Acta Biochimica Polonica

Vol. 53 No. 1/2006, 73–76

on-line at: www.actabp.pl

Regular paper

Cyclic dermorphin tetrapeptide analogues obtained *via* ring-closing metathesis*

Irena Berezowska, Nga N. Chung, Carole Lemieux, Brian C. Wilkes and Peter W. Schiller✉

Laboratory of Chemical Biology and Peptide Research, Clinical Research Institute of Montreal, Montreal, Quebec, Canada; ✉e-mail: schillp@ircm.qc.ca

Received: 06 November, 2005; accepted: 12 December, 2005
available on line: 23 February, 2006

The dermorphin-derived cyclic tetrapeptide analogues H-Tyr-c[D-Cys-Phe-Cys]NH_2 and H-Tyr-c[D-Cys-Phe-D-Cys]NH_2 are opioid agonists at the μ and δ receptor. To enhance the metabolic stability of these peptides, we replaced the disulfide bridge with a bis-methylene moiety. This was achieved by solid-phase synthesis of the linear precursor peptide containing allylglycine residues in place of the Cys residues, followed by ring-closing metathesis. In the case of the peptide with L-configuration in the 4-position both the *cis* and the *trans* isomer of the resulting olefinic peptides were formed, whereas the *cis* isomer only was obtained with the peptide having the D-configuration in position 4. Catalytic hydrogenation yielded the saturated -CH_2-CH_2- bridged peptides. In comparison with the cystine-containing parent peptides, all olefinic peptides showed significantly reduced μ and δ agonist potencies in the guinea pig ileum and mouse vas deferens assays. The -CH_2-CH_2-bridged peptide with L-configuration in the 4-position was equipotent with its cystine-containing parent in both assays, whereas the bis-methylene analogue with D-configuration in position 4 was 10–27-fold less potent compared to its parent. The effect of the disulfide replacements with the -CH=CH- and -CH_2-CH_2- moieties on the conformational behavior of these peptides was examined by theoretical conformational analysis which provided plausible explanations in terms of structural parameters for the observed changes in opioid activity.

Keywords: opioid peptides, dermorphin, cyclic dermorphin analogues, ring-closing metathesis, opioid activity profile *in vitro*

The replacement of the disulfide bridge in cystine-containing biologically active peptides with two methylene groups is of considerable interest because the resulting dicarba analogues are metabolically more stable. In the past, this structural modification was synthetically demanding, as it required the replacement of cystine with diaminosuberic acid in a cumbersome multi-step synthesis (Keller & Rudinger, 1974). Recently, the use of ring-closing metathesis (RCM) has been shown to be a relatively straightforward procedure for the preparation of a dicarba analogue of oxytocin (Stymiest *et al.*, 2003).

All naturally occurring opioid peptides are linear peptides showing more or less pronounced selectivity for μ, δ or κ opioid receptors. Cyclic analogues of the enkephalins, dermorphin and dynorphin have been prepared in efforts to improve their selectivity for a particular opioid receptor class. Furthermore, cyclic peptide analogues are structurally more rigid than their linear counterparts and, for that reason, are more suitable for conformational studies aimed at determining their bioactive conformation. Two different types of cyclic analogues of the N-terminal tetrapeptide segment of dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) have been described (Schiller *et al.*, 1987). Cyclic lactam analogues were prepared through substitution of Orn and Asp residues in the 2- and 4-position of the peptide sequence and subsequent amide bond formation between the side-chain amino and carboxy-

*Presented at the 18th Polish Peptide Symposium, 4–8 September, 2005, Wrocław, Poland.

Abbreviations: Dic, 1,3-diisopropylcarbodiimide; ES-MS, electrospray mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; MVD, mouse vas deferens; RCM, ring-closing metathesis; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

lic acid functions (e.g. H-Tyr-c[D-Orn-Phe-Asp]NH_2). Disulfide-bridged cyclic analogues were obtained by substitution of Cys residues in the 2- and 4-positions, followed by disulfide bond formation (e.g. H-Tyr-c[D-Cys-Phe-Cys]NH_2). These various cyclic tetrapeptides behaved as opioid agonists and showed more or less pronounced μ receptor selectivity (Schiller *et al.*, 1987).

In the present study, we describe the synthesis of dicarba analogues of H-Tyr-c[D-Cys-Phe-L(or D)-Cys]NH_2 (Fig. 1) by RCM between allylglycine residues substituted for the Cys residues. The *in vitro* opioid activity profiles of the new analogues were determined in the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays, and their low-energy conformations were determined by theoretical conformational analysis based on molecular mechanics calculations.

MATERIALS AND METHODS

Analytical methods. Precoated plates (silica gel 60 F_{254}, 250 μm; Merck, Darmstadt, Germany) were used for ascending TLC in the following solvent systems (all by vol.); (I) *n*-BuOH/AcOH/H_2O (4:1:1); (II) *n*-BuOH/pyridine/AcOH/H_2O (15:10:3:12). Preparative reversed-phase HPLC was performed on a Vydac 218-TP1022 column (22 × 250 mm) with a linear gradient of 10–25% acetonitrile in 0.1% TFA over 35 min at a flow rate of 13 mL/min. Analytical reversed-phase HPLC was performed on a Vydac 218-TP54 column (5 × 250 mm) with a linear gradient of 10–25% acetonitrite in 0.1% TFA over 30 min at a flow rate of 1.5 mL/min. The same column was also used for the determination of the capacity factors K′ under the same conditions. Molecular masses of the compounds were determined by electrospray mass spectrometry on a Hybrid Q-Tof mass spectrometer interfaced to a MassLynx 4.0 data system.

Peptide synthesis. The linear precursor peptide amides were prepared by the manual solid-phase technique using Fmoc-protection for the α-amino group and t-butyl protection for the hydroxyl group of tyrosine, and 1,3-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) as coupling agents. The peptides were assembled on a Rink amide Novagel resin according to a published protocol (Chen *et al.*, 2004). After completion of peptide assembly, cyclization between the two allylglycine residues was achieved using the second generation Grubbs catalyst benzylidene[1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene]dichloro(tricyclohexylphosphine) ruthenium (Ahn *et al.*, 2001). The catalyst in 10-fold molar excess was added to the peptide resin suspended in CH_2Cl_2 under a flow of argon. The reaction mixture was refluxed for 48 h and then, after cooling to room temperature, DMSO (50 equiv. relative to the catalyst) was injected and the mixture was stirred for another 24 h. The peptide resin was filtered and washed successively with DMSO, CH_2Cl_2 and MeOH. Fmoc protection was removed by 30% piperidine/DMF treatment and the cyclic olefinic peptides were cleaved from the resin by TFA treatment in the usual manner. The yield of the cyclization reaction was 73% and 75% for the D,D- and the D,L-peptide, respectively. In the case of the peptide with L-configuration in the 4-position of the peptide sequence a 3:1 mixture of *cis* and *trans* isomers was obtained, whereas the *cis* isomer only was formed with the peptide having the D-configuration in the 4-position. The crude peptides were purified by preparative reversed-phase HPLC and were found to be at least 98% pure, as assessed by HPLC and TLC. Catalytic hydrogenation of the cyclic olefinic peptides (the *cis* isomer in the case of the D,L-peptide) was performed with 10% Pd/C in EtOH at 40°C for 18 h (p_{H2} = 45 psig). The resulting -CH_2-CH_2- bridged peptides were obtained in 85–87% yield and were purified by preparative HPLC. Analytical parameters of the peptides are presented in Table 1.

Pharmacological testing *in vitro*. The guinea pig ileum (GPI) and mouse vas deferens (MVD) bioassays were used to determine μ and δ agonist potencies, respectively. These assays have been described in detail elsewhere (Schiller *et al.*, 1978; DiMaio *et al.*, 1982). A 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 (Waterfield *et al.*, 1979).

Theoretical conformational analysis. All calculations were performed using the molecular modeling software SYBYL, version 7.0 (Tripos Associates, St. Louis, MO, USA). The standard SYBYL force field was used for energy calculations, and a dielectric constant of 78 was chosen to simulate an aqueous environment. The molecular mechanics calculations were carried out as previously described for other cyclic dermorphin analogues (Wilkes & Schiller, 1990), using a stepwise approach in order to find low-energy structures of the peptides studied. Briefly, the bare ring structure was constructed for each peptide containing only atoms directly attached to the ring, along with associated hydrogen atoms. A systematic grid search was performed on the bare ring structure and the resulting structures were minimized. All conformers having an energy 3 kcal/mol higher than that of the lowest energy ring structure were discarded. Next, the exocyclic tyrosine residue and the phenylalanine side chain were linked to each low-energy ring structure and a second systematic grid search was performed on the exocyclic

Table 1. Analytical parameters of dicarba analogues of H-Tyr-c[D-Cys-Phe-D(or L)-Cys]NH_2

Compound	R_F (I)	R_F (II)	K′	ES-ML *m/e*
1	0.74	0.82	1.76	494
2	0.70	0.80	1.55	494
3	0.71	0.81	1.58	496
5	0.72	0.79	1.94	494
6	0.70	0.79	1.74	496

rotatable bonds. The resulting conformations were grouped into low-energy families, and the lowest-energy conformation of each family was minimized. The conformations obtained for each peptide were then ranked in order of increasing energy.

RESULTS AND DISCUSSION

In comparison with the parent peptide H-Tyr-c[D-Cys-Phe-Cys]NH_2 (**4**), the *cis* and *trans* isomers (**1** and **2**) showed 7- and 2.5-fold lower μ agonist potency in the GPI assay, respectively, and slightly higher δ agonist potency in the MVD assay (Table 2). The corresponding bis-methylene analogue (**3**) was equipotent with the parent **4** in both assays and, consequently, displayed similar μ receptor selectivity [IC_{50}(MVD)/IC_{50}(GPI) ≅10]. This result indicates that, unlike in the case of oxytocin (Keller & Rudinger, 1974; Stymiest *et al.*, 2003) and of a somatostatin analogue (Nutt *et al.*, 1980), replacement of the disulfide bridge with a bis-methylene moiety does not result in an activity drop.

The *cis* isomer of the olefinic peptide with D-configuration in position 4 (**5**) was 9 times less potent than its corresponding parent H-Tyr-c[D-Cys-Phe-D-Cys]NH_2 (**7**) in both assays. The corresponding -CH_2-CH_2- bridged peptide (**6**) also showed about 10-fold lower potency than parent **7** in the GPI assay and even weaker (27-fold lower) potency in the MVD assay. Thus, in the case of H-Tyr-c[D-Cys-Phe-D-Cys]NH_2 (**7**), replacement of the disulfide bond with both a -CH=CH- (*cis*) and a -CH_2-CH_2- bond was detrimental to opioid activity.

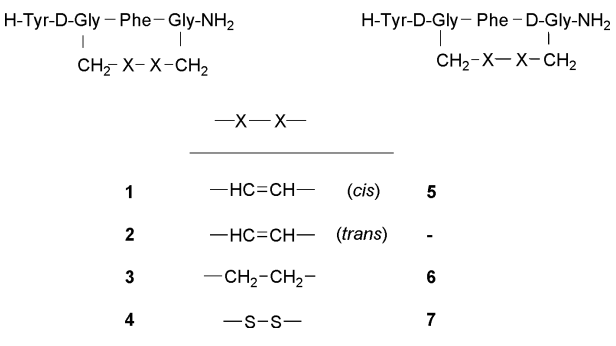

Figure 1. Dicarba analogues of H-Tyr-c[D-Cys-Phe-D(or L)-Cys]NH_2.

In the theoretical conformational analysis, the systematic grid search of the bare ring structures of cyclic peptides **1–7** yielded significantly different numbers of low-energy conformers (within 3 kcal/mol of the lowest-energy structure) within this series of compounds. The numbers of low-energy conformations found were as follows: 5, 7 and 7 for the olefinic peptides **1**, **2** and **5**; 15 and 11 for the -CH_2-CH_2-bridged peptides **3** and **6**; and 10 and 10 for the disulfide-bridged parent peptides **4** and **7**. These results indicate that, in comparison with the cystine-containing parent peptides, the ring structures of the olefinic peptides are more rigid, whereas those of the bis-methylene analogues have equal or even higher structural flexibility. The enhanced structural rigidity of the olefinic peptides may be responsible for their decreased opioid activity, as optimal conformational adaptation to the receptor topography may not be possible. After addition of the exocyclic tyrosine residue and the phenylalanine side chain to the low-energy conformers, and subsequent grid search and energy minimization, the lowest-energy conformer of the -CH_2-CH_2-bridged analog with L-configuration in position 4 (**3**) showed excellent spatial overlap of the peptide ring structure, the Tyr^1 residue and the Phe^3 side chain with the corresponding moieties of the lowest-energy conformer of the H-Tyr-c[D-Cys-Phe-Cys]NH_2 parent peptide **4** (Fig. 2). Since the N-terminal amino group, the Tyr^1 phenolic ring and the Phe^3 phenyl group are the essential pharmacophoric groups in the interaction with opioid receptors, this result may explain the equipotency observed with peptides 3 and 4 at the μ and δ opioid receptors. On the other hand, the poor spatial overlap seen between the lowest-energy conformers

Table 2. Guinea pig ileum (GPI) and mouse vas deferens (MVD) assay of dicarba analogues of H-Tyr-c[D-Cys-Phe-D(or L)-Cys]NH_2

Compound	GPI	MVD	MVD/GPI
	IC_{50} [nM]	IC_{50} [nM]	IC_{50} ratio
1	436 ± 97	460 ± 44	1.06
2	162 ± 17	444 ± 36	2.74
3	76.5 ± 5.5	655 ± 68	8.56
4	64.7 ± 11.9	740 ± 187	11.4
5	176 ± 10	246 ± 17	1.40
6	208 ± 16	786 ± 98	3.78
7	20.0 ± 3.6	28.8 ± 1.7	1.44

Data represent the mean ± S.E.M. from 3–6 independent experiments.

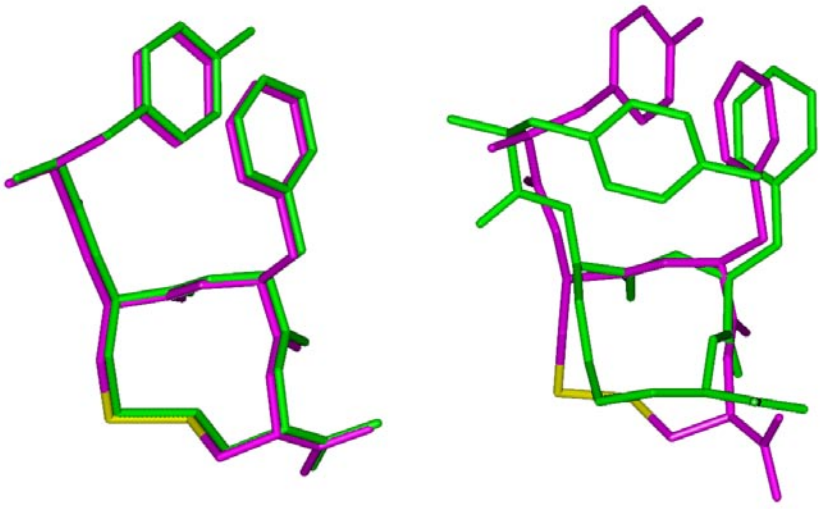

Figure 2. Spatial overlap of lowest-energy conformers of bis-methylene analogues and corresponding disulfide-bridged parent peptides.
Left panel; Analogue **3** (green) and parent peptide **4** (magenta). Right panel; Analogue **6** (green) and parent peptide **7** (magenta).

of the bis-methylene analogue with D-configuration in the 4-position (**6**) and its disulfide-bridged parent (**7**) (Fig. 2) may explain the drastically reduced potency of the former peptide.

Acknowledgements

This work was supported by grants from the Canadian Institutes of Health Research (MOP-5655) and the National Institute on Drug Abuse, NIH, USA (DA004443).

REFERENCES

Ahn YM, Yang KL, Georg GI (2001) A convenient method for the efficient removal of ruthenium by-products generated during olefin metathesis reactions. *Org Lett* **3**: 1411–1413.

Chen H, Chung NN, Lemieux C, Zelent B, Vanderkooi JM, Gryczynski I, Wilkes BC, Schiller PW (2005) [Aladan^3]TIPP: a fluorescent δ opioid antagonist with high δ receptor binding affinity and δ selectivity. *Biopolymers (Peptide Science)* **80**: 325–331.

DiMaio J, Nguyen TM-D, Lemieux C, Schiller PW (1982) Synthesis and pharmacological characterization *in vitro* of cyclic enkephalin analogues: effects of conformational constraints on opiate receptor selectivity. *J Med Chem* **25**: 1432–1438.

Keller O, Rudinger J (1974) Synthesis of [1,6-α,α′-diaminosuberic acid]oxytocin ('Dicarba'-oxytocin). *Helv Chim Acta* **57**: 1253–1259.

Nutt RF, Veber DF, Saperstein R (1980) Synthesis of nonreducible bicyclic analogs of somatostatin. *J Am Chem Soc* **102**: 6539–6545.

Schiller PW, Lipton A, Horrobin DF, Bodanszky M (1978) Unsulfated C-terminal 7-peptide of cholecystokinin: a new ligand of the opiate receptor. *Biochem Biophys Res Commun* **85**: 1332–1338.

Schiller PW, Nguyen TM-D, Maziak LA, Wilkes BC, Schiller PW (1987) Structure-activity relationships of cyclic opioid peptide analogues containing a phenylalanine residue in the 3-position. *J Med Chem* **30**: 2094–2099.

Stymiest JL, Mitchell BF, Wong S, Vederas JC (2003) Synthesis of biologically active dicarba analogues of the peptide hormone oxytocin using ring-closing metathesis. *Org Lett* **5**: 47–49.

Waterfield AA, Leslie FM, Lord JAH, Ling N, Kosterlitz HW (1979) Opioid activities of fragments of β-endorphin and of its leucine^{65}-analogue. Comparison of the binding properties of methionine- and leucine-enkephalin. *Eur J Pharmacol* **58**: 11–18.

Wilkes BC, Schiller PW (1990) Conformation-activity relationships of cyclic dermorphin analogues. *Biopolymers* **29**: 89–95.