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Short communication

Oxytocin with N^5,N^5-dimethylglutamine and N^4,N^4-dimethylasparagine in the 4 and 5 positions: synthesis, pharmacological properties and mass spectral analysis

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Summary — The analog of oxytocin [4-(N^5,N^5-dimethylglutamine), 5-(N^4,N^4-dimethylasparagine)]-oxytocin was synthesized and its structure was fully characterized by liquid secondary mass ion spectroscopy. The analog was found to be almost inactive in the uterotonic, anti-diuretic, pressor and milk ejection assays.

Résumé — **Oxytocine substituée en position 4 et 5 par la N^5,N^5-diméthylglutamine et la N^4,N^4-diméthylasparagine: synthèse, propriétés pharmacologiques et analyse par spectroscopie de masse ionique.** *Le présent travail décrit la synthèse et la caractérisation par spectroscopie liquide de masse secondaire de la [(*N^5,N^5*-diméthylglutamine)-4, (*N^4,N^4*-diméthylasparagine]-5 ocytocine. Cet analogue, par rapport à l'activité biologique de l'ocytocine, est presque inactif.*

oxytocin / analog / synthesis / pharmacology / mass spectral analysis

Introduction

In the proposed 'biologically active' conformation of oxytocin at the uterine receptor, the tyrosyl side chain, together with the carboxamide groups of the side chains of Gln in position 4 and Asn in position 5 as well as the C-terminal glycinamide in position 9, form a hydrophilic cluster [1]. The asparagine residue contributes an 'active element' [2] and that was experimentally supported by the synthesis of [5-(N^4,N^4-dimethylasparagine)]-oxytocin [3] which possesses a potency of only 4.6 ± 0.5 units/mg in comparison to 546 ± 18 units/mg for oxytocin, but had identical intrinsic activity. On the other hand, [4-(N^5, N^5-dimethylglutamine)]-oxytocin [4] exhibited a reduced ability to maximally stimulate uterine contraction as compared to oxytocin, although the carboxamide of Gln^4 is not proposed as an active element. To further explore the effect(s) of a simultaneous dimethylation of the carboxamide groups of Gln^4 and Asn^5 on the biological profile of oxytocin, the synthesis of [4-(N^5,N^5-dimethylglutamine), 5-(N^4,N^4-dimethylasparagine)]-oxytocin was undertaken.

Results and Discussion

The synthesis of [4-(N^5,N^5-dimethylglutamine, 5-(N^4, N^4-dimethylasparagine)]-oxytocin was performed by a stepwise elongation of Boc-Asp[$N(CH_3)_2$]-Cys(Bzl)-Pro-Leu-Gly-NH_2 [3]. Boc-Glu[$N(CH_3)_2$]-OH [4] was pre-activated with DCC (dicyclohexylcarbodiimide), mediated with 1-hydroxybenzotriazole [5] and coupled to the partially deprotected pentapeptide. Thereafter, the peptide chain (Table I) was elongated with the active succinimide esters [6]. Finally, the protecting groups were removed by reduction with sodium in liquid ammonia [7], the disulfide was formed by oxidation with 1 equivalent of 1,2-diiodoethane [8] and the desired product was purified by methods previously described [4].

Full characterization of the synthesized analog was achieved by liquid secondary ion mass spectrometry (liquid SIMS) [9]. Since the classical amino acid analysis gives the ratio for Asp and Glu only and not for the dimethylated derivatives, the mass spectral analyses were needed to establish that the side chain carboxamide groups of Gln^4 and Asn^5 remain dimethylated through the last stages of preparation and purification.

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Table I. Yields and physical data of intermediate compounds of the synthesis of [4-(N^5,N^5-dimethylglutamine), 5-(N^4,N^4-dimethylasparagine)] oxytocin.

No.	Compound	Yield (%)	mp (°C)	$[\alpha]^{25}$
1	Boc-Ile-Glu $N(CH_3)_2$-Asp[$N(CH_3)_2$]--(S-Bzl)Cys-Pro-Leu-Gly-NH_2	51	159—162	— 50.8° (*c*1, DMF)
2	Boc-(O-Bzl)Tyr-Ile-Glu[$N(CH_3)_2$]-Asp[N ($CH_3)_2$]-(S-Bzl)Cys-Pro-Leu-Gly-NH_2	76	185—187	— 44.8° (*c*1, DMF)
3	Z-(S-Bzl)Cys-(O-Bzl)Tyr-Ile-Glu-[$N(CH_3)_2$ Asp[$N(CH_3)_2$]-(S-Bzl)Cys-Pro-Leu-Gly-NH_2	70	209—210	— 54.4° (*c*1, AcOH)
4	[4-(N^5,N^5-dimethylglutamine), 5-(N^4,N^4-dimethylasparagine)]oxytocin	36	n.d.	— 4.5° (0.5, AcOH)

Positive ion liquid secondary ion mass spectral analyses were done on a CEC (Dupont) 21-110 mass spectrometer equipped with a focal plane electro-optical ion detector [10] and a Phrasor cesium ion gun. The cesium gun was mounted on a flange exterior to the source housing and the cesium beam focused in the center of the ion source by an einsel lens mounted inside the source housing. Spectra were taken at 6 kV acceleration potential and a primary ion beam energy of 10 kV. Results are reported as nominal *m/z* values.

Samples were prepared by dissolving 20 μg of the peptide in 50% aqueous acetic acid directly on the brass tip of a direct insertion probe. Approximately 1 μl of glycerol was added to the solution and the solvent was removed in the vacuum lock of the mass spectrometer. Spectra of the reduced form were obtained by removing the probe from the mass spectrometer and adding a molar excess of solid dithiothreitol directly to the sample on the probe. Complete conversion to dithiol required approximately 15 min.

The disulfide gave a prominent protonated molecular ion at *m/z* 1063. Dithiothreitol was used to reduce the disulfide linkage. This was accomplished by adding the solid reagent directly to the sample on the probe tip. The course of the reduction was observed by monitoring the shift of the molecular ion from *m/z* 1063 to *m/z* 1065, and was complete in 15 min.

Sequence information is found primarily in two fragment ion series, B and Y'' [11]. The nomenclature of Roepstorff and Fohlman is used to designate fragment ion series [12]. Although sequence information was abundant in the spectra for both the disulfide and reduced forms of the peptide (Fig. 1), series ions are more prominent and easier to assign in the case of the dithiol. The N-terminal and C-terminal series overlap and together define the entire sequence of the peptide. For the cyclic disulfide, cleavage of peptide bonds at positions 1—5 results in fragments only if accompanied by reduction of the disulfide bond.

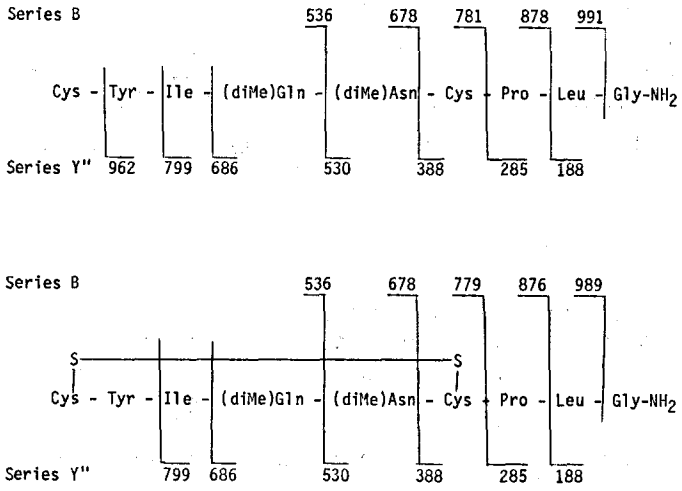

Fig. 1. Sequence fragments in the liquid secondary ion mass spectra of the dithiol and disulfide forms of [4-N^5,N^5-dimethylglutamine), 5-(N^4,N^4-dimethylasparagine)]-oxytocin.

Table II. Biological activities of oxytocin with its 4- and 5-position dimethylated analogs[a].

Compound	*In vitro* rat uterotonic	Rat anti-diuretic	Rat pressor	Milk ejection
Oxytocin	546	2.7	3.1	474 ± 16
Asp[$N(CH_3)_2$][5]oxytocin	4.6[b]	—[e]	—[e]	—
Glu[$N(CH_3)_2$][4]oxytocin	3.01[b]	n.d.	n.d.	—
Glu[$N(CH_3)_2$][4],Asp[N ($CH_3)_2$][5]oxytocin	< 0.37 (0.5)[c,d]	0.1	0.1[d]	0.1

[a]Agonist activities are expressed in units/mg.
[b]Without Mg^{2+}.
[c]With Mg^{2+}.
[d]No inhibitory activity to oxytocin-induced uterotonic or pressor responses were noted.
[e]See [3].

Consequently, intensities of these ions are much less than those observed for the same ions in the spectrum of the reduced form. As expected, the dithiol series B members corresponding to position 6—8 (m/z 781, 878, 991) are two mass units higher than those in the spectrum of the disulfide.

The biological activities of the synthesized analogs [13] compared with those of oxytocin are summarized in Table II. The anti-diuretic potency of [5-N^4,N^4-dimethylasparagine)-4-(N^5,N^5-dimethylglutamine)]-oxytocin as well as its pressor activity are almost undetectable. Moreover, this analog shows no inhibitory activity of the uterotonic or pressor responses to oxytocin. On the other hand, [5-(N^4,N^4-dimethylasparagine)]- and [4-(N^5,N^5-dimethylglutamine)]-oxytocin show 4.6 U/mg and 3.01 U/mg of uterotonic activity, respectively. Their intrinsic activities are 1 and < 1, respectively. The additivity of the bulky methyl groups at positions 4 and 5 on the biological properties is apparent. Characteristically, the uterotonic activity of [5-(N^4,N^4-dimethylasparagine)-4-(N^5,N^5-dimethylglutamine)]-oxytocin is almost undetectable either in the presence or absence of Mg^{2+} ions. It is not possible to say whether the negligible uterotonic activity of this analog is due to the loss of affinity or to reduced intrinsic activity.

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