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ORIGINAL ARTICLE

Synthesis and biological activity of oxytocin analogues containing unnatural amino acids in position 9: structure activity study

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Abstract We report the solid phase synthesis and some pharmacological properties of 24 oxytocin (OT) analogues. Basic modifications at position 9 (introduction of L- or D-β-(2-thienyl)-alanine [L- or D-Thi], or L- or D-3-Pyridylalanine [L- or D-3-Pal]) were combined with D-tyrosine(OEthyl) [D-Tyr(Et)] or D-1-naphthylalanine [D-1-Nal] in position 2 and β-mercaptopropionic acid (Mpa) in position 1 modifications in altogether 14 analogues. Additionally, 8 analogues having α-aminoisobutyric acid [Aib] or D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (D-Tic) or diethylglycine (Deg) in position 9 and D-Tyr(Et) or D-1-Nal or D-Tic in position 2 and Mpa or Pen ($\beta\beta$-dimethylcysteine) in position 1 were prepared. Two of these analogues have one more modification in position 6, i.e. Pen. Furthermore, two analogues having Mpa in position 1 and D-Tyr(Et) or D-1-Nal in position 2 were prepared for comparison purposes. The analogues were tested for rat uterotonic activity in vitro, in the rat pressor assay and for binding affinity to human OT receptor. The analogue having the highest anti-oxytocic activity was [Mpa^1, D-Tyr(Et)^2, Deg^9]OT ($pA_2 = 8.68 \pm 0.26$); this analogue was also selective.

Keywords Oxytocin antagonists · Position 9 · Unnatural amino acids · Biological activity · Binding affinity

Abbreviations

OT	Oxytocin
Mpa	β-Mercaptopropionic acid
[Mpa^1]OT	Deamino-oxytocin
Aib	α-Aminoisobutyric acid
Deg	Diethylglycine
1-Nal	1-Naphthylalanine
3-Pal	3-Pyridylalanine
Pen	$\beta\beta$-Dimethylcysteine
Thi	β-(2-Thienyl)-alanine
Tic	1,2, 3, 4, -Tetrahydroisoquinoline-3-carboxylic acid
Tyr(Et)	Tyrosine(OEthyl)
Fmoc	9-Fluorenylmethoxycarbonyl
Bu^t	*t*-Butyl
Trt	Trityl
DIC	Diisopropylcarbodiimide
HOBt	1-Hydroxybenzotriazole
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
TBAF	Tetrabutylammonium fluoride
TFA	Trifluoroacetic acid
HPLC	High-performance liquid chromatography
ESI–MS	Electrospray ionization–mass spectrometry

Abbreviations of common amino acids are in accordance with the recommendations of IUPAC-IUB Joint Commission on Biochemical Nomenclature: Arch Biochem Biophys 206 (1988) v–xxii, J Biol Chem 264 (1989) 668–673, J Peptide Sci 12 (2006) 1–8.

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Introduction

Oxytocin (OT), a physiologically important nonapeptide hormone and neurotransmitter containing a 20-membered tocin ring (from Cys-1 to Cys-6) and an acyclic tripeptide tail (from Pro-7 to $GlyNH_2$-9) (see Fig. 1) regulates several physiological functions, such as milk ejection, uterine contractions, vascular and cardiac relaxation, interferes with salt and water balance, and is known to play a role in social and reproductive behaviour and emotions (Gimpl and Fahrenholz 2001; Lippert et al. 2003). Subsequent studies have demonstrated synthesis of OT mRNA and the peptide in several peripheral tissues, including ovary, endometrium or deciduas (Lefebvre et al. 1992).

The multiple established and proposed actions of OT are all mediated by one type of OT receptor (Gimpl and Fahrenholz 2001), a member of the super-family of seven-transmembrane G-protein coupled receptors (GPCRs) (Phaneuf et al. 1993), which has no subtypes but is structurally related to the vasopressin receptors. The effect on uterine contractions is of major pharmacological importance because OT is the strongest uterotonic agent known and is commonly used in obstetrical practice to speed up labour.

Preterm birth is defined by the World Health Organization as birth occurring prior to 37 weeks gestation. Preterm labour occurs in up to 20% of all human pregnancies and leads to preterm delivery with a variety of neonatal health problems, e.g. neurosensory deficits, respiratory distress syndrome, low body weight, subnormal height, while can also possibly result in death of the newborn (Hall et al. 1997; Moster et al. 2008). Drugs with a variety of pharmacologic actions, but not specific, have been developed to suppress contractions associated with preterm labour. These include magnesium sulphate, beta-adrenergic agonists (terbutaline and ritodrine), prostaglandin synthetase inhibitors (indomethacin and rofecoxib) or calcium channel blockers (nifedipine) (Gyetvai et al. 1999; Giles and Bisits 2007). To date, the use of most of these drugs is compromised by side effects and limited efficacy. Thus, there is a need for optimization of existing therapeutic options and discovery of new drugs for the management of preterm labour.

OT receptor antagonists represent relatively new class of tocolytics under investigation. They afford greater specificity and can be expected to exhibit improved efficacy and risk profiles (Thornton et al. 2001; Lamont 2003; Åkerlund 2006; Allen et al. 2006). Such compounds would allow more effective treatment of preterm labour with a lower risk of side effects. Moreover, the efficacy of the OT antagonist Atosiban (trade name Tractocile) to inhibit premature uterine contractions in humans is indicative of a role for OT in human labour (Goodwin et al. 1994; Romero et al. 2000; Moutquin et al. 2001). It should be noted that a number of peptide oxytocin antagonists with higher affinity and selectivity for the human oxytocin receptor than atosiban have been synthesized over the last 20 years. These analogues are promising new candidates for discovery and design potential tocolytic agents for the prevention of premature labour (Manning et al. 2008). Furthermore, barusiban is a new OT receptor antagonist with a high affinity for the human oxytocin receptor, currently being developed for the treatment of preterm labour, which is under investigation (Nilsson et al. 2003; Reinheimer et al. 2005; Reinheimer et al. 2006; Thornton et al. 2009). Moreover, in a view of the widespread OT-related actions, OT antagonists may not only be studied as promising inhibitors of preterm labour but may also prove useful in the treatment of dysmenorrhoea, benign prostatic hyperplasia and psychiatric illnesses such as anxiety, sexual dysfunctions, eating disorders, etc. (Borthwick 2006).

Generally, inhibitors of the uterotonic activity of OT have been produced by the introduction of bulky β-carbon substituents into position 1 and/or by substitution of L-tyrosine in position 2 of OT with an aromatic D-amino acid. Previous structure–activity studies in our laboratory revealed that minimal structural changes of the OT molecule could provide quite potent antagonists (Lebl 1987; Assimomytis et al. 1994; Assimomytis et al. 1996). Furthermore, the importance of conformational flexibility for agonist activity and relative conformational rigidity and steric constraints for antagonism has been emphasized (Urry and Walter 1971; Hill et al. 1990; Lebl et al. 1992).

The proper orientation and the sequence of the C-terminal tripeptide are critical for obtaining high potency OT analogues (Ting et al. 1980; Hruby 1986; Fragiadaki et al. 2003). Therefore, structural modifications of the side-chain moieties in the C-terminal tripeptide might lead to analogues with variable biological properties at different OT target tissues. Moreover, synthesis of analogues of biologically active peptides in which structural modifications should enhance resistance towards enzymatic cleavage of peptide bonds, resulting in prolonged course of action, is of great importance. In this regard, OT analogues with varying ring sizes wherein sulphur is replaced by carbon (1,6-carba analogues) showed significantly improved metabolic lifetimes and increased biological stability (Stymiest et al. 2005). It has also been reported that modification of the C-terminal tripeptide side chain influences the chymotryptic cleavage of Tyr-Ile peptide bond in the OT molecule (Hlaváček and Frič 1989).

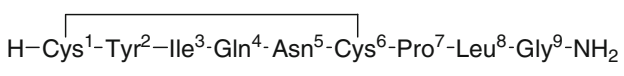


Fig. 1 Amino acid sequence of oxytocin

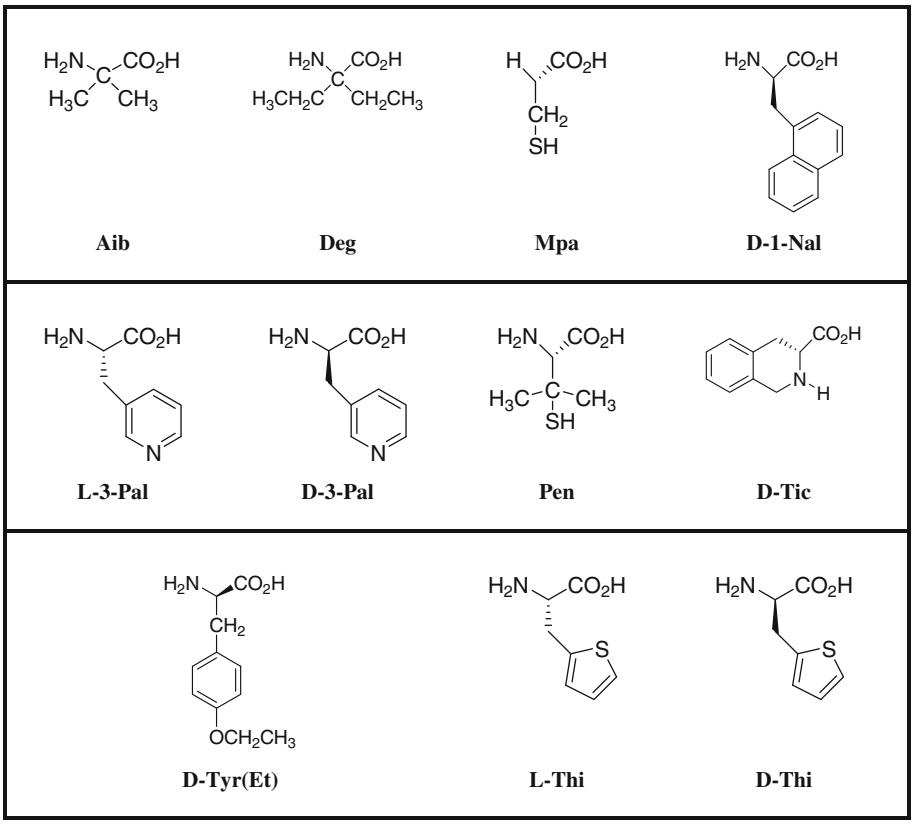


Fig. 2 Structure of the unnatural amino acids used in the present study

In continuing our work aimed at the design of selective OT antagonists (Fragiadaki et al. 2003, 2007), we investigated the effectiveness of modifications in position 9 by unnatural amino acids (Fig. 2). Here, we present the synthesis of twenty-four new OT analogues which contain in position 9 the following residues: L- or D-β-(2-thienyl)-alanine (Thi), L- or D-3-pyridylalanine (3-Pal), α-aminoisobutyric acid (Aib), D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (D-Tic), and diethylglycine (Deg) alone or in combination with D-tyrosine(OEthyl) [D-Tyr(Et)], D-1-naphthylalanine [D-1-Nal] and D-Tic in position 2 (Fig. 3, analogues **1–6, 8–17,** and **19–24**). Two analogues have one more modification in position 6, i.e. $\beta\beta$-dimethylcysteine [Pen] (**13** and **16**). For comparison purposes we have synthesized peptides [Mpa^1, D-Tyr(Et)^2] (analogue **7**) and [Mpa^1, D-1-Nal^2]OT (analogue **18**). The structures of the analogues are summarized in Fig. 3. All 24 analogues were tested for biological potency in rat uterotonic test in vitro and rat pressor test and for binding affinity to human OTR.

Materials and methods

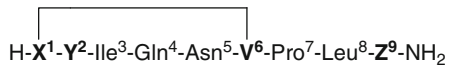
Materials

All 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids were obtained from CBL Patras; Rink Amide MBHA resin from Nova Biochem; the derivative *S*-Trityl-β-mercaptopropionic acid [Mpa(Trt)] was prepared according to the literature (Photaki et al. 1979). Peptide reagents were purchased from Bachem AG and Nova Biochem. All solvents and reagents used for solid-phase synthesis were of analytical quality and used without further purification.

Peptide synthesis and purification

The analogues were synthesized by Fmoc solid phase methodology (Fields and Noble 1990) utilizing either Rink Amide MBHA resin (Rink 1987) or 2-chlorotrityl-chloride resin (Barlos et al. 1991) bearing a Rink-Bernatowitz linker (Bernatowitz et al. 1989) as solid support to provide the peptide amide. Fmoc-protected amino acids were used with the Trityl group (Trt) [Asn, Gln, Cys, Pen] and the *t*-Butyl group (Bu^t) [Tyr] as side-chain protection groups. Stepwise synthesis of a peptide analogue was achieved with diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) as coupling agents in dimethylformamide (DMF) (Köning and Geiger 1970; Sarantakis et al. 1976) in 2.0 (Fmoc-amino acid), 2.2 (DIC) and 3.0 (HOBt) molar excess for 2 h at room temperature. Completeness of the reaction was monitored by the Kaiser test (Kaiser et al. 1970), except in the case of Pro and Tic residues where the end of the reaction was monitored by the Chloranil test (Vojkovsky 1995). The Fmoc groups were removed by treatment with 20% piperidine in

Fig. 3 Structure of oxytocin analogues



Analogue	X^1	Y^2	V^6	Z^9
1				L-Thi
2				L-3-Pal
3				D-3-Pal
4	Mpa			L-Thi
5	Mpa			L-3-Pal
6	Mpa			D-3-Pal
7	Mpa	D-Tyr(Et)		
8	Mpa	D-Tyr(Et)		L-Thi
9	Mpa	D-Tyr(Et)		D-Thi
10	Mpa	D-Tyr(Et)		L-3-Pal
11	Mpa	D-Tyr(Et)		D-3-Pal
12	Mpa	D-Tyr(Et)		Aib
13	Mpa	D-Tyr(Et)	Pen	Aib
14	Mpa	D-Tyr(Et)		Deg
15	Mpa	D-Tyr(Et)		D-Tic
16	Mpa	D-Tyr(Et)	Pen	D-Tic
17	Mpa	D-Tic		Aib
18	Mpa	D-1-Nal		
19	Mpa	D-1-Nal		L-Thi
20	Mpa	D-1-Nal		D-Thi
21	Mpa	D-1-Nal		L-3-Pal
22	Mpa	D-1-Nal		D-3-Pal
23	Mpa	D-1-Nal		Aib
24	Pen	D-1-Nal		Aib

DMF for 30 min. Cleavage of peptide-linker bond and removal of the side chain protecting groups were accomplished using solution of trifluoroacetic acid (TFA)/1,2-ethanedithiol/triethylsilane/water (94:2.5:2.5:1:1, v/v, 15 ml/g peptide resin) in 4 h at room temperature. The released peptides were precipitated upon concentration of solvent and addition of cold diethyl ether. The formation of the disulphide bridge (cyclization) was mediated either by the dimethylsulphoxide (DMSO)/water method in a solution of 20% (DMSO)/H_2O for 18–30 h at room temperature (Tam et al. 1991) or via the carbon tetrachloride/tetrabutylammonium fluoride (CCl_4/TBAF) (Maruyama et al. 1999) method in a solution of 20% CCl_4 in acetonitrile in the presence of 150 µL of 1 M TBAF in dry tetrahydrofuran for 30 min. In both methods of cyclization we used 1.5 mL of the oxidative solution per mg of crude linear peptide. Completeness of the formation of the disulfide bond was monitored either by the Ellman's test or by analytical HPLC.

The products were purified by gel filtration chromatography on Sephadex G-15 using 25% acetic acid as the eluent with the exception of Thi residue containing peptides for which 40% acetic acid was used. Final purification was achieved by semi-preparative HPLC (Mod.10 ÄKTA, Amersham Biosciences, Piscataway, USA) on reversed-phase support LICHROSORB C_{18} (5 µm, 250 × 8 mm) with a linear gradient from 5 to 85% acetonitrile (0.1% TFA) for 30 min at a flow rate of 1.5 ml/min and UV detection at 230 and 254 nm. The appropriate fractions were pooled and lyophilized. All products gave single spots on thin layer chromatography (Merck pre-coated silica gel plates, type G_{60}-F_{254}) in the solvent systems: (A) 1-butanol:acetic acid:water (4:1:5, upper phase), (B) 1-butanol:acetic acid:water:pyridine (15:3:10:6) and (C) acetonitrile: water (5:1). Analytical HPLC (Pharmacia LKB-2210) equipped with a Nucleosil 100 C_{18} column (5 µm; 250 × 4.6 mm) produced single peaks with at least 98% of the total peptide peak integrals. The solvent system used was the same as that

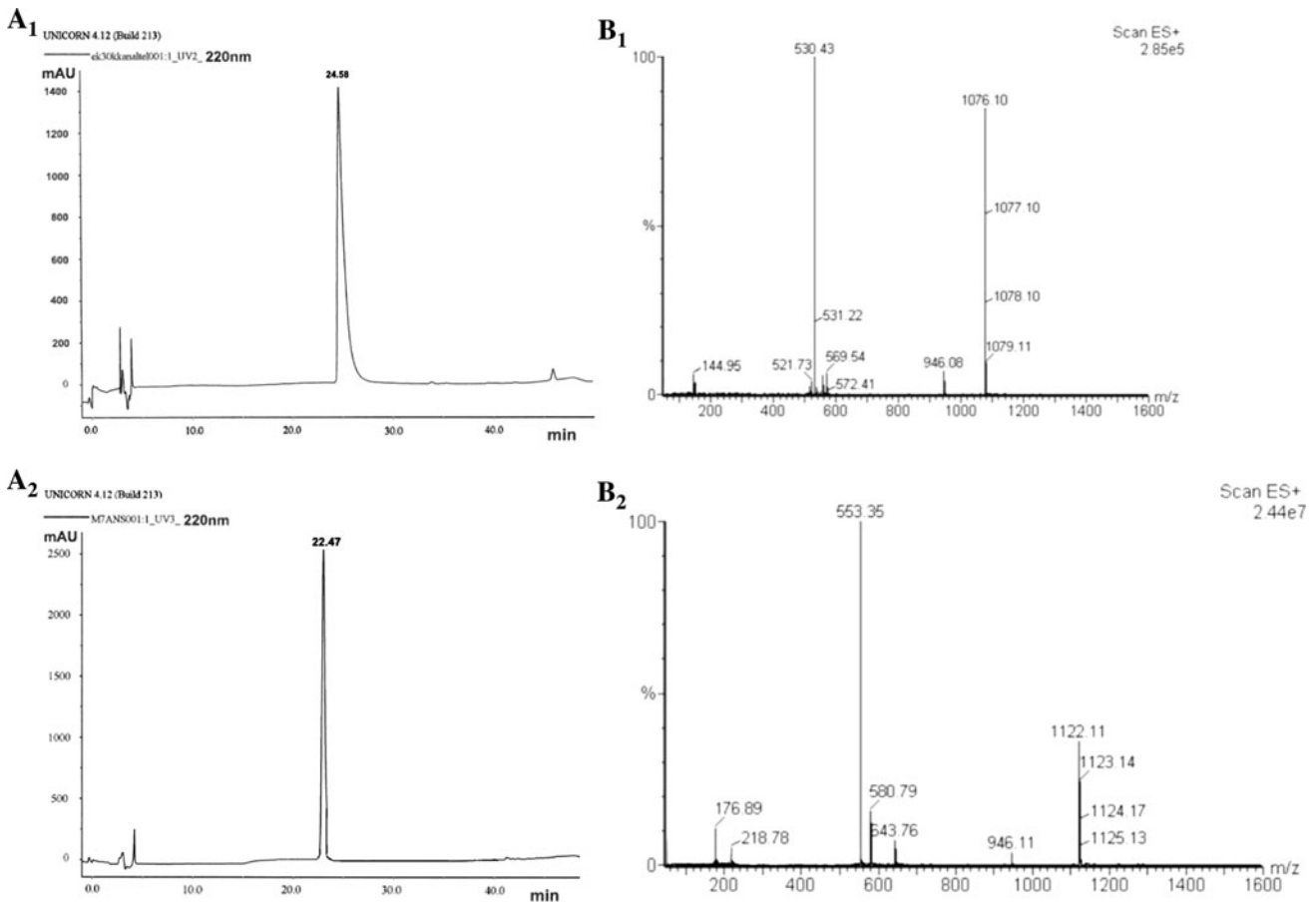


Fig. 4 **a** Analytical HPLC chromatograms of pure analogues [Mpa[1], D-Tyr(Et)[2], Deg[9]]OT (**A_1**) and [Mpa[1], D-Tyr(Et)[2], D-Tic[9]]OT (**A_2**) [UV detection at 220 nm]; **b** Mass spectra of analogues [Mpa[1], D-Tyr(Et)[2], Deg[9]]OT (**B_1**) and [Mpa[1], D-Tyr(Et)[2], D-Tic[9]]OT (**B_2**) resulting from ESI–MS analysis

for the semi-preparative HPLC. The final verification of the peptide sequence was achieved by Electrospray Ionisation-Mass Spectrometry (ESI–MS, Micromass-Platform LC instrument). An example of analytical HPLC-chromatograms of pure peptides and ESI–MS spectra are shown in Fig. 4 for analogues **14** and **15**. The physicochemical properties of the new analogues are summarized in Table 1.

Biological assay methods

The uterotonic activity was determined in vitro on an isolated strip of rat uterus in the absence of magnesium (Holton 1948; Munsick 1960; Slaninová 1987). In principle, cumulative dosing was applied in most experiments, i.e. doses of the standard (in the presence or absence of analogues) or of the analogue were added successively to the uterus in the organ bath, in doubling concentrations and at 1 min intervals without the fluid being changed, until the maximal response was obtained. The agonistic activity was determined by comparing the threshold doses of oxytocin with the analogue. In cases where the analogues were not able to reach the same maximal response as oxytocin, the single dosing procedure was employed. The inhibitory potencies are expressed as pA_2 values. The pA_2 values represent the negative logarithm to the base 10 of the molar concentration of an antagonist which reduces the response to 2 *x* units of the agonist to the response to *x* units of the agonist (Slaninová 1987). Each analogue was tested on uteri from four to five different rats. Pressor activity was determined on phenoxybenzamine-treated male rats (Dekanski 1952). The responses to standard doses of oxytocin or vasopressin were stable for several hours, without problems with tachyphylaxis.

Binding affinity determination

Determination of binding affinity to human OTR was performed basically as described (Fahrenholz et al. 1984) using tritiated oxytocin from NEN Life Science, Boston, MA, USA. In brief, a crude membrane fraction of HEK OTR cells, i.e. HEK cells having stable expressed human

Table 1 Physiochemical properties of oxytocin analogues used in the present study

Analogues		Yield[a]	HPLC[b]	TLC, R_f^c			MW	$[M + 1]^{+}$ [d]
		(%)	t_R (min)	A	B	C		
1	[L-Thi^9] OT[e]	58	18.47	0.54	0.58	0.55	1,104.32	1,105.15
2	[L-3-Pal^9]OT	69	14.09	0.50	0.55	0.52	1,099.28	1,100.13
3	[D-3-Pal^9]OT	67	13.71	0.51	0.56	0.50	1,099.28	1,100.25
4	[Mpa^1, L-Thi^9]OT	56	19.48	0.68	0.70	0.55	1,089.31	1,090.15
5	[Mpa^1, L-3-Pal^9]OT	68	17.69	0.62	0.65	0.49	1,084.27	1,085.02
6	[Mpa^1, D-3-Pal^9]OT	66	19.89	0.60	0.63	0.50	1,084.27	1,085.18
7	[Mpa^1, D-Tyr(Et)^2]OT	70	22.96	0.48	0.67	0.35	1,020.23	1,021.30
8	[Mpa^1, D-Tyr(Et)^2, L-Thi^9]OT	55	25.07	0.81	0.76	0.51	1,089.31	1,090.20
9	[Mpa^1, D-Tyr(Et)^2, D-Thi^9]OT	57	28.94	0.87	0.73	0.52	1,089.31	1,090.25
10	[Mpa^1, D-Tyr(Et)^2, L-3-Pal^9]OT	63	20.10	0.77	0.70	0.59	1,112.32	1,113.28
11	[Mpa^1, D-Tyr(Et)^2, D-3-Pal^9]OT	65	22.79	0.76	0.71	0.57	1,112.32	1,113.24
12	[Mpa^1, D-Tyr(Et)^2, Aib^9]OT	66	25.12	0.77	0.71	0.50	1,049.26	1,050.18
13	[Mpa^1, D-Tyr(Et)^2, Pen^6, Aib^9]OT	63	30.44	0.67	0.73	0.46	1,077.32	1,078.30
14	[Mpa^1, D-Tyr(Et)^2, Deg^9]OT	68	24.58	0.74	0.72	0.70	1,077.32	1,078.10
15	[Mpa^1, D-Tyr(Et)^2, D-Tic^9]OT	63	22.47	0.73	0.70	0.48	1,123.34	1,124.17
16	[Mpa^1, D-Tyr(Et)^2, Pen^6, D-Tic^9]OT	61	28.97	0.76	0.71	0.51	1,151.40	1,152.16
17	[Mpa^1, D-Tic^2, Aib^9]OT	64	23.89	0.66	0.60	0.48	1,017.22	1,018.20
18	[Mpa^1, D-1-Nal^2]OT	69	26.68	0.68	0.76	0.55	1,026.23	1,027.20
19	[Mpa^1, D-1-Nal^2, L-Thi^9]OT	56	29.32	0.83	0.75	0.51	1,123.37	1,124.31
20	[Mpa^1, D-1-Nal^2, D-Thi^9]OT	55	31.09	0.89	0.74	0.59	1,123.37	1,124.18
21	[Mpa^1, D-1-Nal^2, L-3-Pal^9]OT	65	23.74	0.29	0.61	0.68	1,118.33	1,119.15
22	[Mpa^1, D-1-Nal^2, D-3-Pal^9]OT	63	24.97	0.46	0.75	0.61	1,118.33	1,119.15
23	[Mpa^1, D-1-Nal^2, Aib^9]OT	64	24.55	0.67	0.86	0.55	1,055.27	1,056.13
24	[Pen^1, D-1-Nal^2, Aib^9]OT	62	21.59	0.64	0.60	0.34	1,098.34	1,099.23

[a] Yields were calculated on the basis of the amino acid content of the resin. All peptides were at least 98% pure

[b] For elution conditions, see the Experimental Section

[c] Solvent systems and conditions are given in the Experimental Section

[d] Data obtained by ESI–MS

OT receptor (kindly donated by Dr. G. Gimpl (Gimpl et al. 1997)), was incubated with [^{3}H]OT (2 nM) and various concentrations of peptides (0.1–10,000 nM) for 30 min at 35°C. The total volume of the reaction mixture was 0.25 ml and the buffer used was 50 mM HEPES at pH 7.6 containing 10 mM $MnCl_2$ and 1 mg/ml bovine serum albumin. The reaction was terminated by quick filtration on a Brandel cell harvester. Binding affinities were expressed as IC_{50} values calculated from the binding curves using GraphPad Prism 3.02.

Results

Peptide synthesis and purification

All analogues shown in Table 1 were synthesized either manually or automatically on the Rink Amide MBHA resin or the 2-chlorotrityl-chloride resin bearing a Rink-Bernatowitz linker as solid support using standard coupling procedures and Fmoc/Bu^t strategy. Except for the cases of the Fmoc-Gln(Trt)-OH coupling to Fmoc-Cys(Trt)-OH or Fmoc-Pen (Trt)-OH whereby the coupling reaction lasted 1 h longer, couplings were complete within 2.5 h.

The overall yield of the syntheses of the OT analogues was in the range 55–70% (calculated on the amount of linker initially coupled to the resin). Higher yields were obtained using the Rink Amide MBHA as solid support. The purification of the Thi residue containing analogues gives lower yields than those of the rest of the analogues because of their insolubleness.

The formation of the disulfide bond (cyclization) was carried out according to the following two oxidized methods: 1: dimethylsulphoxide (DMSO)/water method and 2: carbon tetrachloride/tetrabutylammonium fluoride (CCl_4/TBAF) method. The cyclization was quantitative by

Table 2 Biological activities of oxytocin analogues

Analogues		Biological activity			
		Uterotonic in vitro		Pressor	
		Agonistic (IU/mg)	Antagonistic (pA_2)	Agonistic[b] (IU/mg)	Antagonistic (pA_2)
I	**Oxytocin**[a]	**546**		**3.10**	
1	[L-Thi^9] OT	4.3 ± 0.8		0	0
2	[L-3-Pal^9]OT	0.25		0	
3	[D-3-Pal^9]OT	~4	~7.0	0	0
II	**[Mpa^1]OT[a] (deamino-oxytocin)**	**803**			
4	[Mpa^1, L-Thi^9]OT	5.5 ± 0.9		0	<5.8
5	[Mpa^1, L-3-Pal^9]OT	~1.1	~7.0	0	0
6	[Mpa^1, D-3-Pal^9]OT	~7.0	~7.2		
III	**[D-Tyr(Et)^2]OT[a]**	0	**7.36**		
7	[Mpa^1, D-Tyr(Et)^2]OT		7.82 ± 0.07	0	0
8	[Mpa^1, D-Tyr(Et)^2, L-Thi^9]OT		8.35 ± 0.26	0	0
9	[Mpa^1, D-Tyr(Et)^2, D-Thi^9]OT		8.61 ± 0.14	0	6.19 ± 0.33
10	[Mpa^1, D-Tyr(Et)^2, L-3-Pal^9]OT		7.82 ± 0.08	0	0
11	[Mpa^1, D-Tyr(Et)^2, D-3-Pal^9]OT		8.13 ± 0.07	0	0
12	[Mpa^1, D-Tyr(Et)^2, Aib^9]OT		8.22 ± 0.19	0	0
13	[Mpa^1, D-Tyr(Et)^2, Pen^6, Aib^9]OT		8.37 ± 0.21	0	0
14	[Mpa^1, D-Tyr(Et)^2, Deg^9]OT		8.68 ± 0.26	0	0
15	[Mpa^1, D-Tyr(Et)^2, D-Tic^9]OT		8.37 ± 0.21	0	0
16	[Mpa^1, D-Tyr(Et)^2, Pen^6, D-Tic^9]OT		8.28 ± 0.22	0	0
17	[Mpa^1, D-Tic^2, Aib^9]OT		6.75 ± 0.35		~5.60
18	[Mpa^1, D-1-Nal^2]OT		7.92 ± 0.21	0	0
19	[Mpa^1, D-1-Nal^2, L-Thi^9]OT		7.83 ± 0.17	0	0
20	[Mpa^1, D-1-Nal^2, D-Thi^9]OT		8.08 ± 0.13	0	0
21	[Mpa^1, D-1-Nal^2, L-3-Pal^9]OT		7.02 ± 0.12	0	0
22	[Mpa^1, D-1-Nal^2, D-3-Pal^9]OT		7.77 ± 0.15	0	0
23	[Mpa^1, D-1-Nal^2, Aib^9]OT		8.48 ± 0.08	0	0
24	[Pen^1, D-1-Nal^2, Aib^9]OT		8.36 ± 0.23	0	0
IV	**[Mpa^1, D-Tyr(Et)^2, Thr^4, Orn^8]OT (Atosiban)[a]**	**0**	**8.29 ± 0.05**	**0.02 ± 0.02**	**0**

[a] The biological assays for the oxytocin analogues **1-24** were performed as outlined in the text. The values are averages ± SEM of at least three experiments. The biological activities of the other analogues reported here as references are taken from the literature: for oxytocin and deamino-oxytocin, see Hill et al. (1990), for [D-Tyr(Et)^2]OT see Ježek et al. (1994) and for Atosiban, see Melin et al. (1986). If no SEM is given for the reference compound, it means that it was not given in the original papers

[b] 0 means no activity up to the dose 0.16 mg/kg of exp. animal

[c] Means no activity or very low inhibitory activity $pA_2 < 6$

both applied methods, whereas using the carbon CCl_4/TBAF method the reaction time for achieving completion of the cyclization was 10 min, the yields and purity of the crude peptides, according to their chromatographic profiles, were lower because of the presence of residual TBAF left after solvent evaporation.

Finally, ESI mass spectrometry revealed that the purified peptides were the desired products and their purity determined by analytical HPLC was higher than 98%.

Biological activity (rat)

Biological evaluation of the new analogues (**1–24**) is summarized in Tables 2 and 3. In analogues **1–6** amino acid Gly in position 9 of oxytocin and deaminooxytocin was replaced by unnatural amino acids with a heterocyclic ring in side-chain, e.g. L-Thi or L- or D-3-Pal. Analogue **1** ([Thi^9]OT) and analogue **2** [L-3-Pal^9]OT were found to be agonists in the uterotonic in vitro assay with a potency approximately 130 and 2,200 times, respectively, lower

Table 3 Binding affinities of oxytocin analogues

Analogues		Binding affinity[a] IC_{50} (nM)
	Oxytocin	2.7 ± 0.2
1	[L-Thi^9] OT	570 ± 149
2	[L-3-Pal^9]OT	4,740 ± 287
3	[D-3-Pal^9]OT	393 ± 221
4	[Mpa^1, L-Thi^9]OT	145 ± 58
5	[Mpa^1, L-3-Pal^9]OT	1,636 ± 128
6	[Mpa^1, D-3Pal^9]OT	174 ± 99
7	[Mpa^1, D-Tyr(Et)^2]OT	148 ± 26
8	[Mpa^1, D-Tyr(Et)^2, L-Thi^9]OT	33.6 ± 4.9
9	[Mpa^1, D-Tyr(Et)^2, D-Thi^9]OT	301 ± 3
10	[Mpa^1, D-Tyr(Et)^2, L-3-Pal^9]OT	92 ± 2
11	[Mpa^1, D-Tyr(Et)^2, D-3-Pal^9]OT	33 ± 5
12	[Mpa^1, D-Tyr(Et)^2, Aib^9]OT	150 ± 34
13	[Mpa^1, D-Tyr(Et)^2, Pen^6, Aib^9]OT	80 ± 6
14	[Mpa^1, D-Tyr(Et)^2, Deg^9]OT	98.0 ± 9.2
15	[Mpa^1, D-Tyr(Et)^2, D-Tic^9]OT	46 ± 15
16	[Mpa^1, D-Tyr(Et)^2, Pen^6, D-Tic^9]OT	25 ± 3
17	[Mpa^1, D-Tic^2, Aib^9]OT	1,800 ± 150
18	[Mpa^1, D-1-Nal^2]OT	34.5 ± 2.9
19	[Mpa^1, D-1-Nal^2, L-Thi^9]OT	32.4 ± 15.8
20	[Mpa^1, D-1-Nal^2, D-Thi^9]OT	16.2 ± 5.7
21	[Mpa^1, D-1-Nal^2, L-3-Pal^9]OT	155 ± 15
22	[Mpa^1, D-1-Nal^2, D-3-Pal^9]OT	37.5 ± 6.5
23	[Mpa^1, D-1-Nal^2, Aib^9]OT	44 ± 14
24	[Pen^1, D-1-Nal^2, Aib^9]OT	142 ± 18

[a] The values given are averages ± SEM from three experiments performed in duplicates

than that of the native hormone. D-diastereoisomer containing 3-Pal (analogue **3**) was found to be a partial agonist in the oxytocic assay, with agonistic potency approximately 140 times lower than that of the native hormone (16 times higher than that of the L-counterpart) and weak antagonist with pA_2 value about 7.0. The analogues **1**–**3** showed no pressor activity.

It has been known that substitution of the N-terminal cysteine by a residue without the amino group, i.e. β-mercaptopropionic acid (Mpa) (Hope et al. 1962), enhances the biological activity. Literature data indicate that the neurohypophyseal hormones lacking the N-terminal amino group are inactivated quite slowly (Grzonka et al. 1983) and that the first amino acid plays a decisive role in receptor binding (Tóth et al. 1999). Therefore, we synthesized analogues **4**–**6**. Replacement of Cys^1 by Mpa^1 in analogue **1** did not affect the agonistic activity (analogue **4**) in the uterotonic test; negligible antagonistic activity in the pressor test was detected. In analogue **2**, this change led to partial agonism (analogue **5**) in the oxytocic assay, with agonistic potency approximately 500 times lower than that of the native hormone and weak anti-oxytocin activity (pA_2 value about 7.0). The potency of analogue **3** was not affected (analogue **6**).

Previously reported studies suggested that the D-configuration and hydrophobicity of the aromatic amino acid in position 2 and the proper orientation of the C-terminal glycine carboxamide are critical for obtaining high potency OT analogues (Flouret et al. 1991; Lebl et al. 1992). We have thus synthesized the analogues **8**–**11** and analogues **19**–**22** having D-Tyr(Et) and D-1-Nal in position 2, respectively, and the above-mentioned modifications in positions 1 and 9. Analogues **7** and **18** were synthesized for comparison reasons.

Upon substitution of the residue in position 2 by D-Tyr(Et) or D-1-Nal the analogues **4**–**6** lost the agonistic activity and become strong antagonists (analogues **8**–**11,** $pA_2 = 7.82$–8.61). It is notable that in the pressor test only analogue **9** showed a weak antagonistic activity ($pA_2 = 6.19 \pm 0.33$).

As can be seen from the table, most of the analogues having in position 2 D-1-Nal—a more compact residue than D-Tyr(Et)—showed slightly lower oxytocin antagonism in the uterotonic test, ensuring the major role of D-aromatic amino acid in position 2. Furthermore, all analogues were selective, showing no pressor activity.

In an attempt to further investigate the role of the residue in position 9, we synthesized analogues **12**–**16** and **23**–**24** with residues without a heterocyclic ring in the side chain, e.g. the conformationally interesting lipophilic residues Aib, Deg and D-Tic, without a heteroatom in side-chain. Analogues having combined substitutions in position 1 (Mpa), in position 2 D-Tyr(Et) and in position 9 Aib or Deg or D-Tic retain the oxytocin antagonistic potency. The highest one had analogue **14** ([Mpa^1, D-Tyr(Et)^2, Deg^9]OT, $pA_2 = 8.68 \pm 0.26$). It is notable that further modification with Pen^6 (peptides **13** and **16**) led to no change in the inhibitory potency, ensuring the role of the folding of the tail portion above or below the plane of the ring. Similarly, further Pen^1/Mpa^1 exchange (analogues **23** and **24)** led to no change in inhibitory potency ($pA_2 = 8.48 \pm 0.08$ and $pA_2 = 8.36 \pm 0.23$, respectively).

Finally, D-Tic^2 modification in analogue **12** resulted in an antagonism with substantially lower potency ($pA_2 = 6.75 \pm 0.35$, analogue **17**).

Among the analogues having Aib in position 9, the Tic^2 analogue had considerably lower anti-oxytocic potency than the D-1-Nal and D-Tyr(Et) analogues.

Binding affinity (human receptor)

Table 3 summarizes the binding affinities of the analogues to human OTR permanently expressed on HEK cells. As can be seen, introduction of the conformationally

constrained residues with an aromatic heterocyclic ring at the side chain (L-Thi or L- or D-3-Pal) decreased the binding affinity significantly. The lowest affinity from these analogues with a single modification had analogue **2** ([L-3-Pal^9]OT, IC_{50} = 4,740 ± 287 nM), the highest analogue **3** ([D-3-Pal^9]OT, IC_{50} = 393 ± 221 nM).

Deamination in position 1 of analogues **1**–**3** increased the affinity slightly (compare analogues **1** and **4, 2** and **5, 3** and **6**).

Further modification using introduction of D-Tyr(Et)^2 significantly increased the affinity (compare peptides **4** and **8**; **5** and **10**; **6** and **11**). Replacement of the L-Thi in position 9 with the D-counterpart (analogue **9**) surprisingly decreased the binding affinity to the OT receptor (compare IC_{50} = 33.6 ± 4.9 nM and 301 ± 3 nM for **8** and **9**, respectively).

On the other hand, the D-1-Nal^2/D-Tyr(Et)^2 exchange in analogues **8**–**11** had an inconsistent effect; in the case of analogues **8**, **10** and **11** there was almost no change in affinity (compare with analogues **19**, **21** and **22**, respectively), in the case of the D-Thi^9 analogue there was strong decrease of affinity (compare peptides **9** and **20**). In analogue **12** it improved the affinity about 3.5 times (compare IC_{50} = 150 and 44 nM for analogues **12** and **23**, respectively). Furthermore, replacement of Mpa^1 with Pen in analogue **23** decreased the affinity about three times (compare IC_{50} = 150 and 44 nM for analogues **23** and **24**, respectively).

Modifications of analogue **7** by the introduction of Aib or Deg or D-Tic also influenced the binding affinity insignificantly. The values lie in the range of 46–150 nM.

Finally, analogue **17** with the combination of deamination, D-Tic^2 and Aib^9 modifications, exhibited significantly low binding affinity (IC_{50} = 1,800 nM). This finding is consistent with that previously observed by Fragiadaki et al. (2007), ensuring the role of the side chain of the residue in position 2 for binding to the uterotonic receptor.

Discussion

We have synthesized 24 analogues of OT with the aim to study the influence of modifications in position 9 on the activity of OT and some of its analogues as deamino[D-Tyr(Et)^2]OT or deamino[D-1-Nal^2]OT. We have introduced into position 9 amino acids both containing a heterocyclic ring in the side chain (Thi, 3-Pal) and lipophilic residues without a heteroatom in the side-chain (Aib, Deg and D-Tic). Structural modifications of the side-chain moieties in the C-terminal tripeptide might lead to analogues with variable biological properties at different OT target tissues. Such variations in biological activity could result from conformational changes influencing interactions of the analogues with the receptor, from steric demands, or from changed susceptibility of the molecules to enzymatic cleavage (Hlaváček and Frič 1989).

We have evaluated the potency of the new analogues in uterotonic in vitro test, pressor test and affinity to human OTR stable expressed on the surface of HEK cells. The comparison of the results of biological activity and binding affinity has to be performed with caution as the tests were performed with rats and the binding experiments using human OTR.

As can be seen from the Table 2, the substitution of L-Thi^9, L-3-Pal^9 or D-3-Pal^9 for Gly^9 in OT or deamino OT (analogues **1**–**6**) leads to drastic losses in agonistic activity. The L-diastereoisomers of 3-Pal (analogues **2** and **5**) showed the highest decrease in agonistic activity. Analogues **1** and **4** with L-Thi^9 showed no inhibitory potency compared to analogues with L- or D-3-Pal^9, an amino acid residue with slightly larger (one methylene group) heterocyclic ring. Our findings are consistent with those previously reported which suggested that the C-terminal tripeptide sequence and especially the proper orientation of the C-terminal glycine carboxamide are critical for obtaining high potency OT agonist analogues (Ting et al. 1980; Hruby 1986; Fragiadaki et al. 2003).

Analogues having combined substitutions in position 1 (Mpa), in position 2 [D-Tyr(Et)] and in position 9 with residues having more compact side chains than Gly exhibited in most of the cases slightly higher anti-OT potency in comparison to the [Mpa^1, D-Tyr(Et)^2]OT (analogue **7**) which has no modification in position 9. In the case of analogues with D-1-Nal^2—more rigid and lipophilic side chain than that of D-Tyr(Et)^2—the same modifications in position 9 did not lead to substantial change in the antagonistic potency in comparison to analogue **18** having no change in position 9. Only in the case of analogue **21** having L-3-Pal in position 9, the antagonistic activity was decreased. If we order the studied amino acids introduced into position 9 according to their effect on antagonistic activity, than in the [Mpa^1, D-Tyr(Et)^2] series it would be (from the best till the worst): Deg = D-Thi > L-Thi = Aib = D-Tic > D-3-Pal > L-3-Pal = **Gly** and in the [Mpa^1, D-1-Nal^2] series: Aib > D-Thi > **Gly** = L-Thi = D-3-Pal > L-3-Pal.

The D-Tic substitution in position 2 seems to have no advantage in comparison to D-Tyr(Et) or D-1-Nal in producing antagonists (both the potency in rat uterotonic test and affinity to human OTR are substantially lower). This finding ensures the role of the side chain of the residue in position 2 for binding to the uterotonic receptor and it is consistent with data previously observed (Fragiadaki et al. 2007).

The binding affinities to human OTR more or less mirror the biological activities. There seems to be a species difference in the preference of the receptors for D-1-Nal^2 analogues. The D-1-Nal^2 modification generally improves

the binding affinity to human OTR in comparison to the D-Tyr(Et)^2 or D-Tic^2 substituted analogues.

The fact that almost all the analogues are inactive in the pressor test shows that receptors mediating the pressor activity are not at all tolerant to the conformational changes arising from replacement of the naturally occurring glycyl residue in position 9.

Additional substitutions of Cys in position 6 by a residue with a more restricting and bulky side chain did not significantly influence the antagonistic biological activity. The introduction of Pen^6 seems not to change the folding of the tail portion above or below the plane of the ring.

Conclusion

These results confirm the fact that the proper topological arrangement in the C-terminal part of the molecule is crucial for the agonistic activity and much less important for the antagonistic activity. Of all the 24 analogues, the [Mpa^1, D-Tyr(Et)^2]OT analogues **14** and **23** containing Deg and Aib in position 9, respectively, are the most promising leads for the synthesis of potent and selective OT antagonists as they show potent anti-OT activity in the rat uterotonic test in vitro, high affinity to human OTR and at the same time exhibit no rat pressor or anti-diuretic (data not shown) activities.

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