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Original article

Synthesis and biological activity of oxytocin analogues containing conformationally-restricted residues in position 7

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Abstract

We report the solid-phase synthesis and some pharmacological properties of twenty oxytocin (OT) analogues. Basic modifications at position 7 (introduction of α-aminoisobutyric acid [Aib], L- or D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid [L/D-Tic], L-α-*t*-butylglycine [Gly(Bu^t)] and pipecolic acid [Pip]) were combined with D-Tyr(Et)[2], L/D-(*p*Et)Phe[2], D-Tic[2], and Mpa[1] modifications and their various combinations in a total of 14 analogues. Additionally, two analogues having one more modification in position 3, i.e. Gly(Bu^t), and three analogues having glycine in position 9 substituted by D-Tic or Aib, were prepared. 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 antioxytocic activity was [Mpa[1], D-Tyr(Et)[2], D-Tic[7], Aib[9]]OT having $pA_2 = 8.31 \pm 0.19$; this analogue was also selective.

Keywords: Oxytocin analogues; α-Aminoisobutyric acid; 1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid; L-α-*t*-Butylglycine; Pipecolic acid; Biological activity

1. Introduction

The nonapeptide oxytocin (OT)[1], see Fig. 1, has been viewed as a hypothalamic neuropeptide that is released into general circulation from the neural lobe of the pituitary, inducing uterine contractions during parturition and milk ejection during lactation [1]. However, in recent years the classical concept of OT action has greatly expanded because of the discovery of novel sites of expression of the gene encoding the OT receptor (OTR), on the one hand, and the revealing of novel sites of OT production on the other. The widespread distribution of OT receptors in the brain and the specific behavioral effects of centrally-applied OT have firmly established OT as a central neurotransmitter with roles in reproductive and social behaviors [1,2].

The multiple established actions of OT are all mediated by one type of OT receptor [1], which is a member of the G-protein coupled receptor super family [3]. 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 labor.

Abbreviations: OT, oxytocin; Mpa, β-mercaptopropionic acid; [Mpa[1]]OT, deamino-oxytocin; Aib, α-aminoisobutyric acid; Gly(Bu^t), L-α-*t*-butylglycine; L/D-(*p*Et)Phe, L- or D-phenylalanine (*p*-ethyl); Pip, pipecolic acid; Tic, 1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid; D-Tyr(Et), D-tyrosine (*O*-ethyl); Fmoc, 9-fluorenylmethoxycarbonyl; Bu^t, *t*-butyl; Trt, trityl; DIC, diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, dimethylformamide; DMSO, dimethylsulfoxide; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionisation-mass spectrometry.

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[1] 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 and J. Peptide Sci. 12 (2006) 1–8.

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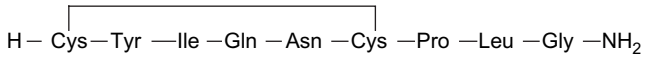

Fig. 1. Amino acid sequence of oxytocin.

OT antagonists, in contrast with currently used tocolytic agents (beta-adrenergic agonists, magnesium sulfate, prostaglandin synthetase inhibitors or calcium channel blockers), afford greater specificity and can be expected to exhibit improved efficacy and risk profiles [4]. Such compounds would allow more effective treatment of preterm labor with a lower risk of side effects. Moreover, the efficacy of the OT antagonist atosiban to inhibit premature uterine contractions in humans is indicative of a role for OT in human labor [5,6].

Generally, inhibitors of the uterotonic activity of OT have been produced by the introduction of bulky β-carbon substituents in 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 [7–9]. Furthermore, the importance of conformational flexibility for agonist activity and relative conformational rigidity and steric constraints for antagonism has been emphasized [10–12]. 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. It has been reported that modification of the C-terminal tripeptide side chain influences the chymotryptic cleavage of Tyr–Ile peptide bond in the OT molecule [13].

Position 7 is occupied in all neurohypophyseal peptides isolated from animals by a proline residue. Bibliographic data suggest that synthetic modifications or substitutions at this position could yield hormone analogues in which one or more of the biological activities of the parent hormone are highly accentuated in terms of potency relative to other activities. Peptides with interesting activity profiles are potentially of great therapeutic value. It has been proposed that side chains of Ile^3 and Pro^7 are involved in the recognition and binding of the hormone by the uterine receptor [14]. OT analogues with modifications at position 7 have substantial oxytocic potency, but their pressor and antidiuretic activities are exceedingly low [15]. These characteristics have made analogues with modifications at position 7 desirable for further investigation.

In continuing our work aimed at the design of selective OT antagonists [16], we investigated the effectiveness of modifications in position 7 by unnatural imino and amino acids. The proper orientation and the sequence of the C-terminal tripeptide are critical for obtaining high potency OT analogues [16–18]. 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. Here we present the synthesis of 19 new OT analogues and a re-synthesis of one [19] which contain the following residues in position 7: pipecolic acid [19], L- or D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid [L/D-Tic], α-aminoisobutyric acid [Aib] and L-α-*t*-butylglycine [Gly(Bu^t)] alone or in combination with D-tyrosine(*O*-ethyl) [D-Tyr(Et)], D-Tic and L- or D-phenylalanine(*p*-ethyl) [L/D-(*p*Et)Phe] in position 2 (Table 1, analogues **1–5**, **7–10**, **13**, **14**, **16** and **18–20**). In addition, the preparation of [$Gly(Bu^t)^3$, $Gly(Bu^t)^7$]OT (analogue **6**) and [Mpa^1, D-$Tyr(Et)^2$, $Gly(Bu^t)^3$, $Gly(Bu^t)^7$]OT (analogue **15**) is described. Finally the synthesis of three analogues with additional substitution of Gly^9 by D-Tic (analogues **11** and **17**) or Aib (analogue **12**) is described. All 20 analogues were tested for binding affinity to human OTR and for biological potency in rat uterotonic test in vitro and rat pressor test.

2. Materials and methods

2.1. Materials

All 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and 2-chlorotrityl-chloride resin with Rink-Bernatowitz linker were obtained from CBL Patras; L/D-(*p*Et)Phe was kindly donated by Ing. M. Zertova from IOCB ASCR, Prague; the derivatives *S*-trityl-β-mercaptopropionic acid [Mpa(Trt)] and Fmoc-L/D-(*p*Et)Phe were prepared according to the literature [20,21]. 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.

2.2. Peptide synthesis and purification

The analogues were synthesized by Fmoc solid-phase methodology [21] utilizing a 2-chlorotrityl-chloride resin [22] as solid support bearing a Rink-Bernatowitz linker to provide the peptide amide [23]. Fmoc-protected amino acids were used with the trityl group (Trt) [Asn, Gln, L-Cys] and the *t*-butyl group (Bu^t) [Tyr] as side-chain protecting groups. Stepwise synthesis of a peptide analogue was achieved with diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) as coupling agents in dimethylformamide (DMF) [24,25] in 3 (Fmoc-amino acid), 3.3 (DIC) and 4.5 (HOBt) molar excess for 2 h at room temperature. Completeness of the reaction was monitored by the Kaiser test [26], except in the case of Pro, Pip and Tic residues where the end of the reaction was monitored by the Chloranil test [27]. The Fmoc groups were removed by the treatment with 20% piperidine in DMF for 30 min. Cleavage of peptide-linker bond and removal of the side-chain protecting groups were accomplished in 4 h at room temperature using a solution (15 ml/g peptide resin) of trifluoroacetic acid (TFA)/1,2-ethanedithiol/triethylsilane/anisole/water (90:5:2:2:1, v/v). The obtained peptide was precipitated upon concentration of solvent and addition of diethyl ether. The formation of the disulfide bridge was performed in 25% dimethylsulfoxide (DMSO)/H_2O for 24–36 h at room temperature [28]. The solution was frozen and lyophilized to give the final crude oxidized product.

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

Entry	Analogues	Yield[a] (%)	Melting point (°C)	HPLC[b] t_R (min)	TLC, R_f[c]			MW	$[M+1]^{+}$[d]
					A	*B*	*C*		
1	[Pip^7]OT[e]	55	221–223	13.65	0.35	0.58	0.41	1021.21	1022.05
2	[L-Tic^7]OT	70	248 decomp.	13.84	0.34	0.59	0.40	1070.91	1072.13
3	[D-Tic^7]OT	65	255 decomp.	13.46	0.43	0.54	0.46	1070.91	1072.17
4	[Aib^7]OT	73	215–217	13.71	0.38	0.62	0.41	995.18	996.10
5	[Gly(Bu^t)^7]OT	80	242 decomp.	16.36	0.52	0.72	0.76	1023.23	1024.02
6	[Gly(Bu^t)^3, Gly(Bu^t)^7]OT	88	198–200	16.20	0.44	0.71	0.77	1023.23	1024.08
7	[Mpa^1, L-Tic^7]OT	61	195–197	13.75	0.50	0.67	0.57	1055.93	1056.16
8	[Mpa^1, D-Tic^7]OT	60	201–203	16.21	0.52	0.69	0.43	1055.93	1056.05
9	[Mpa^1, D-Tyr(Et)^2, L-Tic^7]OT	60	185–188	18.94	0.42	0.73	0.57	1083.25	1084.25
10	[Mpa^1, D-Tyr(Et)^2, D-Tic^7]OT	63	192–1941	20.10	0.47	0.76	0.59	1083.25	1084.25
11	[Mpa^1, D-Tyr(Et)^2, D-Tic^7, D-Tic^9]OT	59	251 decomp.	31.82	0.76	0.83	0.74	1184.06	1185.06
12	[Mpa^1, D-Tyr(Et)^2, D-Tic^7, Aib^9]OT	62	261 decomp.	28.06	0.65	0.76	0.77	1110.13	1111.11
13	[Mpa^1, D-Tyr(Et)^2, Pip^7]OT	57	194–196	18.05	0.45	0.72	0.62	1034.27	1035.03
14	[Mpa^1, D-Tyr(Et)^2, Gly(Bu^t)^7]OT	86	178–180	23.59	0.32	0.61	0.67	1036.27	1037.03
15	[Mpa^1, D-Tyr(Et)^2, Gly(Bu^t)^3, Gly(Bu^t)^7]OT	89	251 decomp.	23.74	0.29	0.61	0.68	1036.27	1037.06
16	[Mpa^1, D-Tyr(Et)^2, Aib^7]OT	62	183–185	18.33	0.46	0.75	0.61	1008.22	1009.08
17	[Mpa^1, D-Tyr(Et)^2, Aib^7, D-Tic^9]OT	61	165–168	28.02	0.76	0.77	0.78	1110.13	1111.11
18	[Mpa^1, (*p*Et)L-Phe^2, Aib^7]OT	70	209–211	18.68	0.44	0.60	0.59	992.60	993.55
19	[Mpa^1, (*p*Et)D-Phe^2, Aib^7]OT	67	216–218	18.48	0.47	0.58	0.61	992.26	993.60
20	[Mpa^1, D-Tic^2, Aib^7]OT	60	203–205	14.69	0.52	0.66	0.41	976.87	977.02

[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.
[e] Re-synthesized analogue.

All the products were purified by gel filtration chromatography on Sephadex G-15 using 25% acetic acid as the eluent. Final purification was achieved by semi-preparative high-performance liquid chromatography (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 10 to 75% acetonitrile (0.1% TFA) for 35 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. The crude mixture of diastereoisomers (analogues **18/19**) was separated by HPLC [Vydac C_{18}, semi-preparative column, (A) water–0.1% TFA, (B) 80% acetonitrile–0.1% TFA] under isocratic conditions (A:B, 60:40) for 20 min (3 ml/min) [29]. Fractions were collected, pooled and lyophilized, yielding pure analogues **18** and **19** (according to analytical HPLC). All products gave single spots on thin layer chromatography (TLC, 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 particle size; 250 × 4.6 mm) produced single peaks with at least 98% of the total peptide peak integrals. The final verification of the peptide sequence was achieved by electrospray ionisation-mass spectrometry (ESI-MS, micromass-platform LC instrument). Analytical HPLC chromatograms and ESI-MS spectra for analogues **6** and **12** are shown in Fig. 2. The physicochemical properties of the new analogues are summarized in Table 1.

2.3. Biological assay methods

The uterotonic activity was determined in vitro on an isolated strip of rat uterus in the absence of magnesium [30–32]. 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 average 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 [32]. Each analogue was tested on uteri from 4 to 8 different rats. Pressor activity was determined on phenoxybenzamine-treated male rats [33]. The responses to standard doses of oxytocin or vasopressin were stable for several hours, without problems with tachyphylaxis.

2.4. Binding affinity determination

Determination of binding affinity to human OTR was performed basically as described [34] using tritiated oxytocin from NEN Life Science, Boston, MA, USA. In brief, a crude

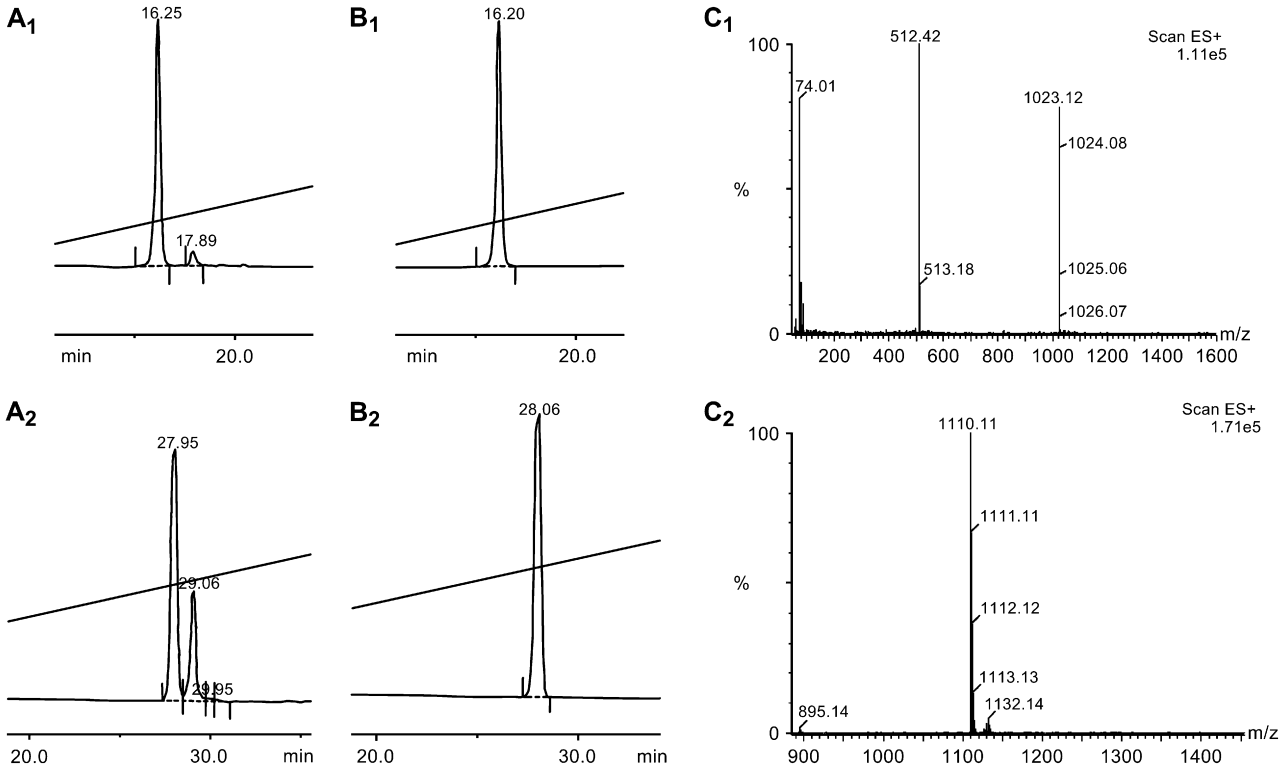

Fig. 2. *A*: Analytical HPLC chromatograms of crude analogues [Gly(Bu^t)^3, Gly(Bu^t)^7]OT (A_1) and [Mpa^1, D-Tyr(Et)^2, D-Tic^7, Aib^9]OT (A_2) previously chromatographed on a semi-preparative C18 column; *B*: Analytical HPLC chromatograms of analogues [Gly(Bu^t)^3, Gly(Bu^t)^7]OT (B_1) and [Mpa^1, D-Tyr(Et)^2, D-Tic^7, Aib^9]OT (B_2) upon re-chromatography on the same column; *C*: Mass spectra of analogues [Gly(Bu^t)^3, Gly(Bu^t)^7]OT (C_1) and [Mpa^1, D-Tyr(Et)^2, D-Tic^7, Aib^9]OT (C_2) resulting from ESI-MS analysis.

membrane fraction of HEK OTR cells, i.e. HEK cells having stable expressed human OT receptor (kindly donated by Dr. G. Gimpl, [35]), 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.

3. Results

3.1. Peptide synthesis and purification

The use of DIC/HOBt as coupling reagent and the 2-chlorotrityl-chloride resin bearing a Rink-Bernatowitz linker as solid support leads to overall yield of the synthesis of the OT analogues in the range of 55−89%. The synthesis of analogues **6**, **7**, **14** and **15** containing the Gly(Bu^t) residue gives higher yields than those of the rest of the analogues (Table 1). ESI mass spectrometry revealed that the purified peptides were the desired products, the purity of which was higher than 98% as determined by analytical HPLC.

The configuration of analogues **18** and **19** was assigned according to the paper of Lebl et al. who postulated that the D-diastereoisomer has higher retention time in comparison to the L-diastereoisomer [36].

3.2. Biological activity (rat)

Biological evaluation of the new and re-synthesized analogues (**1**−**20**) is summarized in Tables 2 and 3. Amino acid Pro in position 7 of oxytocin and deamino-oxytocin was replaced by several unnatural conformationally-restricted imino (Pip or L- or D-Tic) and amino [Aib or Gly(Bu^t)] acids. With the exception of the Pip (pipecolic acid or homoproline) analogue, all other modifications decreased biological activities significantly. The re-synthesized analogue [Pip^7]OT (analogue **1**), with slightly larger (one methylene group) heterocyclic ring than Pro, was found to be an agonist in the uterotonic in vitro assay with a potency approximately seven times lower than that of the native hormone.

The analogue **2** modified in position 7 with the interesting conformational restricted and bulky residue L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (L-Tic) was found to be a partial agonist in the oxytocic assay with agonistic potency approximately 250 times lower than that of the native hormone and weak antagonist with pA_2 value about 5.6. D-Diastereoisomer containing Tic (analogue **3**) showed pure anti-OT potency, approximately 20 times higher than that of the L-counterpart ($pA_2 = 7.00$).

Table 2
Biological activities of oxytocin analogues

Entry	Analogues	Biological activity			
		Uterotonic in vitro		Pressor	
		Agonistic (IU/mg)	Antagonistic (pA_2)	Agonistic[a] (IU/mg)	Antagonistic (pA_2)
I	Oxytocin[b]	546		3.10	
1	[Pip^7]OT	79.80 ± 24.10		0	0
2	[L-Tic^7]OT	~2.00	~5.60	0	0[c]
3	[D-Tic^7]OT	0	7.00 ± 0.30	0	0[c]
4	[Aib^7]OT	1.70		0	0
5	[Gly(Bu^t)^7]OT	1.80 ± 0.40		0	0
6	[Gly(Bu^t)^3, Gly(Bu^t)^7]OT	0	6.13 ± 0.20	0	0
II	[Mpa^1]OT[b] (deamino-oxytocin)	803			0
7	[Mpa^1, L-Tic^7]OT	~3.50	7.45 ± 0.28	0	0[c]
8	[Mpa^1, D-Tic^7]OT	~1.00	8.00 ± 0.10	0	0
III	[D-Tyr(Et)^2]OT[b]	0	7.36		
9	[Mpa^1, D-Tyr(Et)^2, L-Tic^7]OT	0	7.80 ± 0.09	0	0[c]
10	[Mpa^1, D-Tyr(Et)^2, D-Tic^7]OT	0	8.20 ± 0.19	0	0[c]
11	[Mpa^1, D-Tyr(Et)^2, D-Tic^7, D-Tic^9]OT	0	8.07 ± 0.04	0	0
12	[Mpa^1, D-Tyr(Et)^2, D-Tic^7, Aib^9]OT	0	8.31 ± 0.19	0	0
13	[Mpa^1, D-Tyr(Et)^2, Pip^7]OT	0	7.90 ± 0.20	0	0
14	[Mpa^1, D-Tyr(Et)^2, Gly(Bu^t)^7]OT	0	7.93 ± 0.16	0	0
15	[Mpa^1, D-Tyr(Et)^2, Gly(Bu^t)^3, Gly(Bu^t)^7]OT	0	6.45 ± 0.18	0	0
16	[Mpa^1, D-Tyr(Et)^2, Aib^7]OT	0	7.75 ± 0.10	0	0
17	[Mpa^1, D-Tyr(Et)^2, Aib^7, D-Tic^9]OT	0	7.87 ± 0.08	0	0
IV	[(pEt)D-Phe^2]OT[b]		8.15		
V	[Mpa^1, (pEt)D-Phe^2]OT[b]		8.06		$pA_2 = 6.82$
18	[Mpa^1, (pEt)L-Phe^2, Aib^7]OT	0	7.40 ± 0.06 [d]	0	0
19	[Mpa^1, (pEt)D-Phe^2, Aib^7]OT	0	8.30 ± 0.24[d]	0	$pA_2 = 6.50$
20	[Mpa^1, D-Tic^2, Aib^7]OT	0	6.91 ± 0.11	0	0
VI	[Mpa^1, D-Tyr(Et)^2, Thr^4, Orn^8]OT (atosiban)[b]	0	8.29 ± 0.05	0.02 ± 0.02	0

[a] 0 means no activity up to the dose 0.16 mg/kg of exp. animal.
[b] The biological assays for the oxytocin analogues **1–20** were performed as outlined in the text. The values are averages ± SEM of at least three experiments. The biological activities of other analogues reported here as references are taken from the literature: for oxytocin and deamino-oxytocin, see Ref. [10], for [(pEt)D-Phe^2]OT and [Mpa^1, (pEt)D-Phe^2]OT see Ref. [43], for [D-Tyr(Et)^2]OT see Ref. [44] and for atosiban, see Ref. [45]. If no SEM is given for the reference compound, it means that it was not given in the original papers.
[c] 0 means, in this case, no activity or very low inhibitory activity $pA_2 < 6$.
[d] The value for the mixture [Mpa^1, (pEt)L/D-Phe^2, Aib^7]OT was 8.00 ± 0.14; according to HPLC, the L- and D-diastereoisomers were in the mixture in the ratio 1:3.

Analogues **4** ([Aib^7]OT) and **5** ([Gly(Bu^t)^7]OT) with aliphatic hydrophobic bulky side chains in position 7 were found to be low potent agonists, more than 300 times weaker than OT in the uterotonic assay. The great decrease in the agonistic potency of analogues **2**, **4** and **5** suggests that modifications at position 7 by conformationally less restricted yet lipophilic residues induce such conformational changes in the peptide backbone that cause markedly different distribution of the elements necessary for the binding of agonists and intrinsic activity. Analogue **1** was found to be selective due to no activity in the pressor assay. It is interesting that further modification of analogue **5** [replacement of Ile^3 by Gly(Bu^t)^3] led to the change in the character of the activity, i.e. to the weak antagonist **6** ($pA_2 = 6.13 \pm 0.20$).

It has been known that substitution of the N-terminal cysteine by a residue without the amino group, i.e. β-mercaptopropionic acid (Mpa) [37], enhances the biological activity. Literature data indicate that the neurohypophyseal hormones lacking the N-terminal amino group are inactivated quite slowly [38] and that the first amino acid plays a decisive role in receptor binding [39]. In accordance with these observations, we synthesized analogues **7** and **8**. Replacement of Cys^1 by Mpa^1 in analogue **2** slightly affected the agonistic activity (analogue **7**), while in analogue **3** this led to partial agonism (analogue **8**) in the oxytocic assay, with agonistic potency approximately 550 times lower than that of the native hormone. On the other hand, the anti-oxytocin activity was significantly enhanced in both analogues (analogue **7** with $pA_2 = 7.45 \pm 0.28$ and analogue **8** with $pA_2 = 8.00 \pm 0.10$). Both analogues were selective, showing no pressor activity.

Studies of the role played by the tyrosine residue in position 2 have led to the generalization that the D-configuration and hydrophobicity of this aromatic amino acid are important for the antagonistic activity [40,12]. According to these observations, we synthesized analogues **9–20** with further substitution of Tyr^2 by D-Tyr(Et)^2 or D/L-(pEt)Phe^2 or D-Tic^2 and of Cys^1 by Mpa^1. The antagonistic potency of analogues **9** ($pA_2 = 7.80 \pm 0.09$) and **10** ($pA_2 = 8.20 \pm 0.19$) with D-Tyr(Et)^2 was less significantly enhanced than we expected. Analogues having combined substitutions in position 1 (Mpa), in position

Table 3
Binding affinities of oxytocin analogues

Entry	Analogues	Binding affinity[a] IC_{50} [nM]
	Oxytocin	2.7 ± 0.2
1	[Pip^7]OT[b]	7 ± 1*
2	[L-Tic^7]OT	130 ± 56
3	[D-Tic^7]OT	730 ± 250
4	[Aib^7]OT	75 ± 13*
5	[Gly(Bu^t)^7]OT	1380 ± 250
6	[Gly(Bu^t)^3, Gly(Bu^t)^7]OT	$51\,700 \pm 5350$*
7	[Mpa^1, L-Tic^7]OT	103 ± 30
8	[Mpa^1, D-Tic^7]OT	380 ± 63*
9	[Mpa^1, D-Tyr(Et)^2, L-Tic^7]OT	176 ± 60
10	[Mpa^1, D-Tyr(Et)^2, D-Tic^7]OT	51 ± 8*
11	[Mpa^1, D-Tyr(Et)^2, D-Tic^7, D-Tic^9]OT	25 ± 3
12	[Mpa^1, D-Tyr(Et)^2, D-Tic^7, Aib^9]OT	80 ± 6
13	[Mpa^1, D-Tyr(Et)^2, Pip^7]OT	40 ± 3*
14	[Mpa^1, D-Tyr(Et)^2, Gly(Bu^t)^7]OT	1002 ± 158
15	[Mpa^1, D-Tyr(Et)^2, Gly(Bu^t)^3, Gly(Bu^t)^7]OT	7850 ± 379
16	[Mpa^1, D-Tyr(Et)^2, Aib^7]OT	485 ± 105
17	[Mpa^1, D-Tyr(Et)^2, Aib^7, D-Tic^9]OT	55 ± 12
18	[Mpa^1, (*p*Et)L-Phe^2, Aib^7]OT	n.d.[c]
19	[Mpa^1, (*p*Et)D-Phe^2, Aib^7]OT	n.d.[c]
20	[Mpa^1, D-Tic^2, Aib^7]OT	8500 ± 1421*

[a] The values given are averages ± SEM from three experiments performed in duplicates if not having asterics; asterics means average from two experiments carried out in duplicates and the difference of the average.
[b] Re-synthesized analogue.
[c] At the time of testing the binding affinities, no pure samples of **18** and **19** were available; only binding affinity of the L/D mixture could be determined and it amounts to 31 ± 5 nM.

2 [D-Tyr(Et)] and in position 7 (D/L-Tic) retain the antagonistic potency; however, the effects of the individual substitutions are not additive.

Replacement of the amino acid at position 2 with D-Tyr(Et) in analogues **1**, **4** and **5** and additional substitution of Cys^1 by Mpa^1 lead to the potent antagonists **13**, **16** and **14**. These analogues exhibited comparable antagonistic potency as analogues **9** and **10**. Analogue **15**, with the same substitutions, also exhibited a slight increase in inhibitory potency in comparison to analogue **6** ($pA_2 = 6.45 \pm 0.18$ and $pA_2 = 6.13 \pm 0.20$, respectively). It is notable that further modification of analogue **10** with D-Tic in position 9 (analogue **11**) might slightly decrease the inhibitory potency ($pA_2 = 8.07 \pm 0.04$), while Aib modification in the same position (analogue **12**) did not affect anti-OT potency ($pA_2 = 8.31 \pm 0.19$). Analogue **17**, with additional substitution in position 9 by D-Tic, exhibited the same inhibitory potency as analogue **16** ($pA_2 = 7.87 \pm 0.08$ and $pA_2 = 7.75 \pm 0.10$, respectively).

Analogues **18**–**20**, substituted with L/D-(*p*Et)Phe^2 or D-Tic^2 in position 2, were synthesized in an attempt to further enhance the anti-OT potency. However, analogue **18** with L-stereoisomer of (*p*Et)Phe^2 exhibited slightly lower inhibitory potency (7.40 ± 0.06) while analogue **19** with D-stereoisomer of (*p*Et)Phe^2 showed slightly higher anti-OT potency (8.30 ± 0.24) in comparison to analogue **16**. It is notable that in the pressor test analogue **19** showed a weak antagonistic activity ($pA_2 = 6.50$). Finally, D-Tic^2 modification in analogue **20** resulted in an antagonism with substantially lower potency ($pA_2 = 6.91 \pm 0.11$).

3.3. Binding affinity (human receptor)

Table 3 summarizes the binding affinities of the analogues to human OTR permanently expressed on HEK cells. As could be expected, Pip^7 modification leads only to a slight decrease of the binding affinity ($IC_{50} = 7$ nM, analogue **1**). On the other hand, the introduction of smaller but compact residues, such as Aib or Gly(Bu^t), or conformationally constrained and bulky L- or D-Tic, into position 7, decreased the binding affinity to the OT receptor significantly. The lowest affinity from these analogues with a single modification had analogue **5** ([Gly(Bu^t)^7]OT, $IC_{50} = 1380$ nM) and the highest analogue **4** ([Aib^7]OT), $IC_{50} = 75$ nM). Additional modification of analogue **5** by the introduction of [Gly(Bu^t)] in position 3 (analogue **6**) caused a further sharp decrease in the affinity ($IC_{50} = 51\,700$ nM). These findings are consistent with those previously observed by Walter et al. [14], ensuring the role of the side chain of the residue in positions 7 and 3 for binding to the uterotonic receptor.

Deamination in position 1 of analogues **2** and **3** (analogues **7** and **8**) improved the affinity only slightly (compare $IC_{50} = 130$ and 103 nM for **2** and **7** and $IC_{50} = 730$ and 380 nM for **3** and **8**, respectively). Further modification using D-Tyr(Et)^2 improved the affinity only in the case of analogue **10** (compare $IC_{50} = 380$ and 51 nM for analogue **8** and **10**, respectively). Additional modification of analogue **10** by the introduction of D-Tic in position 9 (analogue **11**) improved the affinity slightly (compare $IC_{50} = 51$ and 25 nM for analogue **10** and **11**, respectively), while the introduction of Aib in the same position (analogue **12**) led to almost no change in the affinity (compare $IC_{50} = 51$ and 80 nM for analogue **10** and **12**, respectively).

Simultaneous deamination and replacement of Tyr^2 with D-Tyr(Et)^2 had an inconsistent effect: in analogues **1** and **4**, it decreased the affinity about six times (compared to values for **13** and **16**, respectively); in the case of analogues **2** and **5**, there was almost no change of binding to the receptor (compared to values for **9** and **14**, respectively) and, in the case of analogues **3** and **6**, there was an increase in binding affinity (compared to values for **10** and **15**, respectively). Furthermore, replacement of Gly^9 with D-Tic in analogue **17** improved the affinity remarkably (compare $IC_{50} = 485$ and 55 nM for analogues **16** and **17**, respectively).

The L/D-(*p*Et)Phe^2 change (mixture of analogues **18** and **19**) showed higher binding affinity to the receptors ($IC_{50} = 31$ nM) than in analogue **16**. Finally analogue **20**, with the combination of deamination, D-Tic^2 and Aib^7 modifications, exhibited significantly low binding affinity ($IC_{50} = 8500$ nM).

4. Discussion

We have synthesized 20 analogues of OT with single modification in position 7 (**1**–**5**) or combined modifications in position 1 (deamination), position 2 [D-Tyr(Et) or D/L-(*p*Et)Phe or D-Tic], positions 7 and 9 (**6**–**20**) and evaluated their potency in uterotonic in vitro test, pressor test and affinity to human OT

receptor 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, replacement of Pro^7 by unnatural sterically constrained amino or imino acids influenced biological activities significantly. L-diastereoisomers of Tic (analogues **2**, **7** and **9**) showed more than two orders of magnitude lower or no agonistic activity, and lower inhibitory potency than the D-counterparts (analogues **3**, **8** and **10**). Our findings are consistent with those previously reported which suggested that the structure of the C-terminal tripeptide sequence and mainly the proper arrangement of this are critical for obtaining high agonistic potency [18,12,17]. Furthermore, these results confirm the fact that the orientation of β-turn at position 7 of the analogues is crucial for the appearance of biological activity and for the binding with the receptor. The great decrease in the agonistic potency of analogue $[Gly(Bu^t)]^7$OT indicates that a lipophilic and compact residue in position 7 induces more significant conformational change of the C-terminal part of the molecule than does an identical residue in position 8 or 9 [16]. Replacement of the amino acid at position 2 with D-Tyr(Et) or L/D-(*p*Et)Phe leads to almost the same degree of antagonistic potency as that without the changes in position 7. This points to the fact that position 7 is less important for antagonism. The influence of the substitutions in positions 2 and 7 is thus not additive but they do not cancel the effect of one another. The D-Tic substitution in position 2 seems to have no advantage in comparison to D-Tyr(Et) or L/D-(*p*Et)Phe in producing antagonists (both the potency in rat uterotonic test and affinity to human OTR are substantially lower).

The fact that almost all the analogues are inactive in the pressor test shows that receptors responsible for pressor activity are not at all tolerant to the conformational changes arising from replacement of the naturally occurring prolyl residue in position 7. Furthermore, in comparison to D-(*p*Et)Phe^2 substitution, the D-$Tyr(Et)^2$ modification has the advantage that the analogues are devoid of pressor antagonism.

Additional substitution of Ile in position 3 by a residue with a shorter and compact side chain dramatically influences the binding affinity. This result seems to support the idea that the proper topological arrangement of the binding elements at the corner positions of the two β-turns (3 and 7) makes analogues more or less ''visible'' to the receptor.

Analogues **1**–**3**, **7** and **8** are interesting also from the point of view of the *cis*/*trans* orientation of the peptide bond between positions 6 and 7. The orientation of this bond and its relationship to agonism and antagonism has been studied; however, no direct relationship was found [41,42]. Analogue **1** has L-pipecolic acid (a homoproline) in position 7 and, in the uterotonic test, it is a pure agonist. Analogue **2** (L-Tic^7) can be considered as an analogue of **1**, having substituted the aliphatic ring with an aromatic ring. This causes a change to partial agonism. Deamination of analogue **2** further enhances the antagonistic potency ($pA_2 \sim 5.60$ and $pA_2 = 7.45 \pm 0.28$ of analogues **2** and **7**, respectively). On the other hand, analogue **3** with D-Tic in position 7 is a pure antagonist with higher antagonistic potency than **2**. Deamination of analogue **3** results in analogue **7**, which regained partial agonistic character, but with strongly enhanced antagonistic potency. It would therefore be very interesting to determine the ratio of the *cis*/*trans* conformer of these analogues by 2D NMR.

5. Conclusion

Our studies showed that the replacement of Pro^7 in OT molecule by unnatural sterically constrained amino or imino acids influenced biological activities significantly. Of all 20 analogues, analogues **10**–**12** containing D-Tic in position 7 are the most promising lead 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 OT receptor and at the same time exhibit no rat pressor or antidiuretic activity (data not shown).

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