Journal of Peptide Science
J. Peptide Sci. **8**: 314–326 (2002)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.390

Antagonists of Oxytocin Featuring Replacement with Modified β-Mercaptopropionic Acids at Position 1†

GEORGE FLOURET,[a]* TADEUSZ MAJEWSKI,[a] LAJOS BALASPIRI,[a] WILLIAM BRIEHER,[a] KEVIN MAHAN,[a] OLIVIER CHALOIN,[a] LAIRD WILSON Jr[b] and JIRINA SLANINOVÁ[c]

[a] Department of Physiology, Northwestern University Medical School, Chicago, Illinois 60611, USA
[b] Department of Obstetrics and Gynecology, University of Illinois Medical Center, Chicago, Illinois 60612, USA (Deceased March 2000)
[c] Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic 166 10 Prague 6, Czech Republic

Received 28 February 2002
Accepted 4 March 2002

Abstract: Twenty analogues were synthesized of [Pmp^1, D-Trp^2, Arg^8]oxytocin, PA, (Pmp = β,β-pentamethylene-β-mercaptopropionic acid), a potent antagonist of the uterotonic effect of oxytocin in the rat (uterotonic test *in vitro*, $pA_2 = 7.77$) and in the baboon. Systematic substitution of Pmp^1 was made with β-mercaptopropionic acids featuring replacement of the 4-methylene group of the cyclohexyl ring of Pmp with isosteric O, S, NH or with C=O. Since the more hydrophilic NH and C=O substitutions showed a sharply decreased antagonistic potency (rat uterotonic test *in vitro*), additional modifications were made to reduce their hydrophilicity. Acylation of the NH group with various acyl groups, and ketalization or thioketalization of C=O with more or less bulky substituents led to a partial restoration of potency, the *N*-carbamyl- and the 2-mercapto-2-adamantaneacetyl analogues being equipotent with PA. Internal cyclization by amidation of the NH-group with Gly-9, resulted in a bicyclic analogue, (cyclo 1–9)[(HN)Pmp^1, Gly^9]PA which was equipotent with PA. When Pen-6 was introduced into the bicyclic derivative instead of Cys-6, to reduce the flexibility of the rings, the resulting (cyclo 1–9)[(HN)Pmp^1, Pen^6, Gly^9]PA had somewhat better potency ($pA_2 = 8.17$) in the uterotonic test and no detectable activity in the antidiuretic assay. In the case of substitution of PA with β,β-(3-thiapentamethylene)-β-mercaptopropionic acid, (S)Pmp, there was also an increase in inhibitory potency in the uterotonic test ($pA_2 = 8.08$); the analogue had extremely weak antidiuretic activity. To establish the importance of the steric effects of the Pen-6 substitution, analogues

Abbreviations: Ac_2O, acetic anhydride; AcOH, acetic acid; BOP reagent, (benzotriazolyl-1-oxy)-tris(dimethylamino) phosphonium hexafluorophosphate; *n*-BuOH, *n*-butanol; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; EtOAc, ethyl acetate; EtOH, ethanol; For, formyl; Hex, hexane; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MeCN, acetonitrile; ONp, 4-nitrophenyl ester; OR, optical rotation. OT, oxytocin; OTA/OTAs, oxytocin antagonist/antagonists; PA, [Pmp^1, D-Trp^2, Arg^8]oxytocin; Meb, 4-methylbenzyl; PITC, phenylisothiocyanate; Pmp, β,β-pentamethylene-β-mercaptopropionic acid; iPrOH, isopropanol; PTC, phenylthiocarbamyl; Pyr, pyridine; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; TLC, thin layer chromatography; Tos, p-toluenesulfonyl; Abbreviations used comply with recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* 1989; **264**: 688–673.)

*Correspondence to: Dr. George Flouret, Northwestern University Medical School, Department of Physiology, 303 East Chicago Avenue, Chicago, IL 60611, USA; e-mail: g-flouret@nwu.edu
† Preliminary results were presented in part at the 22nd European Peptide Symposium in Interlaken, Switzerland (13–19 September 1992) and in the Proceedings of the Symposium [41], and at the 23rd EPS in Braga, Portugal (4–10 September 1994) and in the Proceedings of the Symposium [42].

Contract/grant sponsor: National Institute of Child Health and Human Development; Contract/grant number: HD-22567.

Contract/grant sponsor: Abbott Laboratories.

[Pen^6]PA and [(S)Pmp^1, Pen^6]PA were made and found to be very potent, with a pA_2 of 8.72 and 8.86, respectively. The high potency of the latter analogue and its extremely weak action in the diuretic assay makes it an attractive candidate for studies on the inhibition of the biological effects of oxytocin and for the prevention of preterm labour. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: oxytocin; antagonists; uterotonic; β,β-pentamethylene-β-mercaptopropionic acid

INTRODUCTION

There is great interest in the design of inhibitors of preterm labour, since premature birth remains the major factor contributing to perinatal mortality and morbidity [1]. The neurohypophysial hormone oxytocin (OT) seems to be involved in human parturition, although it is unclear whether it is involved in the initiation of labour [2,3]. Whereas the use of β-adrenergic agonists in the treatment of preterm labour is questioned as unsatisfactory [4], an OT antagonist (OTA) has been successfully used to inhibit the uterine contractions of premature labour [5,6].

Considerable effort has been made in designing specific OT analogues [7,8] or non-peptide mimetics [9]. Some of the first potent antagonists of the neurohypophysial hormones oxytocin and [Arg8]vasopressin (AVP) used β-mercaptopropionic acid (Mpa) in position 1 in addition to other changes [10]. This was followed by the synthesis of other β,β-dialkyl-β-Mpa-1-substituted analogues with higher antagonistic potencies [11]. In subsequent attempts, β,β-pentamethylene-β-mercaptopropionic acid (Pmp) was used to prepare a strong OTA [12]. Since then, Pmp has become the preferred substituent at position 1 to maintain high antagonistic potency. Substitution with Mpa-1 was used in combination with other substituents leading to [Mpa^1, D-Tyr(Et)^2, Thr^4, Orn^8]OT (Atosiban) [13] which was sufficiently potent to inhibit preterm labour in clinical studies [5,6] and was approved (trade name Tractocile) for clinical use in Europe in January 2000 (*Pharm J.* **264** (7–100): 871).

In our laboratories [Pmp^1, D-Trp^2, Phe^3, Ile^4, Arg^8]oxytocin was synthesized [14] ($pA_2 = 7.51$) and found to: (a) inhibit uterine contractions of an oestrus rat in response to OT; (b) inhibit milk-let down in the lactating rat; (c) delay labour in the rat and to inhibit labour contractions in the baboon. In addition, this antagonist can inhibit *in vitro* uterine contractions in response to OT in uterine tissue obtained from women at caesarean section [15]. Later, we showed that this OTA inhibits spontaneous uterine contractions and labour in baboons [16]. Subsequently, we developed [Pmp^1, D-Trp^2, Arg^8]OT (PA, or parent antagonist, Figure 1) which is more potent ($pA_2 = 7.77$) and more specific than the previous one [14,17]. More recently, further increases in the potency of OTAs were achieved by the substitution of PA with an additional Trp residue in the tail sequence [18,19].

Attempts have been made to modify Pmp by making 4-alkyl-1-mercaptocyclohexaneacetic acids [20,21], and introducing them instead of Pmp at position 1 of AVP antagonists.

Twenty new OTAs have been prepared (Table 1) and their structures are summarized in Figure 2. To prepare the new analogues the synthesis of

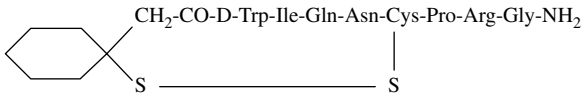
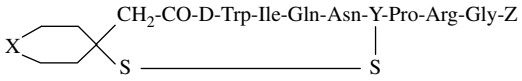


Figure 1 Structure of [β,β-pentamethylene-β-mercaptopropionyl^1, D-Trp^2, Arg^8]oxytocin PA, or parent antagonist.



Analog	X	Y	Z	Analog	X	Y	Z
PA	CH_2	Cys	NH_2				
1	O	Cys	NH_2	11	N*	Pen	-----
2	S	Cys	NH_2	12	C=O	Cys	NH_2
3	NH	Cys	NH_2	13	ethylenedioxymethylene	Cys	NH_2
4	Boc-N	Cys	NH_2	14	ethylenedithiomethylene	Cys	NH_2
5	For-N	Cys	NH_2	15	trimethylenedithiomethylene	Cys	NH_2
6	Ac-N	Cys	NH_2	16	*o*-phenylenedithiomethylene	Cys	NH_2
7	Bac-N	Cys	NH_2	17	Ad**	Cys	NH_2
8	Adac-N	Cys	NH_2	18	Tsa***	Cys	NH_2
9	Carb-N	Cys	NH_2	19	CH_2	Pen	NH_2
10	N*	Cys	-----	20	S	Pen	NH_2

*bicyclic compound, 1-9 lactam ring.

**2-Adamantyl (Ad) instead of cyclohexyl ring.

***Thiosalicylic acid (Tsa) instead of Pmp.

Figure 2 Structure of PA analogues.

Table 1 List of Analogues

No.	Structure	Abbreviation
	[[β,β-Pentamethylene-β-mercaptopropionic acid^1, D-Trp^2, Arg^8] OT	PA
1	[β,β-(3-Oxapentamethylene)-β-mercaptopropionic acid^1]PA	[(O)Pmp^1]PA
2	[β,β-(3-Thiapentamethylene)-β-mercaptopropionic acid^1]PA	[(S)Pmp^1]PA
3	[β,β-(3-Azapentamethylene)-β-mercaptopropionic acid^1]PA	[(HN)Pmp^1]PA
4	[β,β-(N-*tert*-Butyloxycarbonyl-3-azapentamethylene)-β-mercaptopropionic acid^1]PA	[(Boc-N)Pmp^1]PA
5	[β,β-(N-Formyl-3-azapentamethylene)-β-mercaptopropionic acid^1]PA	[(For-N)Pmp^1]PA
6	[β,β-(N-Acetyl-3-azapentamethylene)-β-mercaptopropionic acid^1]PA	[(Ac-N)Pmp^1]PA
7	[β,β-(N-*tert*-Butylacetyl-3-azapentamethylene)-β-mercaptopropionic acid^1]PA	[(Bac-N)Pmp^1]PA
8	[β,β-(N-1-Adamantane acetyl-3-azapentamethylene)-β-mercaptopropionic acid^1]PA	[(Adac-N)Pmp^1]PA
9	[β,β-(N-Carbamyl-3-azapentamethylene)-β-mercaptopropionic acid^1]PA	[(Carb-N)Pmp^1]PA
10	(Cyclo 1–9) [(HN)Pmp^1, Gly^9]PA	
11	(Cyclo 1–9) [(HN)Pmp^1, Pen^6, Gly^9]PA	
12	[β,β-(3-Ketopentamethylene)-β-mercaptopropionic acid^1]PA	[(Keto)Pmp^1]PA
13	[β,β-(Ethylene-3,3-dioxypentamethylene)-β-mercaptopropionic acid^1]PA	[(EDO)Pmp^1]PA
14	[β,β-(Ethylene-3,3-dithiopentamethylene)-β-mercaptopropionic acid^1]PA	[(EDT)Pmp^1]PA
15	[β,β-(Trimethylene-3,3-dithiopentamethylene)-β-mercaptopropionic acid^1]PA	[(TMDT)Pmp^1]PA
16	[β,β-(*o*-Phenylene-3,3-dithiopentamethylene)-β-mercaptopropionic acid^1]PA	[(PDT)Pmp^1]PA
17	[2-Mercapto-2-adamantaneacetic acid^1]PA	[Madac^1]PA
18	[Thiosalicylic acid^1]PA	[Tsa^1]PA
19	[Pen^6]PA	
20	[(S)Pmp^1, Pen^6]PA	

a series of derivatives of Pmp (Table 2) was initially undertaken avoiding the *cis* and *trans* isomerism encountered with the 4-alkyl substituents in the cyclohexane ring. First β,β-(3-oxapentamethylene)-β-(4-methylbenzylmercapto)-propionic acid, (O)Pmp(S-Meb), β,β-(3-thiapentamethylene)-β-(4-methylbenzylmercapto)-propionic acid, (S)Pmp(S-Meb) and β,β-(3-azapentamethylene)-β-(4-methylbenzylmercapto)-propionic acid, (HN)Pmp(S-Meb) were prepared. These three acids may be deemed isosteric with Pmp, with the CH_2 group at position 4 of the cyclohexane ring being replaced by O, S and NH. (O)Pmp and (S)Pmp have been used to make AVP antagonists, but no attempts have been reported to design OTAs with these compounds [22]. Then β,β-(3-keto-pentamethylene)-β-(4-methylbenzylmercapto)-propionic acid, (O=C) Pmp(S-Meb) or (Keto)Pmp(S-Meb) was prepared.

Additional acids were obtained by direct acylation of peptides containing the (HN)Pmp substitution or by preparation of ketals and thioketals of (O=C)Pmp(S-Meb). These acids, and other derivatives related to β-mercaptopropionic acid, were used as a replacement for Pmp in PA. Altogether 16 analogues were prepared in this way. Two bicyclic analogues were also prepared, featuring internal acylation of (HN)Pmp-1 with Gly-9, with one of them having Pen-6 to reduce the flexibility of the peptide rings. Two analogues were prepared to further study the importance of the Pen-6 substitution. The analogues were tested as OTAs of the OT uterotonic action in the rat bioassay *in vitro* [23] in the presence of magnesium ions and some of them were also tested in the rat antidiuretic assay. We report here the physicochemical properties of the new OTAs (Tables 3 and 4) and their biological data (Tables 5 and 6).

Table 2 List of Protected β-Mercaptopropionic Acids and Related Derivatives

No.	Name	Abbreviation
21	β,β-(3-Oxapentamethylene)-β-(4-methylbenzylmercapto)-propionic acid	(O)Pmp(S-Meb)
22	β,β-(3-Thiapentamethylene)-β-(4-methylbenzylmercapto)-propionic acid	(S)Pmp(S-Meb)
23	β,β-(3-Boc-azapentamethylene)-β-(4-methylbenzylmercapto)-propionic acid	(Boc-N)Pmp(S-Meb)
24	β,β-(Ethylene-3,3-dioxypentamethylene)-β-(4-methylbenzylmercapto)-propionic acid	(EDO)Pmp(S-Meb)
25	β,β-(3-Ketopentamethylene)-β-(4-methylbenzylmercapto)-propionic acid	(Keto)Pmp(S-Meb)
26	β,β-(Ethylene-3,3-dithiopentamethylene)-β-(4-methylbenzylmercapto)-propionic acid	(EDT)Pmp(S-Meb)
27	β,β-(Trimethylene-3,3-dithiopentamethylene)-β-(4-methylbenzylmercapto)-propionic acid	(TMDT)Pmp(S-Meb)
28	β,β-(*o*-Phenylene-3,3-dithiopentamethylene)-β-(4-methylbenzylmercapto)-propionic acid	(PDT)Pmp(S-Meb)
29	2-(4-Methylbenzylmercapto)-2-adamantaneacetic acid	Madac(S-Meb)
30	S-(4-Methylbenzyl)-thiosalicylic acid	Tsa(S-Meb)

Table 3 Physicochemical Characteristics of Oxytocin Antagonists

Analogue		TLC[d]						HPLC
No	Name	MW[a]	Yield[b] %	OR[c] deg.	R_fA	R_fB	R_fC	R_fD	Retention time (min)[e]
1	[(O)Pmp^1]PA[f]	1128	25	−95	0.21	0.34	0.15	0.44	3.4
2	[(S)Pmp^1]PA	1144	21	−92	0.22	0.36	0.17	0.54	5.4
3	[(HN)Pmp^1]PA	1127	32	−75	0.10	0.21	0.01	0.26	1.0
4	[(Boc-N)Pmp^1]PA	1227	33	−85	0.31	0.41	0.19	0.66	11.8
5	[(For-N)Pmp^1]PA	1155	31	−74	0.18	0.24	0.13	0.39	3.2
6	[(Ac-N)Pmp^1]PA	1169	28	−83	0.15	0.27	0.11	0.37	3.4
7	[(Bac-N)Pmp^1]PA	1225	50	−74	0.26	0.36	0.20	0.61	8.2
8	[(Adac-N)Pmp^1]PA	1303	23	−52	0.33	0.41	0.23	0.61	45.6
9	[(Carb-N)Pmp^1]PA	1170	17	−84	0.16	0.29	0.11	0.37	3.0
10	(cyclo 1–9)[(HN)Pmp^1, Gly^9]PA	1110	21	−115	0.21	0.32	0.15	0.43	1.2
11	(cyclo 1–9)[(HN)Pmp^1, Pen^6, Gly^9]PA	1137	18	−23	0.35	0.45	0.25	0.58	6.6
12	[(Keto)Pmp^1]PA	1140	4	−90	0.21	0.30	0.14	0.44	3.6
13	[(EDO)Pmp^1]PA	1184	14	−85	0.22	0.15	0.13	0.41	4.8
14	[(EDT)Pmp^1]PA	1216	17	−77	0.27	0.33	0.19	0.55	10.0
15	[(TMDT)Pmp^1]PA	1230	6	−78	0.22	0.40	0.17	0.57	9.8
16	[(PDT)Pmp^1]PA	1264	5	−34	0.31	0.45	0.22	0.59	50.4
17	[Madac^1]PA	1178	27	−132	0.30	0.37	0.20	0.59	12.0
18	[Tsa^1]PA	1106	13	−84	0.25	0.45	0.19	0.51	4.6
19	[Pen^6]PA	1154	28	−44	0.26	0.42	0.19	0.56	16.8
20	[(S)Pmp^1, Pen^6]PA	1172	34	−39	0.27	0.42	0.19	0.56	11.6

[a] FAB/MS gave the MW +1 for all analogues except for **8**, **10** and **14** for which it was identical to the MW, and, for **1** which gave MW +2.
[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).
[d] The composition of solvents A–D is given in the Experimental Section.
[e] The analysis was run isocratically, solvent composition was 50% solvent B, flow rate 1.5 ml/min, in order to determine the relative hydrophilicities by comparing retention times of analogues.
[f] PA, [Pmp^1, D-Trp^2, Arg^8]OT.

Table 4 Amino acid Analyses of Oxytocin Antagonists

Analogue		Amino acid ratios								
No	Name	Asp	Glu	Gly	Arg	Pro	Cys	Ile	Trp[a]	R
1	[(O)Pmp^1]PA	0.95	0.93	1.09	1.00	0.99	0.43	0.90	0.72	0.66[b]
2	[(S)Pmp^1]PA	0.94	0.92	1.10	1.05	1.08	0.64	0.90	0.73	0.25[c]
3	[(HN)Pmp^1]PA	0.94	0.92	1.00	0.98	0.98	0.23	0.90	0.75	—
4	[(Boc-N)Pmp^1]PA	0.94	0.91	1.06	1.06	1.10	0.22	0.90	0.69	—
5	[(For-N)Pmp^1]PA	0.90	1.03	1.05	0.94	1.00	0.29	0.96	0.67	—
6	[(Ac-N)Pmp^1]PA	1.00	1.00	1.00	1.04	1.04	0.22	0.97	0.67	—
7	[(Bac-N)Pmp^1]PA	1.01	0.92	1.01	1.00	1.00	0.24	0.90	0.71	—
8	[(Adac-N)Pmp^1]PA	1.00	1.01	1.00	1.05	1.02	0.24	0.99	0.71	0.85[d]
9	[(Carb-N)Pmp^1]PA	0.90	1.03	0.98	1.03	0.94	0.29	0.96	0.66	—
10	(cyclo 1–9)[(HN)Pmp^1, Gly^9]PA	0.93	0.90	0.94	1.05	0.97	0.29	0.94	0.76	—
11	(cyclo 1–9)[(HN)Pmp^1, Pen^6, Gly^9]PA	0.90	0.94	1.02	1.05	0.97	—	0.95	0.78	0.27[e]
12	[(Keto)Pmp^1]PA	0.99	1.00	0.94	1.10	1.03	0.97	0.90	0.74	—
13	[(EDO)Pmp^1]PA	0.94	1.00	1.00	1.03	1.02	0.52	0.96	0.74	—
14	[(EDT)Pmp^1]PA	0.96	0.90	1.08	1.10	1.01	0.44	0.96	0.66	—
15	[(TMDT)Pmp^1]PA	0.96	0.90	1.10	1.00	1.00	0.47	0.99	0.67	—
16	[(PDT)Pmp^1]PA	1.00	0.91	0.99	1.01	0.90	0.17	0.92	0.85	—
17	[Madac^1]PA	0.93	0.97	1.04	1.05	1.00	0.83	0.99	0.73	—
18	[Tsa^1]PA	0.93	0.90	1.02	1.10	0.92	0.49	1.01	0.84	—
19	[Pen^6]PA	0.96	0.94	1.00	1.02	1.03	—	0.99	0.78	0.31
20	[(S)Pmp^1, Pen^6]PA	0.93	0.99	1.00	1.03	1.01	—	1.02	0.81	0.29

[a] Tryptophan in peptides was estimated by its UV absorption at 280 nm as reported[37]. Values found for Trp suggest that the peptide has several moles of AcOH, TFA and/or H_2O.
[b] The PTC derivative of (O)Pmp-SS-Cys was used to determine the UV absorption and elution time. This PTC derivative was coinjected with PTC-derivatives obtained with the standard amino acid mixture to identify the relative retention times. Values for Cys are low when a derivative of the type of (O)Pmp-SS-Cys can form with some Pmp analogues which in most cases was not detected.
[c] (S)Pmp-SS-Cys.
[d] Adac was estimated by HPLC of the hydrolysis product.
[e] Pen-SS-Pen.

EXPERIMENTAL

Synthesis of β-Mercaptopropionic Acid Derivatives

Preparation of these compounds was patterned after a synthesis of Pmp [24]. As an example, 4-tetrahydropyranone was reacted with triethylphosphonoacetate, yielding ethyl 4-tetrahydropyranylideneacetate. Michael addition of 4-methylbenzyl (Meb) mercaptan and saponification yielded β,β-(3-oxapentamethylene)-β-(4-methylbenzylmercapto)-propionic acid, (O)Pmp(S-Meb), **21** (Table 2), which was used to synthesize the protected amide precursor of analogue **1** (Table 1), by the general solid phase peptide synthesis (SPPS) method. The substituent for analogue **2** was similarly prepared starting with 4-tetrahydrothiopyranone and making (S)Pmp(S-Meb), **22**. For making analogue **4**, (Boc-N)Pmp(S-Meb), **23**, was synthesized from piperidone. Whereas treatment of the (Boc-N)Pmp(S-Meb) protected peptide-amide with HF/anisole led to the (HN)Pmp analogue **3**, treatment with Na in liquid NH_3 led to the (Boc-N)Pmp analogue **4**. Removal of the Boc group in (Boc-N)Pmp(S-Meb)-peptide-resin led to (HN)Pmp(S-Meb)-peptide resin which was suitably acylated, leading to the (For-N)Pmp analogue **5**, (Ac-N)Pmp analogue **6**, (*tert*-butyl-Ac-N) or (Bac-N)Pmp analogue **7** and (1-adamantane acetyl-N)Pmp analogue, (Adac-N)Pmp, analogue **8**. Treatment of analogue **3** with potassium cyanate led to substitution with the carbamyl (Carb) group and formation of the (Carb-N)Pmp analogue **9**.

The (HN)Pmp group was acylated internally by making [(HN)Pmp^1, Gly^9]PA and making a second ring by formation of the 1–9 lactam, in analogue

Table 5 Potency of Competitive Antagonists of Oxytocin Contractile Action in the Rat Uterus *In Vitro* Assay

Analogue		Antioxytocic potency	
No	Name	pA_2	± SEM
	PA[a]	7.77	+0.03
1	[(O)Pmp1]PA	7.20	±0.07
2	[(S)Pmp1]PA	8.08	±0.04; 8.11 ± 0.07[b]
3	[(HN)Pmp^1]PA	4.78	±0.03; 5.2 ± 0.3[b,c]
4	[(Boc-N)Pmp^1]PA	5.29	±0.08
5	[(For-N)Pmp^1]PA	7.46	±0.07[b]
6	[(Ac-N)Pmp^1]PA	7.10	±0.06
7	[(Bac-N)Pmp^1]PA	6.40	±0.02
8	[(Adac-N)Pmp^1]PA	6.04	±0.07
9	[(Carb-N)Pmp^1]PA	7.71	±0.12[b]
10	(cyclo 1–9)[(HN)Pmp^1, Gly^9]PA	7.80	±0.10[b]
11	(cyclo 1–9)[(HN)Pmp^1, Pen^6, Gly^9]PA	8.17	±0.16[b]
12	[(Keto)Pmp^1]PA	5.55	±0.06
13	[(EDO)Pmp^1]PA	7.25	±0.08
14	[(EDT)Pmp^1]PA	7.15	±0.07
15	[(TMDT)Pmp^1]PA	7.00	±0.03
16	[(PDT)Pmp^1]PA	6.82	±0.04
17	[$Madac^1$]PA	7.87	±0.03
18	[Tsa^1]PA	6.10	±0.05
19	[Pen^6]PA	8.72	±0.05
20	[(S)Pmp^1, Pen^6]PA	8.86	±0.09

[a] This analogue [Pmp^1, D-Trp^2, Arg^8]OT, PA, was previously reported [14,17].
[b] Values obtained in the laboratories of J. Slaninová.
[c] The determination of the value is complicated by low agonistic activity; the dose dependency of the antagonistic activity makes a very shallow line.

Table 6 Potency of Most Potent Competitive Antagonists of Oxytocin in the Antidiuretic Assay in the Rat

Analogue		Antidiuretic activity	
No	Name	Agonistic IU/mg	Antagonistic pA_2
	PA[a]		<5.86[a]
2	[(S)Pmp^1]PA	<0.02[b,c]	
11	(cyclo 1–9)[(HN)Pmp^1, Pen^6, Gly^9]PA	0[b,d]	0[b,d]
20	[(S)Pmp^1, Pen^6]PA		<5.75

[a] Biological data for PA ([Pmp^1, D-Trp^2, Arg^8]OT) taken from reference [17].
[b] Values obtained in the laboratories of J. Slaninová.
[c] Very low antidiuretic activity, no antagonism.
[d] No activity at all.

10. We also made another bicyclic 1–9 lactam from [(HN)Pmp^1, Pen^6, Gly^9]PA, leading to analogue **11**.

After synthesis from 1,4-cyclohexanedione *mono*-ethylene ketal, the β,β-(ethylene-3,3-dioxy-pentamethylene)-β-(4-methylbenzylmercapto)-propionic acid, (EDO)Pmp(S-Meb), **24**, was used to prepare analogue **13** which after acid treatment gave the (Keto)Pmp analogue **12**. Treatment of (EDO)Pmp(S-Meb) with acid gave (Keto)Pmp(S-Meb), **25**, which, when treated in the presence of BF_3-etherate/AcOH

with a dithiol, led to a thioketal [25]. In this manner, treatment of **25** with 1,2-ethanedithiol led to *β*,*β*-(ethylene-3,3-dithiopentamethylene)-*β*-(4-methylbenzylmercapto)-propionic acid, (EDT)-Pmp(S-Meb), **26**, used to make analogue **14**; treatment of **25** with 1,3-propanedithiol led to *β*,*β*-(trimethylene-3,3-dithiopentamethylene)-*β*-(4-methylbenzylmercapto)-propionic acid, (TMDT)-Pmp(S-Meb), **27**, used to make analogue **15**; treatment of **25** with 1,2-benzenedithiol led to *β*,*β*-(*o*-phenylene-3,3-dithiopentamethylene)-*β*-(4-methylbenzylmercapto)-propionic acid, (PDT)Pmp-(S-Meb), **28**, used to make analogue **16**.

For making analogue **17**, 2-adamantanone was converted to ethyl 2-(4-methylbenzylmercapto)-2-adamantane acetate. Various conditions for saponification led to small amounts of 2-(4-methyl-benzylmercapto)-2-adamantane acetic acid, Madac(S-Meb), **29**, probably due to steric hindrance favouring elimination of Meb-SH. Preparative HPLC allowed the purification of sufficient Madac(S-Meb) to make analogue **17**. For making analogue **18**, thiosalicylic acid (Tsa) was alkylated to S-(4-methylbenzyl)-thiosalicylic acid, Tsa(S-Meb), **30**. The preparation of analogues **19** and **20** involved standard reagents.

Altogether nine analogues of mercaptopropionic acid, **21–24** and **26–30** were directly incorporated into analogue precursors. Other modifications were made in the stage of protected peptides (in the case of analogues **3** and **5–8**). Analogue **9** was made from analogue **3**, and analogue **12** was made from analogue **13**. Elemental analyses for C, H, N, S were performed by Galbraith Laboratories, Knoxville Tennessee, USA and were within 0.4% of the theoretical value for the proposed structures unless indicated.

***β*,*β*-(3-Oxapentamethylene)-*β*-(4-methylbenzylmercapto)-propionic acid, (O)Pmp(S-Meb), 21.** Following the general procedure of Yim and Huffman [24], tetrahydro-pyran-4-one was reacted with triethylphosphonoacetate, affording ethyl 4-tetrahydro-pyranylidene-acetate as an oil (69% yield) which was subjected to a Michael addition with 4-methylbenzyl-mercaptan and the product subjected to saponification as usual. Crystallization from EtOAc gave (O)Pmp(S-Meb), **21**, (Table 2) in an 81% yield, mp 110°–111 °C. Anal. ($C_{15}H_{20}O_3S$): Calcd.: C, 64.2; H, 7.19; S, 11.4; Found: C, 64.3; H, 7.45; S, 11.2.

***β*,*β*-(3-Thiapentamethylene)-*β*-(4-methylbenzylmercapto)-propionic acid, (S)Pmp(S-Meb), 22.** This intermediate was prepared as for the preceding compound, but starting with tetrahydro-thiopyran-4-one. Crystallization from EtOAc yielded (S)Pmp(S-Meb), **22**, in a 46% yield, mp 114°–115 °C. Anal. ($C_{15}H_{20}O_2S_2$): Calcd.: C, 60.8; H, 6.79; S, 21.6; Found: C, 60.9; H, 6.79; S, 21.2.

***β*,*β*-(3-Boc-azapentamethylene)-*β*-(4-methylbenzylmercapto)-propionic acid, (Boc-N)Pmp(S-Meb), 23.** This compound was made from Boc-piperidone, which was prepared from piperidone by reaction with Boc-anhydride; crystallization from Et_2O-hex gave Boc-piperidone in an 80% yield, mp 73°–74 °C. Anal. ($C_{10}H_{17}O_3N$): Calcd.: C, 60.3; H, 8.59; N, 7.03; Found: C, 60.7; H, 8.87; N, 7.03.

Following the general method Boc-piperidone was converted to ethyl *N*-Boc-piperidylidene acetate; crystallized from iPrOH-hex in a 66% yield, mp 84°–85°. Anal. ($C_{14}H_{23}O_2N$): Calcd.: C, 62.4; H, 8.61; N, 5.20; Found: C, 62.8; H, 8.65; N, 5.17.

The usual method of Yim and Huffman gave (Boc-N)Pmp(S-Meb), **23**, which was crystallized from iPrOH-hex in a 68% yield, mp 115°–117 °C. Anal. ($C_{20}H_{29}NO_4S$): Calcd.: C, 63.3; H, 7.70; N, 3.69; S, 8.45; Found: C, 63.7; H, 7.71; N, 3.73; S, 8.12.

***β*,*β*-(Ethylene-3,3-dioxypentamethylene)-*β*-(4-methylbenzylmercapto)-propionic acid, (EDO)-Pmp(S-Meb), 24.** This intermediate was prepared from 1,4-cyclohexanedione mono-ethylene ketal. Treatment of the product with dicyclohexylamine (DCHA) and crystallization from EtOAc-hex gave the (EDO)Pmp(S-Meb).DCHA salt in an 83% yield, mp 142°–143 °C. Anal. ($C_{30}H_{47}NO_4S$): Calcd.: C, 69.6; H, 9.15; N, 2.71; S, 6.19; Found: C, 69.4; H, 8.93; N, 2.87; S, 6.32.

The DCHA salt was treated with citric acid and extracted with Et_2O, the extracts were washed with H_2O, saturated salt, dried (Na_2SO_4) and evaporated to dryness. The residue was crystallized from EtOAc-hex giving (EDO)Pmp(S-Meb), **24**, in a 65% yield, mp 105°–106 °C. Anal. ($C_{18}H_{24}O_4S$): Calcd.: C, 64.3; H, 7.19; S, 9.53; Found: C, 64.1; H, 7.27; S, 9.14.

***β*,*β*-(3-Ketopentamethylene)-*β*-(4-methylbenzylmercapto)-propionic acid, (Keto)Pmp(S-Meb), 25.** A solution of (EDO)Pmp(S-Meb) in 75% AcOH was kept for 40 min at 105 °C. The reaction was extracted with EtOAc and this solvent washed as above and evaporated to an oil which crystallized from EtOAc-hex yielding (Keto)Pmp(S-Meb), **25**, in

a 95% yield, mp 121°–123 °C. Anal. ($C_{16}H_{20}O_3S$): Calcd.: C, 65.7; H, 6.89; S, 11.0; Found: C, 66.1; H, 7.07; S, 10.9.

β,β-(Ethylene-3,3-dithiopentamethylene)-β-(4-methylbenzylmercapto)-propionic acid, (EDT)-Pmp(S-Meb), 26. (Keto)Pmp(S-Meb) (1.2 g, 4.1 mmol) was dissolved in AcOH (4.5 ml), ethanedithiol (2 ml, 20 mmol) was added, and the solution was treated with BF_3-etherate (1 ml) [25]. After 2 h (EDT)Pmp(S-Meb), **26**, was filtered and washed with AcOH giving a 92% yield, mp 158°–159 °C. Anal. ($C_{18}H_{24}O_2S_3$): Calcd.: C, 58.7; H, 6.56; S, 26.1; Found: C, 58.5; H, 6.68; S, 26.4.

β,β-(Trimethylene-3,3-dithiopentamethylene)-β-(4-methylbenzylmercapto)-propionic acid, (TMDT)Pmp(S-Meb), 27. (Keto)Pmp(S-Meb) was treated as described for the preceding intermediate, but using trimethylene-1,3-dithiol, which gave (TMDT)-Pmp(S-Meb), **27**, in a 90% yield, mp 177°–178 °C. Anal. ($C_{19}H_{26}O_2S_3$): Calcd.: C, 59.7; H, 6.85; S, 25.1; Found: C, 59.8; H, 6.98; S, 25.7.

β,β-(o-Phenylene-3,3-dithiopentamethylene)-β-(4-methylbenzylmercapto)-propionic acid, (PDT)-Pmp(S-Meb), 28. (Keto)Pmp(S-Meb) was treated by the method described above, but using benzene-1,2-dithiol, which gave (PDT)Pmp(S-Meb), **28**, in an 85% yield, mp 146°–147 °C. Anal. ($C_{22}H_{24}O_2S_3$): Calcd.: C, 63.4; H, 5.80; S, 23.1; Found: C, 63.3; H, 5.98; S, 23.1.

2-(4-Methylbenzylmercapto)-2-adamantane acetic acid, (Madac)(S-Meb), 29. Starting with 2-adamantone, the usual methods led to ethyl 2-(4-methylbenzyl-mercapto)-2-adamantaneacetate, Madac(S-Meb)-OEt ester which was crystallized from iPrOH-hex in an 80% yield, mp 85°–86 °C. Anal. ($C_{22}H_{30}O_2S$): Calcd.: C, 73.7; H, 8.43; S, 8.94; Found: C, 73.3; H, 8.73; S, 9.25.

Saponification of the preceding ester [24] gave very impure (Madac)(S-Meb) which was purified by preparative HPLC with a gradient of 0–100% B over 90 min. Crystallization from iPrOH gave 2-(S-Meb-mercapto)-2-adamantaneacetic acid, (Madac)(S-Meb), **29**, in a 9% yield, mp 196°–198 °C. Anal. ($C_{20}H_{26}O_2S$): Calcd.: C, 72.7; H, 7.93; S, 9.70; Found: C, 72.8; H, 7.99; S, 10.1.

S-(4-Methylbenzyl)-thiosalicylic acid, Tsa(S-Meb), 30. Thiosalicylic acid (5 g, 32.5 mmol) was treated with EtOH (50 ml), Cs_2CO_3, (10.6 g, 32.5 mmol) in H_2O (20 ml), and KOH (3.7 g, 65 mmol) to pH 9–10. Evaporation to dryness gave a residue, which was dissolved in DMF (33 ml), Meb-Cl (10.5 g, 65 mmol) was added and the reaction was kept at 50 °C for 1 h. Extraction with EtOAc and washing with H_2O, saturated salt, drying (Na_2SO_4) and evaporation to dryness, gave a solid residue. Crystallization (EtOH) gave Tsa(S-Meb)-OMeb, 4.06 g, in a 34% yield, mp 115°–118 °C. Anal. ($C_{23}H_{22}O_2S$): Calcd.: C, 76.2; H, 6.12; S, 8.84; Found: C, 76.1; H, 6.12; S, 8.77.

To Tsa(S-Meb)-OMeb (1.09 g, 3 mmol) was added EtOH (12 ml) and 45% KOH (1.2 ml). After 15 min the solution was acidified with 2N HCl to pH 2. The precipitate was filtered, air dried and crystallized from EtOH, yielding 555 mg of Tsa(S-Meb), **30**, in a 72% yield, mp 190°–192 °C. Anal. ($C_{15}H_{14}O_2S$): Calcd.: C, 69.7; H, 5.46; S, 12.4; Found: C, 69.5; H, 5.21; S, 12.8.

Synthesis of Protected Peptides

All protected peptide precursors of the antagonists were prepared manually by solid phase peptide synthesis (SPPS) [26] using the Boc-amino acid strategy [27] and methodology previously described [17]. For protection of side chain functionalities, Boc-Arg(Tos) and Boc-Cys(Meb) or Boc-Pen(Meb) were used. We started with Boc-Gly-resin (0.7 mmol of Boc-Gly/g) prepared on a 200–400 mesh chloromethylated resin (BioRad), 1% cross-linked with divinylbenzene, by esterification with the caesium salt of the respective Boc-amino acid [28]. The Boc-Gly-resin (0.5–0.7 mmol/g) was taken manually through the required number of coupling cycles as previously modified [19]. Completion of the coupling step was monitored by means of the ninhydrin test, which usually gave a negative response [29]. When unprotected Boc-D-Trp was introduced at position 2, Boc-groups were removed with 30% TFA in DCM containing 1% mercaptoethanol, 5% anisole [19]. The appropriate acid to be incorporated at position 1 of the peptide was incorporated in a 3-fold excess in DMF solution by activation with DCC and HOBt. The protected peptides were removed from the resin, by ammonolysis for 3 days with MeOH saturated with ammonia [30]. The resin was removed by filtration, and extracted three times with hot DMF. The combined mixture of methanol filtrate and the DMF extracts were pooled and evaporated to dryness, and the residue was dissolved in DMF and the protected peptide amide was precipitated by addition to EtOH : pet ether, yielding 400–600 mg of products. Since TLC analysis of protected peptides usually showed one major component with

minor impurities, they were used directly for removal of blocking groups and purification of the free analogues.

Analogue Synthesis

Protected peptides were freed from blocking groups on side chain functionalities by reduction with Na in liquid ammonia [31] or by treatment with liquid HF–anisole [32] and the disulphydryl peptides were converted in very dilute solution [33] to the cyclic disulphide by oxidation with potassium ferricyanide [34]. The free peptides were subjected to gel filtration on Sephadex G-15 [35] to free them from small by-products and salts and were further purified by preparative HPLC [17]. The solvent systems used both for analytical and/or preparative HPLC were: (A) 0.05% TFA; (B) 60% MeCN–40% solvent A or (C) MeCN containing 0.05% TFA. Peptide purity was also monitored by TLC on silica gel G pre-coated Uniplates (0.25 mm, Analtech). 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). Peptides were detected with Ehrlich reagent or chlorine–tolidine [27]. The molecular weight of each peptide was determined by mass spectrometry (FAB/MS) and the peptide purity was monitored by TLC, HPLC (Table 3) and amino acid analysis (Table 4). For amino acid analysis, peptides were hydrolysed with 6N 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 [36], as previously described [17]. The Trp residues were estimated from the UV absorption of the peptides at 280 nm [37]. The optical rotations of peptides were measured with a Rudolph polarimeter (precision ±0.01°).

(Cyclo S^1-S^6)(S)Pmp-D-Trp-Ile-Gln-Asn-Cys-Pro-Arg-Gly-NH$_2$, ((S)Pmp^1, D-Trp^2, Arg^8)OT, (2, Table I). (S)Pmp(Meb)-D-Trp-Ile-Gln-Asn-Cys(Meb)-Pro-Arg(Tos) Gly-NH_2 assembled by the SP method as described above (250 mg) was dissolved in anhydrous liquid ammonia (200 ml) freshly 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 water (2 l), the pH was adjusted to 7.0 with concentrated ammonium hydroxide and the solution was titrated with 0.01N potassium ferricyanide, until a permanent yellow colour resulted and then a 20% excess of potassium ferricyanide solution was added [34]. After 20 min, the ferrocyanide and ferricyanide salts were removed by stirring for 10 min with AG1 X-2 (Cl^-) ion exchange resin (20 g) and then passing the suspension through a column containing additional ion exchange resin (20 g), with three H_2O (50 ml) washings. The combined filtrate and washings were lyophilized. Analysis of the solid obtained containing the peptide 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, followed by final purification by preparative HPLC. A gradient was run from 0 to 50% B over 50 min, eluting at a rate of 5 ml/min, monitoring the eluent at 280 nm. The purer fractions determined by analytical HPLC, were pooled and lyophilized, yielding antagonist **2** (24 mg). The analogue molecular weight was determined by FAB/MS, and the analogue purity was established by TLC in four separate solvent systems and by analytical HPLC (Table 3) and by amino acid analysis (Table 4).

This procedure, with only minor variations, was used to prepare all the OTAs or their precursors, except for the thioketal analogues **14–16** and the thiosalicylic acid analogue **18** for which deprotection with liquid HF-anisole was performed by methods previously described [17].

For purification of analogues, an isocratic concentration X for solvent B was determined, which would lead to an elution time of 20–30 min. The preparative run was performed with a gradient of 0-X% solvent B for 1h to several hours, suitably chosen to lead to the removal of impurities. For amino acid analyses (Table 4) the Picotag method was used [36]. 1-Adamantylacetic acid was quantified by HPLC using a standard of this acid and monitoring at 220 nm. All analogues gave the expected amino acid analysis ratios ±10%. D-Tryptophan was estimated in peptides by UV spectrophotometry at 280 nm [37]. The lower values found for tryptophan suggest that a peptide may have several moles of AcOH, TFA and/or H_2O, as has been our previous experience [37].

Analogue 3, ((HN)Pmp^1)PA, and acylated peptides thereof (4–9). Treatment of the (Boc-N)Pmp protected-peptide amide with Na/liq NH_3, led to

the (Boc-N)Pmp analogue **4**. Treatment of the (Boc-N)Pmp protected-peptide amide with HF/anisole led to the (HN)Pmp analogue **3**. Removal of the Boc group and neutralization by the usual method led to the (HN)Pmp-peptide resin, which was acylated with For-ONp in DMF, leading to the (For-N)Pmp-peptide resin and from this resin to analogue **5**. Treatment of the (HN)Pmp-peptide resin with a solution of DMF : Ac_2O : DIEA (8 : 1 : 1) by the usual methods led to the (Ac-N)Pmp analogue **6**. Similarly, *tert*-butyl acetic acid and 1-adamantyl acetic acid were activated by DCC in the presence of 1-HOBt in DMF solution and were coupled to (HN)Pmp-peptide-resin leading to the (Bac-N)Pmp analogue **7** and (Adac-N)Pmp analogue **8**, respectively. Treatment of **3** (90 mg) with a solution of potassium cyanate (150 mg, 1.8 mmol) in H_2O (450 µl) and DMF (525 µl) for 1 h, followed by purification by preparative HPLC led to 58 mg of the (Carb-N)Pmp analogue **9**.

Cyclic analogues 10 and 11. The analogues [(HN)Pmp^1, Gly^9]PA and [(HN)Pmp^1, Pen^6, Gly^9]PA were made by the usual methods. A solution of [(HN)Pmp^1, Gly^1]PA (113 mg, 0.1 mmol) in DMF (500 ml) was treated with $NaHCO_3$ (1 mmol), and to the resulting solution was added BOP reagent (155 mg, 0.35 mmol). After 16 h, the reaction was evaporated to a solid residue and purified by preparative HPLC yielding 59 mg of analogue **10**. Analogue **11** was similarly prepared starting with [(HN)Pmp^1, Pen^6, Gly^9]PA (70 mg, 61 mmol), yielding 34 mg of product.

((Keto)Pmp^1)PA and Ketals Thereof (12–16). The [(EDO)Pmp^1]PA, **13**, was made by the usual methodology. Treatment of **13** with 1N HCl for 15 min quantitatively hydrolysed the ketal, and after neutralizing to pH 7 the solution was lyophilized and the product was purified by preparative HPLC yielding the (Keto)Pmp analogue **12**. The thioketals (EDT)Pmp, **26**, (TMDT)Pmp, **27** and (PDT)PMP, **28**, were used to complete the peptides by SPPS and to make their respective analogues **14–16**, which could only be successfully made when HF-anisole was used for protecting group removal.

Analogues 17 and 18. Madac(S-Meb) or Tsa(S-Meb) (3 mmol) were coupled to D-Trp-Ile-Gln-Asn-Cys(Meb)-Pro-Arg(Tos)-Gly-resin (1 mmol), in DMF solution by treatment with 1-HOBt (3 mmol) and DCC (3 mmol), overnight until the reaction was complete. Thereafter the usual methods led to the analogues. However, **18** could only be prepared by removal of the blocking groups with HF-anisole by the usual methods, Na in liquid ammonia gave a very complex mixture from which the product could not be isolated. The physicochemical properties, amino acid analyses and biological properties of analogues **1–18** are shown in Tables 3–6.

(1-L-Amino-3,4-dithiahexane-5,5-(3-thiapentamethylene)-1,6-dicarboxylic acid, (S)Pmp-SS-Cys. The blocking group of (S)PMP(S-Meb) (1.186 g, 4 mmol) dissolved in liquid ammonia (100 ml) was removed by touching the solution with a sodium stick until a blue colour lasted 15–30 s. Cysteine (1.94 g, 16 mmol) was added and the resulting solution was evaporated in a vacuum to a solid mixture. A solution made by dissolving the residue in water (25 ml) and 2N HCl to pH 7, was treated with 1N potassium ferricyanide (4.3 ml). The solution was treated with AG1-X2 (Cl^-) (90 g) and was added to a column with additional resin (30 g). After collecting the eluent, the resin was washed with additional water (15 ml). The pooled eluent was evaporated to dryness in a vacuum. The residue was dissolved in water (8 ml) and 2N HCl was added to pH 3. The solution was evaporated to dryness in a vacuum and the solid formed dissolved in a minimum volume of hot water. The solid formed was collected, mp 190°–191 °C. Anal. ($C_{10}H_{17}N_1O_4S_3$): Calcd.: C, 38.6; H, 5.50; N, 4.50; S, 30.9; Found: C, 38.6; H, 5.57; N, 4.46; S, 30.8.

(1-L-Amino-3,4-dithiahexane-5,5-(3-oxapentamethylene)-1,6-dicarboxylic acid, (O)Pmp-SS-Cys. This mixed disulphide was prepared by a modification of the above method, employing (O)PMP(S-Meb), yielding (O)Pmp-SS-Cys, mp 196°–197 °C. Anal. ($C_{10}H_{17}N_1O_5S_2$): Calcd.: C, 40.7; H, 5.80; N, 4.74; S, 21.7; Found: C, 40.6; H, 5.70; N, 4.75; S, 21.3.

Bioassays

All but four analogues were tested as antagonists of OT uterotonic action as previously described [15]. Uteri isolated from rats in natural oestrus were maintained in Van Dyke–Hastings solution containing 0.5 mM Mg^{2+}. Isometric contractions in response to graded doses of OT were recorded on a chart and were quantified by integrating the area under the peaks obtained. The pA_2 for each antagonist was determined by the method of Schild [38] in at least four separate assays. Four analogues were tested using a modified method [39] in Munsick solution also containing 0.5 mM Mg^{2+}. Rats in

induced oestrus by the injection of oestrogen 48 h before the experiments were used, the height of the single 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 also calculated according to Schild. To ensure the comparability of the results, two analogues were tested using both procedures, one analogue having a high inhibitory potency (Table 5, analogue **2**) and another having a low activity (Table 5, analogue **3**). There was very good agreement in the values obtained using both methods, especially in the case of the highly active analogue **2**. In the case of the weakly active analogue **3**, determination of its pA_2 was complicated by the observed low agonistic activity. In both cases pure synthetic oxytocin from Bachem was used as a standard. Only the most potent antagonists of the series were also tested in a rat antidiuretic assay performed either on ethanol-anaesthetized rats [15] or conscious rats in a modified Burn's arrangement [39]. An *in vivo* pA_2 in the antidiuretic assay was calculated as the negative logarithm to 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.

RESULTS AND DISCUSSION

A total of 20 analogues were synthesized and evaluated. The results are summarized in Tables 1–6. The parent acid, β,β-pentamethylene-β-mercaptopropionic acid, Pmp, was subjected to substitutions at position 3 of the pentamethylene group by replacing the 3-CH_2 with O, S, NH and C=O (analogues **1–3** and **12**). The O substitution led to a more hydrophilic compound and a weaker antagonist than PA (Table 5, analogue **1**). The much more hydrophilic substituents, NH and C=O, led to extremely weak antagonists (Table 5, analogues **3** and **12**). On the other hand, substitution with S led to analogue **2** with a higher potency ($pA_2 = 8.08$) than that of PA. This is not surprising if it is recalled that the earlier OTAs required elimination of the polar amino group of cysteine-1, among other requirements to attain antagonism.

In attempts to reduce the hydrophilicity of the imino group, several derivatives were prepared with acyl groups of increasing lipophilicity and bulkiness. In general, it was found that acylation of analogue **3** led to lower hydrophilicity (comparative HPLC, Table 3) and to improved antagonistic potency, although the potency did not seem to be related to the lipophilicity of the acyl group or its bulk (analogues **4–9**). The most effective of these substitutions led to the *N*-carbamyl derivative, analogue **9**, with a potency similar to that of PA.

Since Hruby proposed that conformational flexibility may be required for transduction of the biological response of oxytocin [40], we attempted next to acylate the imino group by internal acylation with the carboxyl group of Gly^9, which led to the more rigid bicyclo analogue **10**, which was as potent ($pA_2 = 7.8$) as PA. It is not possible to tell whether it is the reduced flexibility of the PA ring that leads to the improved potency or merely acylation of the imino group, which reduces polarity. Another bicyclic analogue was made, featuring further restriction in the flexibility of the two rings, by introducing penicillamine, a β,β-dimethylcysteine, at position 6 common to both rings. This led to analogue **11** with enhanced potency ($pA_2 = 8.17$) and no detectable activity in the antidiuretic assay (Table 6).

We attempted to improve the lipophilicity of the keto-substitution of analogue **12**, by making ketals and thioketals **13–16**, all of which had lower hydrophilicity (comparative HPLC, Table 3) and were more potent than the keto-analogue, but none was a very potent OTA.

We also made [2-mercapto-2-adamantaneacetic $acid^1$]PA, [$Madac^1$]PA, **17**, $pA_2 = 7.87$, which is as potent as PA but its synthesis is laborious and gives low yields. Analogue **18**, [Tsa^1]PA, was a weak OTA.

Since substitution of PA with (S)Pmp appeared to be the most promising and in view of the good increase in antagonist potency obtained with the substitution with Pen-6 in analogue **11**, analogues [Pen^6]PA, **19** and [(S)Pmp^1, Pen^6]PA, **20** were prepared and found to be very highly potent, with $pA_2 = 8.72$ and 8.86, respectively.

CONCLUSIONS

The significant increase in potency obtained by the use of (S)Pmp^1(analogue **2**, $pA_2 = 8.08$) instead of Pmp^1 in PA ($pA_2 = 7.77$) has led to the use of (S)Pmp as a preferred substituent in the design of OTAs, in attempts to obtain analogues with the high potencies that would be helpful in the treatment of preterm labour. In addition, analogue **2** has good specificity since it is an extremely weak agonist in the antidiuretic assay and shows no antagonistic

action (Table 6), hence the optional modification of Pmp with SPmp is a valuable mode of substitution, raising hopes of designing antagonists with higher specificity of action as purer OT antagonists. The finding that the Pen-6 substitution in the bicyclic analogue **11**, led to a potent OTA, inspired us to the design and synthesis of the potent analogues [Pmp^1, Pen^6]PA **19**, and [(S)Pmp^1, Pen^6]PA **20**, with $pA_2 = 8.72$ and 8.86, respectively. Additionally, analogue **20** had extremely weak activity in the antidiuretic assay (Table 6). These findings raise the expectation that analogue **20** might be useful for studies on the inhibition of the biological effects of oxytocin and clinically in the prevention of preterm labour. Of additional interest for the design of selective OT antagonists is the significant increase in potency in the bicyclic analogue **11**, with no detectable activity in the antidiuretic assay. It will be further studied as a potentially specific oxytocin antagonist.

Acknowledgements

This work was supported in part by grant HD-22567 from the National Institute of Child Health and Human Development, and by a research fellowship from Abbott Laboratories. We thank Noelia Herrera, Marilyn Davis and Malgorzata Plackova for performing the uterotonic and antidiuretic bioassays.

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. 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.
3. Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term and preterm. *Endocrine Reviews* 2000; **21**: 514–550.
4. Leveno KJ, Cunningham FG. β-Adrenergic agonists for preterm labor. *N. Engl. J. Med.* 1992; **327**: 349–351.
5. 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.
6. 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.
7. 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.
8. 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.
9. 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 Publishers: Dordrecht, 1999; 618–622.
10. Schulz H, du Vigneaud V. Synthesis of 1-L-penicillamine-oxytocin, 1-D-penicillamine-oxytocin, and 1-deaminopenicillamine-oxytocin, potent inhibitors of the oxytocic response of oxytocin. *J. Med. Chem.* 1966; **9**: 647–650.
11. Vavrek RJ, Ferger MF, Allen GA, Rich DH, Blomquist AT, du Vigneaud V. Synthesis of three oxytocin analogs related to [1-deaminopenicillamine]oxytocin possessing antioxytocic activity. *J. Med. Chem.* 1972; **15**: 123–126.
12. 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.
13. 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.
14. Flouret G, Brieher W, Mahan K, Wilson L Jr. Oxytocin antagonists featuring D-tryptophan at position 2. In *Peptides*, Jüng G, Bayer E (eds). Walter de Gruyter: New York, 1989; 549–551.
15. 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.
16. 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. Obst. Gynecol.* 1990; **163**: 1875–1882.
17. 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.
18. 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.

19. 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.
20. Lammek B, Rekowski P, Kupryszewski G, Melin P, Ragnarsson U. Synthesis of arginine-vasopressin, modified in positions 1 and 2, as antagonists of the vasopressor response to the parent hormone. *J. Med. Chem.* 1988; **31**: 603–606.
21. Newlander KA, Bryan HG, Callahan JF, Eggleston DS, Moore ML, Yim NCF, Huffman WF, Jackman LM. Synthesis of 4-substituted β,β-pentamethylene-β-mercaptopropionic acids. In *Peptides, Chemistry, Structure and Biology*, Rivier JE, Marshall GR (eds). ESCOM: Leiden, 1990; 283–286.
22. Lammek B, Derdowska I, Wierzba T, Juzwa W. Synthesis of four new antagonists of arginine-vasopressin (AVP) containing thioacids at position 1 and their vasoconstrictor activity towards isolated mesenteric arterial vessels of rats. *Collect. Czech. Chem. Commun.* 1991; **56**: 491–498.
23. Holton P. A modification of the method of Dale and Laidlaw for standardization of posterior pituitary extract. *Br. J. Pharmacol.* 1948; **3**: 328–334.
24. Yim NCF, Huffman WF. A facile synthesis of β-(S-benzylmercapto)-β,β-cyclopentamethylene-propionic acid. *Int. J. Pept. Prot. Res.* 1983; **21**: 568–570.
25. Fieser LF, Fieser M. Ethanedithiol. In *Reagents for Organic Synthesis*. Wiley: New York, NY, 1967; 356–357.
26. Merrifield RB. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 1963; **85**: 2149–2154.
27. Stewart JM, Young JD. In *Solid Phase Peptide Synthesis*, 2nd edn. Pierce Chemical Co: Rockford, IL, 1984; 1–176.
28. Gisin BF. Preparation of Merrifield resins through total esterification with cesium salts. *Helv. Chim. Acta* 1973; **56**: 1476–1482.
29. 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.
30. 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.
31. 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.
32. 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.
33. 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.
34. Hope DB, Murti VVS, du Vigneaud V. A highly potent analogue of oxytocin, desamino-oxytocin. *J. Biol. Chem.* 1962; **237**: 1563–1566.
35. 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.
36. Bidlingmeier BA, Cohen SA, Tarvin TL. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.* 1984; **336**: 93–104.
37. 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.
38. Schild HO. pA, a new scale for the measurement of drug antagonism. *Br. J. Pharmacol.* 1947; **2**: 189–206.
39. 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.
40. Hruby VJ, Chow MS, Smith DD. Conformational and structural considerations in oxytocin receptor binding and biological activity. *Annu. Rev. Pharmacol. Toxicol.* 1990; **30**: 501–534.
41. Flouret G, Majewski T, Brieher W, Balaspiri L, Mahan K, Wilson L Jr. Oxytocin antagonists with analogs of 3-mercaptopropionic acid at position 1. In *Peptides 1992*, Schneider CH, Eberle AN (eds). ESCOM: Leiden, 1993; 700–701.
42. Flouret G, Majewski T, Brieher W, Pak SC, Wilson L Jr, Kar L, Johnson M. Potent antagonists of oxytocin substituted with analogues of β,β-pentamethylene-β-mercaptopropionic acid. In *Peptides 1994*, Maia HLS (ed.). ESCOM: Leiden, 1995; 341–342.

