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Cyclic enkephalin-deltorphin hybrids containing a carbonyl bridge: structure and opioid activity

Małgorzata Ciszewska[1], Katarzyna Ruszczyńska[2], Marta Oleszczuk[3], Nga N. Chung[4], Ewa Witkowska[1], Peter W. Schiller[4], Jacek Wójcik[2] and Jan Izdebski[1✉]

[1]Peptide Laboratory, Department of Chemistry, University of Warsaw, Warszawa, Poland; [2]Laboratory of Biological NMR, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warzsawa, Poland; [3]Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada; [4]Laboratory of Chemical Biology and Peptide Research, Clinical Research Institute of Montreal, Quebec H2W IR7, Canada

Six hybrid *N*-ureidoethylamides of octapeptides in which an N-terminal cyclic structure related to enkephalin was elongated by a C-terminal fragment of deltorphin were synthesized on MBHA resin. The synthetic procedure involved deprotection of Boc groups with HCl/dioxane and cleavage of the peptide resin with 45% TFA in DCM. D-Lys and D-Orn were incorporated in position 2, and Lys, Orn, Dab, or Dap in position 5. The side chains of the dibasic amino function were protected with the Fmoc group. This protection was removed by treatment with 55% piperidine in DMF, and cyclization was achieved by treatment with *bis*-(4-nitrophenyl)carbonate. Using various combinations of dibasic amino acids, peptides containing a 17-, 18-, 19- or 20-membered ring structure were obtained. The peptides were tested in the guinea-pig ileum (GPI) and mouse vas deferens (MVD) assays. Diverse opioid activities were observed, depending on the size of the ring. Extension of the enkephalin sequence at the C-terminus by a deltorphin fragment resulted in a change of receptor selectivity in favor of the δ receptor. The conformational propensities of selected peptides were determined using the EDMC method in conjunction with data derived from NMR experiments carried out in water. This approach allowed proper examination of the dynamical behavior of these small peptides. The results were compared with those obtained earlier with corresponding *N*-(ureidoethyl)pentapeptide amides.

INTRODUCTION

Endogenous opioid peptides, such as enkephalins, endorphins, deltorphins, dynorphins and endomorphins are involved in regulation of many biological processes, among them pain control. However, clinical utility as therapeutic analgesics is limited by their susceptibility to enzymatic degradation and several side effects. Biological activities of opioid peptides are mediated through three major opioid receptor types (μ, δ and ϰ) and the distinct structure of a peptide has an impact on the selectivity of receptor types and consequently on the activity profile.

The analgesic effect of the most effective therapeutic non-peptidic agent, morphine, is mediated through the μ opiate receptor. However, its clinical utility is limited by the side effects, such as respiratory depression and development of tolerance and dependence.

Enkephalins and other δ opiate receptor selective drugs share the morphin's analgesic effect, but have reduced negative properties: respiratory depression (Cheng *et al.*, 1993; Su *et al.*, 1998) and reveal minimal potential for the development of physical dependence (Cowan *et al.*, 1988).

The development of new opioid peptides with increased selectivity for δ opioid receptor seems to be a good avenue to obtaining drugs that may produce only desired physiological responses. One of the methods that could allow reaching this goal is the design of conformationally restricted peptides. The most promising approach to obtaining a selective agonist is cyclization of a linear active peptide which can adopt a conformation able to interact preferentially with one receptor. This is also expected to increase the enzymatic stability and activity of the resulting cyclic peptide.

Peptides can be cyclized through formation of additional bonds, e.g., disulfide, lactone, lactame or formation of bridges between two amino-acid residues, e.g., a carbonyl bridge or oligomethylene group (Davis, 2003; Janecka & Kruszynski, 2005). In several cases incorporation of cyclized opioid peptides has been shown to enhance selectivity for a distinct opioid receptor type. Further increase in selectivity of an active and selective cyclic peptide is also possible as demonstrated by replacement of cysteine by β,β-dimethylcysteine in an enkephalin analog cyclized by formation of a disulfide bridge (Akiyama *et al.*, 1985).

Attempts to increase selectivity of a cyclic peptide by synthesis of chimeric peptides have also been reported: -Val-Gly-NH$_2$, which is a C-terminal fragment of deltorphin address sequence (-Val-Val-Gly-NH$_2$), was added to the above-mentioned enkephalin analogs cyclized by formation of a disulfide bridge between two β,β-dimethylcysteine residues (Misicka *et al.*, 1994).

Previously we described the synthesis, biological activity, and conformational analysis of several highly potent side-chain-to-side-chain cyclized analogs of enkephalin

Abbreviations: CLUST, a program for cluster analysis; EDMC, electrostatically driven monte carlo; gHSQC, gradient heteronuclear single quantum coherence spectroscopy; MBHA, 4-methylbenzhydrylamine; MORASS, multiple overhauser relaxation analysis and simulation; ROESY, rotating frame overhauser enhancement spectroscopy; R.m.s.d., Root mean square deviation

amides (H-Tyr-Gly-Gly-Phe-Met-NH$_2$) in which Gly^2 and Met^5 were replaced by dibasic amino acids and cyclic structure was obtained by incorporation of side-chain amino groups into urea residue (Pawlak *et al.*, 2001; Filip *et al.*, 2005). Using the same synthetic procedure we obtained analogs of an N-terminal segment of deltorphin (H-Tyr-Gly-Phe-Asp-) by introduction of dibasic amino acids in positions 2 and 4 (Filip *et al.*, 2003). We also obtained *N*-(ureidoethyl)amides of enkephalin related to the cyclic amides studied earlier, using a convenient synthetic method developed in this laboratory (Ciszewska *et al.*, 2009).

Most peptides of those three series showed very high potency both in the guinea-pig ileum (GPI) and mouse vas deferens (MVD) assays, which indicated a need to modify their structure to obtain more selective opiates. Addition of an address sequence of deltorphin (-Val-Val-Gly-NH$_2$) to the cyclic structure comprising positions 1–4 resulted in greatly increased δ selectivity. Several such peptides were a hundred times more active in the MVD assay than in the GPI assay (Zieleniak *et al.*, 2008).

In this work we obtained six hybrid peptides containing a message sequence of enkephalin, restricted *via* a urea bridge as described above, and a C-terminal address of deltorphin. To facilitate the synthesis, the peptides were obtained in the form of *N*-(ureidoethyl)amides.

MATERIALS AND METHODS

Peptide synthesis. *General procedure.* The *p*-nitrophenoxycarbonyl derivative of Boc-diaminoethane was obtained as described earlier (Wiszniewska *et al.*, 2005). This compound (0.401 g, 1.25 mmol) was coupled to the MBHA resin (0.25 meq/g, 1% crosslink, 100–200 mesh) in DMF at 60 °C for 48 days. The Boc group was removed by treatment with 15% HCl/dioxane, then Boc- amino acids were successively attached using *N,N*′-diisopropylcarbodiimide as a coupling reagent and 15% HCl/dioxane for deprotection. The side chain amino function was protected by Fmoc group. The Tyr side chain was not protected. The peptidyl-resin was treated with 55% piperidine in DMF with stirring for 50 min to remove the Fmoc groups and washed with DMF. To a stirred suspension of peptidyl-resin in DMF (200 ml) *bis*(*p*-nitrophenyl)carbonate (0.76 g, 0.25 mmol) was added in portions (about 50% + 25% + 12.5% + 12.5%) and stirring was continued for 7 days. DIPEA (0.065 g) was gradually added during this reaction. The peptide was cleaved off by treatment with 55% TFA/DCM for 1 + 20 min. TFA was evaporated under reduced pressure and the residue was lyophilized. The yield of the crude product was 80–140 mg. The products were purified by semi-preparative reversed-phase high performance liquid chromatography using the solvent system: A: 0.05% TFA in water, B: 60% MeCN in A on a Vydac column (Nucleosil 300, C$_{18}$, 5 μm, 10 × 250 mm), flow rate of 2 ml/min, gradient B (15% in 15 min, 15–30% in 15 min, 30–45% in 40 min). Fractions were analyzed on a Vertex Nucleosil-100 C$_{18}$ column (4 mm × 250 mm, 5 μm), flow rate 1 ml/min. For analysis, a linear gradient of 20–80% B was used (1 ml/min, t = 15 min), detection at 220 nm. Fractions were pooled for maximum purity rather than yield. Homogeneous fractions containing one peak were combined and lyophilized. Structures were confirmed by ESI-MS mass spectrometry (Finningan MAT 95S spectrometer, Bremen, Germany). **1**: M calcd. 965.5, obtained 988.6 (M+Na$^+$); **2**: M calcld. 979.5, obtained 1002.6

(M+Na$^+$); **3**: M calcld 993.4, obtained 1016.6 (M + Na$^+$); **4**: M calcld. 951.3, obtained 974.5 (M+Na$^+$); **5**: M calcld. 965.5, obtained 988.6 (M+Na$^+$); **6**: M calcd. 993.4 obtained 1016.4 (M+Na$^+$). The structures of the peptides are presented in Fig. 1.

Bioassays. The guinea-pig ileum (GPI) assay (μ receptor-representative) (Paton, 1957) and the mouse vas deferens (MVD) assay (δ receptor-representative) (Henderson *et al.*, 1972) were carried out as reported in detail elsewhere (Schiller *et al.*, 1978; DiMaio & Schiller, 1980). A log dose-response curve was determined with [Leu^5]-enkephalin as a standard for each ileum and vas preparation and the IC$_{50}$ values of the compounds being tested were normalized according to a published procedure (Waterfield *et al.*, 1979). The results are presented in Table 1.

NMR spectroscopy and theoretical analysis. The methodology used for generation and selection of conformations was previously described for oxytocin and arginine-vasopressin (Liwo *et al.*, 1996). It was applied by us to one of the opioid peptides and described in details in this journal (Sidor *et al.*, 1999) and by others to a number of compounds (Cohen *et al.*, 2002; Masman *et al.*, 2006; 2008). Subsequently we used this methodology with success in several studies of different opioid analogs (Pawlak *et al.*, 2001; Filip *et al.*, 2003; 2005; Ciszewska *et al.*, 2009). In this paper NMR spectra of peptides **2, 3** and **4,** which were sufficiently soluble in water, were recorded on a Varian INOVA 400 MHz and/or a Varian Unity PLUS 500 MHz spectrometer at 25 °C in H$_2$O/D$_2$O (9:1, v/v). For 1D proton spectra 16k points were collected and a spectral width of 6 kHz was used. 2D experiments were measured collecting 2k points using 4.5 kHz spectral width in the proton direction and 256-point increments in the F1 direction. The same spectral width was applied in the F1 direction in homo-nuclear 2D spectra, whereas a width of 25 kHz and 2 kHz for carbon and for nitrogen, respectively, was used for hetero-nuclear correlations. A mixing time of 0.08 s was used in TOCSY measurements and 0.25 s in ROESY experiments. DSS was used as a reference external standard for proton, carbon and nitrogen dimensions (Wishart *et al.*, 1995). Chemical shifts were assigned using TOCSY, COSY, HSQC [^{15}N, ^{1}H], and HSQC [^{13}C, ^{1}H] techniques (Aue *et al.*, 1976; Braunschweiler & Ernst, 1983; Bothner-By *et al.*, 1984; Bax & Davis, 1985; Bax & Freeman, 1985; Palmer III *et. al.*, 1991; Kay *et al.*, 1992) and they are available in Supplementary Materials at www.actabp.pl (Table 1S and 2S). $^3J_{H\alpha HN}$ couplings were obtained from 1D ^{1}H spectra and temperature coefficients were determined with 1D ^{1}H spectra recorded at: 6.8, 11.0, 15.5, 19.1 and 25.0 °C (see Table 3S in Supplementary Materials). Cross-peak volume calculations were done with the SPARKY program (www.cgl.ucsf.edu/home/sparky) using data from the ROESY spectra and are available in Supplementary Materials (Tables 4S, 5S and 6S).

Structures of each peptide were calculated using the EDMC method described by (Liwo *et al.*, 1996), e.g., for each peptide starting from a conformation with random geometry its energy was minimized with the ECEPP/3 force field (Nemethy *et al.*, 1992) and surface model SFROPT (Vila *et al.*, 1991). Thus, the total conformational energy included a sum of terms: electrostatic, nonbonding, hydrogen-bond, torsional and terms accounting for the entropy for loop closing and solvation. The ϕ and ψ angles of the resulting geometry were further perturbed using the Monte Carlo method (Li & Scheraga,

1987) and after energy minimization the obtained conformation was compared with the previous one and accepted or discarded using a geometry and/or energy criterion. If the conformation was accepted the procedure was repeated.

The ensemble of resulting structures for each peptide was clustered into families using the program CLUST (Spath, 1980). Finally, theoretical NOESY spectra were generated for each family of structures with the program MORASS (Meadows, 1994) and statistical weights for each conformation were obtained by fitting a linear combination of generated spectra to the experimental data using the Marquardt method (Marquardt, 1963).

RESULTS AND DISCUSSION

The synthesis was performed using a method based on the observation that *N*-ureidoethyl units could be obtained in the synthesis on MBHA resin by reaction of the resin with *p*-nitrophenyl carbonate of Boc-ethylenediamine and that the aminoethylurea unit formed was stable during treatment with HCl/dioxane used for removal of the Boc group (Wiszniewska *et al.*, 2005). D-Lys or D-Orn were incorporated in position 2, and Lys, Orn, Dab or Dap in position 5 of the peptide sequence using Boc-protected derivatives in the α-amino group and Fmoc protection at the side chain amino group. After removal of the Fmoc group by treatment with 55% piperidine in DMF and cyclization in a reaction with *bis*-(4-nitrophenyl)carbonate the resulting peptide was cleaved from the resin by treatment with 45% TFA in DCM. The products were purified using semi-preparative RP-HPLC. Higher amounts of side products in crude products were observed than for similar syntheses in which cyclization was performed in solution (Pawlak *et al.*, 1997; Filip *et al.*, 2003). The increased amount of side-products may be due to formation of a carbonyl bridge between two peptide chains. In cyclization in solution, this problem was overcome by strong dilution of the reaction mixture. Fortunately, using semi-preparative HPLC, we were able to isolate the desired products. All samples for biological studies and NMR measurements were free of any contamination, as judged from analytical HPLC and MS spectra. The chemical formulas of the peptides are presented in Fig. 1. *In vitro* opioid activity profiles of the peptides were determined using the GPI and MVD assays (Table 1). The peptides were found to be agonists in both assays. The activities of the peptides varied depending on the size of the ring. The most active was peptide **1** in which an 18-membered ring was
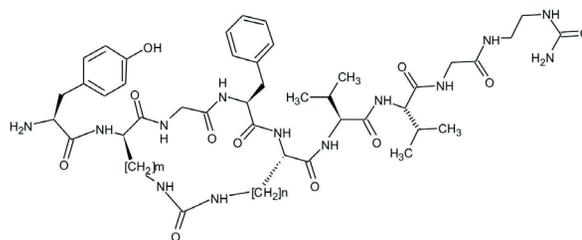
Figure 1. Structural formula of peptides:
1 (D-Lys[2], Dap[5]; m = 4, n = 1); **2** (D-Lys[2], Dab[5]; m = 4, n = 2); **3** (D-Lys[2], Orn[5]; m = 4, n = 3); **4** (D-Orn[2], Dap[5]; m = 3, n = 1); **5** (D-Orn[2], Dab[5]; m = 3,n = 2); **6** (D-Orn[2], Lys[5]; m = 3, n = 4)

formed by incorporation of side-chain amino groups of D-Lys and Dap. It should be recalled that among amides of cyclic enkephalin the most active peptide contained the same ring (Pawlak *et al.*, 2001). It can be seen that the activity of the peptides is high in the MVD assay and relatively lower in the GPI assay.

Since peptide **1** is the most δ-selective agonist in this series and deserves to be studied *in vivo*, it is reasonable to compare (see Table 2) the selectivity of all peptides studied earlier containing the cyclic structure formed by incorporation of side chain amino groups of D-Lys and L-Dap: the GPI/MVD ratios of amide of cyclo[*N*ε,*N*δ-carbonyl-D-Lys[2],Dap[5]]enkephalin and *N*-(ureidoethyl) amide of cyclo[*N*ε,*N*δ-carbonyl-D-Lys[2],Dap[5]]enkephalin were 0.33 (Pawlak *et al.*, 1997) and 1.09 (Ciszewska *et al.*, 2009), respectively, and in the case of the hybrid peptide **1** it was 6.97. This shift in selectivity in favor of δ receptors seems to be important in view of our recent finding (Kotlińska *et al.*, 2009) that even amide of cyclo[*N*ε,*N*δ-carbonyl-D-Lys[2],Dap[5]]enkephalin, which is highly potent both in GPI and MVD assays, *in vivo* studies, with the use of selective opioid receptor antagonists, induced antinociception predominantly through opioid δ receptors.

These results clearly indicate that the increase of selectivity in favor of the δ receptor was achieved by a decrease in the affinity of these peptides for the μ receptor, whereas the δ receptor affinity was more or less unchanged. These results show that in this case the role of the *address* is different from what was expected on the basis of the *message-address* concept, insofar as addition of the *address* sequence did not increase the affinity for the δ receptor.

In our previous studies on deltorphin analogs we observed that elongation of the cyclic N-terminal segment (Filip *et al.*, 2003; Zieleniak *et al.*, 2008) with Val-Val-Gly,

Table 1. GPI and MVD assays of cyclic hybrid peptides

PEPTIDE				GPI		MVD		GPI/MVD
No.	Ring size	D-Daa[2]	Daa[5]	IC$_{50}$ [nM]	Rel. potency	IC$_{50}$ [nM]a	Rel. potency	IC$_{50}$ ratio
1	18	Lys	Dap	12.9 ± 1	19.0 ± 2	1.85 ± 0.1	6.15 ± 0.45	6.97
2	19	Lys	Dab	47.5 ± 5	5.18 ± 0.5	16.9 ± 0.5	0.675 ± 0.019	2.81
3	20	Lys	Orn	30.8 ± 1	7.98 ± 0.4	4.18 ± 5	2.73 ± 0.034	7.37
4	17	Orn	Dap	76.9 ± 3	3.20 ± 0.1	18.5 ± 2	0.617 ± 0.058	4.16
5	18	Orn	Dab	72.1 ± 6	3.41 ± 0.3	20.5 ± 4	0.555 ± 0.109	3.52
6	20	Orn	Lys	71.3 ± 3	3.45 ± 0.2	14.5 ± 1	0.788 ± 0.078	4.92
[Leu5]enkephalin				246 ± 39	1	11.4 ± 1.1	1	21.6

[a]Mean of 3–6 determinations ± S.E.M.

Table 2. GPI/MVD ratio of enkephalin *N*-(ureidoethyl)amides (Ciszewska *et al.*, 2009) and enkephalin/deltorphin hybrid *N*-(ureidoethyl)amides (this work)

Amino acids in positions 2 and 4	Enkephalin ureido-ethylamides	Enkephalin/deltorphin hybrid ureidoethyl-amides
D-Lys, Dap	1.97/1.81 (= 1.09)	12.9/1.85 (= 6.97)
D-Lys, Dab	5.55/6.00 (= 0.92)	47.5/16.9 (= 2.81)
D-Lys, Orn	18.1/22.7 (= 0.79)	30.8/4.18 (= 7.36)
D-Orn, Dap	11.3/14.1 (= 0.80)	76.9/18.5 (= 4.16)
D-Orn, Dab	3.36/11.6 (= 0.29)	72.1/20.5 (= 3.53)
D-Orn, Lys	16.1/28.2 (= 0.57)	71.3/14.5 (= 4.92)
Mean value	9.40/14.1 (= 0.64)	51.9/12.6 (= 4.12)

to obtain the full sequence of deltorphin, also resulted in an increase in selectivity in favor of the δ receptor. This was not only due to a decrease in activity at the μ receptor but also to an increase of the activity at the δ receptor.

It seems that at least in the cases presented in this work, the use of the term *modulator* rather than the commonly used term *address* may be more appropriate for the description of a peptide segment which upon addition to a biologically active peptide changes the receptor selectivity.

For a better description and understanding of the biological results at the molecular level the NMR/EDMC method was employed (Sidor *et al.*, 1999). Temperature coefficients of amide protons obtained from NMR spectra of peptides **2**, **3** and **4** are rather high (Table 3S). Relatively lower values were observed only for amide protons of the Daa[5] residues, similar to what was observed for other opioid peptide analogs (Ciszewska *et al.*, 2009; Zieleniak *et al.*, 2008). This suggests that the amide protons are not engaged in intramolecular hydrogen bonding.

The ensembles of conformations generated and accepted in the EDMC procedure for each peptide are listed in Table 7S in Supplementary Materials. They were clustered into families using an energy threshold of 20 kcal/mol and an r.m.s.d. of 0.15 Å as separation criteria. Numbers of the families are also given in Table 7S. For representatives of each family, NOESY spectra were generated assuming a mixing time of 0.2 s and applying a correlation time of 0.45 ns. The Marquardt convergence criterion value of 10^{-5} was used in calculations of statistical weights of conformations. The conformers obtained for peptides **2**, **3** and **4** using the EDMC/NMR method are presented as their VMD (Humphrey *et al.*, 1996) drawings in Fig. 2 and corresponding selected torsional angles are listed in Table 3.

Inspection of the data presented in Table 3 reveals a large diversity of conformations of the peptides. Their geometries and populations differ significantly. In each case several well populated conformers with substantially varying distances between the aromatic rings of Phe[1] and Tyr[4] were calculated.

A comparison of the r.m.s.d. values calculated for conformers of these peptides and those obtained earlier for their shorter analogs (Ciszewska *et al.*, 2009) indicates that the flexibility of the main rings is similar. The r.m.s.d. values were calculated using the carbon and nitrogen atoms of the main ring for **2**, **3** and **4**,
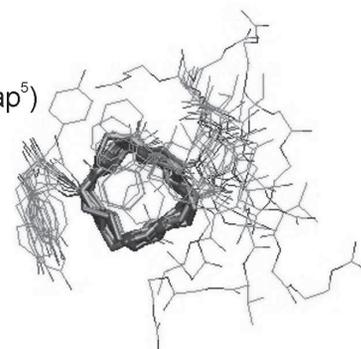
2 (D-Lys[2],Dab[5])
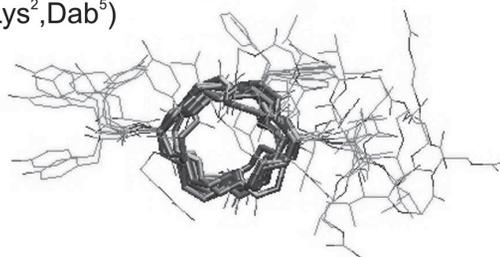
3 (D-Lys[2],Orn[5])
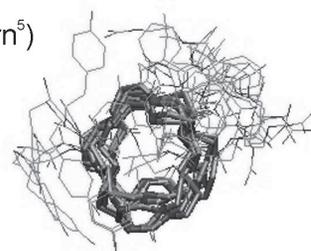
4 (D-Orn[2],Dap[5])

Figure 2. VMD drawings (Humphrey *et al.*, 1996) of the most populated (above 3%) EDMC/NMR calculated conformations of peptides 2, 3 and 4
The structures are superimposed using C and N atoms of the main ring

yielding values of 0.68 Å, 0.80 Å and 0.46 Å, respectively, while the r.m.s.d. values reported for the shorter analogs were 0.75 Å , 0.74 Å and 0.51 Å, respectively. The r.m.s.d. values calculated after superposition in the peptide segment situated between the Tyr and Phe residues were 0.40 for **2**, 0.45 for **3** and 0.27 Å for **4**, which suggests decreased conformational diversity as compared with the whole ring. This propensity of the main ring is common for all types of the peptides analyzed by us earlier (Pawlak *et al.*, 2001; Filip *et al.*, 2005; Ciszewska *et al.*, 2009).

The r.m.s.d. values calculated using all heavy atoms of all residues were 3.73 for **2**, 2.73 for **3** and 2.83 Å for **4**, which indicates a rather high conformational freedom of these peptides. This opens the possibility for additional intramolecular interactions. In fact, for all three peptides NOE contacts were observed between the ring protons of Phe[4] and the CH_3 groups of Val[6]. In addition, visual inspection of the VMD drawings of all calculated conformers shown in Fig. 2 reveals that the conformers of peptide **3** are relatively compact. This is in agreement with the NOE contacts between γCH[3] (Val[7]) and ring (Tyr[1]) protons observed in the spectra of this peptide. It may also be noticed that for a large set of conformers of peptide **4** their C-

Table 3. Parameters for the most populated (populations above 3%) conformations of peptides 2, 3 and 4 found in water

	$\chi_1(1)^a$	$\psi(1)^a$	$\varphi(2)^a$	$\psi(2)^a$	$\varphi(3)^a$	$\psi(3)^a$	$\varphi(4)^a$	$\chi_1(4)^a$	r^b	E^c	pop^d
2 (D-Lys², Dab⁵)											
2-1	−67	−39	148	−135	158	−163	−99	−61	12.6	0.0	22.4
2-2	−64	151	151	−133	136	−112	−153	172	17.4	−0.5	19.7
2-3	170	−58	148	−136	173	172	−86	−57	7.5	−0.7	14.3
2-4	−66	−36	148	−132	136	−113	−152	174	16.7	0.7	9.1
2-5	171	−51	135	−88	−76	72	−162	166	7.2	1.1	6.0
2-6	−59	12	96	−133	103	−34	−62	177	15.3	0.7	5.4
2-7	176	−46	150	−137	162	−97	−152	175	13.4	−0.2	4.8
2-8	−65	153	151	−134	126	−108	−155	171	17.1	−0.4	4.6
2-9	175	−46	150	−141	117	−172	−87	−178	14.3	0.9	3.1
											Σ=89.4
3 (D-Lys², Orn⁵)											
3-1	−174	154	74	47	−161	49	−102	−50	5.4	0.0	19.2
3-2	178	140	152	−140	−174	−153	−61	178	15.2	−2.6	14.1
3-3	177	115	153	−168	161	−146	−62	176	14.6	−1.7	13.0
3-4	−178	158	86	34	−124	−58	−93	−60	9.9	2.1	12.6
3-5	−170	149	82	−78	−62	−40	−68	179	9.2	3.5	11.2
3-6	−172	−29	78	27	−165	41	−73	−173	9.1	−0.3	7.1
3-7	−172	147	81	−75	−76	59	−177	165	7.9	−1.1	5.5
3-8	179	136	78	−147	81	−77	−55	175	12.5	−1.2	4.9
											Σ=87.6
4 (D-Orn², Dap⁵)											
4-1	−176	155	78	39	−160	46	−142	−60	7.7	0.0	16.1
4-2	−176	156	79	34	−139	81	−172	165	9.5	1.5	15.6
4-3	−174	146	73	32	−75	−32	−157	53	9.8	2.0	9.3
4-4	−174	158	86	41	−149	53	−140	−53	6.2	1.3	8.1
4-5	−175	155	76	40	−159	49	−141	40	5.4	1.2	8.1
4-6	−176	154	71	47	−157	45	−148	41	5.6	−0.2	8.1
4-7	−177	152	75	−162	87	−63	−56	174	11.7	0.6	6.8
4-8	−175	154	70	44	−152	45	−145	41	5.5	0.9	6.8
4-9	178	152	86	−133	114	−41	−62	178	12.0	2.9	5.9
4-10	−176	142	77	−152	111	−58	−64	−61	10.3	1.9	4.5
4-11	177	−44	74	37	−147	32	−98	−56	5.3	2.5	4.5
4-12	−176	153	74	54	−178	−38	−53	66	5.7	0.3	4.3
											Σ=90.0

[a]Values of selected torsional angles for the Tyr-Phe "spacer"; [b]distance between tyrosine and phenylalanine ring centers (r in Å); [c]relative calculated energy (E in kcal/mol); [d]relative populations of conformers (pop in %)

terminal segments are directed towards their main ring. These close contacts indicate the presence of additional intramolecular interactions involving aromatic rings and in consequence may have an impact on the possibility of interactions between these rings.

In spite of the differences in distances between the aromatic rings it seems reasonable to assume that these conformations are flexible enough to adopt a conformation permitting interaction with the opioid receptor to induce a biological effect. The similar results for enkephalin-deltorphin hybrids and corresponding enkephalins in the MVD assay support this suggestion.

CONCLUSIONS

The synthesis of N-(ureidoethyl)amides of cyclic enkephalin-deltorphin hybrids was carried out using the method described earlier (Ciszewska et al., 2009). The peptides differed from cyclic enkephalin N-(ureidoethyl)

amides reported earlier in that the amino acid chain was elongated C-terminally with the Val-Val-Gly sequence, the address sequence of deltorphin responsible for its high affinity for δ receptors (Charpentier et al., 1991).

It should be noted that the increased selectivity for δ receptors was mainly result of decreased activity for μ receptors. The activity in the MVD assay of N-ureidoethylamides of cyclic enkephalins and enkephalin-deltorphin hybrids was similar. Since, in most cases, increased selectivity towards δ receptors was obtained as a result of a decrease of the affinity for μ receptors, but not an increase towards δ receptors, it would be reasonable to use the term *modulator* instead of *address* for a sequence that changes the receptor selectivity ratio.

Statistical weights of conformations for three of the six peptides sufficiently soluble in water to permit the recording of NMR spectra were obtained in a global conformational search using the EDMC method in combination with experimental NOE parameters. NOE contacts for all three peptides between their ring protons of Phe⁴ and the CH_3 groups of Val⁶ and for peptide **3** between its ring protons of Tyr¹ and the γCH_3 group of Val⁷ were observed. This finding indicates that a sequence added to a *message*, or to a sequence containing the *message*, is able to introduce additional intra-molecular interactions between the *message* and the rest of the molecule. In the present studies the additional peptide chain interacting with the *message* restricted by ring formation had no effect on the results of the MVD assay.

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