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Synthesis of a Novel Side-chain to Side-chain Cyclized Enkephalin Analogue Containing a Carbonyl Bridge

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Abstract: A novel type of cyclic opiod peptide analogue, cyclo(N^{ε},$N^{\varepsilon'}$-carbonyl-D-Lys^2,Lys^5)enkephalinamide, was prepared from a linear precursor peptide. The peptide was synthesized on the Merrifield resin and also by a combination of the solid-phase technique and the classical method in solution. In both cases the cyclization was performed by reaction of bis(4-nitrophenyl)carbonate with the free side-chain amino groups of the two lysine residues. The described method permits the convenient preparation of novel peptide analogues cyclized via a ureido group incorporating the side-chain amino groups of two α,ω-diamino acid residues. The cyclic enkephalin analogue containing a 21-membered ring structure showed preference for μ over δ opioid receptors in opioid bioassays *in vitro*. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

The lack of selectivity observed with most naturally occurring opioid peptides and with many of their linear analogues is most probably due to their structural flexibility which permits conformational adaptation to more than one opioid receptor type. The conformational space accessible to flexible linear peptides can be limited through introduction of various types of conformational constraints. The most drastic restriction of the overall peptide conformation is achieved through covalent peptide cyclization [1, 2]. Several cyclic enkephalin analogues were prepared with the goal of obtaining compounds with enhanced receptor selectivity as a consequence of the conformational constraints imposed through cyclization between two side-chain groups or between a side-chain group and the C-terminal carboxyl group. Cyclization through covalent linkage of two side-chains has been performed by disulphide bond formation between appropriately substituted half-cystine [3] and penicillamine [4, 5] residues, or by amide bond formation between the side-chain amino group of an α,ω-diamino acid residue and the C-terminal carboxyl group [6, 7] or with the side-chain carboxyl group of a glutamic or aspartic acid residue [8]. A cyclic analogue of [Leu^5]enkephalin containing an azo bridge between the Tyr^1 and Phe^4 aromatic rings has also been

Abbreviations: DIEA, diisopropylethylamine; Z(2-Br), 2-Bromobenzyloxycarbonyl; GPI, guinea pig ileum; MVD, mouse vas deferens.

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reported [9]. In the present paper we describe the synthesis of a side-chain to side-chain cyclized enkephalin analogue (Figure 1) in which ring formation was achieved *via* a ureido group incorporating the ε amino groups of D-Lys^2 and Lys^5

MATERIALS AND METHODS

Amino acid derivatives were purchased from Bachem, Bubendorf, Switzerland. *N-tert*-butyl-*N′*-ethylcarbodiimide was prepared by mixing *N-tert*-butyl-*N′*-ethylthiourea with DCC in a 1 : 1 molar ratio, followed by distillation under reduced pressure. After the second distillation (b.p. 74–76 °C/120 mmHg) the product was homogenous. Yield: 54%.

Peptides were synthesized by the manual solid-phase technique according to the following protocol: (1) deprotection with 55% TFA in CH_2Cl_2 (1 × 1 min, 1 × 15 min); (2) neutralization with 5% DIEA in CH_2Cl_2 (2 × 2 min); (3) coupling with the symmetrical anhydride (3 equiv.) of the Boc-amino acid (60 min). Symmetrical anhydrides were prepared according to the following procedure: the Boc-amino acid (3 mmol) was dissolved in CH_2Cl_2 (10–15 cm^3) and after cooling to 0 °C *N-tert*-butyl-*N′*-ethylcarbodiimide (1.5 mmol) was added. The resulting solution was allowed to stand for 30 min at 0 °C prior to mixing with the peptide resin.

Preparation of Cyclo(N^{ε},$N^{\varepsilon'}$-carbonyl-D-Lys^2,Lys^5)enkephalinamide (Method I)

The linear precursor peptide was synthesized by the solid-phase technique as described above using chloromethylated Merrifield resin (0.6 g; 1.34 meq Cl/g; Bio-Beads S-XI, Bio-Rad Laboratories). The resin was esterified with Boc-Lys[Z(2-Br)] by the cesium salt method. The fully protected peptide was cleaved from the resin by treatment with NH_3 in methanol for a week at room temperature. The resin was separated from the solution by filtration and washed with hot methanol (three times) and DMF (three times). The combined filtrate and washings were evaporated under reduced pressure. Prior to cyclization the N^{ε}-amino groups of the two Lys residues were deprotected by reduction with sodium in liquid ammonia and the resulting peptide amide, Boc-Tyr(Boc)-D-Lys-Gly-Phe-Lys-NH_2 was purified by gel filtration on a Sephadex G-10 column (2.5 × 70 cm) in 0.5 M CH_3COOH. Amino acid analysis of this linear peptide amide gave: Tyr 0.94, Gly 0.93, Phe 1.03, Lys 2.06. Boc-Tyr(Boc)-D-Lys-Gly-Phe-Lys-NH_2 (0.5 mmol) was dissolved in pyridine (500 cm^3) and bis(4-nitrophenyl) carbonate (100 + 50 + 20 = 170 mg; 0.56 mmol within 1 h) was added. The reaction was allowed to continue until free amino groups could no longer be detected (two days) and the solvent was then evaporated under reduced pressure. The solid residue was dissolved in TFA (10 cm^3) and the resulting solution was allowed to stand for 1 h at room temperature. After evaporation to dryness under vacuum, the obtained crude product was purified by partition chromatography on Sephadex G-25 in the following solvent systems (v/v): (1) *n*-BuOH/AcOH/H_2O (4 : 1 : 5); (2) 0.1% AcOH/*n*-BuOH/pyridine (10 : 7 : 3). the product was obtained as a lyophilisate (62.3 mg). Purification to homogeneity was achieved by semi-preparative reversed-phase HPLC on a Waters μ-Bondapak C-18 column (30 × 1 cm) using the isocratic solvent system: 20% CH_3CN/0.03 M CH_3COONH_4 (pH 5). After HPLC purification (two runs) the final yield was 43.6 mg.

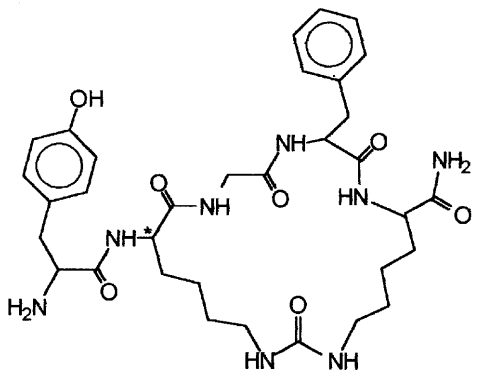


Figure 1 Structural formula of cyclo(N^{ε},$N^{\varepsilon'}$-carbonyl-D-Lys^2,Lys^5)enkephalinamide. The configuration at the position indicated by the star is D.

Preparation of Cyclo(N^{ε},$N^{\varepsilon'}$-carbonyl-D-Lys^2,Lys^5)enkephalinamide (Method II)

The linear fully protected peptide II was synthesized according to the solid-phase synthesis protocols described above, but using the 4-methylbenzydrylamine resin (0.52 g; 0.97 meq./g; 0.5 mmol). While Boc-protection of the terminal α-amino group was retained, Fmoc-deprotection of the ε-amino groups of the side chains of the two Lys residues was achieved by treatment with 50% piperidine in DMF (1 × 20 min; 1 × 30 min). Following washings with DMF, *i*-PrOH and CH_2Cl_2, cyclization was performed

Table 1 Amino Acid Analyses of Peptide Hydrolysates

Product	Duration of the hydrolysis (h)	Tyr	Gly	Phe	Lys	X[a]
I	24	0.90	1.05	1.06	0.78	+
	48	0.98	1.04	0.98	1.46	+
II	24	0.91	1.06	1.03	0.6	+
	48	0.94	1.02	1.04	1.32	+

[a] There was an additional peak which was identified as $N^{\varepsilon},N^{\varepsilon'}$-carbonyl-bis-lysine.

in DMF at room temperature by addition of bis(4-nitrophenyl) carbonate (1 equiv.). Progress of the ring closure was followed by the ninhydrin test (60.3 μmol NH_2/g after 20 h; 50.3 μmol NH_2/g after 22 h; 6.0 μmol NH_2/g after 40 h). After 20 h TEA (2 equiv.) was added. Next, the resin was filtered, washed with DMF ($2 \times 10\,cm^3$) and fresh bis(4-nitrophenyl) carbonate (0.03 equiv.) in DMF was added. The cyclization reaction was complete after two days. After cyclization the N-terminal Boc group was removed as usual by treatment with 50% (v/v) TFA in CH_2Cl_2 (1×1 min, 1×15 min) and then the peptide was cleaved from the resin and completely deprotected by treatment with liquid HF ($10\,cm^3$) in the presence of anisole ($1\,cm^3$) for 1 h at 0 °C. After removal of the HF the resin was extracted three times with ether and subsequently with 50% acetic acid ($4 \times 10\,cm^3$). Then ion exchange resin AG1-X2 (CH_3COO form; Bio-Rad.) was added to the acetic acid extract to remove fluoride ions. The suspension was stirred for 10 min, and the resin was then filtered and washed with 50% acetic acid. The filtrates were combined and lyophilized. The crude product (103 mg) was purified by gel filtration on a Sephadex G-10 column (2.5×70 cm) in 0.5% CH_3COOH, followed by semi-preparative reversed-phase HPLC (two runs) on a Waters μ-Bondapak C-18 column (30×1 cm), using the following isocratic solvent system: 20% CH_3CN/0.03 M CH_3COONH_4 (pH 5). The yield after purification was 27.8 mg.

Characterization of Cyclo($N^{\varepsilon},N^{\varepsilon'}$-carbonyl-D-Lys^2, Lys^5)enkephalinamide

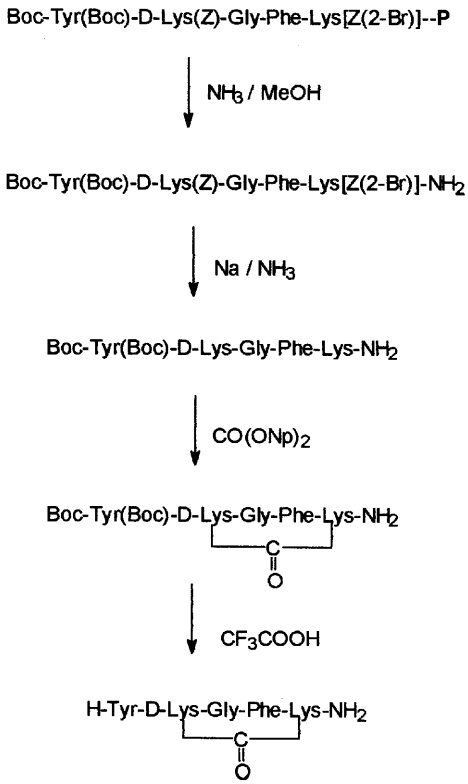
Purity of the products was established by analytical HPLC on a Waters Nova-Pak HR C-18 column ($8 \times 100\,mm^2$) using the solvent system: A = 0.05% TFA, B = 60% CH_3CN/A in a linear gradient mode (0–100% B in 30 min) at a flow rate of $1\,cm^3$/min and with detection at 230 nm ($R_t = 17.35$ min). FAB mass spectrum: MH^+ calc. 667, found 667. For amino acid analysis the products were hydrolized in 6N HCL containing a small amount of phenol for 24 h and 48 h at 110 °C in deaerated and sealed ampules. The hydrolysates were analysed on a Model T 339M Microtechna Amino Acid Analyzer (Table 1).

Bioassays

The GPI [10] and MVD [11] bioassays were carried out as reported in detail elsewhere [7, 12]. A log dose-response curve was determined with [Leu^5]enkephalin as standard for each ileum and vas preparation and IC_{50} values of the compounds being tested were normalized according to a published procedure [13].

RESULTS AND DISCUSSION

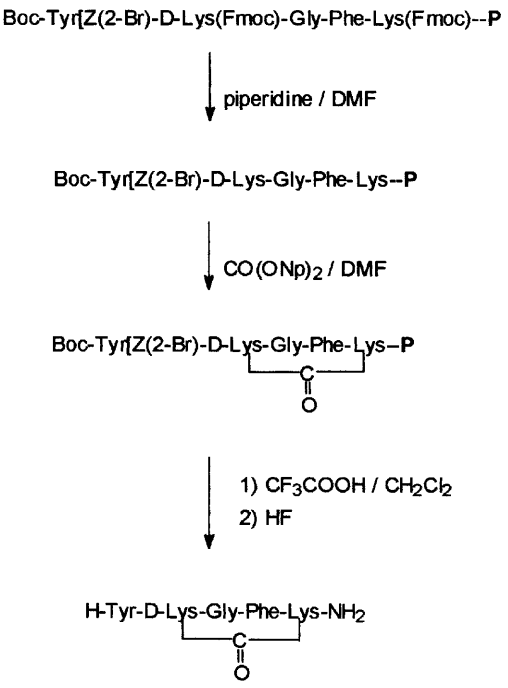
The linear precursor peptide was synthesized by the manual solid-phase technique using *N-tert*-butyl-*N′*-ethylcarbodiimide as reagent for the preformation of the symmetrical anhydrides [14]. Two different variants of the synthesis were performed. In the first variant (Scheme 1), the side-chain amino groups of L-Lys in position 5 and of D-Lys in position 2 were protected with the Z(2-Br) group and the Z group, respectively, and Tyr was protected with two Boc groups. The peptide was cleaved from the resin by treatment with ammonia in methanol and the side-chain protecting groups of the two lysines were removed by reduction with sodium in liquid ammonia. After desalting by gel filtration, a dilute solution of the linear peptide in pyridine was treated with bis(4-nitrophenyl) carbonate. Previously we had used this reagent successfully for the preparation of several urea derivatives [15]. The reaction was rapid at the beginning but took more than a day to go to completion. The product was purified using partition chromatography and HPLC. Amino acid analysis of the homogenous product revealed the presence of the expected amino acids in the proper ratios ($N^{\varepsilon},N^{\varepsilon'}$-carbonyl-bis-lysine was not deter-



Scheme 1 Synthesis of cyclo(N^{ε},$N^{\varepsilon'}$-carbonyl-D-Lys^2, Lys^5)enkephalinamide by a combination of solid-phase and solution methods (Method I).

mined). The structural identity of the product was also confirmed on the basis of its molecular weight, which was determined by FAB mass spectrometry. Dimers that potentially could be formed in this reaction were not identified. However, dimer or oligomer formation cannot be entirely excluded, since some minor side products were removed in the purification process. In the second synthetic variant (Scheme 2), the side chains of both lysines were protected with and Fmoc group. Once the peptide was assembled on the resin, the Fmoc groups were removed by treatment with piperidine and cyclization was accomplished by the reaction of the peptide–resin with bis(4-nitrophenyl) carbonate. The product was cleaved from the resin by treatment with HF. The product was purified as above and was identical to that obtained in synthetic variant 1 by combination of the solid-phase technique (peptide chain assembly) and classical method (peptide cyclization in solution).

The opioid activity profile of the peptide cyclo(N^{ε},$N^{\varepsilon'}$-carbonyl-D-Lys^2,Lys^5)enkephalinamide (Figure 1) was determined in the guinea pig ileum (GPI) assay and in the mouse vas deferens (MVD)



Scheme 2 Synthesis of cyclo(N^{ε}, $N^{\varepsilon'}$-carbonyl-D-Lys^2, Lys^5)enkephalinamide by the solid-phase procedure (Method II).

assay. In comparison with [Leu^5]enkephalin, the analogue showed 12 times higher agonist potency in the GPI assay and was over six times less potent an agonist in the MVD assay (Table 2). Since it is well established that the MVD preparation contains mostly δ-receptors and the GPI preparation largely μ-receptors [10, 11] these results indicate a preference of this cyclic analogue for μ receptors over δ receptors. Increased affinity and selectivity for μ-opioid receptors had previously been observed with enkephalin analogues cyclized by amide bond formation between the side-chain amino group of a D-amino acid residue in position 2 and the C-terminal carboxy function [6, 7] or the side-chain carboxy group of an appropriately substituted 4- or 5-position residue [16, 17]. On the other hand, most of the disulphide bridge-containing cyclic opioid peptide analogues reported to date are δ receptor-selective.

CONCLUSIONS

The method described, based on the reaction of bis(4-nitrophenyl) carbonate with side-chain amino groups of two diamino acid residues, permits the

Table 2 GPI and MVD Assays of Opioid Peptide Analogues

	GPI		MVD		MVD–GPI
Compound	IC_{50}(nM)[a]	Rel. potency	IC_{50}(nM)[a]	Rel. potency	IC_{50} ratio
Cyclo(N^{ε},$N^{\varepsilon'}$-carbonyl-D-Lys^2,Lys^5)enkephalinamide	20.5 ± 2.1	12.0 ± 1.2	73.5 ± 4.3	0.155 ± 0.009	3.59
[Leu^5]enkephalin	246 ± 39	1	11.4 ± 1.1	1	0.0463

[a] Mean of three determinations ± SEM.

relatively convenient preparation of cyclic peptide analogues containing a ureido group within the peptide ring structure. The cyclization can be performed with the linear precursor peptide either in solution or still linked to the resin. Peptides cyclized *via* a carbonyl bridge between two side-chain amino groups represent a novel type of conformationally constrained cyclic analogues.

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