Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 898–902
Published online 12 March 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.1022

JOURNAL OF PEPTIDE SCIENCE

Conformation-activity relationships of *cyclo*-constrained μ/δ opioid agonists derived from the *N*-terminal tetrapeptide segment of dermorphin/deltorphin

SYLWIA RODZIEWICZ-MOTOWIDŁO,* CEZARY CZAPLEWSKI, SYLWIA ŁUCZAK and JERZY CIARKOWSKI

Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland

Received 18 December 2007; Revised 4 January 2008; Accepted 4 January 2008

Abstract: The *N*-terminal tetrapeptide segments of dermorphin (Tyr–D-Ala–Phe–Gly–Tyr–Pro–Ser–NH_2) and deltorphin (Tyr–D-Ala–Phe–Asp/Glu–Val–Val–Gly–NH_2) are agonists at the opioid receptors μ and δ, respectively. [D-Arg^2, Lys^4]-dermorphin-(1–4) amide (Tyr–D-Arg–Phe–Lys–NH_2, DALDA[a]) and [Dmt^1]DALDA (where Dmt is 2′,6′-dimethyltyrosine) are among the most potent and selective μ-agonists reported to date, both *in vitro* (having picomolar μ receptor affinity) and *in vivo*. In this communication, conformation-activity studies of the following four cyclic analogs of DALDA are presented and discussed: the lead peptide S^2,S^4-cyclo (Tyr–D-Cys–Phe–Cys–NH_2), constrained by means of an $S^{4.2}$–$S^{4.4}$ disulfide between Cys^2 and Cys^4; its two *cis* and *trans* $C^{4.2}$–$C^{4.4}$-olefinic dicarba analogs, and the product of saturation of them both. They are potent nonselective or moderately μ-selective opioid agonists *in vitro*.

They have been synthesized and tested earlier [Berezowska I, Chung NN, Lemieux C, Wilkes BC, and Schiller PW, Acta Biochim Polon 53, 2006, 73–76]. We have studied their conformations using NMR and molecular dynamics. With major conformational constraints imposed by the 11-membered ring spanning residues 2–4, they show well defined conformations of this ring, while the exocylic Tyr^1 and Phe^3 side chains still have significant conformational freedom. The more active and selective μ *versus* δ disulfide and saturated dicarba agonists seem to have in common: (i) their ring structures more flexilble than those of the other two and (ii) their ring structures similar to each other and more diverse than those in the other two. Given this and the small size of the peptides having confirmed bioactivity profiles, there is a chance that their conformations determined in solution approach receptor-bound conformations. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: conformation; opioid cyclopeptides; dermorphin/deltorphin; NMR; SAR

INTRODUCTION

The replacement of the disulfide in cystine-containing biologically active peptides with the ethylene bridge is of considerable interest because the resulting dicarba analogs are metabolically more stable. Once the use of ring-closure metathesis has become relatively straightforward [1], it has been feasible to apply this procedure for restraining opioid peptides by means of carbon–carbon equivalents of disulfides [2]. All endogenic opioids are linear peptides showing more or less pronounced selectivity to μ, δ, or κ opioid receptors. Cyclic analogs of the enkephalins, dermorphin, and dynorphin have been prepared in efforts to improve their selectivity for a particular opioid receptor class as being structurally more rigid than their linear counterparts and, accordingly, more suitable for conformational structure-activity studies. Disulfide-bridged cyclic analogs were obtained by introducing Cys residues in the 2- and 4-positions, followed by disulfide bond formation, e.g. S^2,S^4-cyclo(Tyr–D-Cys–Phe–Cys–NH_2). They have been opioid agonists and have shown more or less pronounced μ receptor selectivity [3].

In this work, we have studied conformations of this parent peptide, its two *cis* and *trans* $C^{4.2}$–$C^{4.4}$-olefinic dicarba analogs and the product of saturation thereof, Table 1, using 2D-NMR in H_2O/D_2O and molecular dynamics, MD. A discussion of their structure-activity relationships (SAR) is included.

METHODS

The analogs have been synthesized earlier and found to be potent, nonselective or moderately μ-selective opioid agonists *in vitro* [2,3]. A summary of their biological activity is given in Table 1.

NMR Experiment

All NMR measurements were performed in the H_2O/D_2O solutions, with peptide concentrations about 3 mM on the Varian Unity 500 Plus Spectrometer operating at 500 MHz resonance

Nonstandard abbreviations: DALDA, Tyr-D-Arg-Phe-Lys-NH2; Dmt, 2′,6′-dimethyltyrosine; SAR, structure-activity relationships; TAV-MD, molecular dynamics utilizing the time-averaged constraints; ROESY, rotational nuclear overhauser effect spectroscopy; TOCSY, total correlated spectroscopy; DQF-COSY, double quantum filtered - correlation spectroscopy; RMS, root-mean-square.

*Correspondence to: Sylwia Rodziewicz-Motowidło, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland;
e-mail: sylwia@chem.univ.gda.pl

Table 1 The analogs studied and their biological activities[a]

Tyr-D-Ala-Phe-Ala-NH_2 (–X ~ X–)	No	GPI (μ-selective) EC_{50} (nM)	MVD (δ-selective) EC_{50} (nM)	MVD/GPI EC_{50} ratio
–S–S–	**1**	64.7 ± 11.9	740 ± 187	11.4
–HC=CH–(*cis*)	**2**	162 ± 17	444 ± 36	2.74
–HC=CH–(*trans*)	**3**	436 ± 97	460 ± 44	1.05
$-CH_2-CH_2-$	**4**	76.5 ± 5.5	655 ± 68	8.56

[a] Ref. 2.

frequency with sodium 3-trimethylsilyltetra-deuterium-propionate (TSP), as internal standard. All 2D spectra were recorded at 305 K. The assignment of the proton chemical shifts was accomplished by means of 2D proton spectra TOCSY (80 ms), ROESY (150 ms), and DQF-COSY. Due to problems with solubility, productive sets of 2D spectra exhibiting utilizable cross-peaks could successfully be collected only for analogs **1** and **3**, Table 1. All data were processed using ACDLabs [4] and XEASY [5] software. The $^3J_{HN\alpha H}$ coupling constants were obtained from the DQF-COSY spectra. Temperature dependences of the chemical shifts of NH protons ($\Delta\delta/\Delta T$) were calculated from the 1D spectra recorded at 275, 296, 305, 313, and 323 K.

NMR Structure Calculations of 1 and 3

In our study, we used ~3 ns Molecular Dynamics utilizing the Time-Averaged Constraints (TAV-MD) procedure, dedicated to structure determinations of small flexible peptides [6]. Prior to the productive MD routine operations, including parameterization of new moieties, TAV-constrained model-building, energy minimization, thermalization, etc. were performed. All molecular dynamic simulations were carried out using the AMBER ver. 8.0 software [7]. The all-atom AMBER force field and explicit H_2O molecules as a solvent were used. Each set of conformations from the TAV-MD trajectories was clustered into 5–6 families of conformations.

Molecular Dynamics Simulations

Prior to the structure computations, the new unknown to AMBER structure elements pertinent to the positions 2 and 4 in analogs **1–4** were parameterized as recommended in the AMBER ver. 8 manual [8]. The parameterized new units were used both in resolving the NMR-constrained structures (as shown above), and also in the MD of analogs **2** and **4**, whose 2D NMR spectra were of too poor quality for structure solving based on the NMR-derived constraints. The latter analogs **2** and **4** were submitted to unconstrained MD with starting structures taken at random under otherwise identical conditions as for analogs **1** and **3** (also shown above). Analogs **1** and **3** were also submitted to identical unconstrained MD for reference (given below). Likewise, as for **1** and **3**, each set of conformations of **2** and **4** from the unconstrained MD trajectories was also clustered into 5–6 families of conformations.

RESULTS

Analogs 1 and 3, NMR Supported

The TOCSY, DQF-COSY, and ROESY spectra allowed unambiguous chemical shift assignments requisite for the determinations of other NMR parameters like homonuclear scalar and dipolar couplings, amide NH temperature shift coefficients and the dipolar connectivities. The ^1H chemical shifts, the $^3J_{NH\alpha H}$ coupling constants and the amide NH temperature coefficients for analogs **1** and **3** are given in Tables 2 and 3, respectively. The values of the scalar $^3J_{HN\alpha H}$ couplings for the analogs, both **1** and **3** are compatible with bent structures (Tables 2 and 3, respectively), likewise, are the numbers and types of the connectivities of the i, $i+1$ type, not shown. On the other hand, only one NH temperature coefficient, viz. that for Cys^4 in **1** clearly indicates a possibility of occurrence of a stable intramolecular H-bond. Imposing both the time-averaged connectivities and the $^3J_{HN\alpha H}$ restraints into

Table 2 ^1H chemical shifts, $^3J_{NH\alpha H}$ couplings and amide NH temperature coefficients for analog **1**

Residue	NH	α-CH	β-CH	ArH	$^3J_{NH\alpha H}$ (Hz)	$-\Delta\delta/\Delta T$ (ppb/K)
Tyr^1	—	n/d	3.062, 3.218	2,6H 6.795 3,5H 6.802	—	—
D-Cys^2	8.431	4.476	2.429, 3.011	—	6.03	6.4
Phe^3	8.417	4.792	3.091, 3.309	2,6H 7.399 3,5H 7.263	8.77	4.7
Cys^4	8.375	4.341	3.085, 3.291	—	7.62	2.8

DOI: 10.1002/psc

Table 3 ^{1}H chemical shifts, $^{3}J_{NH\alpha H}$ couplings and amide NH temperature coefficients for analog **3**

Residue	NH	α-CH	β-CH	ArH	γ-CH	$^{3}J_{NH\alpha H}$ (Hz0	$-\Delta\delta/\Delta T$ (ppb/K)(Hz)
Tyr[1]	—	3.874	3.276, 3.421	2,6H 7.128 3,5H 6.880	—	—	—
D-Ala[2a]	8.264	4.451	2.147, 2.258	—	5.331	6.38	6.0
Phe[3]	8.485	4.065	3.207, 3.238	2,6H 7.399 3,5H 7.263	—	5.85	6.5
Ala[4a]	7.502	4.138	2.211, 2.377	—	5.427	6.39	6.4

[a] Compare residue definitions in Table 1.

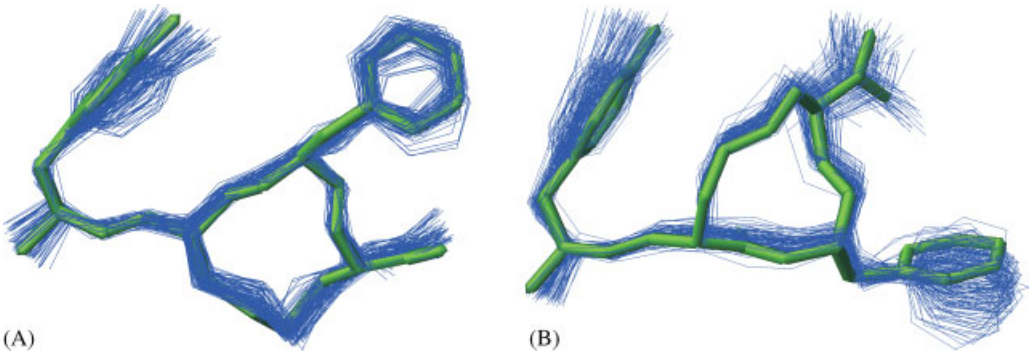

Figure 1 The cluster of representative MD-TAV-derived conformers for analog **1** meeting distance and torsion angle restraints is shown in (A), while that for analog **3** is shown in (B). The aromatic rings of Tyr[1] and Phe[3] in the former are located at an interacting distance on the same side of the ring spanning residues 2–4, while in the latter both the aromatic rings are far apart from one another.

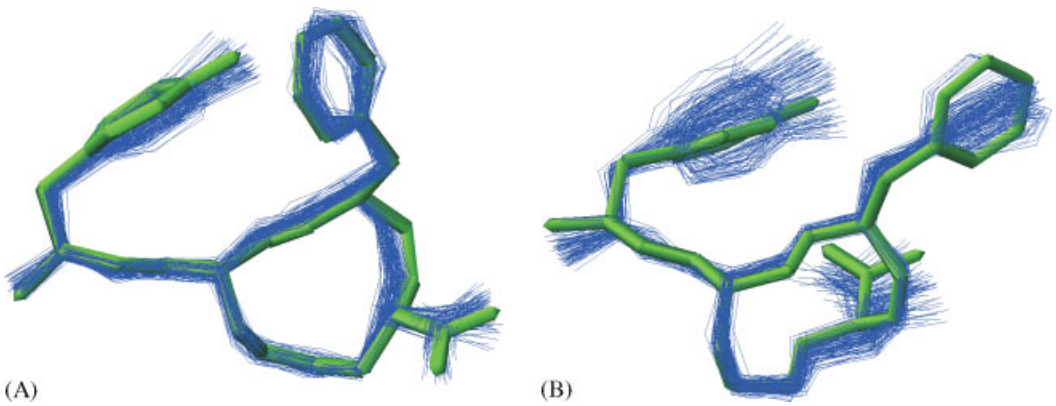

Figure 2 The cluster of representative conformers for analog **2**, derived from unrestrained MD, is shown in (A), while that for analog **4** is shown in (B). The aromatic rings of Tyr[1] and Phe[3] in the former are located far apart from one another, while in the latter, both the rings are at an interacting distance on the same side of the ring spanning residues 2–4. Interestingly, despite lack of restraints imposed during the MD, the results of unconstrained MD for **2** and **4** are quite similar to those yielded by the NMR-constrained MD for **3** and **1**, respectively.

the productive TAV-restrained MD runs has led to several clusters of conformers, the majority of which, for the analogs **1** and **3,** are shown in Figure 1.

Analogs 2 and 4, NMR Unsupported

The analogs **2** and **4** turned out to be not soluble enough to give 2D ^{1}H-NMR spectra warranting the distance and torsion angle restraints necessary for structure solving based on NMR. Hence, these two opioids were submitted merely to unconstrained MD (as shown above), whose productive trajectories (2.9 ns totally) provided material for clustering the obtained structures into the families of conformations. The clusters of representative conformers of analogs **2** and **4** are shown in Figure 2.

DOI: 10.1002/psc

Comparison Among the Four Analogs

Since only analogs **1** and **3** were analyzed using NMR and TAV-restrained MD, and the other two, **2** and **3**, by unconstrained MD, the results from both techniques were not directly comparable. Thus, for the sake of comparability among the four we have also done unconstrained MD for **1** and **3**. Interestingly, both **1** and **3** converged to similar conformations in the constrained and unconstrained MD. For example, constrained and unconstrained MD simulations converged to RMS values equal to 0.78 and 0.84 Å for **1** and **3**, respectively (backbone atoms based), and the respective structures overlapped quite well with their rings. The overlap in **1** would be even better if not the opposite chiralities of the disulfide resulting from both simulations, as in Figure 3. The comparison among the four unconstrained simulations has shown slightly but still clearly larger flexibilities of **1** and **4**, having the rings closed with saturated bridges, than those of **2** and **3**, having these rings spanned with either *cis* or *trans* olefin. This can be seen from the averaged variabilities of the final 1-ns of unconstrained trajectories, equal to 0.41, 0.24, 0.29, and 0.31 Å for **1, 2, 3**, and **4**, respectively (backbone atoms based).

The typical relaxed structures of **1** and **4** compare well with their rings, Figure 4(A), and they are diverse from each of **2** and **3**, as in Figure 4(B) and (C), respectively. While the ring atoms-based RMS is equal to 1.7 Å for the overlap of **1** and **4**, it is equal to 2.3 and 2.9 Å, i.e. considerably larger for the overlaps of **1** with **2** and **1** with **3**, respectively. A direct difference between the rings of **2** and **3** is even larger, their RMS being slightly over 3.0 Å.

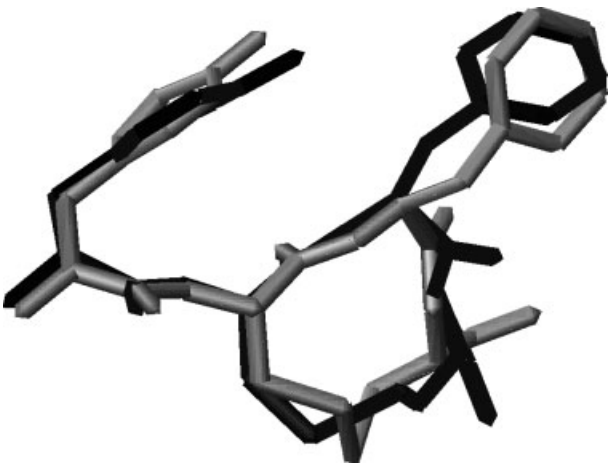

Figure 3 The overlapping structures of **1**, optimized using NMR-derived constraints (dark) and unconstrained MD (gray). Overlap was done using all ring atoms, RMS = ~0.8 Å. The only noteworthy difference between the ring structures consists of the opposite chiralities of the disulfides.

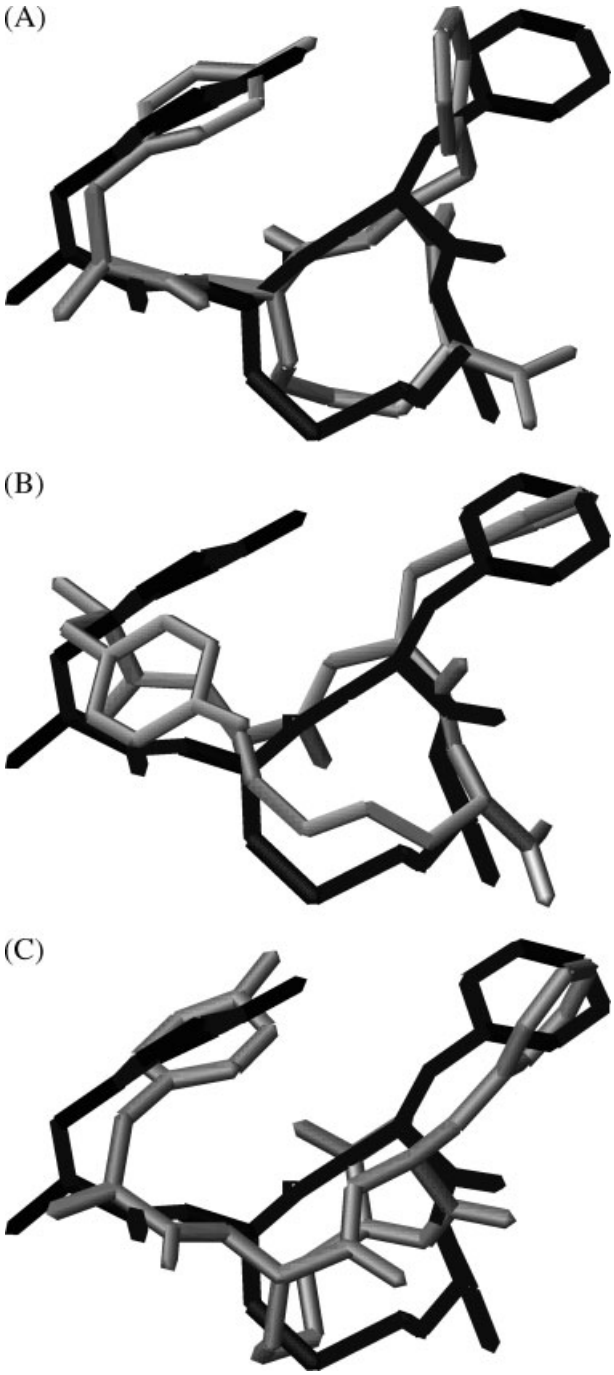

Figure 4 The overlapping optimized structures of **1** (experimental, dark) and the successive other analogs **2**–**4**. Overlaps were done using all rings atoms. (A) With **4**, derived by unconstrained MD, gray, RMS = 1.7 Å. (B) With **2**, derived by unconstrained MD, gray, RMS = 2.3 Å. (C) With **3**, derived by unconstrained MD, gray, RMS = 2.9 Å.

DISCUSSION

A comparison among **1**–**4** indicates more flexibility in the rings of **1** and **4** than in **2** and **3**. Simultaneously, these rings of **1** and **4** take up like shapes and differ from those of **2** and **3**. Although the former observation may seem trivial in view of the fact that the **1** and **4**

DOI: 10.1002/psc

rings are locked by saturated and those of **2** and **3** by olefinic units, the latter observation is not. Given flexibilities of Tyr^1 as a whole and of the Phe^3 side chain in the analogs studied, the similarities of the ring conformations of **1** and **4** (RMS = 0.85 Å, as shown above) clearly correlate with their higher μ agonist activities and μ *versus* δ selectivities in contrast to **2** and **3** (Table 1) having dissimilar ring conformations, as shown in Figure 4. The ring structures of **1** and **4** seem to compare favorably with those derived earlier using Sybyl (Tripos Associates, St. Louis, MO, USA) force field [2]. It is meaningful that the results from two diverse approaches, viz MD driven by NMR data for **1** and unconstrained MD for **4**, converge into similar structure, which correlates with their similar biological activities. Simultaneously, we observe that the structures of **1** and **4**, while being more μ-active and μ-selective, are more compact than the other two. Whether or not the structures of the rings in **1** and **4** make an adequate support for potent μ-active and μ-selective agonists still remains to be resolved since the most active and selective μ agonists to date 2′,6′-dimethyltyrosine Dmt–D-Arg–Phe–Lys–NH_2 ([Dmt^1]DALDA) has no ring, yet has three cationic centers instead, and is about 50 times more active and twice as selective as **1** and **4** [9,10]. Recent works indicate that other not clearly resolved structural features like the *C*-terminal ending (Val–Val–Gly–NH_2 in deltorphin-like [11] against Tyr–Pro–Ser–NH_2 in dermorphin-like [12] peptides) or its lack altogether [13] are also important, suggesting that frequently 'signal' could hardly be dissociated from 'message' [11,12].

Acknowledgements

This work was supported in part by the Polish Ministry of Science and Higher Education, grants BW/8000-5-0376-07 and DS/8372-4-0138-7. We are indebted to Dr Peter W. Schiller from IRCM (Montreal, Quebec, Canada H2W 1R7) for providing us with the analogs **1**–**4** for the NMR measurements. The calculations were carried out in TASK in Gdańsk, Poland.

REFERENCES

1. Stymiest JL, Mitchell BF, Wong S, Vederas JC. Synthesis of biologically active dicarba analogues of the peptide hormone oxytocin using ring-closing metathesis. *Org. Lett.* 2003; **5**: 47–49.
2. Berezowska I, Chung NN, Lemieux C, Wilkes BC, Schiller PW. Cyclic dermorphin tetrapeptide analogues obtained via ring-closing metathesis. *Acta Biochim. Pol.* 2006; **53**: 73–76.
3. Schiller PW, Nguyen TM, Maziak LA, Wilkes BC, Lemieux C. Structure-activity relationships of cyclic opioid peptide analogues containing a phenylalanine residue in the 3-position. *J. Med. Chem.* 1987; **30**: 2094–2099.
4. Copyright © 1994–2004 Advanced Chemistry Development, Inc. ACD/Labs, Toronto, Canada M5C 1T4.
5. Bartels C, Xia T, Billeter M, Güntert P, Wüthrich K. The program XEASY for computer-supported NMR spectral analysis of biological macromolecules. *J. Biomol. NMR* 1995; **6**: 1–10.
6. Pearlman DA. How is an NMR structure best defined? An analysis of molecular dynamics distance-based approaches. *J. Biomol. NMR* 1994; **4**: 1–16.
7. Case DA, Darden TA, Cheatham TE III, Simmerling CL, Wang J, Duke RE, Luo R, Merz KM, Wang B, Pearlman DA, Crowley M, Brozell S, Tsui V, Gohlke H, Mongan J, Hornak V, Cui G, Beroza P, Schafmeister C, Caldwell JW, Ross WS, Kollman PA, *AMBER 8*. University of California: San Francisco, CA, 2004.
8. Pigache A, Cieplak P, Dupradeu F-Y. *R.E.D. Ver 1.0 User's Manual and Tutorial*. The Scripps Research Institute: San Diego, CA, 2003.
9. Schiller PW, Nguyen TM, Chung NN, Lemieux C. Dermorphin analogues carrying an increased positive net charge in their "message" domain display extremely high μ-opioid receptor selectivity. *J. Med. Chem.* 1989; **32**: 698–703.
10. Schiller PW, Nguyen TM, Berezowska I, Dupuis S, Weltrowska G, Chung NN, Lemieux C. Synthesis and in vitro opioid activity profiles of DALDA analogues. *Eur. J. Med. Chem.* 2000; **35**: 895–901.
11. Zieleniak A, Rodziewicz-Motowidło S, Rusak Ł, Chung NN, Czaplewski C, Witkowska E, Schiller PW, Ciarkowski J, Izdebski J. Deltorphin analogs restricted *via* a urea bridge: structure and opioid activity. *J. Pept. Sci.* 2008 (in press). DOI: 10.1002/psc.1010.
12. Witkowska E, Nowakowski M, Oleszczuk M, Filip K, Ciszewska M, Chung NN, Schiller PW, Wójcik J, Izdebski J. Ureido group containing cyclic dermorphin(1–7) analogues: synthesis, biology and conformation. *J. Pept. Sci.* 2007; **13**: 519–528.
13. Filip K, Oleszczuk M, Wójcik J, Chung NN, Schiller PW, Pawlak D, Zieleniak A, Parcińska A, Witkowska E, Izdebski J. Cyclic enkephalin and dermorphin analogues containing a carbonyl bridge. *J. Pept. Sci.* 2005; **11**: 347–352.