
**SYNTHESIS AND PROPERTIES OF "HORMONOGENE"
AND "INHIBITOROGENE" TYPE OXYTOCIN ANALOGS***

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N^α-Glycyl, diglycyl and triglycyl [2-D and [2-L-*p*-ethylphenylalanine]oxytocin analogs were synthesized by the solid phase technology utilizing racemic *p*-ethylphenylalanine. Analogs containing this amino acid of D-configuration were shown to be weak uterotonic antagonists both in vitro and in vivo tests; the compound containing triglycyl residue in position 1 was shown to have prolonged time course of inhibitory action. Analogs containing the L-amino acid were shown to be inhibitors of the uterotonic activity of oxytocin in vitro, but uterotonic agonists with prolonged effect in vivo.

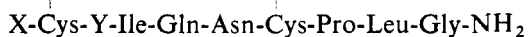
Biological activities of "homonogenes" were described by the Rudinger's group in the sixties (see e.g. ref.¹, for review see ref.²). It is assumed that the prolonged course of biological action is caused by gradual splitting of some part of the molecule, thus leading to generation of the active (or more active) molecule. In the case of oxytocin and vasopressin analogs*** it was shown that the hormonogene character of their molecules may be achieved by acylation of the N-terminal amino group by a short peptide chain which can be cleaved by aminopeptidases. We were interested to see if this principle would work also in the case of inhibitors.

It has been shown that introduction of a D-aromatic *p*-substituted amino acid into position 2 of oxytocin resulted in formation of inhibitors of oxytocin uterotonic activity⁴. Therefore, we have synthesized analogs II–IV, i.e. acylated analogs of the potent uterotonic inhibitor I ($pA_2 = 8 \cdot 15$). At the same time, since the synthesis was performed using racemic *p*-ethylphenylalanine, analogs V–VII containing this amino acid of L-configuration have been obtained.

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*** All the chiral amino acids, mentioned in this work, are of the L-series unless stated otherwise. The nomenclature and symbols of the amino acids and peptides obey the published recommendations³; Phe(*p*Et) denotes the *p*-ethylphenylalanine moiety.



<i>I</i> ,	X = NH ₂ ;	Y = D- <i>p</i> -ethylphenylalanine
<i>II</i> ,	X = Gly;	Y = D- <i>p</i> -ethylphenylalanine
<i>III</i> ,	X = Gly-Gly;	Y = D- <i>p</i> -ethylphenylalanine
<i>IV</i> ,	X = Gly-Gly-Gly;	Y = D- <i>p</i> -ethylphenylalanine
<i>V</i> ,	X = Gly;	Y = L- <i>p</i> -ethylphenylalanine
<i>VI</i> ,	X = Gly-Gly;	Y = L- <i>p</i> -ethylphenylalanine
<i>VII</i> ,	X = Gly-Gly-Gly;	Y = L- <i>p</i> -ethylphenylalanine

The peptides were synthesized by solid phase methodology on *p*-methylbenzhydrylamine resin using the *p*-methylbenzyl group for cysteine side chain protection and Boc-group for temporary α -amino group protection. All couplings were monitored using bromophenol blue (BB) method⁵. After cleavage with liquid HF and cyclization by K₃Fe(CN)₆ (ref.⁶), the peptides were desalted on a Sephadex G-15 column and separation of diastereoisomers was achieved by RP HPLC on a Vydac C-18 column. For the determination of the aromatic amino acid configuration we have employed enzymatic digestion using L-amino acid oxidase, followed by amino acid analysis⁷. In one case the results obtained were verified by a new methodology using acid hydrolysis followed by RP HPLC and chiral TLC separation⁸. Analogs *II*–*VII* were characterized by TLC, electrophoresis, RP HPLC, amino acid analysis and FAB MS data (Table I).

Prepared analogs were examined in uterotonic tests *in vitro* and *in vivo*, as well as in pressor test (max. administered dose $2 \cdot 10^{-2}$ mg/rat, Table II). All the analogs were found inactive in the pressor test. Analogs containing D-Phe(Et) were found to be less potent inhibitors in the uterotonic *in vitro* test than the non-acylated analog *I*. This finding was to be expected, taking into account the structure–activity knowledge collected hitherto^{9–11}. The results of the test performed *in vivo* were more interesting. Based on our preliminary experiments¹² with analog *IV* (prepared originally by acylation of compound *I* by triglycine), we have expected prolonged inhibitory activity in the case of all analogs *II*–*IV*. However, the prolonged character of the biological response was observed with compound *IV* only. This fact may be explained by different ways of enzymatic degradation of compounds *II*, *III* and *IV*. The analogs *II* and *III* may be processed by the enzymes (aminopeptidases or aminodipeptidases) due to the D-amino acid in position 2 more slowly or not at all, in comparison to the compound *IV*. The quantity of the generated peptide *I* may never reach such a concentration to express its activity. Degradation of analog *IV* into compounds *II* and *III* which both possess measurable inhibitory potency, may be responsible for its prolonged inhibitory potency. This hypothesis should be proved by model enzyme experiments.

Analogs *V*–*VII* were obtained as “side products” of the synthesis of the D-amino

acid containing analogs. However, their biological evaluation afforded results pointing to their potential practical application. In the *in vitro* uterotonic test, all the analogs were found to be inhibitors of a potency comparable to potencies of analogs containing the D-amino acid *II–IV*. However, in *in vivo* uterotonic test, a prolonged agonistic activity was found. The longer the amino-terminal peptide chain is, the lower is the activity – this corresponds well with the findings of Rychlík¹³ and Pliška¹⁴ in their studies of oxytocin acylated at the amino terminus. In our case, the finding was surprising since we supposed that splitting the peptide chain from the amino terminus should generate a compound with inhibitory activity as reported by Rudinger¹⁵ and Pliška¹⁶, even though it was first reported as a weak agonist by Zhuze et al.¹⁷.

However, [L-Phe(Et)²]OXT was not previously studied in the *in vivo* test. We have resynthesized this analog and we were able to show that it actually is an *in vivo*

TABLE I
Characteristics of analogs *II–VII*

Compound	E^{His} E^{Gly}	R_P		Formula (M.w.)	Calculated/Found			
		S1	S2		% C	% H	% N	(M + H) ⁺
		S3	S4					
<i>II</i>	0.28	0.14	0.03	$C_{47}H_{73}N_{13}O_{12}S_2$	47.99	6.41	14.85	1 076.5
	0.68	0.09	0.62	.TFA.2 H ₂ O (1 226.4)	48.25	6.79	14.60	1 077
<i>III</i>	0.30	0.13	0.03	$C_{49}H_{76}N_{14}O_{13}S_2$	47.07	6.41	14.50	1 133.5
	0.70	0.08	0.63	.TFA.2.5 H ₂ O. .AcOH (1 352.5)	47.43	6.92	14.21	1 134
<i>IV</i>	0.29	0.11	0.03	$C_{51}H_{79}N_{15}O_{14}S_2$	47.78	6.27	15.20	1 190.5
	0.70	0.06	0.64	.TFA.H ₂ O.AcOH (1 382.5)	47.84	6.45	15.32	1 193
<i>V</i>	0.29	0.12	0.03	$C_{47}H_{73}N_{13}O_{12}S_2$	48.29	6.36	14.36	1 076.5
	0.68	0.09	0.62	.TFA.H ₂ O.AcOH (1 268.4)	48.01	6.58	14.03	1 079
<i>VI</i>	0.31	0.08	0.03	$C_{49}H_{76}N_{14}O_{13}S_2$	48.69	6.24	15.00	1 133.5
	0.70	0.08	0.63	.TFA.AcOH (1 307.4)	48.23	6.61	14.68	1 134
<i>VII</i>	0.30	0.07	0.03	$C_{51}H_{79}N_{15}O_{14}S_2$	47.17	6.33	15.00	1 190.5
	0.70	0.06	0.64	.TFA.2 H ₂ O.AcOH (1 400.5)	46.87	6.57	14.71	1 193

uterotonic agonist. Analogs *V–VII* have a favourable spectrum of biological activities – they are not active in the pressor test and their antidiuretic activity is negligible. Therefore, they may be suggested as a suitable replacement for Carbetocin ([Tyr-(Me)²]dCOT-1) which is used for its prolonged uterotonic activity in veterinary practice (for review see¹⁸) and was also proposed for use in human medicine.

EXPERIMENTAL

General Methods

Thin-layer chromatography (TLC) was carried out on silica gel coated plates (Silufol, Kavalier, Czechoslovakia) in the following systems: 2-butanol–98% formic acid–water (10 : 3 : 8) (S1), 2-butanol–25% ammonia–water (85 : 7.5 : 7.5) (S2), 1-butanol–acetic acid–water (4 : 1 : 1) (S3), 1-butanol–acetic acid–pyridine–water (15 : 3 : 10 : 6) (S4). Paper electrophoresis was performed in a moist chamber in 1M 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 after TLC and electrophoresis were detected with ninhydrin or by the chlorination method¹⁹. Samples for the amino acid analysis were hydrolyzed with 6M HCl at 105°C for 20 h or with a mixture of propionic acid–hydrochloric acid (1 : 1) at 160°C for 15 min and analysed on an Amino acid analyzer T 339 (Mikrotechna Praha, Czechoslovakia) or D-500 analyzer (Durrum Corp., U.S.A.). Fast atom bombardment mass spectra were obtained on a ZAB-EQ spectrometer (BG Analytical Ltd., Manchester) 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.). HPLC purities of products were determined on the Vydac 218TP54 column. Preparative liquid chromatography was carried out on the above described equipment using a Vydac 218TP510 column (5 µm, 250 × 10 mm). Before use, all amino acid derivatives were subjected to the ninhydrin test²⁰.

TABLE II
Biological activities (rat) of oxytocin analogs

Compound	Uterotonic activity		Pressor activity
	in vitro	in vivo	
<i>I</i>	$pA_2 = 8.15$	$pA_2 = 7.7$	—
<i>II</i>	$pA_2 = 7.1$	$pA_2 = 6.0$	0
<i>III</i>	$pA_2 = 7.1$	$pA_2 = 5.8$	0
<i>IV</i>	$pA_2 = 6.6$	$pA_2 = 6.2^a$	0
<i>V</i>	$pA_2 = 7.1$	5.6^b	0
<i>VI</i>	$pA_2 = 6.9$	1.6^b	0
<i>VII</i>	$pA_2 = 6.6$	1.1^b	0

^a Prolonged inhibitory activity; ^b I.U./mg, prolonged agonistic activity.

Solid-phase peptide synthesis was performed on a home-made semi-automatic synthesizer. A cycle for incorporating each amino acid residue into the growing peptide chain consisted of the following: 1) cleaving the Boc group with 50% trifluoroacetic acid in dichloromethane containing 5% anisole, one treatment for 2 min, a second one for 20 min; 2) washing with dichloromethane (2×, 1 min/wash); 3) washing with isopropanol (3×, 1 min/wash); 4) washing with dichloromethane (3×, 1 min/wash); 5) neutralization with 5% diisopropylethylamine in dichloromethane (3× 2min); 6) washing with dichloromethane (2×, 1 min/wash); 7) washing with dimethylformamide (3×, 1 min/wash); 8) adding of the Boc-protected amino acid derivative in dimethylformamide, followed by HOBt, bromophenol blue solution⁵, and by diisopropylcarbodiimide under stirring until the color of bromophenol blue disappeared (2–20 min); 9) washing with dimethylformamide (3×, 1 min/wash); 10) washing with dichloromethane (3×, 1 min/wash); 11) washing with isopropylalcohol (3×, 1 min/wash); 12) washing with dichloromethane (3×, 1 min/wash).

Gly₃-[D- and Gly₃-[L-Phe(Et)²]OXT

A) *p*-Methylbenzhydrylamine resin (1% of divinylbenzene; 2 g, 0.7 mmol NH₂/g) was acylated consecutively by Boc-Gly, Boc-Leu, Boc-Pro, Boc-Cys(pMB), Boc-Asn, Boc-Glu, Boc-Ile, Boc-DL-Phe(Et), Boc-Cys(pMB) and by Boc-Gly. Approximately one third of the resin was removed (resin A, 1.26 g), and the rest was acylated by further Boc-Gly. The resin was then divided in two parts (resin B, 1.33 g) and to one of them, another Boc-Gly was coupled (resin C, 1.15 g). The resins A, B, and C were washed and dried. The resin C was mixed with 0.5 ml of anisole and 0.5 ml of ethanedithiol and treated with 10 ml of liquid HF at 0°C for 1 h. Then HF was evaporated, scavengers were extracted by ethyl acetate and the product was dissolved in 30% acetic acid. Lyophilisation afforded 430 mg of the product which was dissolved in 600 ml of water, the pH was adjusted to 7.5 with aqueous ammonia and 0.01M K₃Fe(CN)₆ was slowly added until the yellow color persisted. After another 30 min of stirring, Amberlite IR-45 (Cl⁻ form, 30 ml) was added and the stirring continued for 30 min. Ion exchange resin was filtered off, the filtrate was lyophilized, dissolved in 5 ml of 3M AcOH and gel-filtered over Bio-Gel P-4 in 3M AcOH. Fractions containing monomer were purified on Vydac C-18 (250 × 10 mm) using a gradient of methanol in 0.05% trifluoroacetic acid (0 to 30% in 2 min and 30 to 50% in 20 min). Lyophilization of the appropriate fractions afforded two analogs IV and VII containing D- and L-*p*-ethylphenylalanine. The characteristics are given in Table II, together with characteristics of other analogs prepared in the same way from resins A and B. Configuration of the aromatic amino acid was verified by enzymatic digestion using L-amino acid oxidase⁷ and by our methodology⁸. The compound IV was compared by RP HPLC with the product obtained by acylation of [D-Phe(Et)²]OXT with triglycine.

B) *o*-Nitrobenzenesulfonyl-glycyl-glycyl-glycine (0.45 g) was dissolved in DMF (3 ml), the solution was cooled to -10°C and N-hydroxysuccinimide (0.15 g) and dicyclohexylcarbodiimide (0.27 g) were added. After stirring for 1 h at -10°C and overnight at room temperature, the urea was filtered off, the solution was evaporated and the residue was triturated with petroleum ether and ether. The product was dried and used for acylation without further purification.

[D-Phe(Et)²]OXT (4 mg) was dissolved in water (0.3 ml), the pH of the solution was adjusted to 8 with 1M NaOH, and active ester of the protected tripeptide prepared as described above (Nps-Gly₃-ONSu, 10 mg), in dimethylformamide (0.3 ml) was added. After 2 h of mixing at room temperature, another portion of the active ester (14 mg) was added. The reaction mixture did not contain the starting oxytocin analog (according to HPLC analysis — Zorbax ODS, 25 × 0.4 cm, 65% methanol in 0.1% trifluoroacetic acid) after overnight stirring. The solution

was concentrated in vacuum and the residue was dissolved in 1 ml of methanol. After the addition of 2.3M HCl in methanol (60 μ l), the mixture was stirred for 5 min, evaporated and dissolved in 3M acetic acid. The solution was applied to the column of Partisil ODS (50 \times 0.9 cm) and the product was eluted with the gradient of methanol in 0.1% trifluoroacetic acid (40 to 60% in 120 min). Lyophilization of the appropriate fractions afforded 2.3 mg of the compound which didn't show any differences when compared (TLC, electrophoresis, HPLC) with the product of the above described synthesis.

o-Nitrobenzenesulfonyl-glycyl-glycyl-glycine

o-Nitrobenzenesulfonyl-glycyl-glycine (0.90 g) was dissolved in DMF (15 ml) together with N-hydroxysuccinimide (0.42 g) and dicyclohexylcarbodiimide (0.72 g). After stirring for 16 h, the urea was filtered off and the solution was mixed with the solution of glycine (0.785 g) in water (16 ml). The pH of the solution was adjusted to 8.5 with 1M NaOH and the solution was stirred for 16 h at room temperature. Solution was evaporated to dryness, the residue was dissolved in KHSO₄ buffer (pH 2, 40 ml) and extracted by ethyl acetate (5 \times 60 ml). The organic extract was washed by water, dried by Na₂SO₄, evaporated and dried in vacuum. Yield 0.9 g (83%). *R_F* 0.62 (S1), 0.05 (S2), 0.55 (S3), 0.38 (S4). A sample of the product was transformed to dicyclohexylammonium salt; m.p. 157–160°C. For C₂₄H₃₇N₅O₆S (523.7) calculated: 55.05% C, 7.12% H, 13.37% N; found: 54.86% C, 7.26% H, 13.15% N.

Pharmacological Methods

Uterotonic activity *in vitro* was determined on an isolated strip of rat uterus^{21,22}. For determination of the uterotonic activity *in vivo* estrogenized rats in ethanol anaesthesia were used²³. Pressor activity was determined on pithed rats²⁴. Inhibitory potencies were expressed by the *pA*₂ values^{25,26}.

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