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Deltorphin analogs restricted via a urea bridge: structure and opioid activity‡

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Abstract: Eight cyclic heptapeptides related to the full sequence of deltorphin have been synthesized. The synthesis of linear peptides containing diamino acid residues in positions 2 and 4 was carried out on a 4-methylbenzhydrylamine resin. Depending on protection procedures, the N-protected peptide-resins or N-protected peptide amides with free amino groups in the side chains were obtained, which were subsequently treated with bis-(4-nitrophenyl)carbonate to form a urea unit. Opioid activities of the peptides were determined in the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays. Several compounds showed high δ opioid agonist potency and high selectivity for δ receptors. The results were compared with those obtained earlier for respective 1–4 deltorphin analogs. The conformations of these peptides have been studied using 2D-NMR in H_2O/D_2O and molecular dynamics. We observed that the backbone rings had well defined conformations, while the Tyr and Phe side chains and the *C*-terminal tail had significant conformational freedom. The bioassay data and conformational parameters of these peptides were compared with those of previously described, corresponding 1–4 deltorphin analogs. This comparison permitted an assessment of the role of the *C*-terminal peptide segment in defining the conformation and receptor interaction of the *N*-terminal portion and provided insight into the relationship between the putative bioactive conformations and bioactivity. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

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

INTRODUCTION

The deltorphins, Tyr-D-Ala-Phe-(Asp/Glu)-Val-Val-Gly-NH_2, are the most selective naturally occurring opioid agonists for δ -receptors. These peptides were isolated from the skin of the South American frog *Phyllomedusa bicolor* [1]. Another amphibian opioid peptide, dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), and its variants were found in the skin of frogs belonging to the family *Phyllomedusa.* Dermorphins bind preferably to μ opioid receptors [2]. Since these two peptide families contain a common 1–3 *N*-terminal sequence, the receptor specificity is due to the different *C*-terminal sequences.

In our previous papers, we described the synthesis, biological activity and conformation of several highly potent side-chain to side-chain cyclized 1–4 dermorphin/deltorphin analogs containing a carbonyl bridge, which links the two side-chain amino groups to form an ureido moiety [3,4]. Also, we recently described a series of dermorphin analogs, in which the cyclic structure was elongated with the native sequence of dermorphin [5]. The elongation resulted in a decrease in the activity in the guinea pig ileum (GPI) assay and, to a lesser extent, in the mouse vas deference (MVD) assay. NMR studies revealed that addition of the *C*-terminal sequence influenced the conformation of the *N*-terminal fragment, known to be mainly responsible for the interaction with the receptors.

In this work we designed and synthesized eight new deltorphin analogs which contain an *N*-terminal cyclic structure, as in peptides described earlier, and the *C*-terminal sequence of native deltorphin. The aim of this work was two-fold: firstly, to shift the selectivity in favor of the δ receptor; secondly, to understand the molecular basis of the impact of the address part of the peptide on the activity and selectivity. We determined their opioid activity using the GPI and MVD assays, and their conformations using 2D-NMR in H_2O/D_2O and molecular dynamics simulations. An attempt was made to correlate structures and biological activities.

METHODS

Synthesis of Peptides

The linear protected peptides were prepared on a 4-methylbenzhydrylamine resin according to standard procedures. α-Amino functions were Boc protected

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‡ This paper is dedicated to the memory of Professor Miklos Bodanszky.

except for Fmoc-Tyr in synthesis *a*, and the side chains were blocked with the following groups: Tyr with *t*-But; D-Daa, Daa, Z(2Cl) in synthesis *a* (peptides **1–8**); in addition, in synthesis *b* peptides **3**,**4**,**5** and **6** were obtained with the use of Tyr with Z(2Br); D-Daa, Daa with Fmoc. In synthesis *a*, the linear precursors Fmoc-D-Daa-Phe-Daa-Val-Val-Gly-NH_2 were cyclized by reaction of bis(4-nitrophenyl)carbonate with the free amino groups of the peptide in DMF as described earlier [3]. In synthesis *b* the carbonyl bridge was preformed on the resin. The terminal α-amino group was protected with the Boc-group and the side-chain amino functions of D-Daa[2] and Daa[4] with the Fmoc group. The side-chain amino groups were deprotected by treatment with 55% piperidine in DMF. Cyclization on the resin was performed with bis(4-nitrophenyl)carbonate (1 equiv) in DMF and DIEA (2 equiv). The carbonyl bridge formation was usually complete after 5–7 days. The Boc protecting group of the tyrosine α-amino group was removed prior to cleavage with HF. The cyclic peptides were cleaved from the resin by treatment with liquid HF in the presence of anisole (10%) for 1 h at 0 °C. The HF was removed under reduced pressure, and the residue was treated with cold Et_2O, extracted with 50% acetic acid and lyophilized. In general, better results were obtained with the use of method *a*.

The cyclic analogs were purified to homogeneity by semipreparative reversed-phase (RP)-HPLC on column, using the following solvent system: A = 0.05% TFA in water, B = 60% MeCN in A. The purity of the products was assessed by HPLC, using a Vertex column Nucleosil-100 C_{18} (4 × 250 mm, 5 μm), at a flow rate of 1 ml/min. and detection at 220 nm. Homogeneous fractions containing one peak were combined and lyophilized. Structures were confirmed by ESI-MS spectra (a Finningan MAT 95S spectrometer, Bermen, Germany). **1**: $C_{43}H_{64}O_9N_{10}$, M calcd 865,04, obtained $(M+Na)^+$ 887,6; **2**: $C_{42}H_{62}O_9N_{10}$, M calcd 851,01, obtained $(M+Na)^+$ 873,6; **3**: $C_{41}H_{60}O_9N_{10}$, M calcd 836,0, obtained $(M+H)^+$ 837,6, $(M+Na)^+$ 859,7; **4**: $C_{40}H_{58}O_9N_{10}$, M calcd 821,95, obtained $(M+Na)^+$ 845,5; **5**: $C_{42}H_{62}O_9N_{10}$, M calcd 851,01, obtained $(M+Na)^+$ 873,7; **6**: $C_{41}H_{60}O_9N_{10}$, M calcd 836,98, obtained $(M+Na)^+$ 859,5; **7**: $C_{40}H_{58}O_9N_{10}$, M calcd 822,10, obtained $(M+Na)^+$ 845.7; **8**: $C_{39}H_{56}O_9N_{10}$, M calcd 807,90, obtained $(M+Na)^+$ 831,7. The structures of the peptides are presented on Figure 1.

Bioassays

The GPI [6] and MVD [7] bioassays were carried out as reported in detail elsewhere [8,9]. A log dose-response curve was determined with [Leu5]-enkephalin as standard for each GPI and MVD preparation, and the IC50 values of the compounds being tested were normalized according to a published procedure [10]. The results are presented in Table 1.

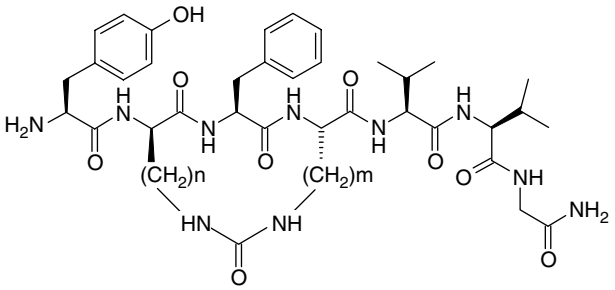

Figure 1 Deltorphin Analogs: **1,** {[H-Tyr-D-Lys(&[1])-Phe-Lys(&[2])-Val-Val-Gly-NH_2][&[1]CO&[2]]}; **2,** {[H-Tyr-D-Lys(&[1])-Phe-Orn(&[2])-Val-Val-Gly-NH_2][&[1]CO&[2]]}; **3,**{[H-Tyr-D-Lys(&[1])-Phe-Dab(&[2])-Val-Val-Gly-NH_2][&[1]CO&[2]]}; **4**, {[H-Tyr-D-Lys(&[1])-Phe-Dap(&[2])-Val-Val-Gly-NH_2][&[1]CO&[2]]}; **5**, {[H-Tyr-D-Orn(&[1])-Phe-Lys(&[2])-Val-Val-Gly-NH_2][&[1]CO&[2]]}; **6,** {[H-Tyr-D-Orn(&[1])-Phe-Orn(&[2])-Val-Val-Gly-NH_2][&[1]CO&[2]]}; **7,** {[H-Tyr-D-Orn(&[1])-Phe-Dab(&[2])-Val-Val-Gly-NH_2][&[1]CO&[2]]}; **8,** {[H-Tyr-D-Orn(&[1])-Phe-Dap(&[2])-Val-Val-Gly-NH_2][&[1]CO&[2]]}.

Table 1 GPI and MVD assay of cyclo(N^{ω}, N^{ω}-carbonyl-D-Daa[2], Daa[4])deltorphin(1–7)-NH_2 analogs

Compound				GPI		MVD		GPI/MVD
No	Ring size	Daa[2]	Daa[4]	IC_{50} (nM)[a]	Rel. potency	IC_{50} (nM)[a]	Rel. potency	IC_{50} ratio
1	18	Lys	Lys	888 ± 99	0.277 ± 0.031	11.8 ± 1.6	0.966 ± 0.131	75.3
2	17	Lys	Orn	>10 000	<0.0246	67.0 ± 6.9	0.168 ± 0.017	>149
3	16	Lys	Dab	65.4 ± 9.6	3.76 ± 0.55	0.640 ± 0.043	17.8 ± 1.2	102
4	15	Lys	Dap	25.4 ± 2.0	9.69 ± 0.76	0.483 ± 0.065	23.6 ± 3.2	52.6
5	17	Orn	Lys	1020 ± 250	0.240 ± 0.058	2.73 ± 0.028	4.18 ± 0.43	374
6	16	Orn	Orn	159 ± 23	1.55 ± 0.22	0.814 ± 0.054	14.0 ± 0.93	88
7	15	Orn	Dab	460 ± 9	0.535 ± 0.010	106 ± 7	0.108 ± 0.007	4.34
8	14	Orn	Dap	>10 000	<0.0246	27.1 ± 3.1	0.421 ± 0.048	>369
[Leu[5]]enkephalin				246 ± 39	1	11.4 ± 1.1	1	21.4

[a] Mean of 3–6 determinations ± SEM.

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NMR Experiments

NMR spectra of deltorphin analogs were recorded on the Varian Unity 500 Plus spectrometer operating at 500 MHz resonance frequency. The proton chemical shifts were referenced to the H_2O frequency measured with respect to internal sodium 3-trimethylsilyltetradeuteriumpropionate (TSP). The following spectra were recorded: 1D (at 10, 20, 30, 40, 50 °C) and 2D: DQF-COSY, TOCSY (80 ms) (Figure 2), NOESY (100 ms) and ROESY (200 ms) (Figure 3) at 32 °C. The samples were dissolved in H_2O/D_2O (9v : 1v) (**3** and **7**) or in H_2O/D_2O (9v : 1v) with addition of CH_3COOH (**1, 2, 5** and **6**). Analogs **4** and **8** were not sufficiently soluble in any solvent and NMR spectra of these analogs could not be collected. The concentration of the investigated peptides was between 3 and 9 mM. All NMR data were processed and cross-peak volume calculations were done using the XEASY program [11]. $^3J_{HN\alpha H}$ coupling constants were obtained from the DQF-COSY spectra.

Parameterization

Eight new moieties were absent in the original AMBER force field. They were parameterized in the present study according to the recommendations in the AMBER 8.0 manual [12]. Molecular orbital calculations for four conformations per each residue at the *ab initio* RHF/6-31G* level using the GAMESS molecular orbital program package [13] and subsequent conformational-averaging of the charges over the residue using the R.E.D.I I. procedure [14] were carried out. All bond angle parameters were obtained by analysis of a crystallographic database [15]. The data for the charges over the residue are available on request from SR-M (sylwia@chem.univ.gda.pl).

Molecular Dynamics Simulations With/Without NMR-Derived Constraints

The structures of **1–3** and **5–7** were refined using 5.0 ns productive MD runs, utilizing the Time-Averaged

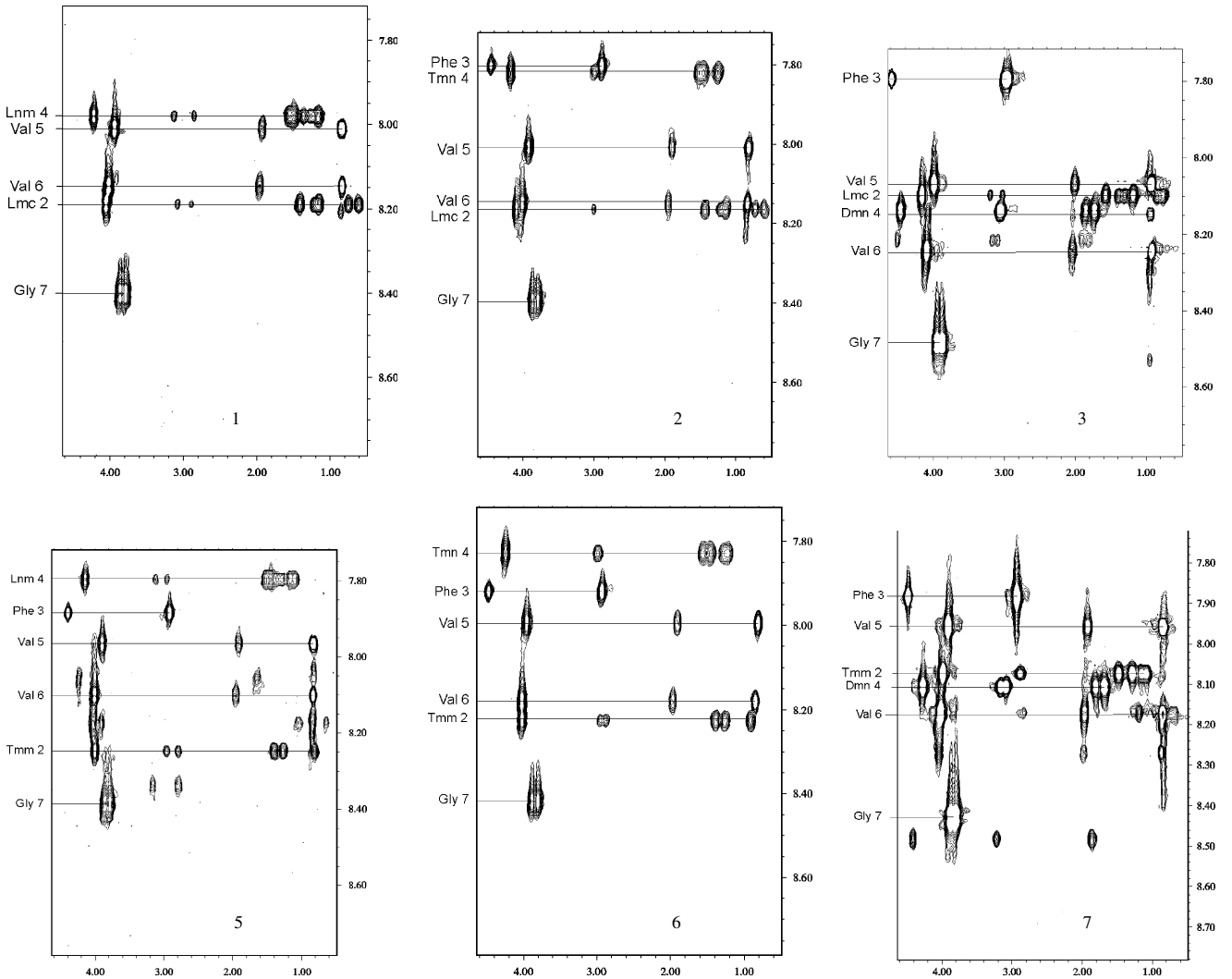

Figure 2 Amino acid spin systems on TOCSY spectrum (diagnostic region) of **1–3** and **5–7** analogs.

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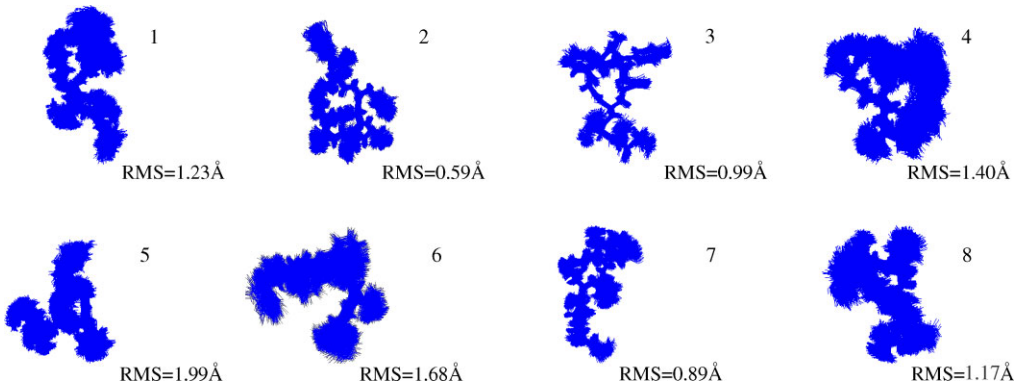

Figure 3 Clusters of representative MD-TAV derived conformers for **1**–**8**. Carbon atoms were superposed. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

Restraints procedure (TAV-MD) and interproton distances calculated from the ROE intensities and torsion angle values determined from vicinal couplings, dedicated to structure determinations from NMR of small flexible peptides [16]. MD simulations were carried out using the AMBER, ver. 8.0 software [12]. The system was initially minimized for 2000 steps without any constraints. Thereafter, the system was subjected to MD under constant pressure without restraints (20 ps) and then under constant volume and for 5 ns using time-averaged distance and dihedral angle restraints derived from the NMR data. The interproton distances were restrained with the force constants $f = 20$ kcal/(mol $\times$ Å^2), and the dihedral angles with $f = 2$ kcal/(mol $\times$ rad^2). The improper dihedral angles centered at C_α atoms were restrained with $f = 50$ kcal/(mol $\times$ rad^2). The geometry of the peptide groups was kept fixed according to NMR data (all *trans*) ($f = 50$ kcal/(mol $\times$ rad^2)). These runs comprised simulations in solution under periodic boundary conditions in the NVT ensemble. Throughout the simulations the solute and solvent were coupled to a constant-temperature ($T = 298$ K) heat bath. All hydrogen-containing bonds were constrained using the SHAKE algorithm, allowing time steps of 0.001 ps. Ewald summation [17] was used for electrostatic interactions with a cutoff of 9 Å. Coordinates were saved every 200 steps. Analogs **4** and **6** were submitted also to a 5.0 ns MD, nonrestrained by NMR data, with starting structures taken at random, under otherwise identical conditions as for the other compounds. Each set of conformations from the MD trajectories was clustered into 5 to 6 families of conformations. The results obtained were analyzed using the Carnal and Ptraj programs from the AMBER package [15]. Molecular structures were drawn with the graphics program MOLMOL [18].

RESULTS AND DISCUSSION

Protected linear peptides containing the full sequences of deltorphin and basic amino acid residues in positions 2 and 4 were synthesized by the solid-phase method. The cyclic peptides were obtained by using two different protocols. In the first one, the *t*-butyl protecting group of the hydroxyl group of tyrosine was removed, and the peptide was cleaved from the resin by treatment with liquid HF. Fmoc protection of the terminal α-amino group was retained and Z-deprotection of side-chain amino groups was achieved. The crude linear peptides were cyclized by reaction with bis(4-nitrophenyl)carbonate in DMF to form peptides containing a 14-, 15-, 16-, 17- or 18-membered ring structure. The Fmoc group was removed by treatment with piperidine and homogeneous peptides were obtained by preparative RP-HPLC. Using this method, peptides **1**–**8** were obtained.

In the second protocol, after removal of the Fmoc groups protecting the side-chain amino groups, the linear peptide was cyclized with bis(4-nitrophenyl)carbonate in DMF on the resin. Cleavage of the cyclic peptides from the solid support was performed by treatment with liquid HF. Using this protocol, we successfully obtained **3**, **4**, **5** and **6.** Molecular weights of peptides were determined by ES-MS. The structures of the peptides are depicted in Figure 1.

The *in vitro* opioid activity profiles were determined in the GPI and MVD assays. The results are presented in Table 1. All analogs showed high or very high potency in the MVD assay. The data indicate that the heptapeptides containing D-Lys and D-Orn and 14-, 15-, and 16-membered rings are the most potent analogs, as it was also the case with the shorter tetrapeptide counterparts. Analogs **3**, **4**, **5** and **6** showed higher agonist potency in the MVD assay than enkephalin. On the other hand, five of eight analogs were less active than enkephalin in the GPI assay. As a result, most of these peptides are selective δ -receptor agonists. The shift of specificity is caused by addition of the *C*-terminal portion of native deltorphin to cyclic peptides containing only the *N*-terminal portion, which themselves are agonists with dual affinity for both μ and δ -receptors. The ratios between the IC_{50}s of the

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1–4 dermorphin analogs and their 1–7 counterparts are presented in Table 2.

The NMR spectra indicated that **3**, **5** and **7** exist in a major and a minor conformation, in contrast to **1**, **2** and **6,** which showed only one conformationally averaged component, Figure 2. The existence of two sets of signals for **3**, **5** and **7** is not due to *cis-trans* isomerization around a peptide bond, since sequential assignments of the major species indicated all *trans* peptide bonds. Signal assignments for the minor species were not possible and there was a lack of NOEs for these forms. Therefore, the calculation of the 3D structures for minor conformers was not performed. The ^{1}H chemical shifts of the common amino acid residues in equivalent positions were found to be slightly different (in the range of 0.5 ppm) for each analog (Table 4), suggesting small differences between the 3D-structures of the investigated analogs. Moreover, the chemical shifts of some amino acids of these analogs were different from those observed for the same residues in the corresponding tetrapeptide (1–4)-analogs of deltorphin [3]. A comparison of the corresponding hepta- and tetrapeptide analogs, for which the data are available, is presented in Table 3. These results suggest that addition of the *C*-terminal portion can change the conformation of the *N*-terminal cyclic portion. This may influence opioid activity and receptor selectivity. The same observations had been made in our studies on dermorphin analogs [5]. The vicinal coupling constants of the analogs were in the range of 6–8 Hz (Table 4), indicating bent or unstructured conformations of the backbones. The $-\Delta\delta/\Delta$T NH proton coefficients (Table 4) indicated a low probability for the formation of stable intramolecular H-bonds involving amide backbone protons. Only the NH protons of residues 4 were engaged in hydrogen-bond formation. Similarly, low temperature coefficients were found for the amide proton of the Daa^4 residue in other dermorphin analogs [4], suggesting common features with the cyclic dermorphin(1–7) analogs containing the ureido group. In addition, the strongest hydrogen bonds were formed by NH protons in Phe^3 and Orn^4 of **6**, whose $-\Delta\delta/\Delta$T coefficients equal to 2.7 and 1.7 ppb, respectively. The coefficients determined for the short analogs [3] are indicative of fast exchange with the solvent, as they are 3 to 4 ppb higher then the corresponding coefficients for the longer peptides. This observation suggests that a substantial number of conformations of the heptapeptides may be engaged in intramolecular hydrogen bonding.

All calculated structures are shown in Figure 3. For **1**–**3** and **5**–**7** the NMR constraints were used. The structures of **4** and **8** were calculated with no NMR constraints. Therefore, these two peptides appear to be more flexible than the others (Figure 3), especially in the side-chain regions. Peptides **3**, **4**, **6** and **8** have compact structures. They form a β-bend in the *C*-terminal segment, which leads to a characteristic 'U'-like shaped structure. Peptides **1**, **2**, **5** and **7** are less compact with the *C*-terminus extended and directed away from the peptide ring structure. The values of the radii of gyration and total accessible surface areas (data not shown) are the smallest for **3**, **4** and **6**, which confirms their compact structure (see Figures 3 and 4, and Table 5).

It is worth mentioning that the U-shaped structures were also observed among the most active and selective cyclic agonists derived from the *N*-terminal

Table 2 Comparison of the opioid activities of 1–7 deltorphin and 1–4 [3,4] dermorphin analogs

Analogue	Amino acides in position 2 and 4	GPI 1–4/1–7 IC_{50} ratio	MVD 1–4/1–7 IC_{50} ratio
1	D-Lys, Lys	1.295	83.983
2	D-Lys, Orn	<0.002	0.70298
3	D-Lys, Dab	0.018	7.8437
4	D-Lys Dap	0.163	40.3727
5	D-Orn, Lys	0.012	5.8242
6	D-Orn, Orn	0.030	14.0049
7	D-Orn, Dab	0.003	0.0125
8	D-Orn, Dap	<0.0003	18.4323

Table 3 Dowfield shifts (in ppm) of proton chemical shifts in the NMR spectra of deltorphin analogs in D_2O (this work) in comparison with the corresponding proton shifts determined with deltorphin analogs 1–4 containing the same ring [4]

Main ring	D-Lys^2,Dab^4 **3**	D-Orn^2,Dab^4 **7**
Tyr^1		
H_α	−0.022	0.029
H_β	0.035; −0.071	0.051; 0.024
H_{26}; H_{34}	0.25; −0.293	0.302; −0.171
D-Daa^2		
H_α	0.001	0.081
H_β	−0.009; −0.389	0.1; −0.1
H_γ	−0.02; −0.01	0.106; 0.056
H_δ	0.193; −0.079	1.667
H_ε	−0.06; 0.05	na
Phe^3		
H_N	0.092	0.161
H_α	−0.007	0.011
H_β	0.107; −0.033	0.147; 0.037
H_{26};H_{35}	0.207; −0.061	0.231; −0.01
Daa^4		
H_N	0.227	0.319
H_α	−0.127	−0.054
H_β	0.085; 0.005	0.191; 0.019
H_γ	na	0.068; 0.081

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Table 4 ^{1}H chemical shifts [ppm], $^{3}J_{NH\alpha H}$ coupling constants and amide NH temperature coefficients (ppb/K) of the deltorphin analogs: **1**–**3** and **5**–**7**. The NH temperature coefficients for **2** were not measured

Peptide ►	**1**	**2**	**3**	**5**	**6**	**7**
Tyr^{1}						
H_α	4.100	4.082	4.152	4.079	4.094	4.101
H_β	3.163; 2.886	3.164; 2.884	3.195; 3.021	3.147; 2.902	3.173 2.914	2.916; 3.159
H_{26}	7.062	—	6.890	7.067	7.08	6.838
H_{35}	6.798	—	7.143	6.803	6.829	7.071
***D*-Daa^{2}** ►	*D*-Lys	*D*-Lys	*D*-Lys	*D*-Orn	*D*-Orn	*D*-Orn
H_N	8.191	8.162	8.102	8.248	8.226	8.089
H_α	4.051	4.824	4.159	4.001	4.014	3.989
H_β	1.223 1.193	1.440 1.138	1.589	1.401; 1.276	1.394 1.267	1.48
H_γ	0.787 0.617	0.733 0.602	0.86; 0.77	0.824	0.939 0.905	1.164; 1.084
H_δ	1.42 1.16	1.231 1.156	1.197; 1.399	2.466; 2.791	2.944	1.283
H_ε	3.071 2.897	3.001	3.24; 2.99	—	—	—
$^{3}J_{NH\alpha H}$	7.2	8.4	7.8	8.1	7.1	7.4
$-\Delta\delta x/\Delta T$	6.6	—	7.1	5.5	3.6	5.6
Phe^{3}	—	—	—	—	—	—
H_N	7.649	7.801	7.808	7.886	7.922	7.899
H_α	4.532	4.438	4.577	4.388	4.462	4.489
H_β	2.888	2.889	2.953	2.978	2.926	2.943
H_{26}	7.224	7.225	7.163	7.238	7.214	7.129
H_{35}	7.082	7.087	7.311	7.096	7.261	7.260
$^{3}J_{NH\alpha H}$	7.2	7.0	—	7.0	7.2	8.6
$-\Delta\delta x/\Delta T$	5.2	—	10.3	5.7	2.7	6.9
Daa^{4} ►	Lys	Orn	Dab	Lys	Orn	Dab
H_N	7.982	7.822	8.143	7.799	7.831	8.111
H_α	4.214	4.165	4.477	4.144	4.234	4.274
H_β	1.365; 1.269	1.532; 1.444	1.705; 1.855	1.545; 1.447	1.566; 1.464	1.809; 1.681
H_γ	1.17; 1.18	1.261; 1.237	3.046	1.175; 1.103	1.297; 1.230	3.172; 3.089
H_δ	1.577; 1.496	2.995; 2.888	—	1.368; 1.299	2.974; 2.876	—
H_ε	3.125; 2.856	—	—	3.118; 2.95	—	—
$^{3}J_{NH\alpha H}$	8.5	9.2	8.2	7.1	9.5	8.2
$-\Delta\delta x/\Delta T$	4.7	—	—	3.6	1.7	4.9
Val^{5}						
H_N	8.009	8.008	8.071	7.965	7.966	7.971
H_α	3.934	3.913	3.867	3.89	3.948	3.914
H_β	1.926	1.905	2.003	1.912	1.907	1.925
H_γ	0.854	0.825	0.94	0.853	0.818	0.872
$^{3}J_{NH\alpha H}$	7.2	9.1	8.0	8.2	8.3	6.5
$-\Delta\delta x/\Delta T$	6.9	—	—	6.6	5.2	6.8
Val^{6}						
H_N	8.148	8.147	8.248	8.101	8.181	8.194
H_α	4.016	3.999	4.076	4.01	4.017	4.011
H_β	1.96	1.953	2.042	1.955	1.969	1.969
H_γ	0.849	0.837	0.93	0.853	0.855	0.858
$^{3}J_{NH\alpha H}$	7.4	8.0	7.8	7.1	9.5	7.6
$-\Delta\delta x/\Delta T$	8.4	—	10.7	5.3	6.0	8.7
Gly^{7}						
H_N	8.399	8.392	8.44	8.387	8.418	8.445
H_α	3.856; 3.785	3.857 3.779	3.94 3.86	3.850; 3.776	3.876 3.795	3.875; 3.08
$\Sigma^{3}J_{NH\alpha H}$	11.8	9.3	10.2	10.8	9.5	11.1
$-\Delta\delta x/\Delta T$	8.6	—	9.0	5.9	3.6	8.8
NH_2	7.336; 6.946	6.946; 7.334	7.014; 7.418	7.328; 6.944	7.358; 6.973	7.349; 6.953

tetrapeptide segment of dermorphin/deltorphin, in which the heterodetic ring was formed either by a disulfide bond between D-Cys^{2} and Cys^{4}, or among their dicarba analogs (Rodziewicz, unpublished).

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Table 5 Structural features of deltorphin analogs

Analogue	Atoms (number) in the cyclic part	Distance (Å) between the aromatic rings	Backbone conformation[c]	Distance (Å) between nitrogen and the phenyl ring	Aromatic rings on the same side of cyclic ring
1	18	8.2	–	4.60	–
2	17	10.6	–	7.5[d]	–
3	16[a]	5.8[b]	+	7.92[d]	+
4	15[a]	6.5[b]	+	9.11[d]	+
5	17	9.9	–	10.9	–
6	16[a]	9.4	+	6.96	±[e]
7	15	8.3	–	9.1[d]	–
8	14	7.8	–	9.36[d]	–

[a] Number of atoms in the cyclic part equals to 15 or 16 in the most δ-active and/or δ-selective analogs **3**,**4** and **6**.
[b] Aromatic rings are closer than 7 Å to each other.
[c] Backbone conformation is 'U'-shaped and compact.
[d] Distance between the *N*-terminal nitrogen and the phenyl ring in the range of 8.2 ± 1.0 Å [19,20].
[e] Aromatic rings are situated in the plane of the peptide ring structure.

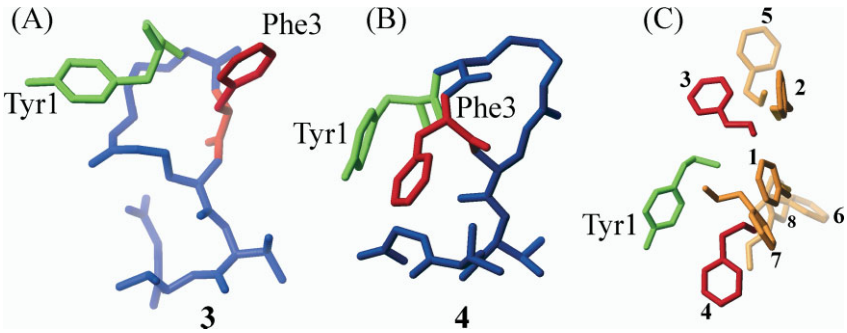

Figure 4 Conformations of (A) **3** and (B) **4** with Tyr^1 and Phe^3 aromatic rings exposed. (C) Distribution of the Tyr^1-Phe^3 orientation among the analogs 1–8 with Tyr^1 side chains fixed in a common place.

It is known that the distances between the two aromatic rings and between the *N*-terminal nitrogen and the phenyl ring are critical for opioid activity [19-22]. In addition Nikiforovich *et al.* [21,23] and Mierke *at al.* [24] suggested that the Tyr and Phe side chains in the bioactive conformation are in close proximity to each other on the same side of the heterodetic ring. Careful analysis of our structures (Table 5) shows that all these requirements are fulfilled by two of eight analogs, viz **3** and **4**.

Further examination of the calculated structures indicates the possibility of the existence of various types of hydrogen bonds. Such hydrogen bonds may include typical NH···OC bonds as well as NH···π type hydrogen bonds. In the peptides examined, the Daa^4 residue is involved in this type of hydrogen bonding (data not shown). For most of the analogs, the NH proton of the Daa^4 residue forms hydrogen bonds with the CO oxygen of Daa^2, stabilizing the β-turn structure in the heterodetic ring.

CONCLUSIONS

Previously, we described the highly active cyclic dermorphin/deltorphin analogs comprising the *N*-terminal portion of the two natural peptides, considered to be the *message* sequence. In this article, we report a series of analogs in which the *N*-terminal sequence (*message*) of the previously studied analogs was elongated with the native *C*-terminal segment of deltorphin (*address*). The resulting peptides were tested in the GPI and MVD assays. A dramatic increase in δ agonist potency was observed in the MVD assay as compared to the corresponding 1–4 analogs. The results of the NMR studies indicate that extension with the *C*-terminal segment results in changes in the ring conformation, as judged from changes of chemical shifts, suggesting that addition of the *address* segment not only changes receptor preference but also affects the receptor binding affinity of the message segment. Similar observations were made upon elongation of the cyclic 1–4 dermorphin/deltorphin analogs with the *C*-terminal dermorphin segment [5].

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