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Ala-Walk Analogs of Oxytocin – HPLC-Based Conformational Studies

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Introduction

Oxytocin (OXT) is one of the first peptide hormones the structure of which was defined [1] and the importance of individual amino acid residues was studied by systematic replacements and modifications (for review see [2]). Interest in oxytocin was recently renewed after its important behavioral activities were discovered (see e.g. [3]). Conformation of oxytocin and its analogs was studied extensively (see e.g. [4,5]) utilizing various physico-chemical methods. We have shown that HPLC can be used for conformational studies as well [6,7].

Results and Discussion

We have synthesized analogs of oxytocin cycl1-6(Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2) with all but 1 and 6 positions consecutively substituted with alanines. Analogs were prepared by standard solid phase synthesis on Rink resin. After cleavage and precipitation, analogs were oxidized by diluted solution of hydrogen peroxide. For RP-HPLC studies the solution of analogs was repeatedly reduced by dithiothreitol and oxidized by hydrogen peroxide to verify the elution position of reduced and oxidized forms. Since [Ala^5]OXT did not follow the trend of the remaining analogs and showed unexpected behavior - disulfide containing cyclic form eluting later than reduced linear bis-sulfhydryl form - we confirmed these structures by mass spectroscopy.

From the previous studies of oxytocin analogues it was apparent that the asparagine residue in position 5 has a very unique behavior in NMR studies. Especially its chemical shift temperature dependence was reversed from the normal positive trend. As the HPLC retention can reflect contribution of hydrophobicity of side chains of individual amino acid residues to the

Table 1. Relationship of retention times of cyclic and linearized Ala-walk analogs of OXT

Analog	*AA* [a]	*k' cyclic*	*k' linear*	*Ratio* [b]	*Pred.* [c]	*A/OXT (lin)* [d]	*A/OXT (cyc)* [e]	*Diff (lin)* [f]	*Diff (cyc)* [g]	*COSI CE* [h]
OXT		2.891	2.982	0.970						
[Ala^2] OXT	Tyr	2.527	2.664	0.949	0.86	0.89	0.87	0.03	0.01	-0.02
[Ala^3] OXT	Ile	2.182	2.473	0.882	0.80	0.83	0.75	0.03	-0.05	**-0.08**
[Ala^4] OXT	Gln	2.964	2.982	0.994	1.09	1.00	1.03	-0.09	-0.06	0.03
[Ala^5] OXT	Asn	3.091	3.000	1.030	1.12	1.01	1.07	-0.11	-0.05	**0.06**
[Ala^7] OXT	Pro	2.773	2.782	0.997	0.96	0.93	0.96	-0.03	0.00	0.03
[Ala^8] OXT	Leu	2.336	2.345	0.996	0.74	0.79	0.81	0.05	0.07	0.02
[Ala^9] OXT	Gly	2.882	3.091	0.932	1.07	1.04	1.00	-0.03	-0.07	-0.04

[a]Amino acid replaced by Ala; [b]Ratio of k' of cyclic and linear forms; [c]Expected ratio of retention times of analog to oxytocin (Average values from [8] and [9]); [d]Ratio of retention times of analog to oxytocin (linear); [e]Ratio of retention times of analog to oxytocin (cyclic); [f]Difference between observed and predicted k' ratios of linear forms of analog; [g]Difference between observed and predicted k' ratios of cyclic forms of analog; [h]Coefficient of side chain exposure.

Table 2. Uterotonic activities of studied analogs

Analog	*Uterotonic activity in vitro*		*Reference*
	This study[a]	*Literature*[a]	
[Ala^2]OXT	<1% OXT	N.A.	Buku [10]
[Ala^3]OXT	<1% OXT		
[Ala^4]OXT	~2% OXT	36 I.U.	Guttmann [11]
[Ala^5]OXT	<1% OXT	<0.05 I.U.	Guttmann [11]
[Ala^7]OXT	<1% OXT	22 I.U.	Walter [12]
[Ala^8]OXT	~20% OXT	141 I.U.	Jaquenoud [13]
[Ala^9]OXT	<1% OXT	0.25 I.U.	Dutta [14]

[a]*Activity of OXT is 450 I.U.*

retention on the reversed phase, we speculated that contribution of asparagine to the retention time could be indicating its exposure to the molecular surroundings. We studied both cyclized and linear versions of all analogs and correlated retention times with relative hydrophobic contributions of individual side chains. As can be seen from the data in Table 1, contribution of tyrosine in position 2, glutamine in position 4, and amino acids in the carboxy terminal part of the molecule of oxytocin follow the predicted trend calculated from the literature [10,11] in both cyclic and linear form. However, the difference of predicted retention values of cyclic and linear forms of [Ala^3]OXT and [Ala^5]OXT are significantly larger. We call this value "COSICE" (coefficient of side chain exposure). A large negative value of COSICE means that the side chain being replaced (in our case isoleucine in [Ala^3]OXT) is very significantly more "buried" inside of the constrained cyclic form of the molecule than in the linear form. On the other hand, a large positive value of COSICE means that this side chain (asparagine in position 5) is being "super-exposed" in the cyclic form of the molecule.

The effect of super exposure of asparagine residue, and therefore importance for the interaction of oxytocin with its receptor, correlates with the biological activity of all analogs of oxytocin substituted in position 5 (for the list of analogs see [4]). Any attempt to replace asparagine resulted in an almost complete loss of biological activity. On the other hand, analogs replacing amino acids with small COSICE value retain some residual biological activities. Significant loss of activity of analogs with replaced C-terminal glycine is again predicted from slightly increased COSICE value explained by interaction of the tripeptide Pro-Leu-Gly-NH_2 with the cyclic part of the molecule which protects the glycine amide from the interaction with the environment.

Uterotonic activities *in vitro* (Table 2) of the prepared analogs were determined to confirm earlier published results - significant activity was found only in Ala^4 and Ala^8 oxytocin. Differences from published values can be attributed to the different methodologies used in different laboratories.

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