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Analogs of oxytocin containing a pseudopeptide Leu-Gly bond of *cis* and *trans* configuration*

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Dedicated to the memory of Prof. Choh Hao Li

Analogs of deamino-oxytocin wherein the Leu-Gly peptide bond has been replaced by a tetrazole moiety or by a double bond of *trans* configuration were synthesized and their biological activities evaluated. *Trans* double bond was found to be the most appropriate substitution for the amide bond (uterotonic activity 24% of the deamino-oxytocin). In the case of all three analogs low but prolonged galactogogic activity was found and the ratio of uterotonic *in vitro* and *in vivo* activity was surprisingly high (ranging from 4.5 to 20).

Key words: modified peptide bonds; oxytocin analogs; tetrazole replacement; *trans* substituted double bond

The tetrazole moiety and the *trans* double bond are the most appropriate models for fixing the orientation of the peptide bond in a *cis* or *trans* configuration, respectively. The recently synthesized tripeptides IV–VI (2, 3) containing these replacements of the Leu-Gly amide bond have been incorporated into deamino-oxytocin to test the influence of this amide bond orientation on its biological activities. Peptides containing these modifications (I–III) are expected to be stabilized against enzymatic degradation and therefore they may in principle exhibit a prolonged time course of action.

RESULTS AND DISCUSSION

We have recently described (4) the synthesis of deamino-oxytocin analogs containing the CH_2-NH replacement of the Leu-Gly peptide bond by the condensation of the *N*-terminal hexapeptide containing the preformed disulfide bond with the *C*-terminal tripeptide amide. This synthetic approach was pioneered by Mühlemann *et al.* (5). We have used the dicyclohexylcarbodiimide/*N*-hydroxybenzotriazole coupling method for the synthesis of deamino-oxytocin and diphenyl phosphoryl azide mediated condensation (6) for the synthesis of analogs. The first method has the disadvantage of insoluble reaction side products, which may be a complication in reactions run on an extremely small scale. Therefore we decided to use diphenyl phosphoryl azide for this condensation, since this reacts rather fast and the resulting reaction mixture does not contain a precipitate. The condensation was tested on the preparation of deamino-oxytocin, which was obtained in good yield and purity. The course of the reaction was followed by reversed-phase HPLC and the product was recovered by preparative chromatography after complete disappearance of the starting hexapeptide was observed. Purity of the products was checked by chromatography (t.l.c., HPLC), and for characterization amino acid analysis and mass spectroscopy were used. In all cases side product formation was observed. According to the evidence (e.g., the same molecular mass and fragmentation and the same amino-acid analysis as the main product) its structure might have been the diastereomeric peptide containing D-cysteine in position 6 as a result of racemization during the coupling step.

Biological activities of the analogs prepared together with those of some reference compounds are given in Table 1. As can be seen, the highest *in vitro*

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Abbreviations: All optically active amino acids are of the L configuration. Symbols and abbreviations are in accordance with recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*European J. Biochem.* (1984) 138, 9–37). Nomenclature of peptide backbone modifications follows the suggestions of Spatola (1).

TABLE I

Biological activities (IU/mg) of oxytocin and deamino-oxytocin analogs containing modified Leu-Gly peptide bond

	Analog[a]	Replacement of CONH	Activity				Distance[b] C'-C' (nm)	Ref.
			Uterotonic		Galactogogic	Pressor		
			in vitro	*in vivo*				
	[Mpa^1]OXT		803		541	1.44	38	7
	[Leu ψ(CH_2S)Gly$^{8-9}$]OXT	CH_2S	10.2		15.5	< 0.01	42	8
	[Mpa^1, Glc^9]OXT	COO	134	54	108	0.35	38	9, 10
	[Sar^9]OXT	$CON(CH_3)$	36		55	< 0.01	38	11
	[Mpa^1, Leu ψ(CH_2NH)Gly$^{8-9}$]OXT	CH_2NH	22.3	1.5	10.0	< 0.02	37	4
I.	[Mpa^1, Leu ψ(1,5-CN_4)Gly$^{8-9}$]OXT	[c]	104 ± 10 (n = 5)	11.5 ± 2.1 (n = 6)	16.8 ± 3.0 (n = 4)	< 0.02	24	[f]
II.	[Mpa^1, Leu ψ(2,5-CN_4)Gly$^{8-9}$]OXT	[d]	24.8 ± 6.0 (n = 5)	5.3 ± 1.1 (n = 5)	5.4 ± 1.0 (n = 4)	< 0.02	48	[f]
III.	[Mpa^1, Leu ψ(CH = CH)Gly$^{8-9}$]OXT	CH = CH[e]	200 ± 34 (n = 5)	11.7 ± 3.0 (n = 4)	22.5 ± 1.0 (n = 4)	< 0.02	39	[f]

[a]Mpa = 3-mercaptopropionic acid, Glc = glycolic acid, Sar = sarcosine.
[b]Based on the standard geometry in fully extended form.
[c]1,5-Substituted tetrazole ring.
[d]2,5-Substituted tetrazole ring.
[e]*Trans* configuration.
[f]This work.

uterotonic potency was found for the analog containing the double bond of *trans* configuration. It is even higher than the activity of the analog containing the COO group instead of the CONH in this position. The analog containing the ester group (9, 10) instead of the amide bond has up to now been the only one where replacement of the *C*-terminal Leu-Gly peptide bond results in higher potency than deaminotocinamide (i.e., the analog not containing *C*-terminal tripeptide at all). This is additional evidence that the presence of the Leu-Gly amide bond is not essential (9) for biological activity, and that the originally suggested (12) hydrogen bond between this amide hydrogen and the cysteine carbonyl in position 6 is not important for the formation of the so called biologically active conformation of oxytocin (13). However, the proper orientation of the *C*-terminal glycine carboxamide is of extreme importance for biological potency. Substitution of the Leu-Gly peptide bond by the double bond of *trans* configuration places the terminal carboxamide at approximately the same place as in unmodified peptide. In Table I the distance between the α-carbons of the Leu and Gly residues is given in nm for all analogs with modifications of this amide bond. As can be seen from the results, only a value close to the value calculated for the amide group gives rise to activity that is higher than that observed for the *N*-terminal hexapeptide amide. Although the sarcosine-containing analog has the correct value for this distance, the likelihood of a *cis-trans* equilibrium makes this analog only moderately potent. Analog I with the fixed *cis* peptide bond replacement (i.e., the 1,5-substituted tetrazole moiety) has higher activity than analog II with the 2,5-substituted tetrazole, even though the C^{γ}-C^{β} distance in II is closer to that of the unmodified peptide than that of I. Compounds I and III display clear agonist activity, however compound II is a partial antagonist – after expressing agonist activity, it inhibits itself and also inhibits further doses of oxytocin.

In all the new compounds reported here, a rather unusual drop of uterotonic potency was observed when tested *in vivo*. The *in vivo* activity is 5–20 times lower than the activity *in vitro*. The largest difference between the *in vitro* and *in vivo* activity is seen in the case of compound III. This is not a usual trend with analogs of neurohypophyseal hormones (for the recent list see (14)). Most importantly, there is no prolongation of the activity.

However, the galactogogic activity of all prepared compounds (even if of the same order of magnitude as the uterotonic *in vivo* activity and lower than in the case of ester bond replacement) is extremely prolonged (see for example Fig. 1). The dose-response curves (see Fig. 2) of oxytocin and of the analogs are parallel if the maximal intramammary pressure is taken as a measure of the effect. However, the slope of the dose-response curves of the analogs is much steeper than that of oxytocin if the whole effect (integrated) is taken as a measure of activity. The response lasts 3–50 times longer. The compound with the least prolonged effect is compound III.

Since the analogs should be stabilized against enzymatic degradation, the difference in the *in vivo* and *in vitro* uterotonic activity might be explained either by the chemical instability of the analogs in the blood stream or by a change in their distribution processes. However, the prolonged galactogogic activity (measured *in vivo*) is an argument in favor of the second option. Analogs may be transported into the so-called storage compartment (15), from where they are gradually released, causing a prolonged galactogogic activity. The release may be too quick for eliciting the prolonged response in the *in vivo* uterotonic test and at the same time slow enough to cause prolongation of the galactogogic test. The situation is exactly the opposite to the behavior of deamino-oxytocin, which is considerably prolonged in the uterotonic *in vivo* test, but not prolonged in the galactogogic test (16).

In view of this situation it was interesting to compare the lipophilicity of the prepared compounds. Due to the limited amount of prepared analogs only retention characteristics obtained from reversed phase HPLC could be used for this purpose (for a discussion of this method of lipophilicity comparison and its pharmacological consequences see (17)). According to the results obtained (see Table 2) we found that the analog most closely resembling deamino-oxytocin

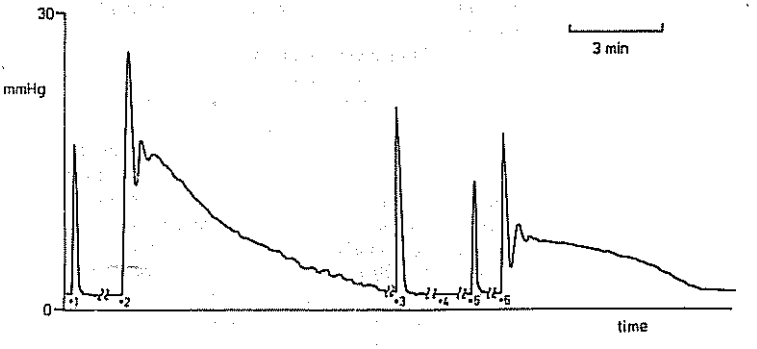


FIGURE 1

Effect of oxytocin and compound II on intramammary pressure of female rat 10 days after delivery – record of a typical experiment. 1, 3, and 5 doses of oxytocin $2.75 \cdot 10^{-7}$, $5.5 \cdot 10^{-7}$, and $1.37 \cdot 10^{-7}$ mg per rat, respectively. 2, 4, and 6 doses of compound II, $4 \cdot 10^{-5}$, $5 \cdot 10^{-6}$, and $2 \cdot 10^{-5}$ mg per rat, respectively.

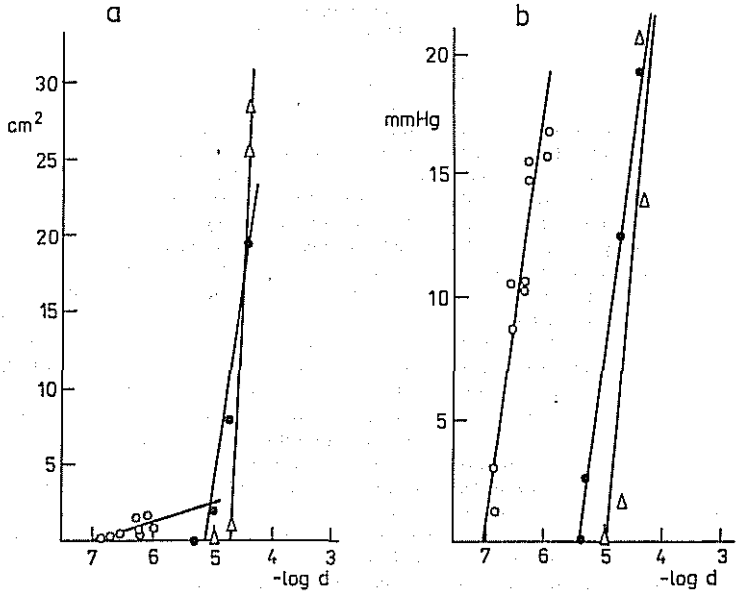


FIGURE 2

Dose-response curves for oxytocin (○) and compounds I (●), and II (△) – a typical experiment. a) the whole response (i.e., integrated area under the pressure recorder trace) taken as a measure of the effect; b) the maximal intramammary pressure taken as a measure of the effect (the standard way of evaluating the potency)

TABLE 2

Characteristics of analogs prepared in this paper

Analog	R_f S1 S3	S2 S4	k'	Amino acid analysis Asp Cys	Glu Ile	Pro Tyr	M + H Found Calc.
I	0.41	0.32	2.81	1.03	1.01	0.96	1017.6
	0.35	0.67		1.16	1.00	0.89	1017.4
II	0.36	0.23	4.23	1.02	0.96	0.94	1017.7
	0.30	0.63		1.23	1.00	0.93	1017.4
III	0.37	0.26	8.17	0.98	1.02	0.92	974.6
	0.32	0.64		0.83	1.00	0.94	974.4

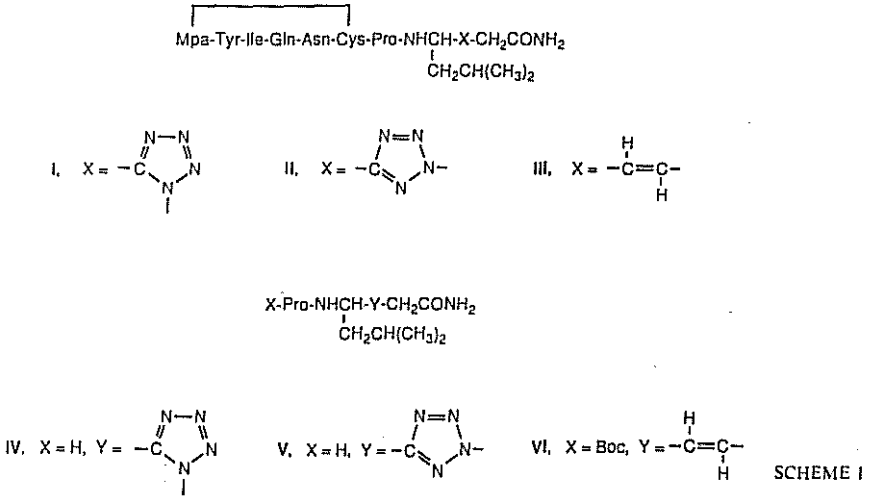
'Retention on RP HPLC – mobile phase 50% MeOH in 0.05% trifluoroacetic acid (under the same conditions deamino-oxytocin has k = 3.16).

chromatographically was analog I, the *C*-terminal tripeptide geometry of which differs the most. On the other hand, the most active analog III possessed a significantly increased lipophilicity. This may further support the idea mentioned earlier that slow release from the storage compartment may be the reason for the great difference between *in vivo* and *in vitro* potency of analog III.

The pressor activity was almost eliminated in all analogs with a modified 8—9 peptide bond. The only exception was the analog with the ester bond. The same holds for analogs prepared in this study. Therefore, the pressor receptor (V_1) may not tolerate a more hydrophobic molecule, or it may need some hydrogen bond accepting group (CO group).

EXPERIMENTAL PROCEDURES

Thin-layer chromatography was carried out on silica gel plates (Silufol, Kavalier, Czechoslovakia) in the following systems: 2-butanol-98% formic acid-water (75:13.5:11.5) (S1), 2-butanol-25% aqueous ammonia-water (85:7.5:7.5) (S2), 1-butanol-acetic acid-water (4:1:1) (S3), and 1-butanol-pyridine-acetic acid-water (15:10:3:6) (S4). The compounds were detected by the chlorination method (18). Samples for amino acid analyses were hydrolyzed with 6 M HCl at 105° for 20 h and analyzed on a Durrum 500 automatic analyzer. High performance liquid chromatography (HPLC) was performed on an SP-8700 instrument equipped with an SP-8400 detector (Spectra-Physics,



SCHEME I

Santa Clara, CA, USA). Fast atom bombardment mass spectra were obtained on a ZAB-EQ spectrometer (VG Analytical Ltd., Manchester) with xenon at 8 kV as the bombarding gas.

Deamino-oxytocin

Prolyl-leucyl-glycinamide (2 mg) was added to the solution of deaminotocinoic acid (5, 19) (1.2 mg) dissolved in dimethylformamide (20 μL). After the addition of diphenyl phosphoryl azide (2 μL) and diisopropylethylamine (2 μL) the mixture was shaken at room temperature. The reaction was followed by reversed phase HPLC (column Separon SI C18, 15 × 0.4 cm, MeOH-0.05% TFA (1:1)). After the disappearance of the deaminotocinoic acid the solution was diluted with 3 M AcOH (300 μL) and injected onto a column of Vydac C18 peptides and Proteins (250 × 10 mm) equilibrated with 0.05% trifluoroacetic acid. Elution was carried out with a gradient from 0.05% TFA to 20% MeOH in 2 min and to 50% MeOH in 32 min. The product was concentrated *in vacuo* and lyophilized. Lyophilizate (0.7 mg) was compared with a standard preparation of deamino-oxytocin (Spofa, Czechoslovakia) by t.l.c., HPLC, and mass spectroscopy and found to be identical.

[Mpa^1, Leu ψ[1,5-CN$_4$]Gly$^{8-9}$]OXT (I)

Deaminotocinoic acid (1.9 mg) and tripeptide IV (3.2 mg) were dissolved in dimethylformamide (32 μL) and the solution was cooled to 0°. After the addition of diphenyl phosphoryl azide (3 μL) and diisopropylethylamine (3 μL) the resulting mixture was shaken at room temperature for 3 h. The reaction was followed by HPLC and worked up as described above. Lyophilization afforded the product as a white fluffy powder (0.95 mg, 35%). The characteristics of the analog are given in Table 2.

[Mpa^1, Leu ψ[2,5-CN$_4$]Gly$^{8-9}$]OXT (II)

Condensation of deaminotocinoic acid (2 mg) and tripeptide V (3.3 mg) was performed in the same manner as in the case of analog I. The same purification methods afforded 1.2 mg (42%) of compound II, the characteristics of which are given in Table 2.

[Mpa^1, Leu ψ[CH=CH]Gly$^{8-9}$]OXT (III)

Tripeptide VI (6 mg) was dissolved in trifluoroacetic acid (40 μL) and after 30 min the mixture was evaporated to dryness. The residue was dissolved in a solution of deaminotocinoic acid (4.2 mg) in dimethylformamide (70 μL). After alkalization of the solution by diisopropylethylamine (10 μL) and addition of diphenyl phosphoryl azide (10 μL) the mixture was shaken for 4 h at room temperature. The workup and purification was performed as described above and afforded 1.45 mg (24%) of a white fluffy powder, the characteristics of which are given in Table 2. As a side product, eluted earlier in the HPLC purification, the compound with a molecular weight higher by two units (FAB MS) was isolated, which may be the analog containing hydrogenated double bond.

Pharmacological methods

Uterotonic *in vitro* activity was determined on an isolated strip of rat uterus (20, 21). For determination of the *in vivo* activity, estrogenized rats in ethanol anesthesia were used (22). Galactogogic activity was determined on ethanol-anesthetized rats (23, 24), and pressor activity on despinalized rats (25).

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