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Ureido group containing cyclic dermorphin(1–7) analogues: synthesis, biology and conformation

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Abstract: Six cyclic peptides related to dermorphin(1–7) 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, and cyclization was achieved by treatment with bis-(4-nitrophenyl)carbonate to form a urea unit. The peptides were tested in the guinea-pig ileum (GPI) and mouse vas deferens (MVD) assays. Diverse opioid agonist activities were observed, depending on the size of the ring. The results were compared with those obtained earlier for 1–4 dermorphin analogues. The conformations of all six dermorphin analogues were studied. The conformational space of the peptides was examined using the electrostatically driven Monte Carlo method. On the basis of NMR data, an ensemble of conformations was obtained for each peptide. The opioid activity profiles of the compounds are discussed in the light of the structural data. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cyclic opioid peptides; conformation; dermorphin analogues; EDMC; NMR; SAR

INTRODUCTION

Recently, we described the synthesis, biological activity and conformations of several highly potent side-chain to side-chain cyclized opioid tetra- and pentapeptide analogues containing a carbonyl bridge, which links the two side-chain amino groups to form an ureido moiety [1–3]. The sequences of the tetrapeptides [2,3] were related to the common *N*-terminal fragment of dermorphin and deltorphin (Tyr-D-Aaa-Phe-Aaa-NH_2). Some of these peptides showed very high potency both in the guinea-pig ileum (GPI) and the mouse vas deferens (MVD) assay. The naturally occurring 7-peptides isolated from the skin of a South American frog: dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) and deltorphin (Tyr-D-Ala-Phe-Asp/Glu-Val-Val-Gly-NH_2), differ in the amino acid residues in positions 5–7. Dermorphin is μ-opioid-receptor selective, whereas deltorphin is δ-receptor selective. According to the message–address concept, the *N*-terminal segment could be considered as the *message* and the *C*-terminal segment as the *address.*

In this work we designed new analogues in which the *message* sequences, modified as described above, were elongated at the *C*-terminus to obtain peptides related to the full sequence of dermorphin. This made it possible to determine the impact of the addition of the *C*-terminal segment of native dermorphin on the activity and receptor selectivity of the cyclic peptide analogues.

MATERIALS AND METHODS

Synthesis of Peptides

General procedure. α-Amino groups were Boc protected, and the side-chain protection was as follows: Dap and D-Lys, Z; Ser, OBzl; Tyr, Z(2-Br). The linear protected peptides Fmoc-Tyr-D-Daa^2-Phe-Daa^5-Tyr-Pro-Ser-NH_2 were prepared on a 4-methylbenzhydrylamine resin as described earlier [2]. The crude peptides obtained after HF cleavage from the resin were dissolved in DMF (1 mg of peptide/2 ml DMF) and were treated with bis-(4-nitrophenyl) carbonate in DMF to form the cyclic peptides (Figure 1). The products were purified using semi-preparative reversed-phase high performance liquid chromatography (RP-HPLC) according to the described procedure [2] using the solvent system A: 0.05% TFA in water, B: 60% MeCN in A. A Vertex column Nucleosil-100 C_{18} (4 × 250 mm, 5 µm) was used at a flow rate of 1 ml/min with detection at 220 nm. Homogeneous fractions containing one peak were combined and lyophilized. For analytical purity determination, HPLC systems of A with gradients of 30–60% B over 20 min (peptides **1,4** and **6**), 20–80% B over 30 min (peptides **2** and **5**) and 40–80% B over 20 min (peptide **3**) were used. Structures were confirmed by ESI-MS analysis (Finningan MAT 95S spectrometer, Bremen, Germany). **1:** RT 10.7 min, $C_{45}H_{58}O_{11}N_{10}$, M calcd 915.0,

Abbreviations: Dap: α,β-diaminopropionic acid; Dab: α,γ-diaminobutyric acid; EDMC: Electrostatically Driven Monte Carlo; CLUST: a program for cluster analysis; MORASS: Multiple Overhauser Relaxation AnalysiS and Simulation.

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obtained $[M+H]^+$ 915.6, $(M+Na)^+$ 937.5; **2**: RT 18.0 min, $C_{46}H_{60}O_{11}N_{10}$, M calcd 928.6, obtained $[M+H]^+$ 929.6, $[M+Na]^+$ 951.6; **3**: RT 7.9 min, $C_{47}H_{62}O_{11}N_{10}$, M calcd 943.1, obtained $[M+H]^+$ 944.2; **4**: RT 10.3 min, $C_{44}H_{56}O_{11}N_{10}$, M calcd 901.0, obtained $[M+H]^+$ 901.6, $[M+Na]^+$ 923.4; **5**: RT 17.6 min, $C_{45}H_{58}O_{11}N_{10}$, M calcd 915.0, obtained $[M+H]^+$ 915.6, $[M+Na]^+$ 937.6; **6**: RT 11.1, $C_{46}H_{60}O_{11}N_{10}$, M calcd 928.5, obtained $[M+H]^+$ 929.2.

Synthesis of 1. Peptide chain assembly was performed on an MBHA resin (0.25 mmol) as described above using Boc-D-Lys(Fmoc)-OH and Boc-Dap(Fmoc)-OH. The peptide-resin was treated with 55% piperidine in DMF with stirring for 50 min. The resulting product was suspended in DMF (250 ml) and bis(4-nitrophenyl) carbonate (0.25 mmol, 76.05 mg) and DIEA (0.50 mmol, 88.2 µl) were added. The mixture was stirred for seven days. The product was filtered off and washed with DMF (3×) and DCM (3×) and treated with TFA in DCM (1 : 1). The peptide was cleaved from the resin by treatment with liquid HF (10 ml) in the presence of anisole (1 ml) for 1 h at 0 °C. The HF was removed under reduced pressure, and the residue was treated with cold ether, extracted with 50% acetic acid and lyophilized. The yield of pure peptide was lower than that obtained with the use of the general procedure described above.

Bioassays

The GPI [4] and MVD [5] bioassays were carried out as reported in detail elsewhere [6,7]. A log dose–response curve was determined with [Leu^5]-enkephalin as standard for each ileum and vas preparation and the IC_{50} values of the compounds being tested were normalized according to a published procedure [8]. The results are presented in Table 1.

NMR Spectroscopy and Theoretical Analysis

NMR samples of peptides **1–6** were prepared and their spectra were recorded using the conditions and parameters already described [1,10]. The conformational space of each peptide was explored using the EDMC method proposed by Liwo *et al.* [11] and references cited therein. The conformations were subsequently clustered into families using the program CLUST [12], taking all heavy atoms into consideration as well as the rmsd and energy criteria. For the estimation of the population of particular conformational families of each peptide, NOESY spectra were generated for representatives of each family with the program MORASS [13] using the parameters described previously [1]. In this way the statistical weights for each of the conformational families were obtained.

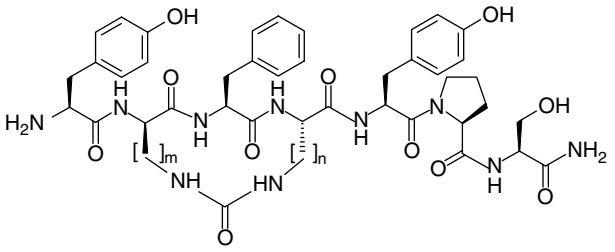

Figure 1 Dermorphin analogues: **1**, {[H-Tyr-D-Lys(&^{1})-Phe-Dap(&^{2})-Tyr-Pro-Ser-NH_2][&^{1}CO&^{2}]}; **2**, {[H-Tyr-D-Lys(&^{1})-Phe-Dab(&^{2})-Tyr-Pro-Ser-NH_2][&^{1}CO&^{2}]}; **3,** {[H-Tyr-D-Lys(&^{1})-Phe-Orn(&^{2})-Tyr-Pro-Ser-NH_2][&^{1}CO&^{2}]}; **4**, {[H-Tyr-D-Orn(&^{1})-Phe-Dap(&^{2})-Tyr-Pro-Ser-NH_2][&^{1}CO&^{2}]}; **5**, {[H-Tyr-D-Orn(&^{1})-Phe-Dab(&^{2})-Tyr-Pro-Ser-NH_2][&^{1}CO&^{2}]}; **6,** {[H-Tyr-D-Orn(&^{1})-Phe-Orn(&^{2})-Tyr-Pro-Ser-NH_2][&^{1}CO&^{2}]}. (The structures are described using abbreviated nomenclature for cyclic peptides [9]).

RESULTS AND DISCUSSION

Protected linear peptides containing the full sequences of dermorphin and basic amino acid residues in positions 2 and 4 were synthesized by the solid phase method. Removal of the Z-group protecting the side-chain amino groups and cleavage of the peptides from the solid support were performed by treatment with liquid HF. Fmoc protection of the *N*-terminal α-amino group was retained under these conditions. The crude 7-peptides were next reacted with bis(4-nitrophenyl)carbonate in DMF to form the cyclic peptides containing a 14-, 15-, 16- or 17-membered ring. The Fmoc group was then removed by treatment with piperidine, and homogeneous peptides were obtained by RP-HPLC purification (Figure 1). The molecular weights of the peptides were confirmed by ES-MS. In an alternative version of the synthesis, in which cyclization was performed with the resin-bound peptide, the yield was lower.

In vitro opioid activity profiles of the compounds were determined using the GPI and in the MVD assays (Table 1). In contrast to natural dermorphin, five of the six analogues were more active in the MVD assay than in the GPI assay. While in native dermorphin the *C*-terminal portion is responsible for the high µ-receptor selectivity, the analogues described here are not only more active in the δ-receptor representative MVD assay but their activities in both assays are also lower than those of the corresponding peptides containing the 1–4 sequences only (Table 2). The activities of the analogues containing D-Lys in position 2 are comparable to that of enkephalin, while the D-Orn^2-analogues are less potent. The ring size does not have an important effect on activity (compare **2** and **6**). Comparison of these results with those obtained earlier for the corresponding analogues containing the sequence 1–4 only [2,3] indicates that elongation of the cyclic tetrapeptides with the *C*-terminal segment of dermorphin decreases biological activity (Table 2) in the GPI assay. In the MVD assay two analogues with D-Lys in position 2 were found to be as potent as their shorter counterparts.

1D and 2D homo- and heteronuclear NMR spectra were recorded for all six compounds in water. Two forms were observed in solution with each peptide with a *trans* or *cis* proline peptide bond at the ratio of *ca* 4 : 1. The full assignment of the peaks was achieved in the ^{1}H and ^{13}C NMR spectra of the *trans* isomer. The proton

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Table 1 GPI and MVD assay of cyclo(N^{ω}, $N^{\omega'}$-carbonyl-D-Daa^2, Daa^4)dermorphin(1–7)-NH_2 analogues

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	15	Lys	Dap	104 ± 13	2.37 ± 0.30	19.6 ± 1.3	0.582 ± 0.039	5.31
2	16	Lys	Dab	186 ± 24	1.32 ± 0.17	85.1 ± 1.9	0.134 ± 0.003	2.19
3	17	Lys	Orn	80.7 ± 1.3	3.05 ± 0.05	49.4 ± 4.7	0.231 ± 0.022	1.63
4	14	Orn	Dap	1440 ± 200	0.171 ± 0.024	475 ± 113	0.0240 ± 0.057	3.03
5	15	Orn	Dab	609 ± 51	0.404 ± 0.034	460 ± 37	0.0248 ± 0.0020	1.32
6	16	Orn	Orn	1630 ± 150	0.151 ± 0.014	2410 ± 230	0.00473 ± 0.00045	0.676
[Leu^5]enkephalin				246 ± 39	1	11.4 ± 1.1	1	21.6

[a] Mean of 3–6 determinations ± SEM.

Table 2 Comparison of the opioid activities of 1–7 and 1–4 dermorphin analogues

Analogue	Amino acids in position 2 and 4	GPI 1–4/1–7 IC_{50} ratio	MVD 1–4/1–7 IC_{50} ratio
1	D-Lys^2, Dap^4	0.090	0.995
2	D-Lys^2, Dab^4	0.006	0.059
3	D-Lys^2, Orn^4	0.193	0.953
4	D-Orn^2, Dap^4	0.002	0.016
5	D-Orn^2, Dab^4	0.003	0.003
6	D-Orn^2, Orn^4	0.007	0.005

chemical shifts for this isomer are listed in Table 3 along with the vicinal coupling constants, $^3J_{H\alpha HN}$, and the temperature coefficients, $\Delta\delta/\Delta T$, of the signals of the amide protons determined in the 1D proton spectra. The ^{13}C chemical shifts for the *trans* isomer of peptides **1**–**6** are presented in Table 4. A complete assignment of the signals obtained for the less populated *cis* isomer was not possible and the number of NOE contacts found for this form was always small. The trial procedure of the calculation of the statistical weights failed for this isomer and, therefore, the conformational analysis for this isomer was not performed. The data for the less populated *cis* isomer are available on request from JW (jacekw@ibb.waw.pl).

A global conformational search was carried for the *trans* isomer of all six compounds followed by fitting to the experimental data. The numbers of the conformations generated and accepted with the EDMC calculation procedure together with those left after the clusterization procedure (with rmsd of 0.1 Å for all heavy atoms) are listed in Table 5.

NOE contacts were utilized to assign statistical weights for representatives of conformational families. For subsequent analysis we chose only those conformations whose relative population was higher than 1%. This resulted in 13–23 conformations with the sum of their statistical weights amounting to 95–98% in each case. The parameters that characterize the chosen conformations are listed in Table 6 and their drawings are shown in Figure 2.

The diversity of conformations is indicated by the rmsd data presented in Table 7. The rmsd values calculated for these structures using heavy atoms of the main ring or the 'spacer' are similar to those obtained previously [1,2]. The numbers given in this Table reflect the large conformational diversity of the exocyclic *C*-terminal tripeptide segment of each peptide.

The dihedral angles shown in Table 6 reveal that it is possible to group all listed structures in five conformations in respect to the dihedral angles $\psi(2)$ and $\varphi(3)$ within the ranges of *ca* ±15 to ±30°. The most populated conformation is that with $\psi(2) = +40$ and $\varphi(3) = -140$. Eighty-four structures out of the 112 considered belong here: e.g. all structures of compound **5**, almost all of compounds **1** and **4** and most of the structures of compound **3** and **6**. Compound **3** partially exists in two conformations with $\psi(2) = +40$ and $\varphi(3) = -110$ or $+50$. Compound **6** and partially compound **2** exist in conformations with $\psi(2) = +40$ and $\varphi(3) = -85$. However, a clear correlation between these data and the compounds' activity is not evident.

A comparison of the conformations obtained for peptides **1**, **2** and **5** with those found earlier by us [2] for the corresponding cyclic tetrapeptides containing residues 1–4 can be made. This reveals that for compounds **1** and **5** a larger distribution of conformations is now observed but within the same range of $\psi(2)$ and $\varphi(3)$ angles. For compound **1** the $1 \rightarrow 3$ aromatic ring distances in some cases are longer (10–13 Å) than those observed earlier, but for compound **5** the distribution of these distances is similar. Interestingly, for compound **2** an additional set of conformations is observed with $\varphi(3) = -140°$ and with much shorter (5–8 Å) $1 \rightarrow 3$ aromatic ring distances. These observations again do not explain the

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Table 3 Proton chemical shifts (ppm) of the *trans* isomer of cyclo(N^{ω}, $N^{\omega'}$-carbonyl-D-Daa^2, Dxx^4)dermorphin(1–7) amides in water at 25 °C

Peptide	**1**(D-Lys^2,Dap^4)	**2**(D-Lys^2,Dab^4)	**3**(D-Lys^2,Orn^4)	**4**(D-Orn^2,Dap^4)	**5**(D-Orn^2,Dab^4)	**6**(D-Orn^2,Orn^4)
Tyr^1						
H_N	na	na	na	na	na	na
$^3J_{H\alpha HN}$	na	na	na	na	na	na
H_α	4.065	4.145	4.144	4.138	4.146	4.137
H_β	2.954; 3.198	2.949; 3.229	2.942; 3.227	2.984; 3.202	2.967; 3.211	2.955; 3.221
H_{26}	7.116	7.126	7.126	7.119	7.116	7.121
H_{35}	6.871	6.869	6.862	6.868	6.876	6.874
D-Daa^2						
$H_N(-\Delta\delta/\Delta T$[ppb])	8.087 (8.8)	8.099 (10.0)	8.226 (8.8)	8.127 (8.6)	8.138 (8.0)	8.267 (10.6)
$^3J_{H\alpha HN}$	6.6 Hz	7.0 Hz	7.7 Hz	6.7 Hz	5.9 Hz	na
H_α	3.977	4.126	4.126	3.972	4.013	4.044
H_β	1.386	1.164; 1.560	1.188; 1.486	1.308; 1.431	1.326; 1.538	1.301; 1.422
H_γ	0.848	0.746; 0.832	0.655; 0.782	1.179	1.126; 1.223	0.953
H_δ	1.301	1.289; 1.386	1.281	2.780; 3.027	2.915	2.909; 2.996
H_ε	2.930; 3.114	3.004; 3.168	3.055	—	—	—
$H_{N\ bridge}$	5.756	5.827	na	6.093	5.812	5.811
Phe^3						
$H_N(-\Delta\delta/\Delta T$[ppb])	7.747 (7.4)	7.775 (8.8)	7.841 (7.4)	7.968 (6.2)	7.905 (9.4)	7.949 (7.0)
$^3J_{H\alpha HN}$	7.0 Hz	7.3 Hz	7.5 Hz	na	na	7.6 Hz
H_α	4.428	4.445	4.417	4.531	4.421	4.436
H_β	2.935	2.761; 2.904	2.849; 2.907	2.912	2.839; 2.924	2.934
H_{26}	7.096	6.966	7.030	7.169	6.972	7.176
H_{35}	7.209	7.091	7.130	7.112	7.127	7.105
H_4	7.216	7.172	7.148	7.178	7.190	7.175
Daa^4						
$H_N(-\Delta\delta/\Delta T$[ppb])	8.213 (7.6)	7.945 (4.2)	7.796 (4.8)	7.947 (5.6)	8.028 (6.0)	7.781 (5.4)
$^3J_{H\alpha HN}$	na	8.8 Hz	8.3 Hz	na	8.0 Hz	8.3 Hz
H_α	4.389	4.395	4.171	4.426	4.273	4.197
H_β	3.079; 3.367	1.623; 1.756	1.428; 1.504	3.172; 3.259	1.613; 1.763	1.421; 1.513
H_γ	—	2.995; 3.009	1.258	—	3.074; 3.138	1.175; 1.244
H_δ	—	—	2.900; 3.028	—	—	2.992
$H_{N\ bridge}$	5.995	5.587		6.106	5.794	5.974
Tyr^5						
$H_N(-\Delta\delta/\Delta T$[ppb])	8.001 (6.8)	8.070 (10.6)	8.070 (10.8)	8.095 (10.6)	7.984 (10.2)	8.018 (10.2)
$^3J_{H\alpha HN}$	7.2 Hz	6.8 Hz	7.3 Hz	7.3 Hz	7.1 Hz	7.5 Hz
H_α	4.683	4.544	4.606	4.620	4.549	4.664
H_β	2.793; 3.068	2.775; 3.072	2,756; 3.061	2.731; 3.038	2.754; 3.050	2.744; 3.052
H_{26}	7.175	7.225	7.188	7.138	7.187	7.154
H_{35}	6.846	6.868	6.861	6.846	6.856	6.852
Pro^6						
H_α	4.421	4.423	4.417	4.416	4.410	4.418
H_β	1.928; 2.258	1.950; 2.275	1.946; 2.269	1.938; 2.263	1.949; 2.264	1.946; 2.265
H_γ	1.991	2.028	2.007	1.993	2.012	2.000
H_δ	3.530; 3.746	3.617; 3.786	3.588; 3.761	3.530; 3.729	3.586; 3.751	3.575; 3.744
Ser^7						
$H_N(-\Delta\delta/\Delta T$[ppb])	8.224 (10.0)	8.255 (12.2)	8.248 (10.8)	8.243 (10.4)	8.248 (11.6)	8.249 (8.0)
$^3J_{H\alpha HN}$	na	7.2 Hz	7.1 Hz	6.7 Hz	7.0 Hz	na
H_α	4.382	4.392	4.391	4.394	4.384	4.393
H_β	3.858	3.864	3.864	3.868	3.856	3.871
NH_2	7.114; 7.522	7.116; 7.547	7.117; 7.542	7.121; 7.536	7.112; 7.539	7.123; 7.544

na – not assigned.

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Table 4 Carbon and nitrogen chemical shifts (ppm) of the *trans* isomer of cyclo(N^{ω}, $N^{\omega'}$-carbonyl-D-Daa^2, Dxx^4)dermorphin(1–7) amides in water at 25 °C

Peptide	**1**(D-Lys^2,Dap^4)	**2**(D-Lys^2,Dab^4)	**3**(D-Lys^2,Orn^4)	**4**(D-Orn^2,Dap^4)	**5**(D-Orn^2,Dab^4)	**6**(D-Orn^2,Orn^4)
Tyr^1						
N_H	na	na	na	na	na	na
C_α	57.50	57.64	57.78	57.51	57.59	57.74
C_β	38.98	38.99	38.97	39.02	39.03	38.95
C_{26}	141.31	141.50	141.46	141.46	141.46	141.47
C_{35}	126.72	126.56	126.44	126.60	126.60	126.64
D-Daa^2						
N_H	126.45	125.91	126.58	125.66	125.30	125.96
C_α	57.69	56.96	56.28	57.33	56.74	56.53
C_β	33.02	32.66	33.25	30.51	30.49	30.93
C_γ	23.09	22.78	23.90	26.23	27.08	28.14
C_δ	32.15	30.56	31.74	41.76	41.49	41.82
C_ε	40.78	41.34	42.01	—	—	—
$N_{H\ bridge}$	125.83	124.45	na	na	na	na
Phe^3						
N_H	119.18	120.85	120.47	120.25	120.67	120.52
C_α	58.18	na	57.82	57.82	na	na
C_β	39.27	39.63	39.17	38.89	39.13	38.65
C_{26}	139.91	139.66	139.72	139.39	139.72	139.39
C_{35}	139.54	139.36	139.36	139.76	139.37	139.80
C_4	137.82	137.75	137.66	137.65	137.71	137.67
Daa^4						
N_H	120.10	125.13	125.86	121.86	126.40	125.15
C_α	56.91	56.06	55.22	56.10	na	55.32
C_β	43.34	33.12	31.84	43.35	33.23	31.71
C_γ	—	39.23	28.53	—	39.72	28.31
C_δ	—	—	41.78	—	—	41.69
$N_{H\ bridge}$	115.86	na	na	118.13	na	na
Tyr^5						
N_H	123.38	122.38	122.48	122.95	121.62	121.85
C_α	na	??	56.26	56.08	na	na
C_β	38.09	37.93	38.10	38.12	38.05	38.14
C_{26}	141.33	141.50	141.45	141.48	141.48	141.45
C_{35}	126.37	126.50	126.45	126.32	126.32	126.29
Pro^6						
C_α	63.50	na	63.31	63.50	na	63.42
C_β	31.90	31.92	31.95	31.92	31.91	31.94
C_γ	27.36	27.44	27.40	27.38	27.43	27.37
C_δ	50.63	50.70	50.68	50.66	50.67	50.65
Ser^7						
N_H	116.67	116.80	116.76	116.73	116.73	116.71
C_α	56.91	58.36	58.18	58.19	58.18	58.20
C_β	63.94	63.98	63.97	63.97	63.96	63.99
NH_2	108.71	108.72	108.64	108.72	108.68	108.69

na – not assigned.

relative decrease in activity of the compounds of the present series.

The rmsd data shown in Table 7 indicate that the flexibility of the main ring as well as the flexibility of the 'linker' depends firstly on the size of the main ring and on the residue type in position 2. The biological activities of peptides with D-Lys in position 2 are higher than those of the D-Orn^2-analogues. This is in correlation with the higher stiffness of the 'linker' observed in the compounds with D-Orn (e.g. lower rmsd, Table 7).The distance between the aromatic rings of Tyr^1 and Phe^3 is thought to be of great importance for opioid activity. In the peptides studied here two other potential aromatic rings interactions are possible, involving the Phe^3 and Tyr^5 or Tyr^1 and Tyr^5 rings. However, a careful inspection of the structures reveals

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Table 5 Number of calculated conformations of the *trans* isomer

Conformations	Compound	**1**(D-Lys^2,Dap^4)	**2**(D-Lys^2,Dab^4)	**3**(D-Lys^2,Orn^4)	**4**(D-Orn^2,Dap^4)	**5**(D-Orn^2,Dab^4)	**6**(D-Orn^2,Orn^4)
Electrostatic	Generated	1315	1535	1093	1125	1394	1034
	Accepted	450	385	295	424	443	353
Random	Generated	12 519	15 616	10 711	10 964	13 787	10 995
	Accepted	2550	2614	2705	2576	2556	2647
Thermal	Generated	0	3	0	0	5	0
	Accepted	0	1	0	0	1	0
Total	Generated	13 834	17 154	11 804	12 089	15 186	12 029
	Accepted	3000	3000	3000	3000	3000	3000
Fraction conf. accept:		0.217	0.175	0.254	0.248	0.198	0.250
Number after clusterization		1413	1088	1557	1103	1157	1185

Table 6 Parameters for the most populated conformations of the *trans* isomer of peptides **1** to **6** (with Populations above 1%) Found in Water

1(D-Lys^2, Dap^4)	$\chi_1(1)$[a]	$\psi(1)$	$\varphi(2)$	$\psi(2)$	$\varphi(3)$	$\chi_1(3)$	$r(1 \rightarrow 3)$[b]	$r(3 \rightarrow 5)$	$r(1 \rightarrow 5)$	E_n[c]	Pop (%)[d]
1_1	−179.4	153.2	133.4	**65.4**	**−142.8**	−56.8	7.2	11.3	12.7	0.00	2.3
1_2	−179.3	154.1	127.7	**66.6**	**−141.4**	−61.9	7.6	10.7	13.1	0.02	6.1
1_3	−65.4	162.8	145.1	**65.2**	**−132.0**	−55.0	7.5	12.9	18.8	0.03	1.1
1_4	−179.8	153.4	132.9	**65.6**	**−142.6**	−57.4	7.2	11.2	12.7	0.15	2.2
1_5	−64.3	159.3	85.2	**25.9**	**−151.8**	−177.2	11.8	8.0	14.6	0.28	3.1
1_6	−68.6	−44.0	88.0	**62.2**	**−78.8**	−59.9	5.1	7.3	11.6	0.92	1.4
1_7	−177.9	151.2	141.4	**63.5**	**−95.8**	−55.3	5.6	7.9	10.9	1.24	1.8
1_8	−179.4	150.9	138.0	**63.4**	**−104.9**	−55.1	5.7	7.5	11.7	1.41	6.6
1_9	−65.3	162.4	144.4	**65.7**	**−132.7**	−55.3	7.6	12.0	18.4	1.58	3.3
1_10	−63.8	162.2	90.8	**53.0**	**−85.3**	−55.7	6.4	9.3	15.3	1.70	1.9
1_11	−66.4	−34.3	145.2	**−109.4**	**50.2**	−61.9	6.9	8.6	15.1	2.09	2.0
1_12	−66.9	−33.8	157.5	**−144.1**	**−169.1**	169.4	13.1	12.0	11.8	2.09	6.5
1_13	−69.1	−43.2	80.3	**60.6**	**−79.4**	−56.2	5.1	11.2	15.3	2.13	10.7
1_14	−173.5	−47.2	97.4	**50.8**	**−70.0**	−71.3	4.0	10.4	12.4	2.23	2.1
1_15	58.0	124.4	59.2	**56.0**	**−128.4**	−58.1	4.8	8.7	13.4	2.23	4.1
1_16	−65.1	162.6	145.1	**64.7**	**−131.9**	−54.9	7.5	13.1	18.9	2.37	5.0
1_17	−64.4	156.3	135.9	**−15.6**	**−102.3**	−59.8	10.4	11.8	15.4	2.56	2.1
1_18	−178.2	156.0	76.7	**44.0**	**−85.1**	−170.6	10.6	6.0	13.4	2.61	7.8
1_19	−64.9	159.7	82.0	**29.4**	**−146.3**	−170.0	11.8	5.7	15.3	2.73	10.8
1_20	−64.2	160.9	81.4	**29.8**	**−137.9**	−174.6	11.7	4.7	9.1	2.78	3.9
1_21	−178.0	155.6	99.3	**58.5**	**−102.7**	−59.4	7.4	7.1	12.8	2.79	9.8
1_22	−70.7	160.5	148.2	**59.5**	**−135.5**	−56.3	7.4	10.8	15.9	2.91	1.8
1_23	74.3	171.3	155.0	**62.8**	**−94.6**	−57.9	7.6	11.3	13.7	2.99	1.7
											Total 98.3
2(D-Lys^2, Dab^4)											
2_1	−174.4	−21.5	91.1	**−134.4**	**−79.6**	−58.3	7.0	11.8	14.0	0.00	22.9
2_2	174.2	−26.1	167.9	**−124.3**	**−137.9**	179.6	7.9	7.7	11.2	1.98	4.1
2_3	−178.2	−32.7	147.4	**−122.8**	**−92.8**	−53.5	5.4	11.0	13.5	3.53	2.5
2_4	−177.3	−21.3	100.5	**−131.8**	**−92.6**	−58.2	7.2	6.8	12.1	3.86	1.1
2_5	−177.9	136.4	100.0	**−127.6**	**−78.5**	−64.9	4.1	11.4	13.7	4.38	1.5
2_6	−61.2	138.0	157.3	**37.0**	**−130.0**	−56.4	11.3	11.7	4.0	4.90	20.6
2_7	177.3	−33.0	157.3	**45.8**	**−157.0**	171.8	10.4	12.0	12.4	5.36	7.3
2_8	178.3	−37.3	143.0	**37.4**	**−137.2**	−62.4	7.5	6.9	8.1	5.37	3.3
2_9	178.8	137.7	103.7	**−123.4**	**−131.2**	−178.4	7.6	4.8	8.0	5.57	2.3
2_10	−178.5	−36.1	80.8	**16.3**	**−142.1**	−55.9	8.4	11.7	12.0	5.85	17.9
2_11	176.2	130.2	152.5	**−121.6**	**−130.5**	−59.9	5.1	6.1	10.8	5.91	5.5

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Table 6 (*Continued*)

1(D-Lys^2, Dap^4)	$\chi_1(1)$[a]	$\psi(1)$	$\varphi(2)$	$\psi(2)$	$\varphi(3)$	$\chi_1(3)$	$r(1 \rightarrow 3)$[b]	$r(3 \rightarrow 5)$	$r(1 \rightarrow 5)$	E_n[c]	Pop (%)[d]
2_12	−171.3	−16.6	158.8	**35.8**	**−138.7**	−58.2	6.2	12.1	13.2	5.93	5.5
2_13	−174.7	−25.8	85.9	**−134.8**	**−137.6**	−138.1	10.8	8.9	14.2	6.15	3.5
											Total 98.0
3(D-Lys^2, Orn^4)											
3_1	−66.0	−36.6	77.2	**35.4**	**−85.1**	−173.6	8.3	9.8	15.8	0.00	1.1
3_2	−60.0	−41.0	151.9	**59.3**	**−142.6**	−178.4	10.0	7.6	16.2	0.13	1.0
3_3	177.6	121.4	147.8	**−33.7**	**−102.9**	−60.0	9.8	11.2	13.1	0.94	6.9
3_4	−157.5	171.2	143.2	**55.0**	**−129.6**	−50.7	5.4	10.5	10.0	1.15	2.6
3_5	−178.6	153.3	136.9	**54.9**	**−105.0**	−56.9	5.9	9.1	12.5	2.38	1.4
3_6	−66.6	−36.7	78.9	**31.0**	**−86.2**	−176.7	8.5	6.4	14.8	2.72	9.2
3_7	−179.0	145.3	151.2	**48.9**	**−95.5**	−56.0	5.6	8.3	10.6	2.74	3.9
3_8	−154.6	171.7	142.5	**53.4**	**−124.3**	−48.9	5.1	12.8	14.0	2.80	2.6
3_9	−63.8	157.5	79.8	**41.1**	**−107.2**	−170.1	11.4	4.8	12.0	2.84	11.3
3_10	−171.4	151.7	87.3	**44.3**	**−81.0**	−60.9	7.7	12.1	16.5	2.89	2.6
3_11	−175.3	154.5	126.5	**−58.5**	**53.6**	−45.0	5.4	8.1	12.0	2.97	4.5
3_12	−174.7	155.6	124.3	**−59.6**	**54.2**	−44.1	5.4	13.2	15.7	3.20	5.2
3_13	−177.2	154.8	121.5	**55.3**	**−89.2**	−54.4	5.6	6.8	11.3	3.27	2.7
3_14	178.4	148.1	150.2	**48.3**	**−102.2**	−55.7	5.6	12.1	15.3	3.61	2.7
3_15	−179.5	135.6	140.9	**−26.2**	**−137.0**	−61.9	9.6	11.6	14.9	3.62	2.9
3_16	177.6	121.1	147.9	**−33.4**	**−103.8**	−59.0	9.8	11.3	13.2	4.11	3.9
3_17	−177.6	−43.8	102.0	**49.1**	**−76.6**	−68.1	4.0	11.7	13.6	4.59	7.3
3_18	−179.6	154.7	93.6	**−55.8**	**55.8**	−48.4	6.6	11.5	14.0	4.64	7.5
3_19	−179.7	143.4	148.5	**49.2**	**−94.4**	−57.9	5.8	5.3	8.2	4.71	1.1
3_20	−65.4	−35.3	75.1	**20.7**	**−95.6**	−176.5	8.2	5.1	12.5	4.93	9.6
3_21	−178.7	156.4	76.7	**37.5**	**−78.3**	−179.4	10.8	6.8	16.9	4.98	7.8
											Total 97.8
4(D-Orn^2, Dap^4)											
4_1	178.5	151.1	147.0	**55.5**	**−106.9**	−53.2	5.8	8.0	10.7	0.00	3.0
4_2	−166.0	167.7	168.2	**59.2**	**−150.6**	177.7	7.2	6.0	11.1	0.19	1.4
4_3	−174.8	157.4	102.9	**49.2**	**−100.0**	−59.4	7.3	10.4	13.5	0.34	7.0
4_4	178.9	151.4	147.4	**55.2**	**−107.0**	−55.7	5.9	7.5	10.9	0.85	1.1
4_5	−176.1	136.3	154.5	**61.6**	**−151.8**	178.8	9.7	5.2	13.4	1.05	5.0
4_6	−67.8	102.8	156.8	**62.1**	**−150.5**	−178.9	12.6	8.1	16.6	1.24	1.3
4_7	−166.7	167.7	167.9	**59.7**	**−148.0**	177.4	7.2	5.2	11.2	1.28	3.6
4_8	−175.5	154.1	82.4	**56.3**	**−142.2**	172.6	11.0	4.5	12.6	1.59	11.6
4_9	72.8	169.9	148.2	**56.8**	**−143.6**	−57.8	8.7	11.7	13.5	1.66	6.1
4_10	64.2	−29.3	154.5	**60.3**	**−149.5**	172.7	6.9	8.2	12.6	1.73	1.7
4_11	−175.5	154.1	82.4	**56.4**	**−142.1**	172.5	11.0	4.5	12.6	1.87	1.6
4_12	−166.7	167.8	167.9	**59.8**	**−148.4**	178.5	7.2	5.1	11.1	2.09	7.8
4_13	−68.0	103.6	157.0	**61.9**	**−151.2**	−177.3	12.5	8.5	16.0	2.13	9.6
4_14	173.9	−56.8	150.3	**57.8**	**−116.8**	−60.1	4.8	7.6	12.0	2.16	8.5
4_15	68.3	169.7	81.6	**55.9**	**−141.7**	173.0	11.8	4.4	14.6	2.20	7.4
4_16	−64.6	160.5	137.3	**−15.9**	**−79.2**	−58.5	9.2	10.5	14.9	2.23	11.1
4_17	74.0	169.4	159.3	**61.7**	**−150.1**	−179.3	11.2	8.1	12.8	2.31	6.0
4_18	68.8	170	79.8	**52.4**	**−141.3**	−178.1	11.8	8.0	14.9	2.36	2.8
											Total 96.6
5(D-Orn^2, Dab^4)											
5_1	−175.5	155.4	83.2	**48.2**	**−126.6**	−173.9	10.8	4.7	13.1	0.00	2.7
5_2	−174.2	157.0	86.0	**50.5**	**−136.7**	−174.0	10.8	4.5	12.7	1.44	4.1
5_3	−175.9	155.1	81.9	**50.4**	**−138.5**	178.2	11.1	6.8	12.4	2.29	5.2
5_4	−175.4	157.5	86.2	**48.7**	**−134.4**	−61.4	8.4	6.4	11.1	2.37	5.0
5_5	−175.8	155.2	84.2	**49.2**	**−146.8**	178.4	11.0	7.8	13.8	2.55	1.8
5_6	−175.1	155.8	86.7	**50.0**	**−145.4**	−171.7	10.9	4.4	13.5	2.61	2.3
5_7	−176.0	155.1	81.7	**48.4**	**−131.9**	177.4	11.0	5.2	14.1	2.97	5.8

(*continued overleaf*)

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Table 6 (*Continued*)

1(D-*Lys*^{2}, *Dap*^{4})	$\chi_1(1)$[a]	$\psi(1)$	$\varphi(2)$	$\psi(2)$	$\varphi(3)$	$\chi_1(3)$	$r(1 \rightarrow 3)$[b]	$r(3 \rightarrow 5)$	$r(1 \rightarrow 5)$	E_n[c]	Pop (%)[d]
5_8	−177.3	155.8	89.3	**49.1**	**−144.1**	63.6	7.5	7.5	14.4	3.14	3.6
5_9	−64.0	156.8	80.8	**49.1**	**−136.1**	−174.1	11.9	4.6	10.0	3.27	13.4
5_10	−69.4	−38.8	81.0	**48.5**	**−140.4**	−171.2	8.8	5.7	13.8	3.88	7.3
5_11	−65.7	−38.0	83.0	**52.3**	**−134.3**	174.3	8.8	7.6	14.3	3.89	6.9
5_12	−173.9	158.0	98.9	**50.3**	**−90.1**	−55.7	6.7	12.0	14.1	4.02	12.7
5_13	−177.9	72.3	67.2	**48.4**	**−132.9**	−63.1	6.6	10.9	16.3	4.38	8.0
5_14	−66.7	−37.7	83.7	**52.4**	**−137.1**	174.2	9.1	5.6	8.7	4.52	7.9
5_15	−63.9	157.3	79.6	**30.2**	**−85.8**	−179.4	11.2	5.3	16.2	4.73	9.0
											Total 95.8
6(D-*Orn*^{2}, *Orn*^{4})											
6_1	−174.7	−38.1	95.0	**−121.1**	**−95.6**	−52.6	6.9	10.1	9.4	0.00	4.4
6_2	−179.6	−31.4	148.1	**−126.4**	**−94.6**	−58.9	5.8	11.8	12.9	0.72	1.3
6_3	−175.8	−24.7	85.4	**−131.6**	**−144.8**	175.6	10.9	5.2	14.4	1.19	1.0
6_4	−176.9	138.7	102.6	**−135.7**	**−76.4**	−69.5	4.0	11.5	13.0	2.51	7.3
6_5	−176.9	138.7	102.6	**−135.7**	**−76.4**	−69.5	5.3	10.4	10.4	2.69	8.5
6_6	−171.4	−24.2	106.8	**−128.8**	**−78.0**	−56.6	8.3	8.9	13.6	3.00	1.2
6_7	−175.5	−23.5	95.8	**−132.7**	**−140.0**	−61.7	5.7	7.2	10.9	3.50	2.6
6_8	−179.2	−28.7	146.6	**−126.3**	**−109.3**	−53.5	7.1	5.0	11.7	3.54	1.4
6_9	−178.1	148.6	91.0	**−135.5**	**−139.9**	−76.7	6.0	12.2	12.8	4.20	2.1
6_10	−161.2	−7.0	165.6	**−132.6**	**−145.3**	−56.2	4.6	8.8	11.3	4.36	1.8
6_11	67.3	−9.4	89.7	**−136.5**	**−83.6**	−61.3	5.7	7.2	8.9	4.51	3.1
6_12	−174.7	−37.8	93.4	**−121.2**	**−95.3**	−53.5	7.9	8.8	14.7	4.81	1.4
6_13	−177.5	−24.4	88.7	**−129.9**	**−143.4**	172.5	7.0	11.5	15.4	5.11	5.5
6_14	−175.2	−29.3	81.1	**−136.5**	**−143.4**	178.6	10.8	5.6	13.8	5.15	8.2
6_15	171.0	−27.7	149.1	**−131.5**	**−142.2**	−56.2	11.1	5.9	16.3	5.17	12.3
6_16	171.9	122.7	152.5	**−126.7**	**−149.6**	−178.4	7.0	10.9	10.7	5.33	9.3
6_17	171.5	122.6	152.1	**−126.6**	**−146.9**	176.7	8.2	5.5	13.2	5.35	1.2
6_18	−179.3	−26.3	101.8	**−129.2**	**−141.9**	−58.6	8.2	5.6	13.0	5.36	8.3
6_19	−179.3	−26.3	101.8	**−129.2**	**−141.9**	−58.6	8.1	7.9	12.9	5.61	3.0
6_20	−63.9	149.2	156.5	**−128.6**	**−147.7**	−64.2	7.3	10.9	16.2	5.68	3.0
6_21	−177.1	−29.7	141.7	**−125.4**	**−106.3**	−54.3	5.8	11.8	14.8	5.72	4.5
6_22	−176.6	138.3	102.1	**−134.8**	**−76.4**	−69.7	4.0	10.6	13.5	5.95	6.2
											Total 97.9

[a] Values of selected torsional angles. Torsional angles of the 'spacer' are given in bold.
[b] Distance between tyrosine and phenylalanine ring centres (r in Å).
[c] Relative calculated energy (E_n in kcal/mol).
[d] Relative populations of conformers (Pop in %).

that in most of them this type of interactions can be excluded.

Examination of the ^{1}H NMR chemical shifts of compounds **1**, **2** and **5** in comparison with those of their corresponding 1–4 analogues already published [2] show that all signals of residues Phe(3) and Daa(4) are shifted upfield, some of them substantially by 0.1 to 0.4 ppm (for the comparison see Table 8). The most shifted is the amide proton of the Daa(4) residue, which is shifted by 0.42, 0.34 and 0.40 ppm in **1**, **2** and **5**, respectively. This finding indicates the existence of additional interactions in the 1–7 peptides, which are absent in their shorter correlates. Since the corresponding proton chemical shifts of residues Phe(3) and Daa(4) of all six 1–7 peptides are very similar (Table 3), this may also be the case for the remaining peptides of this series. On the other hand, rather low temperature coefficients were found for the amide proton of the Daa(4) residue and a slightly lower coefficient for the Phe(3) amide of each 1–7 peptide (Table 3). The coefficients determined for the short analogues [2] are indicative of fast exchange with the solvent and were 2 to 3 ppb higher then those observed with the longer 1–7 peptides. This observation suggests that a substantial number of conformations of the 7-peptides may be engaged in intramolecular hydrogen bonding.

Careful inspection of the calculated structures indicates the possibility of the existence of various types of hydrogen bonds. Such hydrogen bonds may include typical N–H$\cdots$O=C bonds as well as N–H$\cdots\pi$ type hydrogen bonds. One can assume that in the

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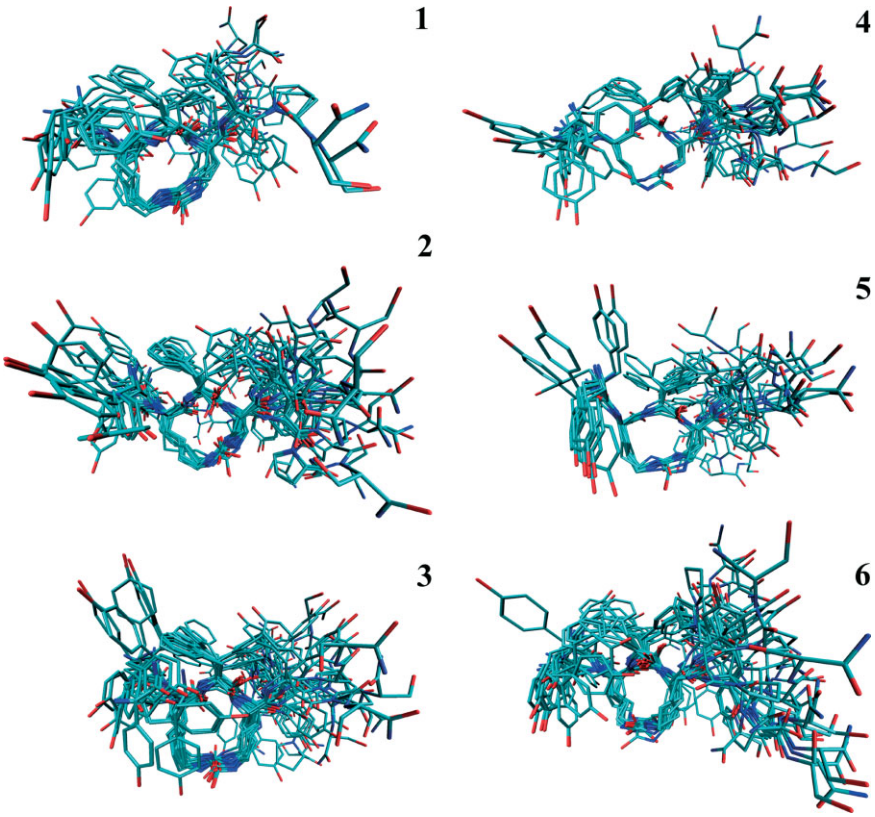

Figure 2 VMD drawings [14] of the most populated (above 1%) EDMC/NMR calculated conformations of the more populated *trans* isomer of dermorphins **1**–**6**. The structures are aligned using C and N atoms of the main ring.

Table 7 Calculated rmsd (in Å) for conformers of the *trans* isomers of peptides **1**–**6** with the populations above 1%

Compound	**1**(D-Lys^2,Dap^4)	**2**(D-Lys^2,Dab^4)	**3**(D-Lys^2,Orn^4)	**4**(D-Orn^2,Dap^4)	**5**(D-Orn^2,Dab^4)	**6**(D-Orn^2,Orn^4)
[a]	3.104	3.265	2.932	2.847	2.753	3.069
[b]	1.819	2.189	1.827	1.733	1.494	2.018
[c]	0.478	0.595	0.576	0.304	0.420	0.528
[d]	0.264	0.314	0.232	0.186	0.058	0.151
[e]	0.038	0.038	0.043	0.036	0.027	0.048
[f]	98.3%	98.0%	97.8%	96.6%	95.8%	97.9%

[a] Using all heavy atoms of all residues.
[b] Using all heavy atoms of residues 1–4.
[c] Using carbon and nitrogen atoms of main ring.
[d] For 'spacer' thyrosine-phenylalnine.
[e] For Cα (2), C′(2), N(3), Cα (3).
[f] The sum of relative populations of conformations above 1%.

longer peptides the Daa(4) residue is involved in this type of hydrogen bonding. From the data presented, the conclusion may be drawn that elongation of the cyclic peptide with the *C*-terminal tripeptide segment of dermorphin causes evident changes of their main ring conformation and introduces additional intramolecular interactions. Since these changes correlate with the decrease in activity of the 1–7 peptides, it is obvious that the *C*-terminal segment does not play the role of an independent *address* part in the molecule.

CONCLUSIONS

Previously we reported the highly active cyclic dermorphin analogues comprising the *N*-terminal segment 1–4 of the natural peptide, which is considered to be a *message* sequence. In this paper we report a series of analogues in which the native sequence of dermorphin 5–7 was added to the previously reported cyclic tetrapeptide structures. The resulting peptides were less active in the GPI and MVD assays than their shorter

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Table 8 Downfield shifts (in ppm) of proton chemical shifts in the spectra of dermorfins 1–7 *trans* isomers in D_2O (this paper) in respect to the corresponding proton shifts determined for dermorphins 1–4 [2] containing the same main ring

Main ring	D-Lys^2,Dab^4	D-Lys^2,Dap^4	D-Orn^2,Dab^4
Tyr^1			
H_α	−0.01	−0.03	−0.02
H_β	0.00; 0.00	0.00; −0.01	0.00; −0.03
H_{26}; H_{35}	0.01; −0.02	0.01; 0.02	0.02; 0.02
D-Daa^2			
H_N	na	0.08	na
H_α	0.03	0.01	0.06
H_β	0.02; 0.04	−0.03	0.04; 0.05
H_γ	0.01; 0.01	0.10	0.05; 0.01
H_δ	0.00; 0.03	0.07	0.04
H_ε	0.01; 0.04	0.07; 0.00	na
$H_{Nbridge}$	0.03	0.13	0.07
Phe^3			
H_N	0.13	0.14	0.16
H_α	0.13	0.05	0.08
H_β	0.16; 0.16	0.11	0.17; 0.14
H_{26}; H_{35}	0.29; 0.28	0.16; 0.14	0.24; 0.28
H4	0.15	0.09	0.12
Daa^4			
H_N	0.42	0.34	0.40
H_α	−0.04	−0.04	−0.05
H_β	0.18; 0.08	0.19; 0.11	0.24; 0.09
H_γ	0.07; 0.05	na	0.10; 0.10
$H_{Nbridge}$	0.06	0.19	0.09

na – Not assigned.

analogues. The NMR study indicated that addition of the *C*-terminal sequence resulted in changes in the ring conformations and introduced additional intramolecular interactions.

These results indicate that addition of an *address* sequence to a modified *message* sequence can produce conformational changes in the *message* and thereby may substantially modify the properties of the resulting peptide.

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