ANTAGONISTIC ANALOGS OF OXYTOCIN WITH SUBSTITUTED PHENYLALANINE OR TYROSINE IN POSITION 2*

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Solid phase method on *p*-methylbenzhydrylamine resin was used for the synthesis of seven analogs of oxytocin with non-coded amino acids in position 2. [L-Phe(4-Me)^2]oxytocin (*I*), [D-Phe(4-Me)^2]-oxytocin (*II*), [L-Phe(2-Me,4-Et)^2]oxytocin (*III*), [D-Phe(2-Me,4-Et)^2]oxytocin (*IV*), [D-Tyr(Me)^2]oxytocin (*V*), [D-Tyr(Et)^2]oxytocin (*VI*) and [L-Tyr(2-Me)^2]oxytocin (*VII*) were synthesized. All analogs with D-stereoisomer of alkyl or alkoxy substituted phenylalanine possess uterus in vitro inhibiting activity. In the case of L-stereoisomers the structure–activity relationship is more complicated. As far as the pressor activity is concerned, the analogs have either very low agonistic activity or low degree of antagonism.

The design of oxytocin inhibitors originates historically from the knowledge that the modification of position 2 leads to compounds having the character of partial agonists or antagonists[1,2], and that the character of the response depends to a considerable extent on conditions under which the test is performed[3]. Generally, inhibitory effect of an analog is accentuated when the calcium content is decreased, or when magnesium is eliminated and the temperature is decreased. An illustrative example is given in refs[2,4] where comparison of the properties of a number of oxytocin analogs modified in the *para*-position of the aromatic amino acid in position 2 in two different testing arrangements is performed. While in one test the analogs function as partial agonists, in the other one they appear as antagonists.

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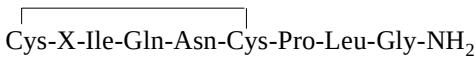
The principle modifications leading to the change of an agonist to antagonist of uterotonic activity in oxytocin molecule* are in detail described for example in ref.[6].

In this paper we continue in our effort to elucidate the effect of substitution of amino acid in position 2 and its configuration on antioxytocin uterotonic activity. The series of analogs containing substituted phenylalanine (L- and D-4-ethylphenylalanine[4,7], L- and D-2-methylphenylalanine[8], L- and D-2,6-dimethylphenylalanine analogs[8]) was supplement by the synthesis of the pair of D- and L-4-methylphenylalanine analogs and D- and L-2-methyl-4-ethylphenylalanine analogs. In the case of analogs having substituted tyrosine in position 2, we performed a new synthesis of the controversial D-*O*-ethyltyrosine analog, we synthesized an analog missing in the series containing D-*O*-methyltyrosine (*V*), and finally we completed the series with the synthesis of analog having 2-methyl substituted tyrosine.

All seven analogs (*I–VII*) described in this paper were synthesized by the solid phase technique on *p*-methylbenzhydrylamine resin.



I	X = L-Phe(4-Me)	*V*	X = D-Tyr(Me)
II	X = D-Phe(4-Me)	*VI*	X = D-Tyr(Et)
III	X = L-Phe(2-Me, 4-Et)	*VII*	X = L-Tyr(2-Me)
IV	X = D-Phe(2-Me, 4-Et)		

D,L-2-Methyl-4-ethylphenylalanine was prepared from D,L-4-ethylphenylalanine via D,L-7-ethyl-1,2,3,4-tetrahydroisochinolin-3-carboxylic acid[8]. 2-Methyltyrosine was prepared from 3,5-dibromotyrosine[9] via 6,8-dibromo-7-hydroxy-1,2,3,4-tetrahydroisochinolin-3-carboxylic acid and via 7-hydroxy-1,2,3,4-tetrahydroisochinolin-3-carboxylic acid[8]. Both amino acids were protected by *tert*-butoxycarbonyl group.

The *tert*-butoxycarbonyl group was used for the α-amino group protection. For the side chain protection of cysteine the 4-methylbenzyl group was used. The protected amino acids were coupled by *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxy-

* All the chiral amino acids, unless otherwise stated, are of the L-series. The nomenclature and symbols of the amino acids and peptides obey the published recommendations[5]: Phe(4-Me) denotes the 4-methylphenylalanine, Phe(2-Me,4-Et) the 2-methyl-4-ethylphenylalanine, Tyr(Me) the *O*-methyltyrosine, Tyr(Et) the *O*-ethyltyrosine and Tyr(2-Me) 2-methyltyrosine.

benzotriazole (HOBt) in dimethylformamide. Side chain protecting group removal was simultaneous with the cleavage of the peptide from the resin using liquid hydrogen fluoride. Sulfhydryl group oxidation was performed by potassium ferricyanide. Because of easy separability of diastereoisomeric peptides by means of RP HPLC (refs[10–17]), the syntheses of analogs *I – IV* were thus performed using racemic amino acids D,L-4-methylphenylalanine or D,L-2-methyl-4-ethylphenylalanine. Peptides containing the appropriate diastereoisomers were separated at the end of the preparation. Formation of both diastereoisomers was accomplished using only 1.1 equivalents of protected racemic amino acid (see ref.[13]). Chirality of the amino acid in the pure peptide was determined in hydrolysates either by digestion, using L-amino acid oxidase[18,19] (digestion time 100 h), or on chiral plates[20]. In HPLC on reverse phase determined *k* value was in both cases lower for the L-diastereoisomer, which is consistent with previous findings[10,11,13–17].

Analogs containing L-amino acid in position 2 are significantly more basic (electrophoresis in pyridine–acetate buffer, pH 5.7) than those containing D-amino acid (see[15–17] and compare[21,22]).

Biological activities of the analogs are given in Table I. Their pressor activity is either negligible or they are weak antagonists. In the uterus in vitro test analogs with alkyl or alkoxy substituted D-phenylalanine possess inhibitory activity (see *II*, *IV*, *V*, *VI* in Table I) similarly to other analogs (see Table II). The 4-methylphenylalanine of D-configuration was found superior in producing an inhibitor. The exceptional agonistic

TABLE I
Biological activities (rat) of oxytocin analogs (I.U./mg or pA_2) with modifications in position 2

Compound	Activity	
	uterotonic in vitro	pressor
OXT	450	5
I	30.3	0.7
II	pA_2 = 8.2	0[a]
III	pA_2 = 7.6	pA_2 = 7.0
IV	pA_2 = 8.0	pA_2 = 6.2
V	pA_2 = 7.7	pA_2 = 6.0
VI	pA_2 = 7.4	0[a]
VII	3.1	0[a]

[a] 0 Means inactive up to the dose 2 . 10^{-2}mg/rat.

activity of [D-Tyr(Et)^2]OXT (ref.[33]) was not confirmed by the new synthesis of the compound.

In the case of analogs with substituted L-phenylalanine the relations are more complicated. The analogs with unsubstituted phenylalanine or 4-substituted phenylalanine (methyl, ethyl, methoxy or ethoxy group) have agonistic activity. However alkyl substitution in 2-position of benzene ring provides analogs with antagonistic activity. It pays also for disubstituted analogs (i.e. 2,4- and 2,6-dialkyl substituents) with the exception of analog having free hydroxyl group in 4-position (i.e. with 2-methyltyrosine). On the other hand analogs with L-3-substituted tyrosine (see Table II) are also inhibitors.

TABLE II
Uterotonic activities (rat) of oxytocin analogs (I.U./mg or pA_2) with L- or D-modifications in position 2

Compound		Uterotonic activity		ref. L/D
		L	D	
OXT		450	6.6	6/23
[Phe^2]OXT		32	pA_2 = 6.00	24/25
[Leu^2]OXT		0.6	pA_2 = 5.23	26/25
[Trp^2]OXT		0.24	pA_2 = 6.87	25/25
[Phe(F_5)^2]OXT		0.01	pA_2 = 6.27	27/27
[Phe(4-Me)^2]OXT		19	–	4/–
	I and *II*	30.3	pA_2 = 8.2	[a]/[a]
[Phe(4-Et)^2]OXT		6.5	pA_2 = 8.15	4/7
[Phe(2-Me)^2]OXT		pA_2 = 6.6	pA_2 = 7.6	8/–
[Phe(2,6-diMe)^2]OXT		pA_2 = 6.2	pA_2 = 7.4	8/–
[Phe(2-Me,4-Et)^2]OXT	*III* and *IV*	*p*A_2 = 7.6	pA_2 = 8.0	[a]/[a]
[Tyr(Me)^2]OXT		≈5	–	31/–
	in D-series *V*	>2	pA_2 = 7.7	26, 32/[a]
[Tyr(Et)^2]OXT		0.15	3.3	4/33
	in D-series *VI*	–	pA_2 = 7.4	–/[a]
[Tyr(2-Me)^2]OXT	in L-series *VII*	3.1	–	[a]/–
[Tyr(3-I)^2]OXT		pA_2 = 7.2	–	28/–
		pA_2 = 7.05	–	29/–
Tyr(3-NO_2)^2]OXT		1.1	pA_2 = 6.28	30/31
[Tyr(3-OH)^2]OXT		23	–	34/–
[Tyr(3-Me)^2]OXT		pA_2 = 6.79	–	29/–

[a] This paper.

We can conclude that the free hydroxyl preserves the agonistic character of the analogues in both L- and D-series. The hydroxyl group in 4-position has an opposite and superior effect to the methyl substituent in 2-position however not to that in 3-position. Meanwhile introduction of 2-Me into phenylalanine leads to the change of agonism into antagonism, introduction of 2-Me into tyrosine leads just to decrease of agonistic activity. Introduction of 3-Me leads to the change from agonism into antagonism already in the case of L-tyrosine. It looks like that the *meta* position in the benzene ring is crucial for the transfer of signal – both its position (L or D series) and slightly electronegative character (oxytocin).

It is evident from Table II, that the potency of oxytocin analogs is inevitably linked to the substitution on the benzene ring of tyrosine. As far as we evaluate the activity data according to the electronic effect of the substituents in 4-position on the electronegativity in 3-position, we can see that in the case of the L-series there is no correlation between the activity and the electronegativity expressed as Hammett constant σ_p (OH, 450 and –0.38; H, 32 and 0; Me, 30.3 and –0.14; Et, 6.5 and –0.13; OMe, 5 and –0.28; substituent, uterotonic activity and Hammett constant, respectively). On the other hand the steric effect is evident from the first sight (OH > Me > Et = OMe > OEt), however only what the magnitude of the agonistic effect is concerned. The change from agonist into antagonist does not occur, the signal transfer is not blocked. The interpretation of data of the analogs with substituent in 2-position are more complicated. Single substituent in 2-position causes the change from agonist into antagonist. Additional alkyl substituents in 2′- or 4-positions have no influence on the quality. However, the electronic effect of hydroxyl group in 4-position is probably much stronger than the steric effect of methyl group in position 2 and the resulting analog with these two substituents has low intrinsic activity. Analogs substituted in 3-position of tyrosine are mostly antagonist (see 3-methyl and 3-*I* in Table II and 3,5-diBr analogs[35]) with the exception of 3-nitro- and 3-hydroxytyrosine analogs which have intrinsic activity. Unfortunately no data about biological activity of 3-substituted analogs derived from phenylalanine were reported.

As far as the D-aromatic amino acid in position 2 is concerned, only the analog with free hydroxyl in 4-position (D-tyrosine) has agonistic activity. In the case of any other substituent in whatever position, the configuration is probably so different that there is no signal transmission and the analogs are antagonists.

EXPERIMENTAL

General methods: Thin-layer chromatography (TLC) was carried out on silica gel coated plates (Silufol, Kavalier, The Czech Republic) in the following system: 1-butanol–acetic acid–pyridine–water (15 : 3 : 10 : 6) (S4) or on silica gel RP with Cu^{2+} and chiral reagent coated plates (Chiralplatte, Macherey–Nagel, Germany) in the system acetonitrile–water–methanol (4 : 1 : 1) (CH). Paper electrophoresis was performed in a moist chamber in 1 M acetic acid (pH 2.4) and in pyridine–acetate buffer (pH 5.7) on Whatman 3MM paper at 20 V/cm for 60 min. Spots in the TLC and electro-

phoresis were developed with ninhydrin or by the chlorination method. Samples for amino acid analysis were hydrolyzed with 6 M HCl at 105 °C for 20 h or with a mixture of acetic acid–hydrochloric acid (1 : 1) at 160 °C for 15 min, and analyzed on an Amino Acid Analyzer T 339 (Mikrotechna Praha, The Czech Republic) or D-500 Analyzer (Durrum Corp., U.S.A.). Optical rotations were determined on a Perkin–Elmer instrument type 141 MCA (Norwalk, U.S.A.) at 22 °C. Fast atom bombardment mass spectra (FAB) were obtained on a ZAB-EQ spectrometer (VG Analytical Ltd., Manchester, U.K.) with xenon at 8 kV as the bombarding gas. High performance liquid chromatography (HPLC) was carried out on an SP-8800 instrument equipped with an SP-8450 detector and SP-4290 integrator (all from Spectra Physics, Santa Clara, U.S.A.). Preparative HPLC was carried out using Vydac 218TP510 column (5 μm, 250 × 10 mm). Purity of the products was determined on Separon SIX C-18 (S) or Vydac 218TP54 (V) columns. Before use, all amino acid derivatives were subjected to the ninhydrin test[36].

Solid phase peptide synthesis: Incorporation of each amino acid residue into the growing peptide chain consisted of the following cycle: 1. cleaving the Boc group by 50% trifluoroacetic acid in dichloromethane containing 5% anisole, 5 min and 30 min; 2. washing with dichloromethane, 2-propanol and dichloromethane; 3. neutralizing by 5% ethyldiisopropylamine in dichloromethane, 2 and 5 min; 4. washing with dichloromethane and dimethylformamide; 5. adding the Boc-protected amino acid derivative in dimethylformamide followed by HOBt, followed by DCC and stirring for 1 – 2.5 h; 6. washing with dimethylformamide, dichloromethane, isopropanol and dichloromethane. The synthesis was monitored using the bromophenol blue method[37].

Nonapeptide Resin

p-Methylbenzhydrylamine resin (Peptides International, 0.79 meq/g, 1.3 g) was suspended in dichloromethane. After washing with 5% ethyldiisopropylamine in dichloromethane and with dimethylformamide, it was coupled with 3 molar excess of Boc-Gly-OH in the presence of *N*-hydroxybenzotriazole and dicyclohexylcarbodiimide in dimethylformamide. The coupling reaction was interrupted after 2 h, the resin was subsequently washed consequently by dimethylformamide (3-times) and dichloromethane (3-times) and the resin substitution was determined by amino acid analysis (0.31 mmol/g). The polymer was then acetylated (5 ml acetic anhydride, 2 ml triethylamine in 50 ml dichloromethane). The following procedure was performed according to the general scheme given in the beginning of the Experimental of this paper (starting from point 1.). Boc-amino acids were coupled to the resin by the DCC/HOBt procedure. All reagents were used in 3 molar excess. The protected derivatives were used in the following order: Boc-Leu-OH, Boc-Pro-OH, Boc-Cys(4-Me-Bzl)-OH (ref.[38]), Boc-Asn-OH, Boc-Gln-OH and Boc-Ile-OH. Amount of the heptapeptide resin: 1.7 g. Amino acid analysis on the resin: Asp 0.90, Glu 1.03, Pro 1.06, Gly 1.02, Cys 0.70, Phe 1.01, Leu 0.98. Following this step the resin was divided into five parts. The first two parts (0.4 g, 0.12 mmol each) were then coupled according to the general scheme with either Boc-D,L-Phe(4-Me)-OH (ref.[13]) or Boc-D,L-Phe(2-Me,4-Et)-OH and with Boc-Cys(4-Me-Bzl)-OH. The last three parts (0.3 g, 0.09 mmol each) were coupled with either Boc-D-Tyr(Me)-OH or Boc-D-Tyr(Et)-OH or Boc-Tyr(2-Me) and with Boc-Cys(4-Me-Bzl)-OH.

Cleavage of the Peptide from the Resin, Oxidation and Purification of Analogs *I* – *VII*

After removal of the Boc-protecting group, the nonapeptide resin was treated with liquid hydrogen fluoride (15 ml, 60 min, 0 °C) in the presence of anisole (1 ml). After the evaporation of hydrogen fluoride, the nonapeptide together with the resin was triturated with ether, filtered, and washed with ethyl acetate. The free peptide was successively extracted with acetic acid, 50% acetic acid, and water, and then lyophilized. The substance was then dissolved in water (900 ml) and the pH of the

solution was adjusted with 0.1 M NaOH to 7.0. Potassium ferricyanide (0.01 M solution) was added to this solution until a stable yellow color persisted. During the oxidation (20 min), the pH was maintained at 7.2 by adding 0.1 M NaOH and then adjusted with acetic acid to 4.5. The solution was then either put on a column of Amberlite CG-50I (50 ml), which was washed with 0.25% acetic acid and the product eluted with 50% acetic acid (150 ml) or to the solution was added in batch an Amberlite IR-45 (Cl^- form) and after stirring (1 h) the resin was filtered off. After freeze-drying, the crude product was purified by HPLC on a Vydac 218TP510 column in a slow gradient running from 30% to 50% methanol in 0.05% trifluoroacetic acid in 60 min and lyophilized. Data *k*, R_F, electrophoretic mobilities, optical rotation values, FAB MS and amino acid analyses are given in Table III.

Pharmacological Methods

All pharmacological tests were performed using Wistar rats weighing 200 – 300 g. The uterotonic potency in vitro was evaluated using the Holton procedure[39] in Munsick[40] solution. Inhibitory activity was characterized by the pA_2 value (ref.[41]). Pressor activity was tested on pithed rat according to refs[42,43].

TABLE III
Physico-chemical and analytical data for analogs *I – VII*

Data	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*
k	1.56[a]	4.19[a]	1.14[b]	2.84[b]	1.41[a]	3.01[a]	1.41[c]
R_F (S4)	0.64	0.64	0.63	0.63	0.64	0.60	0.57
$E_{2.4}^{Gly}$	0.55	0.56	0.53	0.52	0.52	0.51	0.57
$E_{5.7}^{His}$	0.44	0.41	0.36	0.33	0.39	0.42	0.36
$[\alpha]_D$[d]	–20.0	–55.0	–106.2	–51.4	–85.6	–51.1	–63.2
FAB MS[e]	1 005.6	1 005.6	1 033.1	1 033.2	1 021.0	1 035.0	1 021.6
			Amino acid analysis				
Asp	0.97	1.01	1.01	1.04	1.02	1.02	1.02
Glu	1.01	0.90	0.92	1.00	1.07	1.05	0.90
Pro	1.20	1.22	1.05	1.00	1.30	0.97	1.07
Gly	0.97	1.02	1.08	0.93	0.98	1.02	1.04
Cys	1.47	1.96	1.46	1.17	1.22	1.39	1.43
Ile	0.87	0.76	0.94	1.00	0.97	0.89	0.95
Leu	0.96	0.93	1.02	1.06	0.99	1.05	1.02
Phe(*p*-Me)	1.20	1.17	–	–	–	–	–
Phe(*o*-Me, *p*-Et)	–	–	0.94	0.98	–	–	–
Tyr	–	–	–	–	0.83	0.85	–
Tyr(*o*-Me)	–	–	–	–	–	–	1.10

[a] Methanol–0.05% trifluoroacetic acid (1 : 1), Vydac; [b] methanol–0.05% trifluoroacetic acid (6 : 4), Vydac; [c] methanol–0.05% trifluoroacetic acid (4 : 6), Vydac; [d] *c* = 0.1, 1 M acetic acid; [e] for $M + H^+$.

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