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Analogs of oxytocin containing a modified peptide bond*

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Analogs of deamino-oxytocin and deamino-oxypressin containing a CH_2-NH group instead of an amide bond between positions 8 and 9 were synthesized. All tested compounds exhibit significantly lowered biological activities.

Key words: oxytocin analogs; oxypressin analogs; modified peptide bonds

Modification of peptide bonds by pseudoisosterical replacement are generally expected to influence the physicochemical and biological properties of the resulting analogs in several ways (for review see (1)). In the case of neurohypophyseal hormones, the integrity of *C*-terminal tripeptides is known to be very important for their full biological activity (for review see (2, 3)). Cleavage of the Leu-Gly peptide bond was suggested as one of the inactivation pathways for these hormones (for review see (4)). Modification of this peptide bond was already described in the literature (see Table 1), generally leading to analogs exhibiting decreased biological potencies. We have synthesized analogs containing CH_2NH group in place of the leucyl-glycine peptide bond (II and IV), which should be stable to enzymatic splitting of glycine amide but which still have the potential of being a part of the hydrogen-bonding interaction with the receptor. The synthetic approach also enables us to test related analogs containing the bulky and lipophilic benzyloxycarbonyl group attached to the amino-group in the peptide chain (I and III).

RESULTS AND DISCUSSION

The synthesis of the modified carboxyterminal tripeptide of oxytocin V, reported elsewhere (5), was the starting point for the synthesis of the neurohypophyseal hormone analogs. The synthesis of the oxytocin and vasopressin analogs was based on the known possibility of condensing the aminoterminal hexapeptide containing a preformed disulfide bond with the carboxyterminal tripeptide. This condensation proceeds smoothly (6, 7) in contrast to the very difficult condensation of the oxytocin aminoterminal hexapeptide azide containing protected cysteines with the *C*-terminal tripeptide (8).

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

TABLE I

Biological activities of analogs containing modified Leu-Gly peptide bond and of some reference compounds

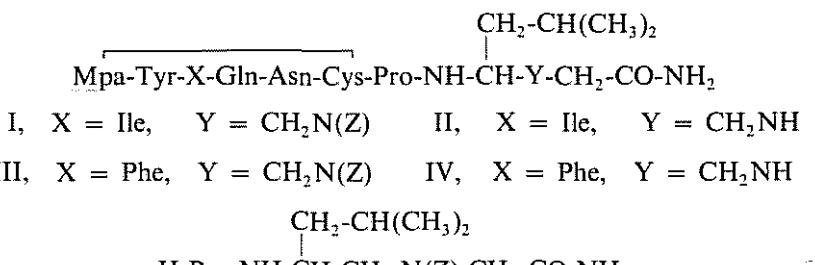
Analog[a]	Replacement of CONH	Uterotonic (I.U./mg) *in vitro*	*in vivo*	Galactogogic (I.U./mg)	Pressor (I.U./mg)	Ref.
[Mpa^1]OXT	CONH	803		541	1.44	11
[Leu^8]VP	CONH	39		–	5.1	12
[Leuψ[CH$_2$S]Gly$^{8-9}$]OXT	CH$_2$S	10.2		15.5	< 0.01	13
[Mpa^1,Leuψ[COO]Gly$^{8-9}$]OXT	COO	134	54	108	0.35	14, 15
[Sar^9]OXT	CON(CH$_3$)	36		55	< 0.01	16
I	CH$_2$NZ	4.3 ± 0.7[b] (n = 5)	0.39 ± 0.1 (n = 4)	1.1[c]	< 0.02	e
II	CH$_2$NH	22.3 ± 4.4[b] (n = 5)	1.5 ± 0.3 (n = 4)	10.0[c]	< 0.02[d]	e
III	CH$_2$NZ	0.1	0.1	0.14	< 0.02	e
IV	CH$_2$NH	0.3	0.1	0.6[c]	< 0.02[d]	e

[a] Mpa = 3-mercaptopropionic acid, Sar = sarcosine. [b] In the presence of 1 mM Mg^{2+}, the activity of the analog decreases 5-10-fold and the analog is not able to produce the same maximal contraction as the standard. In 50% of tests in the absence of magnesium the analog was also unable to produce the same maximal effect as oxytocin. [c] Prolonged. [d] In some tests a very low inhibitory activity was found, $pA_2 \sim 5.5$. [e] This work.

Deaminotocinoic acid (6, 9) was synthesized by solid phase method on a standard Merrifield resin. A model synthesis of deamino-oxytocin by dicyclohexylcarbodiimide-hydroxybenzotriazole condensation of deaminotocinoic acid with Pro-Leu-Gly-NH_2 was performed. This synthesis afforded pure product in reasonable yield (31.3%). However, the same approach with the modified tripeptide V failed. We succeeded in synthesizing the protected nonapeptide with the use of diphenylphosphoryl azide (10) as the coupling agent. Cleavage of the benzyloxycarbonyl protecting group was achieved by liquid hydrogen fluoride in the presence of anisole. The same synthetic strategy was used in the preparation of the oxypressin analog IV.

The biological activities of the prepared compounds are given in the Table 1. As can be seen, replacement of CONH group by CH_2NH decreased biological activities significantly. Even the most active compound II has only about 3.8% of the potency of the parent analog, deamino-oxytocin (17), in the uterotonic *in vitro* test. Analog II is more selective than deamino-oxytocin due to the almost complete elimination of pressor activity. The bulky carbobenzoxy group attached to the backbone of the carboxyterminal tripeptide significantly decreased all tested agonist activities without introducing any sign of inhibitory activity. Very low inhibitory activity in the pressor test was observed in analogs having the free amino group in the backbone (II and IV).

More stable analogs might exhibit prolonged activities due to their longer half-life



in the living organism. Analogs I, II and IV had slightly prolonged galactogogic activity in comparison with the activity of deamino-oxytocin. The galactogogic activity of analogs was decreased significantly more than the uterotonic activity, which was quite unexpected since the exactly opposite behavior was demonstrated in most analogs of oxytocin and vasopressin synthesized up to now (see (2)). Uterotonic activity of compounds I and II determined *in vivo* was an order of magnitude lower than the activity determined *in vitro*, and there were no signs of prolonged activity. Higher *in vitro* activity is quite exceptional, the opposite being more common. The difference in the uterotonic *in vitro* and *in vivo* tests of about one order of magnitude may be partly explained by the influence of magnesium ions. It was found that the *in vitro* uterotonic activity of compounds I and II is decreased 5-10-fold if the test is performed in the presence of 1 mM magnesium.

Modification of the tripeptide side chain has been reported (18) to influence the chymotryptic cleavage of Tyr-Ile peptide bond in the oxytocin molecule. We have compared the enzymatic degradation of deamino-oxytocin and analog II. The cleavage of Tyr-Ile bond is accompanied in deamino-oxytocin by the splitting of the carboxyterminal glycinamide residue (which is about 10 times slower) (19). Nevertheless, we were able to differentiate between these two processes and, contrary to the above mentioned work (18), we have not observed any significant difference in the speed of cleavage of Tyr-Ile peptide bond in deamino-oxytocin and in analog II.

EXPERIMENTAL PROCEDURES

Amino acids used in the synthesis were obtained from Vega Biochemicals (Tucson, AZ), Bachem (Torrence, CA), or were prepared by standard literature methods. All were checked prior to coupling by the ninhydrin test (20). Solid phase synthesis was performed on a Vega 1000 synthesizer. Thin-layer chromatography was carried out on silica gel plates (Silufol, Kavalier, Czechoslovakia) in the 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). Electrophoresis was performed for 1 h on Whatman 3 MM paper in a moist chamber (20 V/cm) in 1 M acetic acid (pH 2.4) or a pyridine-acetate buffer (pH 5.7). The compounds were detected by ninhydrin or by the chlorination method (21). Samples for amino acid analyses were hydrolyzed with 6 M HCl at 105° for 20 h and analyzed on an automatic analyzer (Durrum 500). High performance liquid chromatography (HPLC) was performed on an SP-8700 instrument equipped with an SP-8400 detector (Spectra-Physics, Santa Clara, CA, USA). ^{1}H n.m.r. spectra were recorded on a Bruker 250 MHz spectrometer. Fast atom bombardment mass spectra were obtained on a Varian 311A spectrometer equipped with an Ion Tech Ltd. source with xenon as the bombarding gas. Tripeptide V was prepared as previously described (5).

Deaminotocinoic acid

Boc-Cys(*p*-MeBzl) was coupled to Merrifield's resin (2% DVB) by the cesium salt procedure (22) to obtain a resin with a substitution of 0.55 mM/g (determined by picric test) of amino acid. Using standard solid phase peptide synthesis methods (23), Boc-Asn-ONp and Boc-Gln-ONp were then coupled in the presence of *N*-hydroxybenzotriazole. Boc-Ile, Boc-Tyr(2, 6-Br_2-Z) and Mpa(2, 4-$(CH_3)_2$-Bzl) were coupled by standard DCC/HOBt procedure. Half of the obtained resin (2.44 g) was subjected to cleavage by liquid HF (20 mL, 0°, 60 min) in the presence of anisole (1.5 mL). The product was washed with ethylacetate, extracted with 30% acetic acid and lyophilized. The lyophilizate was dissolved in water (1 L), the pH of the solution was brought to 8.3 with NH_4OH, and 0.01 N $K_3Fe(CN)_6$ was slowly added (80 mL). After mixing at room temperature for 1 h, anion exchange resin (Bio-Rad, Cl^- cycle, 15 mL settled resin) was added and solution was mixed for another $\frac{1}{2}$ h. After filtration, the solution was lyophilized to give

498 mg fluffy powder. Part of the crude product (78 mg) was dissolved in 3 M AcOH and applied to a Bio-Gel P-4 (100 × 1 cm) column. A single symmetric peak was observed and after lyophilization, 28 mg (31.3%) of pure peptide (t.l.c., HPLC) was obtained. Amino acid analysis: Asp 0.96, Glu 0.98, Cys 0.36, Ile 1.04, Tyr 1.02.

Deamino-oxytocin

Deaminotocinoic acid (3.6 mg) was dissolved in dimethylformamide (150 μL). The solution was cooled to 0° and prolyl-leucyl-glycinamide (3.2 mg), dicyclohexylcarbodiimide (5.2 mg) and *N*-hydroxybenzotriazole (2.2 mg) were added. After 2 h mixing at 0° and 4 h at room temperature, the solution was diluted with 1 mL 3 M acetic acid and placed on a Bio-Gel P-4 column (100 × 1 cm). The product was eluted with 3 M acetic acid and lyophilized to give 2.8 mg (55%) of deamino-oxytocin. This product was compared with a standard preparation of deamino-oxytocin (Spofa, Czechoslovakia) and found to be identical.

[Mpa^1,Leuψ[CH$_2$NZ]Gly$^{8-9}$]OXT (I)

Deaminotocinoic acid (15.8 mg) and the tripeptide H-Pro-Leuψ[CH$_2$NZ]Gly-NH$_2$ (V) (16 mg) were dissolved in dimethylformamide (150 μL), cooled to 0°, and after the addition of diphenylphosphorylazide (8.6 μL) and diisopropylethylamine (8 μL) the resulting mixture was shaken in the dark at room temperature for 15 h. The product was precipitated with ether and reprecipitated twice by ether from methanolic solution (the precipitate was separated by centrifugation). The pellet was dissolved in 3 M acetic acid (5 mL) and placed on a column of Bio-Gel P-4 (100 × 1 cm). Elution was performed using 3 M acetic acid and fractions containing peptide material of approximate molecular weight 1000 (calibration by deamino-oxytocin) were combined and injected (16 × 250 μL) onto a HPLC column (Vydac C^{18}-Peptides and Protein - 250 × 10 mm) equilibrated with 0.1% trifluoroacetic acid. Elution was performed with the following gradient: 0–25% acetonitrile for 2 min followed by 25–40% acetonitrile for 30 min. The product was obtained after lyophilization as a white fluffy powder (14.8 mg; 56%). The characteristics of the analog are given in Table 2.

[Mpa^1,Leuψ[CH$_2$NH]Gly$^{8-9}$]OXT (II)

Analog I (8 mg) was mixed with anisole (100 μL) and liquid hydrogen fluoride (2 mL) was distilled into this mixture. After ½ h at 0°, the mixture was evaporated to dryness, dissolved in 3 M acetic acid (5 mL) and the solution injected onto an HPLC column (Vydac C^{18}-Peptides and Protein - 250 × 10 mm) equilibrated with 0.1% trifluoroacetic acid. Elution was performed by the following gradient: 0–15% acetonitrile for 2 min and 15–30% for 23 min. Lyophilization of the appropriate fraction afforded 5.5 mg (78%) of a white fluffy powder of II. Characteristics are given in Table 2.

[Mpa^1,Leuψ[CH$_2$HZ]Gly$^{8-9}$]VP (III)

Condensation of deaminopressinoic acid (24) (15 mg) and tripeptide V was performed in the same manner as in the case of analog I. The

TABLE 2

Characteristics of analogs prepared in this paper

Analog	Rf							Amino acid analysis							M + H	
	S1	S2	S3	S4	$E^{His}_{5.7}$	$E^{Gly}_{2.4}$	k[a]	Asp	Glu	Pro	Cys	Ile/ Phe	Tyr	Leg[b]	Found	Calc.
I	0.28	0.19	0.15	0.55			30.2								1112.4	1112.4
II	0.10	0.01	0.00	0.48	0.36	0.76	5.08	1.02	1.03	1.09	0.34	0.97	0.87	1.03	978.4	978.5
III	0.33	0.23	0.19	0.57			32.3								1147	1146.4
IV	0.11	0.02	0.01	0.50	0.37	0.76	5.90	1.00	0.98	1.05	0.32	1.00	0.92	1.06	1012	1012.4

[a] 50% MeOH in 0.05% trifluoroacetic acid [b] Leg = Leuψ[CH$_2$NH]Gly, eluted at the place of Lys with the color value 26% of the Lys value.

same purification methods afforded 7 mg (27%) of compound III, the characteristics of which are given in Table 2.

[Mpa^1,Leuψ[CH$_2$NH]Gly$^{8-9}$]VP (IV)
Cleavage of the protecting group from analog III (4.8 mg) was performed in the same manner as in the case of analog II. Purification was accomplished as reported for compound II. Characteristics of the product IV (3.7 mg, 87%) are given in Table 2.

Cleavage of analog II and deamino-oxytocin with chymotrypsin
The incubation mixture contained 0.5 mg analog II (or deamino-oxytocin) in 1 mL of 20 mM phosphate buffer pH 7.8 and 60 μL of the solution of chymotrypsin (3 mg/mL) in the same buffer. Mixture was incubated at 28° and analyzed by HPLC on the column Separon SI C18 25 × 0.4 cm (Laboratorni pristroje, Prague) in the system 0.5% trifluoroacetic acid - methanol (1:1).

Pharmacological methods
Uterotonic *in vitro* activity was determined on an isolated strip of rat uterus (25, 26), for determination of the *in vivo* activity, estrogenized rats in ethanol anesthesia were used (27). Galactogogic activity was determined on ethanol-anesthetized rats (28, 29) and pressor activity on despinalized rats (30).

REFERENCES

1. Spatola, A. (1983) in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins* (Weinstein, B., ed.), Vol. 7, pp. 267–357, Marcel Dekker, New York
2. Lebl, M. (1987) in *Handbook of Neurohypophysial Hormone Analogs* (Jošt, K., Lebl, M. & Brtník, F., eds.), Vol. 2, CRC Press, Boca Raton, FL, in press
3. Hruby, V.J. (1986) in *Biochemical Actions of Hormones* (Litwack, G., ed.), Vol. 13, pp. 191–241, Academic Press, New York
4. Jošt, K. (1987) in *Handbook of Neurohypophysial Hormone Analogs* (Jošt, K., Lebl, M. & Brtník, F., eds.), Vol. 1, Part 2, pp. 15–30, CRC Press, Boca Raton, FL
5. Vander Elst, P., Elseviers, M., De Cock, E., Van Marsenille, M. & Van Binst, G. (1986) *Int. J. Peptide Protein Res.* **27,** 633–642
6. Mühlemann, M., Titov, M.I., Schwyzer, R. & Rudinger, J. (1972) *Helv. Chim. Acta* **55,** 2854–2858
7. Jones, W.C., Jr., Nestor, J.J., Jr. & du Vigneaud, V. (1973) *J. Am. Chem. Soc.* **95,** 5677–5679
8. Kaurov, O.A., Martynov, V.F. & Popernatskii, O.A. (1973) *Zh. Obshch. Khim.* **43,** 909–917
9. Hruby, V.J., Smith, C.W., Linn, D.K., Ferger, M.F. & du Vigneaud, V. (1972) *J. Am. Chem. Soc.* **94,** 5478–5480
10. Shiori, T., Ninomiya, K. & Yamada, S. (1972) *J. Am. Chem. Soc.* **94,** 6203–6205
11. Ferrier, B.M., Jarvis, D. & du Vigneaud, V. (1965) *J. Biol. Chem.* **240,** 4264–4266
12. Walter, R., Smith, C.W. & Roy, J. (1976) *Proc. Natl. Acad. Sci. US* **73,** 3054–3056
13. Hlaváček, J., Pospíšek, J., Slaninová, J., Barth, T. & Jošt, K. (1984) in *Peptides 1984,* Proc. 18th European Peptide Symposium (Ragnarsson, U., ed.), pp. 415–418, Almqvist and Wiksell, Stockholm
14. Roy, J., Johnson, M., Gazis, D. & Schwartz, I.L. (1980) *Int. J. Peptide Protein Res.* **16,** 55–60
15. Gazis, D., Roy, J., Roy, U., Glass, J.D. & Schwartz, I.L. (1983) in *Peptides 1982,* Proc. 17th European Peptides Symposium (Bláha, K. & Maloň, P., eds.), pp. 809–812, de Gruyter, Berlin
16. Cash, W.D., McCulloch Mahaffey, L., Buck, A.S., Nettleton D.E., Jr., Romas, C. & du Vigneaud, V. (1962) *J. Med. Pharm. Chem.* **5,** 413–423
17. Ferrier, B.M., Jarvis, D. & du Vigneaud, V. (1965) *J. Biol. Chem.* **240,** 4264–4267
18. Hlaváček, J. & Jošt, K. (1985) in *Proc. 3rd Conference of Chemistry and Biotechnology of Biologically Active Natural Products, 1985,* Vol. 3, pp. 244–250, Bulgarian Academy of Science, Sofia
19. Barth, T. (1977) *Collect. Czech. Chem. Commun.* **42,** 195–199
20. Kaiser, E., Colescott, R.L., Bossinger, C.D. & Cook, P.I. (1970) *Anal. Biochem.* **34,** 595–598
21. Reindel, F. & Hoppe, W. (1954) *Chem. Ber.* **87,** 1103–1107
22. Gisin, B.F. (1973) *Helv. Chim. Acta* **56,** 1476–1482
23. Upson, D.A. & Hruby, V.J. (1976) *J. Org. Chem.* **41,** 1353–1358
24. Ferger, M.F., Jones, W.C., Jr., Dyckes, D.F. & du Vigneaud, V. (1972) *J. Am. Chem. Soc.* **94,** 982–984
25. Holton, P. (1948) *Brit. J. Pharmacol.* **3,** 328–334
26. Munsick, R.A. (1960) *Endocrinology* **66,** 451–458
27. Pliška, V. (1969) *European J. Pharmacol.* **5,** 253–259
28. Bisset, G.W., Clark, B.J., Haldar, J., Harris, M., Lewis, G.P., & Rocha e Silva, M. (1957) *Brit. J. Pharmacol. Chemother.* **31,** 537–545
29. Barth T., Jošt K. & Rychlík I. (1974) *Endocrinol. Exper.* **9,** 35–42
30. Krejčí, I., Kupková, B. & Vávra, I. (1967) *Brit. J. Pharmacol. Chemother.* **30,** 497–502

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