5	PA^{1-7} acid	960.7	34	−6	0.74	0.69	0.70	0.69	19.2
6	PA^{1-6} amide	862.1	22	22	0.74	0.70	0.71	0.82	11.4
7	PA^{1-6} acid	863.3	41	10	0.76	0.71	0.73	0.68	19.2
8	[Lys^8]PA	1144.4	28	−56	0.21	0.37	0.16	0.50	5.0
9	[Orn^8]PA	1130.3	37	−61	0.22	0.39	0.17	0.50	5.2
10	[Dab^8]PA	1116.2	20	−51	0.24	0.40	0.23	0.53	5.2
11	[Dap^8]PA	1102.3	26	−45	0.28	0.43	0.25	0.57	5.6
12	[Cit^8]PA	1173.4	31	−30	0.38	0.47	0.27	0.61	5.6

[a] ESMS gave the calculated MW.
[b] These yields are based on the milliequivalents of starting Boc-amino acid-resin.
[c] OR = Optical rotation. OR was determined as [alpha]D^{27}, in degrees (c 1, 1N AcOH), except for compounds 4 and 6, which were determined in AcOH, and 12, in 5N AcOH.
[d] The composition of solvents A–D is given in the Experimental Part.
[e] The analysis was run isocratically, solvent composition = 38% solvent B, flow rate 1 ml/min, in order to determine the relative hydrophilicities by comparing retention times of analogues.
[f] PA = [(S)Pmp^1, D-Trp^2, Pen^6, Arg^8]OT.

Table 3 Amino Acid Analyses of Oxytocin Antagonists

Analogue No.	Name	Amino acid ratios								
		Asp	Glu	Gly	Arg	Pro	Pen[a]	Ile	Trp[b]	R
1	PA^{1-9} acid	0.99	0.98	1.01	1.00	0.98	0.27	0.95	0.80	—
2	PA^{1-8} amide	0.99	1.00	—	0.92	1.03	0.25	0.94	0.81	—
3	PA^{1-8} acid	0.92	0.92	—	1.00	1.04	0.16	1.00	0.89	—
4	PA^{1-7} amide	0.96	0.90	—	—	0.95	0.29	1.05	0.97	—
5	PA^{1-7} acid	0.94	1.00	—	—	0.99	0.30	0.96	0.83	—
6	PA^{1-6} amide	0.99	0.94	—	—	—	0.38	1.00	0.96	—
7	PA^{1-6} acid	0.99	1.00	—	—	—	0.23	0.96	0.92	—
8	[Lys^8]PA	1.00	0.98	1.02	—	0.90	0.30	1.03	0.78	0.93[c]
9	[Orn^8]PA	0.91	0.90	1.04	—	1.05	0.38	1.03	0.77	1.00[d]
10	[Dab^8]PA	1.00	1.00	0.93	—	1.01	0.20	0.95	0.82	0.99[e]
11	[Dap^8]PA	0.98	0.97	1.00	—	1.00	0.21	1.00	0.79	1.03[f]
12	[Cit^8]PA	0.97	0.96	1.03	—	1.00	0.34	1.04	0.93	1.01[g]

[a] Pen-SS-Pen. Values for Pen are low because a derivative of the type of (S)Pmp-SS-Pen can form which could not be detected.
[b] Tryptophan in peptides was estimated by its UV absorption at 280 nm as reported [37].
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; [g] Detected as Cit 0.83 and Orn 0.18.

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); (D) *n*-BuOH : AcOH : H_2O : Pyr (5 : 1 : 1 : 1). Free or protected peptides were detected with Ehrlich reagent, chlorine and KI-starch, or chlorine-tolidine [31]. The molecular weight of each peptide was determined by ESMS (Table 2), and peptide purity was also assessed by amino acid analysis (Table 3). For amino acid analysis, peptides were hydrolysed with 6 N HCl for 24 h at 110 °C and the resulting amino acid components were derivatized with phenylisothiocyanate and analysed by the Waters Associates Picotag method [40] as previously described [27]. All analogues gave the expected amino acid analysis ratios ± 10%. The Trp residue was estimated from the UV absorption of the peptide at 280 nm [41]. The lower values found for tryptophan suggest that a peptide may contain several moles of AcOH, TFA and/or H_2O, as has been our previous experience [41]. The optical rotations of peptides were measured with a Rudolph polarimeter (precision ± 0.01°).

((S)Pmp[1], D-Trp[2], Pen[6], Lys[8])OT, ([Lys[8]]PA, 8, (Table 1). Example of the synthesis and purification of an analogue. (S)Pmp(Meb)-D-Trp-Ile-Gln-Asn-Pen(Meb)-Pro-Lys(2-Cl-Z) Gly-NH_2, assembled by the SPPS method as described above (450 mg) was dissolved in anhydrous liquid NH_3 (200 ml) distilled from sodium and treated under anhydrous conditions with sodium until a pale-blue colour lasted for about 15–30 s. After evaporation of ammonia in a vacuum, the solid residue was dissolved in 20 ml of 50% AcOH. The clear solution was added to deaerated water (2 l), the pH was adjusted to 7.0 with concentrated ammonium hydroxide and the solution was titrated with 0.01 N potassium ferricyanide, until a permanent yellow colour resulted and then a 20% excess of potassium ferricyanide solution was added [38]. After 20 min, the ferrocyanide and ferricyanide salts were removed by stirring for 10 min with AG1 X-2 (Cl^-) ion exchange resin (15 g) and passing the suspension through a column containing additional ion exchange resin (15 g), followed by three washings using H_2O (50 ml). The filtrate combined with the washings was acidified with AcOH to pH 6 or lower and were lyophilized.

Analysis of the peptide obtained was accomplished on an analytical μBondapak C_{18} column (30 × 0.39 cm), monitoring at 220 nm, and eluting isocratically with 57% solvent B (solvent A, 0.05% TFA; solvent B 60% MeCN-40% of 0.05% TFA), at a rate of 1.5 ml/min. The peptides were freed from scavengers, and/or were desalted by gel filtration on Sephadex G-15, monitoring the eluate by UV spectrophotometry at 254 nm, followed by final purification by preparative HPLC. A linear

gradient was run from 0 to 50% B (preparative solvent B = acetonitrile containing 0.05% TFA) over 120 min, eluting at a rate of 3 ml/min, monitoring the eluent at 254 nm. The purer fractions determined by analytical HPLC, were pooled and lyophilized, yielding antagonist **8** (95 mg) in estimated purity of 95% or better.

This procedure, with only minor variations was used to prepare analogues **2**, **4** and **8**–**12**. Analogue **6**, PA^{1-6} amide, and analogue acids, **1**, **3**, **5** and **7** were removed from the peptide resin and simultaneously freed from blocking groups by treatment with liquid HF-anisole by methods previously described [27].

Biological Evaluation

All analogues were evaluated in a rat uterotonic *in vitro* assay using a modified Holton method [23] in Munsick solution [42] containing 0.5 mM Mg^{++}. Rats in induced oestrus by the injection of oestrogen 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 [43]. Synthetic oxytocin from Bachem or PolyPeptide Labs was used as standard. The most potent antagonists of the series were also tested for their potency in a rat antidiuretic assay performed on conscious rats in a modified Burn's arrangement [44,45] and in the rat pressor test performed on phenoxybenzamine treated male rats [46]. An *in vivo* pA_2 in the antidiuretic and pressor assays was calculated as the negative logarithm of the base 10 of the effective concentration which 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 [47].

RESULTS AND DISCUSSION

The physicochemical characteristics, amino acid analyses and biological activities of the 12 analogues are summarized in Tables 2–4. All the analogues were homogeneous according to HPLC and TLC, their molecular weight determined by ESMS corresponded to the calculated one, and the amino

Table 4 Potency of Competitive Antagonists of Oxytocin Contractile Action in the Rat Uterus *In Vitro* Assay

Analogue No.	Name	Biological activity		
		Antioxytocic $pA_2 \pm SEM$	Pressor $pA_2 \pm SEM$	Antidiuretic pA_2
	PA^{1-9} amide, or PA[a]	8.86 ± 0.09[b]		<5.75[b]
		8.68 ± 0.18[c]	7.47 ± 0.35	
1	PA^{1-9} acid	8.38 ± 0.12	7.00 ± 0.15	0[d]
2	PA^{1-8} amide	8.40 ± 0.11	n.d.[e]	n.d.
3	PA^{1-8} acid	8.53 ± 0.15	n.d.	n.d.
4	PA^{1-7} amide	7.91 ± 0.09	6.51 ± 0.31	n.d.
5	PA^{1-7} acid	8.33 ± 0.18	5.60 ± 0.20	0[d]
6	PA^{1-6} amide	8.49 ± 0.10	6.35 ± 0.15	n.d.
7	PA^{1-6} acid	8.27 ± 0.25	0[f]	0[d]
8	[Lys^8]PA	8.25 ± 0.21	n.d.	n.d.
9	[Orn^8]PA	8.29 ± 0.29	n.d.	n.d.
10	[Dab^8]PA	8.21 ± 0.36	n.d.	n.d.
11	[Dap^8]PA	8.53 ± 0.20	6.90 ± 0.34	0[d]
12	[Cit^8]PA	8.18 ± 0.30	6.95 ± 0.20	n.d.

[a] This analogue [(S)Pmp^1, D-Trp^2, Pen^6, Arg^8]OT, PA, was previously reported [19].
[b] Values obtained from reference [19].
[c] Re-tested for the purpose of this study.
[d] No antidiuretic or diuretic activity is detected up to a dose of 2 mg/kg of the experimental animal, *s.c.*
[e] n.d. means not determined.
[f] No pressor or antipressor activity is detected up to a dose of 0.2 mg/kg of the experimental animal, *i.v.*

acid analyses also agreed with the theoretical composition.

All the new analogues were potent OTAs (Table 4). The PA^{1-8}, **2**, was roughly as potent as PA in the uterotonic assay *in vitro* and the shorter amides, **4** and **6**, were only somewhat weaker. All four acid analogues, **1**, **3**, **5** and **7**, were weaker antagonists than PA but still maintained rather high antagonistic potency. The findings suggest that if PA is administered to an animal and these truncated acids form *in vivo*, they might contribute to the biological action of PA. They could also cross react with the antibodies used for a radioimmunochemical determination of PA and thus interfere with the measurement.

The antipressor activity was low for all tested acids, **1**, **5** and **7**. For PA^{1-6} acid, **7**, it was well below the detection limit. Additionally, PA^{1-9} acid,**1**, PA^{1-7} acid, **5**, and PA^{1-6} acid, **7**, showed no antidiuretic activity. Hence, the PA^{1-6} acid, **7**, with $pA_2 = 8.27$ in the uterotonic test *in vitro*, and no measurable effect in the pressor or antidiuretic tests, is a pure oxytocin antagonist. This remarkable result may be due to the lack of arginine, which means removal of an important similarity to the vasopressin analogues. These findings raise our expectation of designing a very potent, pure antagonist of oxytocin devoid of effects on renal or vascular tissues. In addition, these results make PA^{1-6} acid, **7**, as a small entity, an attractive candidate for further studies on inhibition of OT biological effects and for treatment and inhibition of preterm labour.

Among the analogues with shortened basic amino acid side chain in position 8, analogues **8**–**11**, [Dap^8]PA, **11**, appeared to be the most interesting. This analogue maintained substantially high uterotonic activity ($pA_2 = 8.53$) with no antidiuretic activity and low antipressor activity ($pA_2 = 6.90$). Neutralizing the basic group of [Orn^8]PA, analogue **9**, with an N-carbamoyl group somewhat lowered the uterotonic OTA potency of analogue **12** compared with analogue **9**, and did not bring about great improvement in specificity in the pressor assay.

There are described in the literature several syntheses of the ring of the oxytocin molecule lacking the *C*-terminal Pro-Leu-Gly or Pro-Leu-Gly-NH_2 tail portions, i.e. the so-called tocinamide or tocinoic acid, respectively, substituted with antagonism-inducing moieties, e.g. β,β-diethyl-β-mercaptopropionic acid (Dep), β,β-cyclopentamethylene-β-mercaptopropionic acid (Cpp), β,β-dimethyl-β-mercaptopropionic acid (Dmp), or desaminopenicillamine (dPen) at position 1 [48,49]. These analogues show usually antagonistic qualities in the uterotonic test *in vitro* in the absence of magnesium ions 4 to 6 times lower ($pA_2 = 6.37$–6.68) than non-truncated analogues.

Another analogue [$dPen^1$, Pen^6]tocinoic acid [49], inhibits the uterine activity of OT *in vitro*, in the absence of magnesium ions, with $pA_2 = 7.8$. The presence of magnesium ions in the bathing medium strongly influences the activity of oxytocin analogues [50]. The OTAs usually (not always) display higher antagonistic potency in the absence of magnesium ions in comparison with the potency in the presence of 0.5–1 mM concentration of magnesium ions [48, 14]. The presence of magnesium ions in the bathing medium, however, better simulates physiological conditions. The above mentioned analogue as well as our analogue **6** had also no pressor or antipressor activity, however, it was a very weak agonist in the antidiuretic assay (0.010 ± 0.001 U/mg).

CONCLUSIONS

Our findings lead to the conclusion that the truncated sequences, **1**, **3**, **5** and **7**, with free carboxylic acid at the *C*-terminus may contribute to the observed high biological potency of the parent antagonist. Their interference with the radioimmunoassay procedure is also highly probable. These peptides may be used for the standardization of the raioimmunoassay. In addition, the high potency of these truncated sequences further confirm that it is the ring portion of the antagonist that is primarily responsible for the inhibition of the binding of oxytocin to its uterine receptor.

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REFERENCES

1. Olah KS, Gee H. The prevention of preterm delivery — can we afford to continue to ignore the cervix? *Br. J. Obstet. Gynaecol.* 1992; **99**: 278–280.

2. Creasy RK. Preterm birth prevention: Where we are? *Am. J. Obstet. Gynecol.* 1993; **168**: 1223–1230.
3. Radetsky P. Stopping premature births before it is too late. *Science* 1995; **266**: 1486–1488.
4. Chibbar R, Miller FD, Mitchell BF. Synthesis of oxytocin in amnion, chorion, and decidua may influence the timing of human parturition. *J. Clin. Invest.* 1993; **91**: 185–192.
5. Norwitz ER, Robinson JM, Challis JRG. The control of labor. *N. Engl. J. Med.* 1999; **341**: 660–666.
6. Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term and preterm. *Endocrine Rev.* 2000; **21**: 514–550.
7. Weiss G. Endocrinology of parturition. *J. Clin. Endocrinol. Metabol.* 2000; **85**: 4421–4425.
8. Leveno KJ, Cunningham FG. β-Adrenergic agonists for preterm labor. *N. Engl. J. Med.* 1992; **327**: 349–351.
9. Åkerlund M, Stromberg P, Hauksson A, Andersen LF, Lyndrup J, Trojnar J, Melin P. Inhibition of uterine contractions of premature labour with an oxytocin analogue. *Br. J. Obstet. Gynaecol.* 1987; **94**: 1040–1044.
10. Melin P, Trojnar J, Johanson B, Vilhardt H, Åkerlund M. Synthetic antagonists of the myometrial response to vasopressin and oxytocin. *J. Endocrinol.* 1986; **111**: 125–131.
11. Romero R, Sibai BM, Sanchez-Ramos L, Valenzuela GJ, Veille J-C, Tabor B, Perry KG, Varner M, Goddwin TM, Lane R, Smith J, Shangold G, Creasy GW. An oxytocin receptor antagonist (Atosiban) in the treatment of preterm labor: A randomized, double-blind, placebo-controlled trial with tocolytic rescue. *Am. J. Obstet. Gynecol.* 2000; **182**: 1173–1183.
12. Freidinger RM, Bock MG, Evans BE, Pettibone DJ, Williams PD. Design of novel, nonpeptide oxytocin receptor antagonists. In *Peptide Science — Present and Future*, Shimonishi Y (ed.). Kluwer Academic: Dordrecht, 1999; 618–622.
13. Hruby VJ, Smith CW. Structure-activity relationships of neurohypophyseal peptides. In *The Peptides*, Udenfriend S, Meienhofer J. (eds), vol. 8, *Chemistry, Biology and Medicine of Neurohypophyseal Hormones and their Analogs*, Smith CW (ed.). Academic Press: Orlando, FL, 1987; 77–207.
14. Manning M, Cheng LL, Klis WA, Stoev SA, Przybylski J, Bankowski K, Sawyer WH, Barberis C, Chan WY. Advances in the design of selective antagonists, potential tocolytics and radioiodinated ligands for oxytocin receptors. In *Oxytocin: Cellular and Molecular Approaches in Medicine and Research*, Ivell R, Russell J (eds). Plenum Press: New York, 1995; 559–583.
15. Goodwin TM, Zograbyan A. Oxytocin receptor antagonists update. *Emerg. Concepts Perinat. Endocrinol.* 1998; **25**: 859–871.
16. Manning M, Stoev S, Cheng LL, Wo NC, Chan WY. Design of oxytocin antagonists, which are more selective than Atosiban. *J. Peptide Sci.* 2001; **7**: 449–465.
17. Flouret G, Chaloin O, Slaninová J. Analogues of a potent oxytocin antagonist with truncated *C*-terminus or shorter amino acid side chain of the basic amino acid at position 8. In *Peptides 2002*, Benedetti E (ed.). ESCOM: Leiden; 482–483.
18. Flouret G, Chaloin O, Slaninová J. Novel bicyclic analogues of a potent oxytocin antagonist. In *Peptides 2002*, Benedetti E (ed.). ESCOM: Leiden; 484–485.
19. Flouret G, Majewski T, Balaspiri L, Brieher W, Mahan K, Chaloin O, Wilson L Jr, Slaninová J. Antagonists of oxytocin featuring replacement with modified β-mercapto-propionic acids at position 1. *J. Peptide Sci.* 2002; **8**: 314–326.
20. New drug for delaying premature birth marketed. *Pharmac. J.* 2000; **264**: 871.
21. Vavrek RJ, Ferger MF, Allen GA, Rich DH, Blomquist AT, du Vigneaud V. Synthesis of three oxytocin analogs related to [1-deaminopenicillamine]oxytocin possessing antiooxytocic activity. *J. Med. Chem.* 1972; **15**: 123–126.
22. Nestor JJ Jr, Ferger MF, du Vigneaud V. [1-β-Mercapto-β,β-pentamethylene-propionic acid]oxytocin, a potent inhibitor of oxytocin. *J. Med. Chem.* 1975; **18**: 284–287.
23. Flouret G, Brieher W, Mahan K, Wilson L Jr. Design of potent oxytocin antagonists featuring D-tryptophan at position 2. *J. Med. Chem.* 1991; **34**: 642–646.
24. Wilson L Jr, Parsons MT, Ouano L, Flouret G. A new tocolytic agent: development of an oxytocin antagonist for inhibiting uterine contractions. *Am. J. Obstet. Gynecol.* 1990; **163**: 195–202.
25. Wilson L Jr, Parsons MT, Flouret G. Inhibition of spontaneous uterine contractions during the last trimester in pregnant baboons by an oxytocin antagonist. *Am. J. Obstet. Gynecol.* 1990; **163**: 1875–1882.
26. Flouret G, Brieher W, Majewski T, Mahan K, Wilson L Jr. Improvement in potency of an oxytocin antagonist after systematic substitutions with L-tryptophan. *J. Med. Chem.* 1991; **34**: 2089–2094.
27. Flouret G, Brieher W, Majewski T, Wilson L Jr. Some pharmacological properties of cyclic and linear analogs obtained by substituting each residue of an oxytocin antagonist with D-tryptophan. *Int. J. Peptide Protein Res.* 1991; **38**: 169–175.
28. Flouret G, Chaloin O. Tritium labeling of an oxytocin antagonist. In *Peptides 2000*, Martinez J, Fehrentz J-A (eds). EDK: Paris 2001; 979–980.
29. Holton P. A modification of the method of Dale and Laidlaw for standardization of posterior pituitary extract. *Br. J. Pharmacol.* 1948; **3**: 328–334.
30. Merrifield RB. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 1963; **85**: 2149–2154.
31. Stewart JM, Young JD. In *Solid Phase Peptide Synthesis*, 2nd edn. Pierce Chemical Co. Rockford, IL, 1984; 1–176.
32. Gisin BF. Preparation of Merrifield resins through total esterification with cesium salts. *Helv. Chim. Acta* 1973; **56**: 1476–1482.

33. Kaiser E, Colescot RL, Bossinger CD, Cook PI. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* 1970; **34**: 595–598.
34. Manning M. Synthesis by the Merrifield method of a protected nonapeptide amide with the amino acid sequence of oxytocin. *J. Am. Chem. Soc.* 1968; **90**: 1348–1349.
35. du Vigneaud V, Ressler C, Swan JM, Roberts CW, Katsoyannis PG, Gordon S. The synthesis of an octapeptide amide with the hormonal activity of oxytocin. *J. Am. Chem. Soc.* 1953; **75**: 4879–4880.
36. Sakakibara S, Shimonishi Y. A new method for releasing oxytocin from fully protected nonapeptides using anhydrous hydrogen fluoride. *Bull. Chem. Soc. Jpn* 1965; **38**: 1412–1413.
37. Manning M, Lammek B, Kolodziejczyk AM. Synthetic antagonists of *in vivo* antidiuretic and vasopressor responses to arginine-vasopressin. *J. Med. Chem.* 1981; **24**: 701–706.
38. Hope DB, Murti VVS, du Vigneaud V. A highly potent analogue of oxytocin, desamino-oxytocin. *J. Biol. Chem.* 1962; **237**: 1563–1566.
39. Manning M, Wuu TC, Baxter JWM. The purification of synthetic oxytocin and analogues by gel filtration on Sephadex G-15. *J. Chromatogr.* 1968; **38**: 396–398.
40. Bidlingmeier BA, Cohen SA, Tarvin TL. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.* 1984; **336**: 93–104.
41. White WF, Hedlund MT, Rippel RH, Arnold W, Flouret G. Chemical and biological properties of gonadotropin-releasing hormone synthesized by the solid-phase method. *Endocrinology* 1973; **93**: 96–106.
42. Munsick RA. Effect of magnesium ion on the response of the rat uterus to neurohypophysial hormones and analogues. *Endocrinology* 1960; **66**: 451–457.
43. Schild HO. pA, a new scale for the measurement of drug antagonism. *Br. J. Pharmacol.* 1947; **2**: 189–206.
44. Vávra I, Machová A, Krejí I. Antidiuretic action of 1-deamino-8-D-arginine vasopressin in unanesthetized rats. *J. Pharmacol. Exp. Ther.* 1974; **188**: 241–247.
45. Hrbas P, Barth T, Skopkova J, Lebl M, Jost K. Effect of some oxytocin analogues on natriuresis in rats. *Endocrinol. Exp.* 1980; **14**: 151–157.
46. Debanski J. The quantitative assay of vasopressin. *Br. J. Pharmacol.* 1952; **7**: 567–572.
47. Slaninová J. Fundamental biological evaluation. In *Handbook of Neurohypophyseal Hormone Analogues*, Jost K, Lebl M, Brtnik F (eds). CRC Press: Boca Raton, FL, 1987; Vol. 1, Part 2: 83–107.
48. Lebl M, Jost K, Brtnik F. Tables of analogs. In *Handbook of Neurohypophyseal Hormone Analogues*, Jost K, Lebl M, Brtnik F. (eds). CRC Press: Boca Raton, FL, 1987; Vol 2, Part 2: 127–267.
49. Roy U, Gazis D, Glass JD, Schwartz IL. Desaminopenicillamine tocinoic acid derivative — inhibitors of oxytocin. *Int. J. Peptide Protein Res.* 1986; **28**: 154–162.
50. Slaninová J, Maletinska L, Vondrasek J, Procházka J. Magnesium and biological activity of oxytocin analogues modified on aromatic ring of amino acid in position 2. *J. Peptide Sci.* 2001; **7**: 413–424.

