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Analogues of oxytocin antagonists bearing a ureido group in the amino acid side chain at position 4 or 5‡

GEORGE FLOURET,[a]* OLIVIER CHALOIN,[a] LENKA BOROVICKOVA[b] and JIRINA SLANINOVÁ[b]

[a] Department of Physiology, Northwestern University Medical School, Chicago, Illinois 60611, USA
[b] Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague 6, Czech Republic

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Abstract: Substitution of the side chain carboxamido group at position 4 in the potent oxytocin antagonist (OTA) [ThiaPmp^1, D-Trp^2, Cys^6, Arg^8]-OT, PA, in which ThiaPmp = β,β-(3-thiapentamethylene)-β-mercaptopropionic acid, led to [Orn(Car)^4]-PA, ([Cit^4]-PA), which had uterotonic antagonistic activity equal to that of PA. The same modification at position 5, leading to [Cit^5]-PA, resulted in antagonistic potency more than 10 times lower than that of PA. This paper also describes the same substitutions introduced in the highly potent OTA [Pen^6]-PA (antioxytocic *in vitro* $pA_2 = 8.72$). Analogues of the general formula [U^4-X^5-Pen^6]-PA, in which U = Lys, Orn, Dab, Dap or X = Orn, Dab or Dap, were synthesized by SPPS. Each of these analogues was carbamoylated by treatment with KCNO in DMF-H_2O, yielding the corresponding U(Car)^4 or X(Car)^5 derivatives. In the uterotonic assay, the substitution with the ureido group at Gln^4 results in retention of high antagonistic potency, albeit somewhat lower than that of PA, e.g. [Orn(Car)^4, Pen^6]-PA and [Dab(Car)^4, Pen^6]-PA having $pA_2 = 8.52$ and $pA_2 = 8.42$ respectively. In the pressor assay, [Lys(Car)^4, Pen^6]-PA and [Dab(Car)^4, Pen^6]-PA were somewhat weaker antagonists of arginine vasopressin than [Pen^6]-PA; [Dap(Car)^4, Pen^6]-PA showed only a faint trace of pressor agonistic activity. The substitution with the ureido group at position 5 leads to a significant loss of OTA potency in the *in vitro* uterotonic assay. The [Orn(Car)^5, Pen^6]-PA was the most potent of the series ($pA_2 = 8.05$). An interesting finding is that [Dap(Car)^5, Pen^6]-PA is equipotent with its precursor [Dap^5, Pen^6]-PA (potency in the uterotonic test *in vitro*, $pA_2 = 7.71$ and $pA_2 = 7.68$, respectively). Furthermore, neither [Dap^5, Pen^6]-PA nor [Dap^5, Pen^6, Gly^9]-PA exhibited activity in the antidiuretic or pressor assays. Although these last two analogues show some decrease in antioxytocin potency, they behave as pure oxytocin antagonists, which makes them attractive candidates for further studies on the development of potent and specific OTAs. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: oxytocin; antagonists; ureido group; premature labor

INTRODUCTION

The neurohypophyseal hormones oxytocin (OT) and arginine vasopressin (AVP) have important biological effects in humans in both the central nervous system and peripheral organ systems. Different receptors have been identified in various organs and have been labeled as V_{1a} (vascular), V_{1b} or V_3 (pituitary), V_2 (renal), and OT (uterine) receptors [2–4]. There is continuing interest in the design of highly potent and specific oxytocin antagonists (OTAs) and AVP antagonists at least in part to aid in the definitive characterization of new OT or AVP tissue receptors. Additionally, suitable OTAs would be useful for evaluating the importance of OT in normal and preterm labor. Oxytocin seems to be involved in human parturition, although it has an uncertain role in the initiation of labor [5–8]. Because premature birth is the major factor contributing to perinatal mortality and morbidity [9–11], there has been continued effort in the design of inhibitors of preterm labor. The use of β-adrenergic agents, e.g. ritodrine, is regarded as unsatisfactory [12]. Recently, the OTA Atosiban, [Mpa^1, D-Tyr(Et)^2, Thr^4, Orn^8]OT, has been used, albeit in high doses, to inhibit the uterine contractions of preterm labor [13–15] and has been introduced for clinical use in Europe [16]. Considerable effort has been made in designing specific OT analogues [17–22] with the expectation that they would be more selective for OT receptors and would have less side effects than nonpeptide mimetics [23]. In our laboratories, we prepared [Pmp^1, D-Trp^2, Phe^3, Ile^4, Arg^8]OT [24], which is an OTA in the uterus *in vitro*, $pA_2 = 7.51$, inhibits uterine contractions of an estrus rat in response to exogenous OT and milk let down in the lactating rat

Abbreviations: Abbreviations: AcOH, acetic acid; *n*-BuOH, *n*-butanol; Car, carbamoyl; 2-Cl-Z, 2-Chlorobenzyloxycarbonyl; Dab, L-1,4-diaminobutyric acid; Dap, L-1,3-diaminopropionic acid; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DMF, dimethylformamide; ESI-MS, electrospray ionization-mass spectrometry; EtOAc, ethyl acetate; 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, [ThiaPmp^1, D-Trp^2, Cys^6, Arg^8]OT; PITC, phenylisothiocyanate; Pmp, β,β-(pentamethylene)-β-mercaptopropionic acid; PTC, phenylthiocarbamyl; Pyr, pyridine; ThiaPmp, β,β-(3-thiapentamethylene)-β-mercaptopropionic acid; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; TLC, thin layer chromatography; Tos, *p*-toluenesulfonyl; Z, benzyloxycarbonyl.

*Correspondence to: G. Flouret, Northwestern University Medical School, Department of Physiology, 303 East Chicago Avenue, Chicago, IL 60611, USA; e-mail: g-flouret@northwestern.edu

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and delays labor contractions in rats and baboons. Additionally, this antagonist inhibits *in vitro* uterine contractions in response to OT in uterine strips obtained from women at cesarean section [25] and inhibits spontaneous uterine contractions and labor in baboons [26]. Subsequently, [Pmp^1, D-Trp^2, Arg^8]OT was found to be more potent (uterine *in vitro* test, $pA_2 = 7.77$) and more specific [24,27,28]. We designed progressively more potent OTAs such as [ThiaPmp^1, D-Trp^2, Cys^6, Arg^8]OT, PA, (parent antagonist, Figure 1), with $pA_2 = 8.11$, and its [Pen^6] analogue, [Pen6]-PA, with $pA_2 = 8.72$ [21], which is very promising as a preterm labor inhibitor.

In preliminary studies to determine the suitability of substituting the carboxamide groups of Gln^4 or Ans^5 with the isosteric ureido group, we prepared [Orn(Car)^4]-PA, in which Car is carbamoyl, and [Orn(Car)^5]-PA (Table 1, Figure 2). Encouraged by the results of the bioassays, we extended the studies to the more highly potent [Pen^6]-PA, which also inhibits labor in rats and baboons.

Recognizing that the length of the side chain bearing the ureido group would be critical, we synthesized by SPPS analogues of the general formula [U^4-X^5-Pen^6]-PA in which U = Lys, Orn, Dab or Dap or X = Orn, Dab or Dap. Each of these analogues was carbamoylated by treatment with KCNO in DMF-H_2O, yielding the corresponding U(Car)^4 or X(Car)^5 derivatives (Table 1).

The analogues were tested as OTAs of the OT uterotonic action in the rat bioassay *in vitro* [29] in the presence of magnesium ions, and some of the most potent of them were also tested in the rat antidiuretic assay and/or the pressor assay. We also tested [Dap^5, Pen^6]-PA, which is the smallest of the basic amino acids and closer in length to Asn. We also tested [Dap^5, Pen^6, Gly^9]-PA, since our previous studies [22] suggested that OTA's action is maintained in peptide acids or amides. We report here the synthesis, physicochemical properties and biological data of 11 new OTAs (Tables 2, 3, and 4).

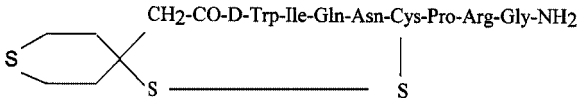
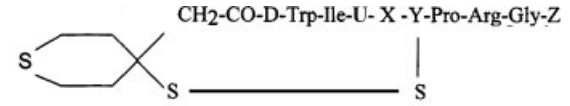


Figure 1 Structure of [β,β-(3-thiapentamethylene-β-mercaptopropionyl^1, D-Trp^2, Cys^6, Arg^8]oxytocin PA, or parent antagonist.



Analogue	U	X	Y	Z
PA	Gln	Asn	Cys	Gly-NH_2
1	Orn(Car)	Asn	Cys	Gly- NH_2
2	Gln	Orn(Car)	Cys	Gly- NH_2
3	Lys(Car)	Asn	Pen	Gly- NH_2
4	Orn(Car)	Asn	Pen	Gly- NH_2
5	Dab(Car)	Asn	Pen	Gly- NH_2
6	Dap(Car)	Asn	Pen	Gly- NH_2
7	Gln	Orn(Car)	Pen	Gly- NH_2
8	Gln	Dab(Car)	Pen	Gly-NH_2
9	Gln	Dap(Car)	Pen	Gly-NH_2
10	Gln	Dap	Pen	Gly-NH_2
11	Gln	Dap	Pen	Gly

Figure 2 Structure of PA analogues.

Table 1 List of Analogues

No.	Structure	Abbreviation
	[ThiaPmp^1, D-Trp^2, Arg^8] OT	PA
	[ThiaPmp^1, D-Trp^2, Pen^6, Arg^8] OT	[Pen^6]-PA
1	[ThiaPmp^1, D-Trp^2, Orn(Car)^4, Pen^6, Arg^8] OT	[Orn(Car)^4]-PA
2	[ThiaPmp^1, D-Trp^2, Orn(Car)^5, Pen^6, Arg^8] OT	[Orn(Car)^5]-PA
3	[ThiaPmp^1, D-Trp^2, Lys(Car)^4, Pen^6, Arg^8] OT	[Lys(Car)^4, Pen^6]-PA
4	[ThiaPmp^1, D-Trp^2, Orn(Car)^4, Pen^6, Arg^8] OT	[Orn(Car)^4, Pen^6]-PA
5	[ThiaPmp^1, D-Trp^2, Dab(Car)^4, Pen^6, Arg^8] OT	[Dab(Car)^4, Pen^6]-PA
6	[ThiaPmp^1, D-Trp^2, Dap(Car)^4, Pen^6, Arg^8] OT	[Dap(Car)^4, Pen^6]-PA
7	[ThiaPmp^1, D-Trp^2, Orn(Car)^5, Pen^6, Arg^8] OT	[Orn(Car)^5, Pen^6]-PA
8	[ThiaPmp^1, D-Trp^2, Dab(Car)^5, Pen^6, Arg^8] OT	[Dab(Car)^5, Pen^6]-PA
9	[ThiaPmp^1, D-Trp^2, Dap(Car)^5, Pen^6, Arg^8] OT	[Dap(Car)^5, Pen^6]-PA
10	[ThiaPmp^1, D-Trp^2, Dap^5, Pen^6, Arg^8] OT	[Dap^5, Pen^6]-PA
11	[ThiaPmp^1, D-Trp^2, Dap^5, Pen^6, Arg^8, Gly^9] OT	[Dap^5, Pen^6, Gly^9]-PA

Table 2 Physicochemical Characteristics of Oxytocin Antagonists

Analogue number	Name	MW Calculated	ESI-MS	Yield[a] (%)	TLC[c]					HPLC retention time (min)[d]
					Or[b] (deg.)	R_fA	R_fB	R_fC	R_fD	
1	[Orn(Car)^4]-PA	1173.4	1173.6	20	−71	0.32	0.41	0.20	0.43	5.4
2	[Orn(Car)^5]-PA	1187.5	1187.5	29	−52	0.33	0.43	0.21	0.47	6.0
3	[Lys(Car)^4, Pen^6]-PA	1215.5	1216.0	27	−27	0.34	0.42	0.20	0.46	8.4
4	[Orn(Car)^4, Pen^6]-PA	1201.5	1201.5	23	−31	0.34	0.43	0.20	0.47	8.8
5	[Dab(Car)^4, Pen^6]-PA	1187.5	1188.0	32	−38	0.35	0.45	0.20	0.51	8.0
6	[Dap(Car)^4, Pen^6]-PA	1173.4	1174.0	31	−40	0.36	0.47	0.24	0.51	7.6
7	[Orn(Car)^5, Pen^6]-PA	1215.5	1216.0	16	−3	0.34	0.41	0.21	0.52	8.8
8	[Dab(Car)^5, Pen^6]-PA	1201.5	1202.0	27	−6	0.35	0.41	0.21	0.54	8.8
9	[Dap(Car)^5, Pen^6]-PA	1187.5	1188.0	45	−38	0.36	0.46	0.22	0.54	8.4
10	[Dap^5, Pen^6]-PA	1144.0	1144.4	60	−55	0.28	0.39	0.17	0.51	7.6
11	[Dap^5, Pen^6, Gly^9]-PA	1145.0	1145.5	28	−71	0.31	0.41	0.20	0.49	12.0

[a] These yields are based on the milliequivalents of the starting Boc-amino acid resin.
[b] OR = optical rotation. OR was determined as $[alpha]_D^{27}$ in degrees (c 1, 1 N AcOH).
[c] The composition of solvents A–D is given in the 'Experimental Section'.
[d] 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. A Waters C18 column (30 × 0.75 cm) was used.

EXPERIMENTAL SECTION

Synthesis of Protected Peptides

The protected peptide precursors of the OTAs were made manually by the SPPS method of Merrifield [30] employing the Boc-amino acid strategy [31] with minor modifications [24,28]. The starting Boc-Gly-Resin (0.5–0.7 mmol, 0.7–1.0 mmol of Boc-Gly/g) was prepared on a 200–400 mesh chloro-methylated resin (BioRad), 1% cross-linked with divinylbenzene, by esterification with the cesium salt of Boc-Gly [32]. The following side chain–protected amino acids were used: Boc-Arg(Tos), Boc-Cys(Meb), Boc-Pen(Meb), Boc-Lys(2-Cl-Z), Boc-Orn(Z), Boc-Dab(Z), and Boc-Dap(Z). Boc-amino acids were purchased from Bachem, except for Boc-Pen(Meb). DCHA, which was obtained from Peptides International, was converted to Boc-Pen(Meb) prior to use. The Boc-Gly-Resin (0.5–0.7 mmol) was taken manually through the required Boc-amino acid–coupling cycles. Progress of coupling was monitored by the Kaiser test [33]. After Boc-D-Trp was introduced at position 2, Boc groups were removed with 30% TFA in DCM containing 1% mercaptoethanol and 5% anisole [28]. ThiaPmp(Meb), was then coupled in threefold excess in DMF solution by activation with DCC and HOBt. The protected peptide amides were removed from the resin by ammonolysis for 3 days with MeOH saturated with ammonia [34]. The resin was filtered and extracted with hot DMF three times. The methanolic filtrate and the DMF extracts were combined and evaporated to dryness, the solid residue obtained was dissolved in DMF, and the protected peptide amide was precipitated by addition to EtOAc-hexane, yielding 400–600 mg of products. Since TLC analysis of protected peptides usually showed one major component with minor impurities, they were used without further purification for removal of blocking groups.

Analogue Synthesis

The blocking groups on side chain functionalities of protected peptide amides were removed by reduction with sodium in liquid ammonia, yielding the free disulfhydryl peptides [35]. In the case of the protected peptide-resin precursor of [Dap^5, Pen^6, Gly^9]-PA, **11**, treatment with liquid HF-anisole [36] using an all-Teflon apparatus (Protein Research Foundation, Osaka, Japan), yielded directly the free analogue precursor as the disulfhydryl [Dap^5, Pen^6, Gly^9]-PA, free of blocking groups. All disulfhydryl peptides were oxidized in very dilute solution [37] to the corresponding cyclic disulfide by treatment with a potassium ferricyanide solution [38]. The free peptides thus obtained were desalted, freed from small by-products, and purified at the same time by preparative HPLC [20]. The solvent systems used both for analytical and/or preparative HPLC were (A) 0.05% TFA and (B) MeCN containing 0.05% TFA. Peptide purity was assessed by HPLC and by TLC (Table 2) on silica gel G precoated Uniplates (0.25 mm, Analtech). After applying a solution of peptide containing 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); (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 [31]. The molecular weight of each peptide was determined by ESI-MS (Table 2) and peptide composition was also determined by amino acid analysis (Table 3). For amino acid analysis, peptides were hydrolyzed with 6 N HCl for 24 h at 110 °C and the amino acid components obtained were derivatized with phenylisothiocyanate and analyzed by the Waters Associates' Picotag method [39] as previously described [24]. The Trp residue was estimated from the UV absorption of the peptide at 280 nm [40]. The optical rotations of peptides were measured with a Rudolph Polarimeter (precision ±0.01°).

Table 3 Amino Acid Analyses of Oxytocin Antagonists

Analogue number	Name	Amino acid ratios									
		Asp	Glu	Gly	Arg	Pro	Cys[a]	Pen[b]	Ile	Trp[c]	R
1	[Orn(Car)^4]-PA	0.90	—	0.98	0.95	0.92	0.93	—	0.99	0.80	1.03[d]
2	[Orn(Car)^5]-PA	—	0.92	1.01	1.05	0.90	0.91	—	0.99	0.78	1.09[d]
3	[Lys(Car)^4, Pen^6]-PA	0.95	—	1.07	1.10	0.95	—	0.49	0.97	0.85	0.95[e]
4	[Orn(Car)^4, Pen^6]-PA	0.96	—	1.00	0.92	0.91	—	0.29	0.96	0.81	1.03[d]
5	[Dab(Car)^4, Pen^6]-PA	0.90	—	1.04[f]	1.09	1.02	—	0.41	0.98	0.83	1.04[f]
6	[Dap(Car)^4, Pen^6]-PA	1.0	—	1.03	0.94	0.96	—	0.31	0.90	0.83	0.99[g]
7	[Orn(Car)^5, Pen^6]-PA	—	0.96	1.00	1.07	0.91	—	0.30	0.97	0.82	0.99[d]
8	[Dab(Car)^5, Pen^6]-PA	—	0.90	0.98	0.96	1.00	—	0.41	0.99	0.86	0.98[h]
9	[Dap(Car)^5, Pen^6]-PA	—	0.92	0.95	1.00	0.94	—	0.35	0.92	0.86	0.97[g]
10	[Dap^5, Pen6]-PA	—	0.93	1.02	0.95	0.94	—	0.35	0.90	0.82	0.92[i]
11	[Dap^5, Pen^6, Gly^9]-PA	—	0.94	1.00	0.94	1.05	—	0.34	0.93	0.81	0.91[i]

[a] This value combines those obtained for Cys and for ThiaPmp-SS-Cys.
[b] Pen-SS-Pen. Values for Pen are low because a derivative of the type of ThiaPmp-SS-Pen can form, which could not be detected.
[c] Tryptophan in peptides was estimated by its UV absorption at 280 nm as we have reported. [37] Values found for Trp suggest that the peptide preparation contains several moles of AcOH, TFA, and/or H_2O.
[d] Detected as the sum of Cit and Orn.
[e] Lys(Car) is partially hydrolyzed and the PITC derivative of unhydrolyzed Lys(Car) coelutes with the PITC derivative of Pro. The sum of the values of Lys(Car) plus Pro (1.70) is added to the value of Lys (0.20) and divided by 2.
[f] Dab is partially hydrolyzed and the PITC derivative of unhydrolyzed Dab(Car) elutes together with that of Gly. The sum of the values of Dab(Car) plus Gly (1.38) is added to the value of Dab (0.70) and the sum is divided by 2.
[g] Detected as the sum of Dap and Dap(Car).
[h] The individual value of Gly plus Dab(Car) (1.33) is added to the value of that of Dab (0.65) and the total is divided by 2.
[i] Dap

(Dap^5, Pen^6)-PA, 10 (Table 1)

ThiaPmp(Meb)-D-Trp-Ile-Gln-Dap(Z)-Pen(Meb)-Pro-Arg(Tos) Gly-NH_2, obtained by the SPPS method as described above (300 mg), was dissolved in liquid ammonia (150 ml) freshly distilled from sodium and treated with sodium until a pale-blue color lasted for about 15–30 sec. After evaporation of ammonia in a vacuum, the solid residue was dissolved in 20 ml of 50% AcOH and 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 color resulted and then a 10% excess of potassium ferricyanide solution was added [38]. After 15 min, the ferrocyanide and ferricyanide salts were removed by stirring for 15 min with AG1 X-2 (Cl^-) ion exchange resin (15 g) and then adding the suspension to a chromatographic column containing an additional 15 g of the same ion exchange resin. The column was washed with three portions of water (50 ml each).

The combined eluents were lyophilized. Analysis of the peptide obtained was accomplished on an analytical μBondapak C_{18} column (30 × 0.39 cm), monitored at 220 nm, and eluted isocratically with 45% solvent B in A (solvent A, 0.05% TFA; solvent B MeCN containing 0.05% TFA), at a flow rate of 1 ml/min. For purification by preparative HPLC, a linear gradient was run from 0 to 40% B in A over 240 min, at a flow rate of 3 ml/min, monitoring the eluent at 254 nm. The purer fractions determined by analytical HPLC were pooled and lyophilized, yielding antagonist [Dap^5, Pen^6]-PA (103 mg), in estimated purity of 95% or better. The analogue molecular weight was determined by ESI-MS, analogue homogeneity was determined by TLC in four separate solvent systems (Table 3) and by analytical HPLC, and the amino acid components were determined by amino acid analysis (Table 4).

This procedure, with only minor variations, was used to prepare OTAs **1–10** or their precursors.

(Dap(Car)^5, Pen^6)-PA, 9 (Table 1)

A solution of the preceding [Dap^5, Pen^6]-PA (90 mg) in DMF (0.525 ml) was treated with a solution of potassium cyanate (150 mg) in water (0.450 ml). After 24 h, the reaction mixture was treated with water (10 ml), filtered, and purified by preparative HPLC running a gradient 0–40% B for 240 min. Suitable portions of the main peak were pooled and lyophilized yielding [Dap(Carb)^5, Pen^6]-PA (71 mg).

(Dap^5, Pen^6, Gly^9)-PA, 11 (Table 1)

The protected resin ThiaPmp(Meb)-D-Trp-Ile-Gln-Dap(Z)-Pen(Meb)-Pro-Arg(Tos)-Gly-Resin (0.3 mmol), precursor of the preceding two peptides **9** and **10**, was treated with liquid HF (10 ml) containing anisole (1 ml) in an ice bath for 1 h. The suspension was evaporated to dryness, the peptide was dissolved with 20% AcOH (20 ml), and the solution was washed with hexane (3 × 20 ml portions) in a separatory funnel. The aqueous solution was oxidized with potassium ferricyanide, and the final solution was lyophilized. The residue, still containing the ferricyanide and the ferrocyanide salts, was desalted and purified directly on preparative HPLC, running a gradient 0–36% B in A for 180 min, which yielded the peptide acid [Dap(Carb)^5, Pen^6, Gly^9]-PA (95 mg).

Table 4 Potency of New Antagonists of Oxytocin in the Rat Uterus *in vitro*, Rat Pressor, and Rat Antidiuretic Assays

Analogue number	Name	Biological activity		
		Antioxytocic $pA_2 \pm SEM$	Pressor $pA_2 \pm SEM$	Antidiuretic pA_2
	PA[a]	8.11 ± 0.07	n.d.[b]	n.d.
1	[Orn(Car)^4]-PA	8.00 ± 0.07	7.18 ± 0.10	n.d.
2	[Orn(Car)^5]-PA	6.83 ± 0.10	0[e]	n.d.
	[Pen^6]-PA[a]	8.72 ± 0.18	7.47 ± 0.35	<5.75
3	[Lys(Car)^4, Pen^6]-PA	8.15 ± 0.10	6.95[c]	n.d.
4	[Orn(Car)^4, Pen^6]-PA	8.52 ± 0.30	7.13 ± 0.05	n.d.
5	[Dab(Car)^4, Pen^6]-PA	8.42 ± 0.29	7.2[c]	n.d.
6	[Dap(Car)^4, Pen^6]-PA	8.05 ± 0.25	0[d]	n.d.
7	[Orn(Car)^5, Pen^6]-PA	8.05 ± 0.10	n.d.	n.d.
8	[Dab(Car)^5, Pen^6]-PA	7.57 ± 0.16	n.d.	n.d.
9	[Dap(Car)^5, Pen^6]-PA	7.71 ± 0.25	<5.40[c]	n.d.
10	[Dap^5, Pen^6]-PA	7.68 ± 0.13	0[e]	0[f]
11	[Dap^5, Pen^6, Gly^9]-PA	7.13 ± 0.31	0[e]	0

[a] Activities of these analogues, PA and [Pen^6]-PA, were previously reported [21].
[b] n.d. means not determined.
[c] Preliminary result.
[d] The compound showed trace agonistic activity of about 0.09 IU/mg, AVP has 412 IU/mg (Lebl M, Jost K, Brtnik F, Tables of analogs, In Handbook of Neurohypophyseal Hormone Analogs, Jost K, Lebl M, Brtnik F. (eds.) CRC Press. Inc., Boca Raton, Florida: 1987, Vol 2, Part 2, 127–267).
[e] No pressor or antipressor activity is detected up to a dose of 0.2 mg/Kg of the experimental animal, *i.v.*
[f] No antidiuretic or diuretic activity is detected up to a dose of 0.2 mg/Kg of the experimental animal, *s.c.*

BIOASSAYS

All analogues were tested in the rat uterotonic *in vitro* assay using a modified Holton method [29] in Munsick solution [41] containing also 0.5 mM Mg^{++}. Rats in estrus induced by an injection of estrogen 48 h before the experiments were used; the height of the single isometric contraction of a uterine strip was measured and cumulative dose response curves were constructed in the absence and in the presence of various concentrations of the analogues. The pA_2 was calculated according to Schild [42]. 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 the rat antidiuretic assay performed on conscious male rats in a modified Burn's arrangement [43,44] and/or in the rat pressor test performed on phenoxybenzamine-treated male rats [45]. In these tests, synthetic AVP from Bachem or Polypeptide Labs was used. *In vivo* pA_2 in the antidiuretic assay was calculated as the negative logarithm of base 10 of the effective concentration which was calculated from the effective dose (a dose of an inhibitor that reduces the effect of 2x dose of standard to the effect of x dose of standard) by dividing it 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 [46].

RESULTS AND DISCUSSION

A total of 11 analogues were synthesized and evaluated. The results are summarized in Tables 1–4. In the rat uterotonic assay, [Orn(Car)^4]-PA, **1**, was equipotent with PA (Table 4). However, when the same modification was accomplished at position 5, the analogue [Orn(Car)^5]-PA, **2**, showed a drop in antagonistic potency more than 10 times in comparison to that of the PA. Encouraged by the finding of equipotency of **1** with PA, we extended the same mode of ureido substitution for carboxamide side chains to the highly potent [Pen^6]-PA. We found that substitution with the ureido group for the carboxamide group of Gln^4 results in good retention of antagonistic potency in the uterotonic assay. Thus, [Orn(Car)^4, Pen^6]-PA, **4** with $pA_2 = 8.52$ and [Dab(Car)^4, Pen^6]-PA, **5** with $pA_2 = 8.42$ may be deemed equipotent with [Pen^6]-PA.

In the pressor assay, [Lys(Car)^4, Pen^6]-PA, **3**, and [Dab(Car)^4, Pen^6]-PA, **5**, show antagonism of AVP lower than expected and lower than [Pen^6]-PA. The analogue [Dap(Car)^4, Pen^6]-PA, **6**, had undetectable antagonistic activity under our experimental conditions, but exhibited a remnant activity as an agonist (about 0.09 IU/mg). In a preliminary test for antidiuretic activity, compounds **1, 2, 4, 5, 6, 10**, and **11** showed no activity with a dose of 0.2 mg/Kg. Further testing was not performed since none of these compounds appeared to have decisive advantage over [Pen^6]-PA.

The substitution with the ureido group at Asn^5 led to a significant loss in antioxytocic potency. Surprisingly, the most potent in this series was [$Orn(Car)^5$, Pen^6]-PA, **7** ($pA_2 = 8.05$); analogues having the smaller substituents, which might be more truly isosteric with Asn^5, were somewhat less potent. An interesting finding was that [$Dap(Car)^5$, Pen^6]-PA, **9** ($pA_2 = 7.71$), is equipotent with its precursor [Dap^5, Pen^6]-PA, **10** ($pA_2 = 7.68$), which has a positive charge at position 5. It is noteworthy that substitutions at position 5 in the agonist series are usually deleterious and result in large losses in potency. No such sensitivity is noted in these antagonists. We may hypothesize that the modification with Pen at position 6 may compensate for the modification in position 5 (compare analogues **2** and **7**).

Since our studies on truncated [Pen^6]-PA analogues [22] showed that both the acid and amide of an analogue showed retention of antagonistic potency, we synthesized [Dap^5, Pen^6, Gly^6]-PA, **11.** In the uterotonic assay, **11** is a slightly weaker OTA than **10**, and neither analogue has activity either in the pressor or in the antidiuretic assays. The finding that [Dap^5, Pen^6]-PA behaves as a more selective OTA than others in this series makes this analogue an attractive candidate for further studies for the development of potent and specific OTAs.

OTAs substituted with Dap^5 or Dab^5 have been previously reported by Chan *et al.* [47]. These analogues also showed significant retention of antioxytocic activity, but they also showed some antipressor activity *in vivo*; no antidiuretic activity as agonists or antagonists was reported. The authors also stressed the need for more selective OTAs free from blocking vascular V1a receptors, which may compromise the patient's ability to maintain blood pressure during hemorrhage. We may conclude that our antagonists, possibly due to the Pen^6 substitution, showed much greater selectivity.

CONCLUSIONS

Our results lead to the conclusion that some ureido substitutions at position 4 of [Pen^6]-PA lead to retention of activity as OTAs, without a decisive advantage in specificity. However, some substitutions at position 5 of [Pen^6]-PA with the basic amino acid Dap led to a decrease in OTA potency in the uterotonic assay, but to an increase in specificity since these analogues are inactive in the pressor and in the antidiuretic assay at the doses tested. These findings raise our expectations of designing a potent but pure antagonist of oxytocin devoid of effects on renal and vascular tissues.

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