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Cyclic pentapeptide analogs based on endomorphin-2 structure: Cyclization studies using liquid chromatography combined with on-line mass spectrometry and tandem mass spectrometry

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ABSTRACT

The cyclization of linear analogs based on endomorphin-2 structure, Tyr/Dmt-D-Lys-Phe-Phe-Asp-NH_2 and Tyr/Dmt-D-Cys-Phe-Phe-Cys-NH_2 (where Dmt = 2′,6′-dimethyltyrosine), resulting in obtaining lactam or disulfide derivatives, was studied using liquid chromatography combined with on-line mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS/MS). In case of cyclization via an amide bond, the formation of the cyclic monomers, cyclic but not linear dimers and even traces of cyclic trimers was observed. Disulfide bridge containing peptides was obtained by the solid-phase synthesis of the linear sequences, followed by either in-solution or on-resin cyclization. In case of the in-solution cyclization, the expected cyclic monomers were the only products. When oxidation of the cysteine residues was performed when the peptides were still on the resin, cyclic monomer and two cyclodimers, parallel and antiparallel, were found. Digestion of the isolated cyclodimers with α-chymotrypsin allowed for their unambiguous identification. The comparison of the cyclic monomer/dimer ratios for analogs with Tyr versus Dmt in position 1 revealed that the presence of the exocyclic Dmt favored formation of the cyclic monomer, most likely due to the increased steric bulk of this amino acid side-chain as compared with Tyr.

1. Introduction

Cyclization is a well recognized strategy in peptide chemistry for generating analogs with improved bioactivities and bioavailabilities. Cyclic peptides are important starting structures for the development of non-peptide mimetics which can be viewed as a new generation of peptide-based drugs.

Endomorphins (EM-1, Tyr-Pro-Trp-Phe-NH_2 and EM-2, Tyr-Pro-Phe-Phe-NH_2), the endogenous ligands of the μ-opioid receptor [19], are strong analgesics with similar to morphine potency but very limited bioavailability after exogenous administration. Among various chemical modifications of endomorphin structure aiming at enhancing their bioactivity [5], cyclization represents an interesting option [11]. However, EMs are difficult to cyclize due to their short, only tetrapeptide sequence and the lack of the reactive side-chain groups. Additionally, both N- and C-termini are important for binding with the opioid receptors and therefore of limited value for the ring closure. Therefore, to obtain cyclic analogs based on endomorphin structure introduction of additional amino acids with functionalized side-chains is a possibility.

Here, the cyclization reaction of a linear EM-2 analogs of a general sequence, Tyr/Dmt-D-Lys-Phe-Phe-Asp-NH_2 and Tyr/Dmt-D-Cys-Phe-Phe-Cys-NH_2 (DMT = 2′,6′-dimethyltyrosine) via either an amide or a disulfide bond, respectively, was studied. The by-products of the cyclization reaction were identified using liquid chromatography combined with on-line mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS/MS).

2. Results

Two pairs of conformationally constrained analogs of a general structure Tyr/Dmt-c[D-Lys-Phe-Phe-Asp]NH_2 and Tyr/Dmt-c[D-Cys-Phe-Phe-Cys]NH_2, cyclized via an amide or a disulfide bridge, respectively, were synthesized. Analogs in each pair differed only by the amino acid residue in position 1 (Tyr or Dmt) (Fig. 1). In the first pair (**1m**, **2m**), side-chains of D-Lys and Asp residues in positions 2 and 5, respectively, were linked by an amide bond. The synthesis was performed by an entirely solid-phase methodology using Fmoc/*t*Bu chemistry with the hyper-acid labile Mtt/O-2Ph*i*Pr groups for the selective amine/carboxyl side-chain protection.

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1m	Tyr-c[D-Lys-Phe-Phe-Asp]NH_2	on-resin cyclization
2m	Dmt-c[D-Lys-Phe-Phe-Asp]NH_2	on-resin cyclization
3m	Tyr-c[D-Cys-Phe-Phe-Cys]NH_2	in solution cyclization
4m	Dmt-c[D-Cys-Phe-Phe-Cys]NH_2	in solution cyclization
5m	Tyr-c[D-Cys-Phe-Phe-Cys]NH_2	on-resin cyclization
6m	Dmt-c[D-Cys-Phe-Phe-Cys]NH_2	on-resin cyclization

Fig. 1. The sequences of the synthesized cyclic analogs.

The LC–MS analysis of a crude mixture **1** (numbers **1–6** represent crude mixtures obtained after cyclization) revealed the presence of two main peaks with retention times 17.0 and 21.3 min (Fig. 2, panel A). Both these peaks were represented by mass spectra characterized by the intensive signals corresponding to 700.345 *m*/*z* value, expected for the $[M+H]^+$ ion. However, a close examination of the spectra revealed different isotopic patterns, typical for +1 and +2 ions (Fig. 2, panels B and C). The fraction eluting at 17.0 min contained the expected cyclic peptide **1m**, while the *m*/*z* value of the fraction observed at 21.3 min suggested that the signals may represent a cyclodimer $[M_2+2H]^{2+}$ (**1d**). To confirm the cyclodimeric structure of this ion and to differentiate it from a possible non-covalent dimer $[2M+2H]^{2+}$ that could be formed during electrospray ionization (ESI), the collision induced dissociation (CID) experiments were performed for *m*/*z* 700.3 ions of the separated fractions.

The fragmentation of *m*/*z* 700.345 parent ion from **1m** resulted in the neutral loss of NH_3 and CO. The 586.303 *m*/*z* signal corresponds to the loss of Asp residue, whereas other fragmentation pathways may lead to the removal of the N-terminal Tyr with the formation of a z-type ion or alternative ring opening and the loss of consecutive Phe residues, as suggested by 520.256 *m*/*z*. The appearance of the signal 491.229 *m*/*z* could be explained by the loss of a Phe residue combined with two NH_3 molecules and CO, leading to a relatively stable ion (insert in Fig. 3 panel A) [4].

The comparison of the MS/MS spectra recorded for **1m** and **1d** evidenced the differences in the fragmentation pattern of both compounds (Fig. 3). For **1d** several daughter ions were found with higher *m*/*z* values than the doubly charged parent ion. Briefly, a series of +1 ions, corresponding to the loss of Asp residue (*m*/*z* 1285.664), Tyr residue and NH_3 (z-type ion, *m*/*z* 1218.6), Tyr and Asp (*m*/*z* 1121.5), and Tyr, Asp and Phe (*m*/*z* 974.5) were observed. The obtained fragmentation patterns, the energy required for the dissociation of ions (20 eV) and elution in separate fractions during LC–MS confirmed without doubt the cyclodimeric structure of **1d**.

The LC–MS spectrum of the crude peptide **1** showed also a small peak at 21.6 min, characterized by a 1050.519 *m*/*z* signal. Deconvolution of this spectrum suggested the presence of a small amount of a trimeric form of this peptide.

The search for ions corresponding to the linear monomeric or dimeric forms in the mixture **1** in the LC–MS chromatogram did not reveal even traces of such compounds.

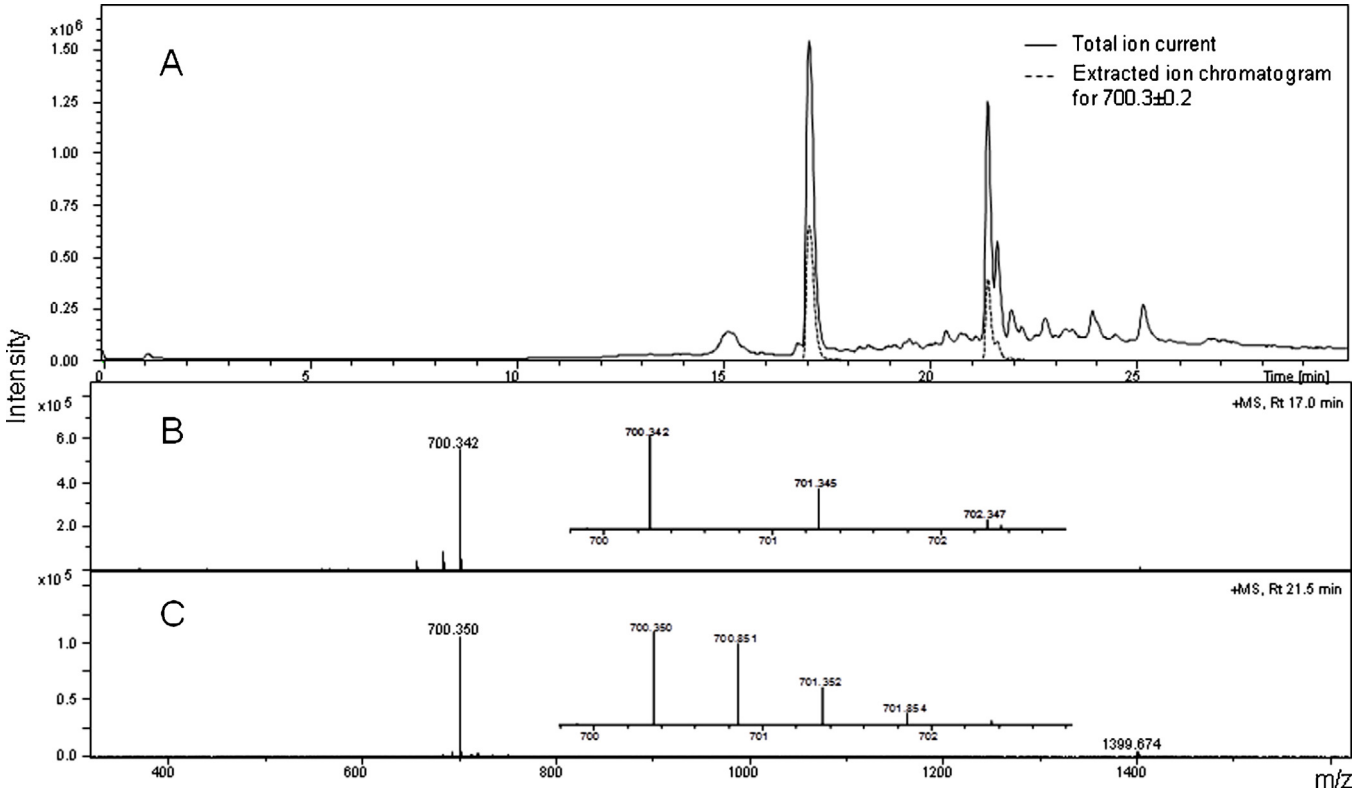

Fig. 2. LC–MS analysis of a crude mixture 1. The total ion chromatogram and the extracted ion chromatograms (panel A). Mass spectra for peaks with retention times 17.0 and 21.3 min (panels B and C, respectively). The isotopic patterns for the most intensive signals are included in insets.