Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 667–672

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHESIS OF OXYTOCIN ANTAGONISTS CONTAINING CONFORMATIONALLY CONSTRAINED AMINO ACIDS IN POSITION 2

Gábor K. Tóth[a,*], Krisztina Bakos[a], Botond Penke[a], Imre Pávó[c], Csaba Varga[d], Gabriella Török[e], Antal Péter[e] and Ferenc Fülöp[b,*]

[a]*Department of Medical Chemistry,* [b]*Department of Pharmaceutical Chemistry,* [c]*Department of Endocrinology, Albert Szent-Györgyi Medical University, Szeged;* [d]*Department of Comparative Physiology,* [e]*Department of Analytical Chemistry, Attila József University, Szeged, Hungary*

Received 17 November 1998; accepted 19 January 1999

Abstract: Analogues of oxytocin containing *D*-Trp, 2-amino-1,2,3,4-tetrahydronaphthalene-1-carboxylic acid (Atc) or 1,2,3,4-tetrahydro-β-carboline-1-carboxylic acid (Car) with *R* or *S* configurations in position 2 were synthetized, and their receptor bindings were tested on isolated guinea-pig uterus, rat liver and rat kidney inner medulla plasma membranes. The peptides were synthetized in the solid phase by using racemates of Car and Atc. The resulting diastereomeric mixtures were separated by means of RP-HPLC. The binding to the oxytocin receptor was somewhat decreased for the Atc isomers and dramatically decreased for both *R*- and *S*-Car, while the *D*-Trp-containing analogue displayed a relatively high receptor affinity. However, the V_1 receptor affinities were almost the same as those of the parent peptide for the Car-containing analogues and dramatically decreased for the *S*-Atc substituted analogue, which has a relatively high OT/V_1 receptor selectivity of 44.5.

Introduction

There has recently been an increasing tendency towards the rational design of highly active and selective analogues of different peptides. The small peptides are usually highly flexible molecules, and the structures observed in solution depend greatly on the environment. Local constraints are introduced, therefore, in order to restrict the conformational freedom of the parent peptide and to stabilize the desired bioactive conformation.

There are different possibilities for the insertion of definite constraints into a peptide backbone: by introduction of a constrained amino acid, such as an N^{α}-methylated amino acid, which will restrict the torsion angle Φ (Φ=-120° ± 20°), or proline (Φ=-70° ± 20°); the introduction of cyclic amino acids, or side-chain cyclized amino acids, such as like Tic, etc.; the formation of mono- or polycyclic structures involving disulphide bridges; cyclization of the side-chain to the backbone, or cyclization of the C-terminal to the N-terminal.[1,2] The recent increasing interest in posttranslationally modified peptides is due in part to the search for conformationally constrained amino acids. Modulation of the flexibility of a peptide backbone from an extended conformation to a β-turn structure is an important breakthrough in the rational design of highly selective and active peptide or peptidomimetic drugs.

Oxytocin is one of the most well known peptide hormones. In the past few decades, hundreds of oxytocin analogues have been synthetized in an attempt to obtain selective and active analogues, and more information about the structure-activity relationships. One of the main modes of action of oxytocin is its uterus-

* Fax: 36 62 420604; Email: fulop@pharma.szote.u-szeged.hu

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00041-4

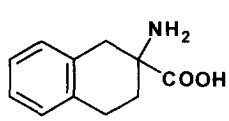
contracting action, and therefore its participation in birth.[3] Preterm birth continues to account for the vast majority of cases of neonatal morbidity and mortality. Currently available tocolytic agents suffer from low uterospecificity and prolong pregnancy only marginally, although postponement of birth by merely a few days may naturally be valuable. Effective, early treatment of vaginosis offers particular promise for the prevention of preterm labour. Oxytocin antagonists afford greater specificity than currently used tocolytics and can be expected to exhibit improved efficacy and risk profiles. Such compounds will allow a more effective treatment of preterm labour, with a lower risk of side-effects.[4]

Although the first oxytocin analogues with antagonistic potencies were described by Law and du Vigneaud in 1960,[5] none of the now numerous synthetized analogues have as yet been applied in human medical practice, probably because of their low receptor specificity or low receptor affinity. An attempt was made to introduce 1-deamino-*D*-Tyr(OEt)^2-Thr^4-Orn^8-oxytocin (atosiban) into medical practice as an oxytocin antagonist in the last decade.[3,6] After the successful use of atosiban in clinical studies to treat women in preterm labour, many other analogues were synthetized. Some of them were more potent than the parent peptide. In addition, the conformational background of the inhibitory properties was studied.[7] The results of Hruby *et al.*[8] and Manning *et al.*[9,10] pointed to the importance of the amino acid in position 2 for antagonistic properties, together with the presence of a basic amino acid in position 8.

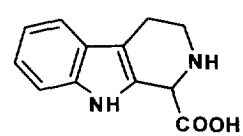
On the basis of this and our previous work,[11,12] we set out to design and synthetize more selective and more potent antagonistic analogues of oxytocin.

Materials and Methods

Synthesis of conformationally constrained Phe and Trp analogues. The racemic 2-amino-1,2,3,4-tetrahydro-naphthalene-1-carboxylic acid (Atc) was prepared from 2-spirohydantointetraline as described earlier.[13,14] The racemic 1,2,3,4-tetrahydro-ß-carboline-1-carboxylic acid (Car) was synthetized from tryptamine with glyoxylic acid according to the literature description.[15] The amino-protecting group (Boc) was incorporated by means of di-tert-butyl dicarbonate in the described manner. The structures of the resulting Boc amino acids were proved by mass spectrometry.



Atc



Car

Fig. 1. The structures of the conformationally constrained amino acids used

Peptide synthesis. The peptides were synthetized by a solid-phase technique, utilizing Boc chemistry.[16] Side-chain-protecting groups were as follows: Arg(Tos), Cys(Meb) and Mpa(Meb). The peptide chains were elongated on *p*-methylbenzhydrylamine (MBHA) resin (0.6-0.8 mmol/g) and the syntheses were carried out on an ABI 430 A automatic peptide synthetizer. Couplings were performed with DCC, with the exceptions of Asn, Gln and Arg, which were incorporated as their 1-hydroxybenzotriazole esters. Amino acid incorporation was monitored via the ninhydrin test.[17] The completed peptide resins were treated with liquid HF/dimethyl

sulphide/*p*-cresol/anisole/*p*-thiocresol (93:4:1:1:1, vol/vol) at 0 °C for 1 h. HF was removed and the resulting free peptides were solubilized in 10% aqueous acetic acid (1 mg/10 ml) and folded by stirring with 0.01 M potassium hexacyanoferrate(III). The completion of disulphide formation was monitored by HPLC. After complete folding, the reaction mixture was filtered, the anions were removed by ion-exchange and the remaining solution was lyophilized.

Peptide purification. The crude peptides were purified by reverse-phase HPLC on a Lichrosorb RP-18 10 μ column (16 x 250 mm). The HPLC apparatus was made by Knauer (Berlin, Germany). The solvent system used was as follows: 0.1% TFA in water, 0.1% TFA, 80% CH_3CN in water, gradient: 20% → 45% B in 1 h, flow 4 ml/min. The appropriate fractions were pooled and lyophilized. The purity was checked by RP-HPLC (Lichrosorb 7 C 18 column; the upper solvent system, gradient: 30% → 45% B in 15 min (**a**), or 35% → 50% B in 15 min (**b**), flow 1.4 ml/min, detection at 220 nm). The products proved to be substantially pure (≥ 97%), with the exception of peptide **4**, which was rather impure (~90%).

Determination of the absolute configuration. The absolute configuration of Atc in the peptide was determined after acidic hydrolysis (6 M HCl, 110 °C, 24 h) of the peptide, applying precolumn derivatization with fluorenyl ethylchloroformate (FLEC).[18] The FLEC derivatives of amino acids were separated on a Eurosphere 100-C_4 column (Knauer, Berlin), using a NaOAc - CH_3CN mobile phase system and fluorescence detection. The chromatograms of standard racemic Atc and the hydrolysed sample are shown in Fig. 2. For the Car derivatives, due to the extreme acid lability of the β-carboline ring, the determination of the absolute configuration failed.

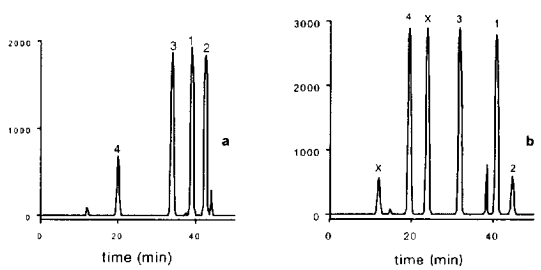


Figure 2. Determination of the absolute configurations of Atc. Column: Eurosphere 100-C_4; flow rate: 0.8 ml/min; detection: fluorescence, Ex. 260 nm, Em. 315 nm; temperature: 40 °C; mobile phase: **A**: 0.01 M NaOAc (pH 4.2) : CH_3CN = 80 : 20, **B**: 0.01 M NaOAc (pH 4.2) : CH_3CN = 20 : 80; gradient 0 →10% **B** in 1 min, 10 → 25% **B** in 30 min, 25 → 100% **B** in 40 min; chromatogram **a**: standard (*S*)-Atc and (*R*)-Atc, chromatogram **b**: hydrolysed peptide peaks: 1. (*R*)-Atc 2. (S)-Atc 3. hydrolysed reagent 4. Gly as standard, x product of hydrolysis of peptides. Carboline analysis: Column: Chiradex 250 x 4 mm I.D., 5 μm particle size (Merck, Darmstadt, Germany); flow rate: 0.5 ml/min; eluent: 0.01 M KH_2PO_4 (pH = 3) : CH_3CN = 95 : 5; detection: 220 nm. Column temperature: ambient. Retention factors: → isomer 1: 3.77; isomer 2: 3.99 (resolution: R_s = 0.67)

Mass spectrometry. The MS experiments were performed with a Finnigan Mat TSQ 7000 tandem mass spectrometer equipped with an ESI source. The results of the MS measurements in all cases agreed well with the calculated values, as shown in Table 1.

Table 1. Sequences, abbreviations, retention times (R_t) and mass spectrometric characterization of the synthetized peptides

No.	Peptide (abbreviation)	Gradient	R_t	Mw calcd	Mw found
1	Mpa-*S*-Atc-Ile-Gln-Asn-Cys-Sar-Arg-Gly-NH_2 (Mpa^1,*S*-Atc^2,Sar^{7}AVT)	a	8'28"	1019.86	1019.2
2	Mpa-*R*-Atc-Ile-Gln-Asn-Cys-Sar-Arg-Gly-NH_2 (Mpa^1,*R*-Atc^2,Sar^{7}AVT)	a	9'27"	1019.86	1019.3
3	Mpa-I-Car-Ile-Gln-Asn-Cys-Sar-Arg-Gly-NH_2 (Mpa^1,I-Car^2,Sar^{7}AVT)*	b	7'17"	1044.86	1044.7
4	Mpa-II-Car-Ile-Gln-Asn-Cys-Sar-Arg-Gly-NH_2 (Mpa^1,II-Car^2,Sar^{7}AVT)*	b	8'01"	1044.86	1044.3
5	Mpa-*D*-Trp-Ile-Gln-Asn-Cys-Sar-Arg-Gly-NH_2 (Mpa^1,*D*-Trp^2,Sar^{7}AVT)	a	6'33"	1032.24	1032.8

*I and II Car corresponds to the two stereoisomers but the determination of the absolute configuration has not been done

Receptor assay. Plasma membranes from 60-65 day pregnant guinea-pig uterus containing 1-1.5 pmol of oxytocin receptor/mg of protein were prepared by a modification of the method of Fuchs *et. al.*[19] Uterine tissue was cut into small pieces and suspended in 10 volumes of buffer containing 10 mM Hepes, 1 mM EDTA and 0.5 mM dithiotreitol. After homogenization with a blazer homogenizer and a glass potter, the suspension was filtered and centrifuged at 1000 x g for 30 min. The supernatant was centrifuged at 165,000 x g for an additional 30 min. The resulting pellet was resuspended in assay buffer: 50 mM Hepes, 10 mM $MnCl_2$, pH 7.6, with a concentration of approximately 10 mg of protein in 1 ml. The affinities of oxytocin and its analogues for the receptor were determined by homologue and heterologue displacement, incubating 10 nM tritiated oxytocin (32 Ci/mmol, NEN) and different concentrations of various peptides at 25 °C for 60 min. Membrane-bound radioactivity (total binding) was separated by filtration through a GF/C glass filter and counted. The apparent dissociation constants (K_d values) were calculated by the iterative nonlinear model-fitting program LIGAND.[20]

Rat kidney inner medulla containing V_2 receptors was prepared by differential centrifugation.[21] 50 g of kidney inner medulla was cut into small pieces and homogenized in 500 ml of buffer containing 250 mM sucrose. After filtration, the liquid was centrifuged for 10 min at 1000 x g, and the supernatant was centrifuged for another 30 min at 15000 x g. The pellet was suspended in 100 mM HEPPS, 10 mM $MgCl_2$ pH 8.2 buffer. The protein content of the suspension was approximately 10 mg/ml.

Rat liver plasma membrane was prepared by the method of Lesko *et al.*[22] Briefly, 40 g of rat liver was cut into small pieces and suspended in 250 ml of 1 mM $NaHCO_3$ and 0.5 mM $CaCl_2$, pH=7.5 solution. After homogenization, the resulting suspension was filtered and the filtrate was centrifuged at 800 x g. The pellet was suspended in 1000 ml of buffer, the suspension was centrifuged again, and the resulting pellet was then suspended in a biphase system consisting of 200 g of 20% dextran 500, 103 g of 30% polyethylene glycol 6000, 333 ml of 0.22 M Na_3PO_4, pH=6.5, and 179 ml of distilled water. After centrifugation at 1000 x g for 15 min, the interphase was collected, suspended in 20 ml of 10 mM HEPPS buffer containing 250 mM sucrose (protein content approximately 1 mg/ml) and stored at -70°C. The receptor assay was similar to that of the oxytocin receptor, using tritiated AVP as radiolabelled ligand.

Results and Discussion

Position 2 in oxytocin seems to be crucial for antagonistic activity. We therefore set out to establish the optimal size of the apolar side-chain and in parallel to find the optimal conformational constraints on the basis of the work of Manning and Lebl.[7,8,10,23-25] The use of 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid for the substitution of tyrosine methyl ether resulted in selective analogues,[9] but the selectivity was not better than that of atosiban. The use of 1 and 2-naphthylalanine resulted in analogues with high uterotonic inhibitory activity, especially in the vasopressin series containing *D*-Har (homoagrinine) in position 8, but the *in vivo* potencies were much lower than the *in vitro* uterotonic activity.[26] Sar^7 AVT derivatives previously proved to be potent and selective antagonistic analogues if the structure of the amino acid in position 2 was appropriate.[11] The high selectivity of the Trp^2 analogue (Mca^1-*D*-Trp^2-Sar^7 AVT undergoes practically no binding to the V_2 receptor, while its affinity for the oxytocin receptor is practically the same as that of the parent peptide) led us to introduce Car, which is a constrained analogue of Trp, in a similar way to 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid being a constrained analogue of phenylalanine.

The resulting analogues (**3** and **4**) displayed practically no binding to the uterotonic receptor, while the V_2 receptor affinity was negligible in both cases. The incorporated conformational constraints probably

completely inhibit the interaction with both receptor types. The binding pocket for the apolar side-chain in position 2 does not seem large enough for the rigid tricyclic structure. Interestingly, the V_1 affinities were relatively high in both cases. Thus, with a 3 orders of magnitude decrease in the OT and V_2 binding, these analogues are selective V_1 agonists. Mpa^1-*D*-Trp^2-Sar^7 AVT was also synthetized. Its uterotonic receptor affinity was found to be almost the same as that of native oxytocin, while the V_2 receptor affinity was significantly decreased, but stronger than that of the Mca^1 analogue, which undergoes practically no binding to the V_2 receptor.[11] The only difference lies in the space-filling properties of the first amino acid (Mpa and Mca, respectively). These data indicate that the first amino acid can have a decisive role in the receptor selectivity. This analogue is a 6 times weaker OT agonist than the Mca^1 analogue, but the V_1 binding is 200 times weaker, and the V_1 binding more than 100 times weaker than that of the Mca^1 peptide.

Table 2. Apparent dissociation constants (K_d values in nanomole) of new oxytocin antagonists to guinea-pig myometral oxytocin, rat liver vasopressin (V_1) receptors and rat kidney V_2 receptors

	peptide	OT	V_2	V_1
	AVP		1.5±0.1	0.65±0.04
	oxytocin	6.7±0.5		
	Mpa^1,*D*-Phe^2,Sar^{7}AVT	8±2	421±120	50.1±6
	Mca^1,*D*-Trp^2,Sar^{7}AVT	6±2	$3x10^5$	3.8±0.6
1	Mpa^1,*S*-Atc^2,Sar^{7}AVT	369.5±87	2290±410	17600±1838
2	Mpa^1,*R*-Atc^2,Sar^{7}AVT	1917.7±1682	5340±640	2640±163
3	Mpa^1,I-Car^2,Sar^{7}AVT	8545±3330	$>10^4$	63.5±4.1
4	Mpa^1,II-Car^2,Sar^{7}AVT	57450±1061	$>10^4$	17.8±4.1
5	Mpa^1,*D*-Trp^2,Sar^{7}AVT	37.6±23.2	4960±510	888±210

Because of the good receptor binding of the *p*-chlorophenylalanine-containing analogues[12] and the reported antagonistic properties of some Tic-containing peptides,[27] we additionally replaced the second amino acid (tyrosine in the original oxytocin molecule) by the conformationally constrained analogue of Phe, Atc.

Both Atc and Car were used as racemic mixtures and, after the completion of the synthesis, the resulting diastereomers were successfully separated by reverse-phase HPLC. The configurations of the individual isomers were determined after acidic hydrolysis by using a chiral compound (FLEC) as chromophoric modifier[18] in the case of the Atc-containing peptides. The earlier eluted peak corresponds to the peptide containing the *S* antipode, and the second peak to that containing the *R* antipode. In the case of the Car isomer-containing peptides, the regular acidic hydrolysis completely destroyed the acid-sensitive norharmane ring. Under milder conditions, *e.g.* on applcation of microwaves or reductive additives which were successfully applied for Trp, the result was practically the same. Since the OT receptor binding for both Car isomers was quite low, we did not make further attempts to determine the absolute configuration. However, we did find appropriate chromatographic conditions for this purpose (see legend to Fig. 2). The OT receptor affinity of Mpa^1-*R*-Atc^2-Sar^7 AVT was 1917 nanomoles, while that of Mpa^1-*S*-Atc^2-Sar^7 AVT was 369 nanomoles, which is moderately less than those of the *D* or *L*-Phe- containing analogues, so the decreased conformational freedom in this case does not support the preferred steric structure.

Although the oxytocic potencies were slightly lower for both antipodes, the even greater decrease in V_2 and V_1 receptor affinity pointed to the possibility of the design and synthesis of selective and potent oxytocin analogues based on determination of the optimal size and steric structure in the second amino acid side-chain in these vasotocin analogues. The *S*-Atc[2] analogue has only 17600 binding to V_1 and 2290 binding to V_2 receptors. Thus, their use for the design of selective OT antagonists could be of interest.

Acknowledgements

This work was supported by the following grants: OTKA 16156 and 14898, and ETT T03/523 and 560. The authors are indebted to Mr Pál Szabó and Mr Zoltán Kele for the MS measurements.

References

1. Hruby, V. J. *Life Sci.* **1982,** *31*, 189.
2. Kazmierski, W.; Wire, W. S.; Lui, G. K.; Knapp, R. J.; Shook, J. E.; Burks, T. F.; Yamamura, H. I.; Hruby, V. J. *J. Med. Chem.* **1988,** *31*, 2170.
3. Akerlund, M.; Carlsson, A. M.; Melin, P.; Trojnar, J. *Acta Obset. Gynecol. Scand.* **1985**, *64*, 449.
4. Keirse, M. J. *Am. J. Obstet. Gynecol.* **1995**, *173*, 618.
5. Law, H. D.; du Vigneaud, V. *J. Amer. Chem. Soc.* **1960,** *82*, 4579.
6. Melin, P.; Trojnar, J.; Johansson, B.; Vilhardt, H.; Akerlund, M., *J. Endocrinol.* **1986**, *111*, 125.
7. Lebl, M.; Hill, P.; Kazmierski, W.; Karaszova, L.; Slaninova, J.; Fric, I.; Hruby, V. J. *Int. J. Peptide Protein Res.* **1990**, *36*, 321.
8. Hruby, V. J.; Chan, W. Y.; Rockway, T. W.; Hlavacek, J.; Ormberg, J. In *Peptides: Design, Synthesis, and Biological Activity* (Channa Basava, G. M. Anantharamaiah eds.), Birkhauser, Boston, **1994**, pp. 199-208.
9. Manning, M.; Cheng, L. L.; Stoev, S.; Bankowski, K.; Przybylski, J.; Klis, W. A.; Sawyer, W. H.; Wo, N. C.; Chan, W. Y. *J. Pept. Sci.* **1995**, *1*, 66.
10. Manning, M.; Nawrocka, E.; Misicka, A.; Olma, A.; Klis, W. A.; Seto, J.; Sawyer, W. H. *J. Med. Chem.* **1984**, *27*, 423.
11. Pávó, I.; Slaninova, J.; Klein, U.; Fahrenholz, F. *J. Med. Chem.* **1994**, *37*, 255.
12. Pávó, I.; Varga, C.; Vecsernyés, M.; Bakos, K.; Tóth, G. K.; Herczeg, J.; Slaninova, J.; Fahrenholz, F. *Hanseatic Endocrine Conference. Stade*, **1995**, A. 104.
13. Schiller, P. W.; Weltrowska, G.; Nguyen, T. M.-D.; Lemieux, C.; Chung, N. N.; Marsden, B. J.; Wilkes, B. C. *J. Med. Chem.* **1991**, *34*, 3125.
14. Rastogi, S. N.; Bindra, J. S.; Anand, N. *Ind. J. Chem.* **1971**, *9*, 1175.
15. Vejdelek, Z. J.; Trcka, V.; Protiva, M. *J. Med. Pharm. Chem.* **1961**, *3*, 427.
16. Merrifield, R. B. *J. Amer. Chem. Soc.* **1963**, *85*, 2149.
17. Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. J. *Anal. Biochem.* **1970**, *34*, 595.
18. Einarsson, S.; Josefsson, B.; Möller, P.; Sanchez, D. *Anal. Chem.* **1987**, *59*, 1191.
19. Fuchs, A. R.; Periyasami, S.; Alexandrova, M.; Soloff, M. S. *Endocrinology* **1983**, *113*, 742.
20. Munson, P. J.; Rodbard, D. *Anal. Biochem.* **1980,** *107*, 220.
21. Fahrenholz, F.; Crause, P. *Biochem. Biophys. Res. Commun.* **1984,** *122*, 974.
22. Lesko, L.; Donlon, D.; Marinetti, G. V.; Hare, J. D. *Biochim. Biophys. Acta* **1973,** *311*, 173.
23. Manning, M.; Miteva, K.; Pancheva, S.; Stoev, S.; Wo, N. C.; Chan, W. Y. *Int. J. Pept. Protein. Res.* **1995,** *46*, 244.
24. Manning, M.; Lowbridge, J.; Seto, J.; Haldar, J.; Sawyer, W. H. *J. Med. Chem.* **1978**, *21*, 179.
25. Bankowski, K.; Manning, M.; Seto, J.; Haldar, J.; Sawyer, W. H. *Int. J. Peptide Protein Res.* **1980**, *16*, 382.
26. Procházka, Z.; Slaninová, J. *Collect. Czech Chem. Commun.* **1995**, *60*, 2170.
27. Majer, P.; Slaninova, J.; Lebl, M. *Int. J. Peptide Protein Res.* **1994**, *43*, 62.