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Highly Potent Side-chain to Side-chain Cyclized Enkephalin Analogues Containing a Carbonyl Bridge: Synthesis, Biology and Conformation

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Abstract: Six novel cyclic enkephalin analogues have been synthesized. Cyclization of the linear peptides containing basic amino acid residues in position 2 and 5 was achieved by treatment with bis(4-nitrophenyl)carbonate. It was found that some of the compounds exibit unusually high μ-opioid activity in the guinea pig ileum (GPI) assay. The 18-membered analogue cyclo(N^{ε},$N^{\beta'}$-carbonyl-D-Lys^2,Dap^5)-enkephalinamide turned out to be one of the most potent μ-agonists reported so far. NMR spectra of the peptides were recorded and structural parameters were determined. The conformational space was exhaustively examined for each of them using the electrostatically driven Monte Carlo method. Each peptide was finally described as an ensemble of conformations. A model of the bioactive conformation of this class of opioid peptides was proposed. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cyclic opioid peptides; conformation; EDMC; NMR; side-chain to side-chain cyclization; structure–activity relationship; synthesis

INTRODUCTION

Cyclization of a biologically active lead linear peptide is known to often improve its biological properties, such as metabolic stability, potency and receptor selectivity. Cyclic peptides are expected to adopt conformations that are better defined than those of their linear counterparts which often exist in a conformation equilibrium. Therefore, conformational studies of cyclic peptides may provide insight into the bioactive conformation. Peptides may be cyclized through a variety of functionalities, e.g. a cysteine bridge, lactones, lactams, and at various positions: *N*-terminus to *C*-terminus, *C*-terminus to side chain, *N*-terminus to side chain, *N*-backbone to *C*-terminus or side-chain to side-chain [1]. Recently, we reported the synthesis and biological activity of the first side-chain to side-chain cyclized enkephalin analogue, cyclo(N^{ε},$N^{\varepsilon'}$-carbonyl-D-Lys^2,Lys^5)enkephalinamide **1**, in which ring formation was achieved via a ureido group incorporating the ε amino group of D-Lys^2 and Lys^5. This cyclic

Abbreviations: EDMC, electrostatically driven Monte Carlo; ECEPPAK, a program for global conformational analysis of peptides; ECEPP/3, empirical conformational energy program for peptides; SRFOPT, a model of fitting to small-molecule free energy hydration with atomic solvation parameters optimized using nonpeptide thermodynamic data; CLUST, a program for cluster analysis; part of ANALYZE; MORASS, multiple overhauser relaxation analysis and simulation; part of ANALYZE; DSS, 2,2-dimethyl-2-silapentatesulfonic acid; gHSQC, gradient heteronuclear single quantum coherence spectroscopy; PPJ-HMQC, pure phase homonuclear J-modulated heteronuclear multiple quantum coherence spectroscopy.

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enkephalin analogue containing 21-membered ring structure showed preference for μ over δ opioid receptor in an opioid bioassay *in vitro* [2].

In order to study structure–activity relationships of cyclic enkephalin analogues of this type, we employed an integrated approach, including synthesis, bioassays, NMR spectroscopy and theoretical calculation. We synthesized five new cyclic enkephalin analogues containing a carbonyl bridge using the same method as in the synthesis of **1**: reaction of bis(4-nitrophenyl) carbonate with the linear peptide containing the two unprotected side chain amino groups. The linear peptides contained D-Lys or D-Orn in position 2 and Lys, Orn, Dab (α,γ-diaminobutyric acid) or Dap (α,β-diaminopropionic acid) in position 5. Thus, 17–20 membered cyclic analogues of enkephalin were obtained. We also determined the *in vitro* opioid activity profiles of these peptides, and studied their conformations using NMR and electrostatically driven Monte Carlo (EDMC) calculation.

MATERIALS AND METHODS

Synthesis of Peptides

The amino acid derivatives Fmoc-Tyr(*t*-But), Boc-D-Lys[Z(2-Cl)], Boc-Lys[Z(2-Cl)], Boc-D-Orn(Z), Boc-Orn(Z), Boc-Dab(Z)·DCHA, Boc-Dap(Z)·DCHA were purchased from Bachem, Bubendorf, Switzerland. *N*-cyclohexyl-*N*′-isopropylcarbodiimide (CIC) was prepared as described before [3].

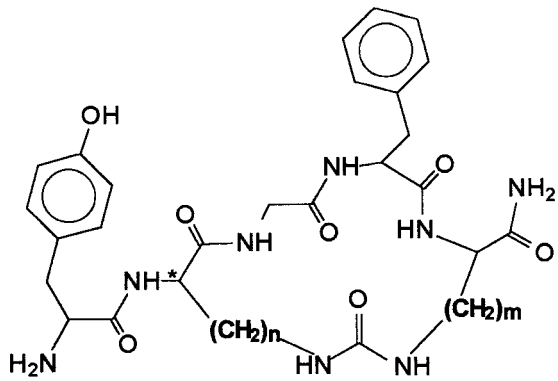
The linear fully protected peptides Fmoc-Tyr(*t*-But)-D-Daa(Z)-Gly-Phe-Daa(Z)-NH_2 were synthesized by the manual solid-phase technique, using a 4-methylbenzhydrylamine resin (0.97 meq/g) according to the following protocol: (1) deprotection with 55% TFA in CH_2Cl_2 (1×1 min,1×15 min), (2) neutralization with 5% DIEA in CH_2Cl_2 (2×2 min), (3) coupling with a mixture of 3 equivalent of the Boc-amino acid (or Fmoc-Tyr(*t*-But)-OH) and 3 equivalent of CIC.

The peptides were cleaved from the resin by treatment with liquid HF in the presence of anisole (10%) for 1 h at 0°C. After removal of the HF, the resins were extracted three times with ether, and subsequently, with 50% acetic acid ($4 \times$). The water filtrates were combined and lyophilized. Fmoc-protection of the terminal α-amino group was retained and Z-deprotection of the side-chain amino groups of Daa^2 and Daa^5 residues was achieved. The *t*-Butyl protecting group of the tyrosine hydroxyl group was removed prior to clevage with HF. The crude linear precursors Fmoc-Tyr-D-Daa_1-Gly-Phe-Daa_2-NH_2 were cyclized by reaction of bis(4-nitrophenyl)carbonate with the free amino groups of the peptide in DMF. The linear peptide (1 equivalent) was dissolved in DMF (1 mg of peptide/2 cm^3 DMF) and DIEA (2 equivalent) and bis(4-nitrophenyl) carbonate ($0.5 + 0.25 + 0.25 + 0.1 = 1.1$ equivalent) were added to the solution. The reaction was allowed to continue until free amino groups could no longer be detected (3–4 days), and the solvent was then evaporated under reduced pressure. The residue was triturated with ether.

The crude cyclic peptides were dissolved in 50% piperidine in DMF to remove the *N*-terminal Fmoc group. After 2 h, the solvents were evaporated under the reduced pressure. The residues were triturated with ether, filtrated off and lyophilized from acetic acid. The cyclic analogues **1**–**6** were purified to homogeneity by semi-preparative reversed-phase high performance liquid chromatography (HPLC) on a Vydac C-18 column (250×10 mm), using the following solvent system: $A = 0.01\%$ TFA/H_2O, $B =$ 60% CH_3CN/A, with detection at 220 nm. Purity of the product was established by analytical HPLC on a Nucleosil column (250×7 mm), using a solvent system identical to that used in the semi-preparative HPLC experiments in a linear gradient mode (0–100% *B* in 30 min) at flow rate of 1 cm^3/min, with detection at 220 nm. Molecular weights of the obtained analogues were determined by LSIM mass spectrometry. The structural formulas of cyclic peptides **1**–**6** are presented in Figure 1.

Bioassay. The guinea pig ileum (GPI) [4] and mouse vas deferens (MVD) [5] bioassay 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 IC_{50} values of the compounds being tested were normalized according to a published procedure [8].

NMR Spectroscopy and Theoretical Analysis. NMR samples were prepared as described elsewhere [9]. NMR spectra were measured at 20°C on a UNITY500 plus (Varian) spectrometer equipped with a gradient generator unit, Performa II, WFG, Ultrashims, and high stability temperature unit using a 5 mm $^1H\{^{13}C/^{15}N\}$ PFG triple probe. Spectra were measured using TOCSY [10–12] and ROESY [13,14] experiments under conditions and with parameters described elsewhere [9]. $\{^1H/^{13}C\}$gHSQC [8–10],



(1) H-Tyr-D-Lys-Gly-Phe-Lys-NH_2 (n=4, m=4)
CO

(2) H-Tyr-D-Orn-Gly-Phe-Lys-NH_2 (n=3, m=4)
CO

(3) H-Tyr-D-Orn-Gly-Phe-Orn-NH_2 (n=3, m=3)
CO

(4) H-Tyr-D-Orn-Gly-Phe-Dab-NH_2 (n=3, m=2)
CO

(5) H-Tyr-D-Orn-Gly-Phe-Dap-NH_2 (n=3, m=1)
CO

(6) H-Tyr-D-Lys-Gly-Phe-Dap-NH_2 (n=4, m=1)
CO

Figure 1 Structural formulas of cyclic analogues of enkephalin containing a carbonyl bridge.

{^{1}H/^{15}N}gHSQC [15–17] and/or PPJ-HMQC [18] experiments were performed in some cases. All spectra were analysed using VNMR 5.1A (Varian) software.

All proton NMR spectra in aqueous solution were calibrated against the water signal [19]. Calibration of the correlation spectra was done for the carbon axis using the external DSS reference signal[13] in {^{1}H/^{13}C} spectra and the NH_3 signal [20] for the nitrogen axis in the {^{1}H/^{15}N} spectra. In DMSO-d_6 solution, the residual solvent signal was used as a reference [21] in both proton and carbon dimensions.

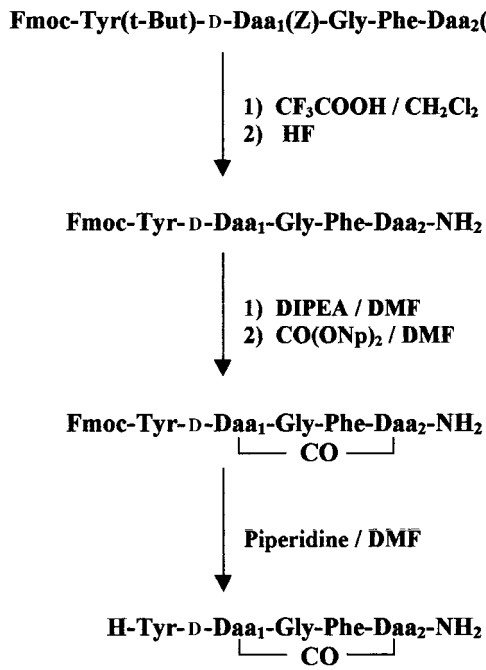
The conformational space of each peptide **1**–**6** was explored using the method previously employed by Liwo *et al.* [22], which is an EDMC method [23,24]. In our calculations, we used the ECEPPAK program that started with a conformation of random geometry whose energy was minimized with the ECEPP/3 force field [25] and surface model SRFOPT [26]. The conformation with minimized energy was subsequently perturbed by changing its torsional ψ and φ angles using the Monte Carlo method [27]. The Piela algorithm [28] was also applied at this stage. The Metropolis criterion [29] was used, when necessary, in the acceptation procedure.

The conformations were subsequently clustered into families by means of the program CLUST [30], using all heavy atoms, and the r.m.s. and energy criterion. The available version of the ECEPPAK program contains solvent parameters for peptide–water interactions. Therefore, conformations generated with parameters accounting for peptide–water interactions were used in comparison with the experimental data obtained for peptide dissolved in water and in DMSO-d_6 as well.

For the estimation of the population of particular conformations in the conformational ensemble of each peptide the NOESY spectra were generated for representative structures of each family with the program MORASS [31,32], using a correlation time of 0.45 ns, a mixing time corresponding to that used in the experiment and a cutoff value of 6.0 Å. A linear combination of the generated spectra was fitted to the experimental spectrum using the Marquardt method [33]. A convergence criterion value of 10^{-5} was used. A separate term with an additional statistical weight of 0.01 was introduced to account for couplings. In this way, the statistical weights for each of the conformational families were found.

RESULTS AND DISCUSSION

A combination of the solid-phase technique and classical method in solution was employed for the synthesis of cyclic analogues, as shown in Scheme 1. The linear fully protected peptides Fmoc-Tyr(*t*-But)-D-Daa(Z)-Gly-Phe-Daa(Z)-NH_2 were synthesized by the manual solid-phase technique using *N*-cyclohexyl-*N*′-isopropyl-carbodiimide as coupling reagent. After complete assembly of the peptide chain, 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 amino groups of side-chains of Daa[2] and Daa[5] residues was achieved. The crude linear peptides Fmoc-Tyr-D-Daa-Gly-Phe-Daa-NH_2 were cyclized by reaction with bis(4-nitrophenyl)carbonate in DMF. After cyclization removal of Fmoc groups were achieved by treatment with 50% piperidine in DMF. The cyclic analogues **1**–**6** were purified by semi-preparative



Scheme 1 Synthesis of cyclo(N^{ε},$N^{\beta'}$-carbonyl-D-Lys^2,Dap^5)enkephalinamides by a combination of solid-phase and solution methods.

reversed-phase HPLC. Homogeneity of the products was verified by analytical HPLC. Molecular weights of the obtained analogues were determined by LSIM mass spectrometry. NMR spectra of the peptides were measured, and all signals were assigned (Tables 1 and 2).

The *in vitro* opioid activity profile of the analogues was determined in the GPI assay and in the MVD assay (Table 3, Figure 2). All analogues showed higher agonist potency in the GPI assay than in the MVD assay. As it is well established that the MVD preparation contains mostly δ-receptors and the GPI preparation largely μ-receptors, these results indicate a preference of these analogues for μ-receptors over δ-receptors. In comparison with [Leu^5]enkephalin, all analogues showed higher μ-agonist potency. The most active peptides contain 17-18 membered rings (Table 3). One of them, **6**, is about 1200 times more active than Leu-enkephalin in the GPI assay, and is one of the most potent μ-agonists among the enkephalin analogues reported to date. It could be noticed that its structure is closely related to that of a similar enkephalin analogue cyclized *via* an amide bond (D-Lys^2 with Glu^5) [34], which also contains an 18-membered ring structure, and was also the most active compound of a series of cyclic lactam analogues. However, the differences in activity cannot just be explained in terms of ring size variation. For example, compound **6** is about 10 times more potent than **4**, even though both compounds contain an 18-membered ring structure.

Chemical shifts of peptides **1**–**6** in DMSO-d_6 and in water were fully assigned using methods described elsewhere [9] and are listed in Table 1 and Table 2, respectively. The amino acid composition and sequence of each new enkephalin analogue was confirmed using TOCSY and ROESY spectra. The ROESY spectra used for the structure calculation were measured using the 0.2 s mixing time. ROE signal intensities were recalculated to NOE ones according to the equation given by Croasmun and Carlson [35]. Vicinal coupling constants within the peptide in water were measured using 1D proton spectra or the PPJ-HMQC heteronuclear technique (data not shown). The temperature coefficients of the signals of the amide and ureido bridge protons were measured. None of those protons were found to be engaged in hydrogen bonding or to be protected from exchange with solvent protons.

It has been shown that EDMC calculations combined with NMR data provide a useful tool for conformational studies of cyclic peptides, especially because they allow us to search the conformational space effectively, and to describe the flexibility of a peptide in a more quantitative way [22]. The calculation procedure outlined in 'Materials and Methods' yielded a large set of conformational families for each peptide studied. The total number of conformations generated for each peptide varied between 8000 and 15000, and the number of those accepted between 2000 and 3000. The detailed procedure is described in Reference [9]. In the clustering procedure, an r.m.s.d. of 0.10–0.40 Å and a ΔE of 10–50 kcal/mol were used. The number of families after clustering varied between 600 and 1060. The total number of NOE contacts used in the analysis was 75 in the case of the DMSO solution, and 63 in the case of the water solution. The NOE spectra allowed the assignment of statistical weights for representative structures of conformational families. The use of coupling constant data did not alter the results and, therefore, we do not report them here. For subsequent analysis, we chose only those conformations whose relative population was higher than 3%. There were 58 such families for all six peptides in DMSO-d_6 and 57 in H_2O. Their populations sum up to *ca* 90% of all conformations in each case. Thechosen conformations are shown in Figures 3 and 4. The parameters that characterize the chosen conformations are listed in Table 4.

Table 1 Proton Chemical Shifts of Cyclo (N^{ω},$N^{\omega'}$-carbonyl-D-Daa^2,Daa^5)enkephalinamides in DMSO-d_6 at 20°C

Peptide	**1** (Lys^2,Lys^5)	**2** (Orn^2,Lys^5)	**3** (Orn^2,Orn^5)	**4** (Orn^2,Dab^5)	**5** (Orn^2,Dap^5)	**6** (Lys^2,Dap^5)
Tyr^1						
H_{α}	3.78	3.35	3.82	4.00	3.98	4.03
H_{β}	2.81; 2.67	2.78; 2.44	2.87; 2.74	2.83; 2.83	2.84; 2.82	2.83; 2.83
H_{26}; H_{35}	6.98; 6.66	6.96; 6.63	7.02; 6.68	7.02; 6.73	7.00; 6.68	7.00; 6.66
H_O	9.27	9.19	9.25	9.34	9.31	9.31
D-Daa^2						
H_N	8.19	7.94	8.31	8.47	8.38	8.41
H_{α}	4.24	4.35	4.38	4.56	4.44	4.30
H_{β}	1.42; 1.42	1.51; 1.40	1.57; 1.39	1.36; 1.46	1.36; 1.48	1.40; 1.40
H_{γ}	1.08; 1.08	1.21; 1.21	1.19; 1.10	1.18; 1.05	1.10; 1.14	1.05; 1.05
H_{δ}	1.26; 1.26	3.00; 2.88	3.00; 2.87	3.06; 2.78	2.94; 2.73	1.27; 1.27
H_{ε}	3.06; 2.82	–	–	–	–	3.02; 2.88
Gly^3						
H_N	8.26	8.10	8.04	8.14	8.29	8.37
H_{α}	4.08; 3.38	3.93; 3.40	3.98; 3.40	4.00; 3.53	4.28; 3.30	4.23; 3.39
Phe^4						
H_N	8.43	8.25	8.17	8.47	8.35	8.39
H_{α}	4.59	4.43	4.31	4.46	4.30	4.30
H_{β}	3.04; 2.75	2.97; 2.76	3.04; 2.85	2.98; 2.80	3.03; 2.82	3.00; 2.80
H_{26}; H_{35}	7.34; 7.29	7.28; 7.29	7.19; 7.25	7.26; 7.27	7.26; 7.26	7.25; 7.25
H_4	7.19	7.19	7.23	7.19	7.19	7.18
Daa^5						
H_N	8.22	8.06	7.83	7.99	8.45	8.32
H_{α}	4.20	4.14	4.15	4.30	4.15	4.25
H_{β}	1.69; 1.50	1.66; 1.49	1.64; 1.44	1.76; 1.69	3.50; 3.00	3.58; 2.86
H_{γ}	1.28; 1.28	1.27; 1.27	1.44; 1.27	2.96; 2.96	–	–
H_{δ}	1.32; 1.32	1.34; 1.34	2.99; 2.92	–	–	–
H_{ε}	2.94; 2.94	2.99; 2.99	–	–	–	–
NH_2	7.00; 7.21	6.99; 7.02	6.96; 6.99	7.06; 7.07	6.81; 7.15	6.83; 7.14
$H_{Nbridge}$	5.66; 5.48	5.79; 5.64	5.70; 5.77	5.89; 5.85	5.71; 5.88	5.55; 5.60

It is well known that the presence of both the Tyr^1 and the Phe^4 residue in enkephalin analogues is required for opioid activity [1]. Therefore, it may be assumed that the conformation of these two residues, as well as the distance between their aromatic rings, is of great importance for the peptide activity. In a cyclic peptide containing the ureido bridge, these conformational factors may be regulated by the size of the main ring. Therefore, in this study, a series of peptides with different size of the main ring structure (17-21 membered) was used. We expected to observe different biological activities and different conformational equilibria within this series.

Inspection of the data presented in Table 4 reveals a large diversity of conformations of the peptide chain found for each peptide. This is the case for the entire peptide chain, and it applies also for the peptide segment situated between the Phe and Tyr residues, the so-called spacer segment. However, the conformational diversity clearly decreases when one goes from peptide **1** to **6**. This can be seen from inspection of Figures 3 and 4, and it is even more evident from the r.m.s.d. data given in Table 5. This tendency is clear for the r.m.s.d. calculated using heavy atoms of the entire molecule, for the r.m.s.d. calculated using carbon and nitrogen atoms of the main ring and for the r.m.s.d. calculated using heavy atoms of the 'spacer'. Even though the peptide is rather flexible in both solvents, the influence of the solvent type on the

Table 2 Proton Chemical Shifts of Cyclo (N^{ω},$N^{\omega'}$-carbonyl-D-Daa^2,Daa^5)enkephalinamides in Water at 20°C

Peptide	**1** (Lys^2,Lys^5)	**2** (Orn^2,Lys^5)	**3** (Orn^2,Orn^5)	**4** (Orn^2,Dab^5)	**5** (Orn^2,Dap^5)	**6** (Lys^2,Dap^5)
Tyr^1						
H_{α}	4.15	4.00	4.13	4.12	4.14	4.13
H_{β}	3.19; 2.95	3.12; 2.94	3.15; 2.92	3.17; 2.93	3.16; 2.96	3.17; 2.96
H_{26}; H_{35}	7.11; 6.86	7.11; 6.85	7.11; 6.86	7.11; 6.86	7.10; 6.84	7.21; 6.95
D-Daa^2						
H_N	8.30	8.43	8.45	8.38	8.31	8.25
H_{α}	4.08	4.02	4.03	4.08	4.14	4.18
H_{β}	1.42; 1.42	1.45; 1.35	0.98; 0.98	1.42; 1.42	1.38; 1.33	1.42; 1.31
H_{γ}	0.93; 0.93	1.09; 0.90	3.00; 1.35	1.17; 0.94	1.07; 0.97	1.02; 0.89
H_{δ}	1.36; 1.36	2.93; 2.87	2.99; 2.99	3.03; 2.83	2.94; 2.87	1.31; 1.31
H_{ε}	3.09; 3.03	–	–	–	–	3.12; 2.94
Gly^3						
H_N	8.26	8.21	8.31	8.35	8.42	8.43
H_{α}	3.99; 3.74	3.91; 3.65	4.00; 3.69	4.10; 3.66	4.18; 3.60	4.05; 3.70
Phe^4						
H_N	8.28	8.15	8.21	8.13	8.04	8.14
H_{α}	4.54	4.28	4.41	4.45	4.50	4.43
H_{β}	3.05; 3.02	3.01; 2.92	3.02; 2.95	3.07; 2.97	3.09; 2.96	3.09; 2.94
H_{26}; H_{35}	7.35; 7.26	7.24; 7.34	7.22; 7.33	7.22; 7.34	7.20; 7.32	7.33; 7.44
H_4	7.29	7.29	7.29	7.29	7.28	7.39
Daa^5						
H_N	8.27	8.12	8.07	8.32	8.23	8.23
H_{α}	4.16	4.03	4.15	4.29	4.36	4.33
H_{β}	1.72; 1.59	1.69; 1.57	1.46; 1.46	1.90; 1.70	3.48; 3.11	3.56; 2.98
H_{γ}	1.35; 1.26	1.30; 1.21	1.68; 1.29	3.11; 3.03	–	–
H_{δ}	1.44; 1.36	1.37; 1.37	2.99; 2.99	–	–	–
H_{ε}	3.05; 3.05	3.00; 3.00	–	–	–	–
NH_2	6.91; 6.59	6.93; 6.55	6.90; 6.42	6.95; 6.53	6.98; 6.69	7.02; 6.64

Table 3 GPI and MVD Assay of Cyclic Opioid-peptide Analogues Cyclo(N^{ω},$N^{\omega'}$-carbonyl-D-Daa^2,Daa^5)enkephalinamides

Compound			Ring size	GPI		MVD		MVD/GPI IC_{50} ratio
No.	Daa^2	Daa^5		IC_{50} [nM][a]	Rel. potency	IC_{50} [nM][a]	Rel. potency	
1	Lys	Lys	21	20.5 ± 2.1[b]	12.0 ± 1.2	73.5 ± 4.3[b]	0.155 ± 0.009	3.59
2	Orn	Lys	20	10.6 ± 1.2	23.2 ± 2.6	41.3 ± 10.8	0.276 ± 0.072	3.90
3	Orn	Orn	19	4.68 ± 0.70	52.6 ± 7.9	20.2 ± 5.7	0.564 ± 0.159	4.32
4	Orn	Dab	18	2.05 ± 0.09	120 ± 5	8.70 ± 0.20	1.31 ± 0.03	4.24
5	Orn	Dap	17	1.64 ± 0.46	150 ± 42	12.5 ± 1.0	0.912 ± 0.073	7.62
6	Lys	Dap	18	0.212 ± 0.01	1160 ± 55	0.651 ± 0.015	17.5 ± 0.04	3.07
[Leu^5]enk				246 ± 39	1	11.4 ± 1.1	1	0.046

[a] Mean of 3-4 determination ± S.E.M.
[b] Reference [2].

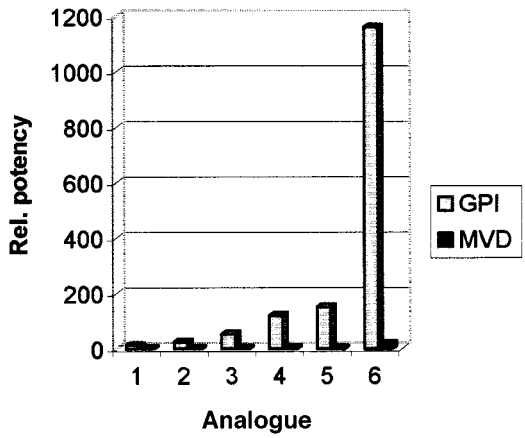


Figure 2 GPI and MVD assay of cyclic opioid-peptides **1**–**6**. Potencies are indicated relative to that of [Leu^5]enkephalin (potency = 1).

conformation flexibility is also evident in the series. It is obvious that water strongly diminishes the conformational diversity.

We calculated the distance between the centres of the two aromatic ring for each conformation. The data are shown in Figure 5 and Table 4. It is evident that the biologically most active peptide **6** has mostly conformations with a large intraring distance. We also calculated Euler angles between aromatic rings, but the orientation of the rings appeared not to be significant.

Analysis of the data in Table 4 for the most active peptide **6** determined in water indicated that the most populated conformers of the peptide are rather similar to each other. In particular, this is the case for the torsional angles of the 'spacer'. This led to the conclusion that the proper conformation of the

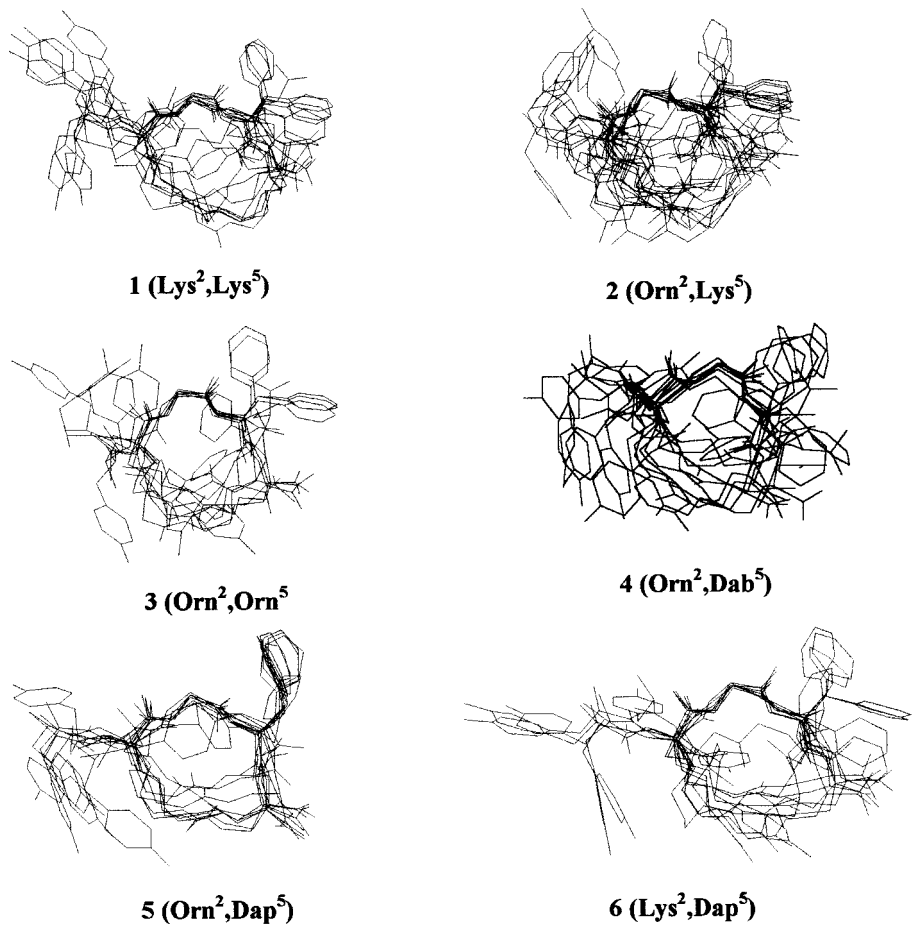


Figure 3 MOLMOL [36] drawings of conformations with a relative population above 3% in DMSO-d_6. The structures are aligned using heavy atoms of the 'spacer' between tyrosine and phenylalanine.

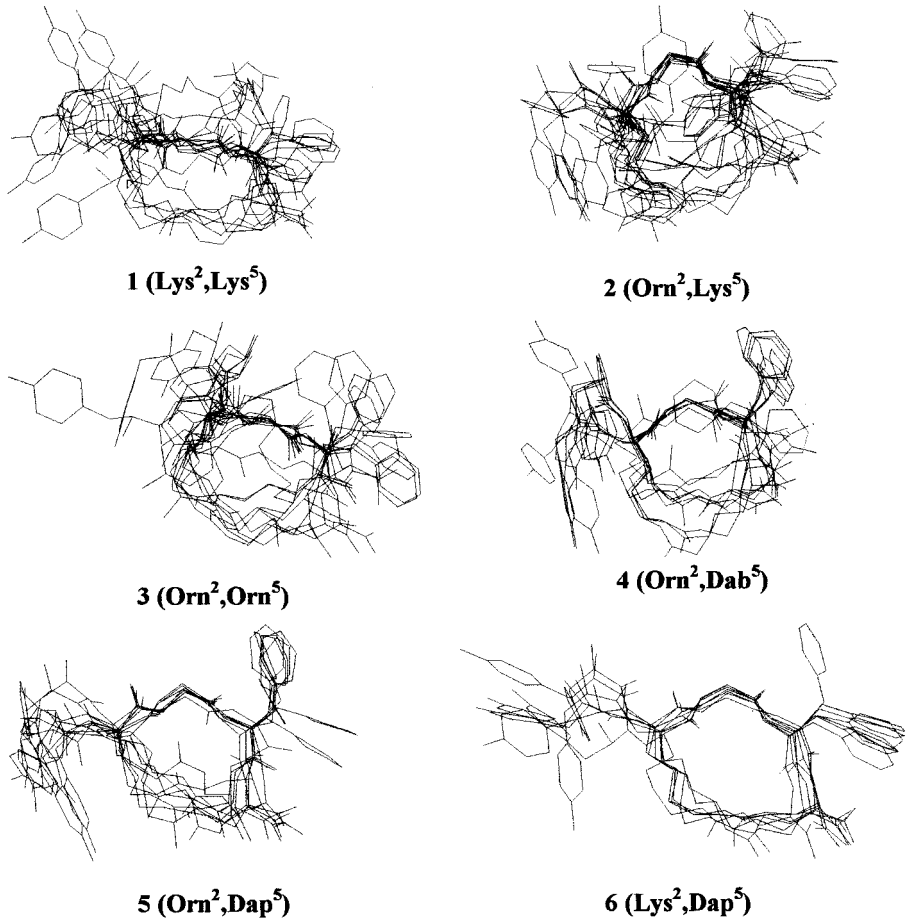


Figure 4 MOLMOL [36] drawings of conformations with relative population above 3% in H_2O. The structures are aligned using heavy atoms of the 'spacer' between tyrosine and phenylalanine.

'spacer' is also important for the very high biological activity observed for peptide **6**. It is very probable that the most populated conformations of analogue **6** closely resemble its bioactive conformation. The proposed bioactive conformation is shown in Figure 6.

Among the conformations of the other peptides examined, only very few have a conformation similar to that which is predominant in the case of **6**. This observation, combined with the information on the distance between the aromatic rings, seems to correlate well with the variation in biological activity observed within this series of compounds. The diagram shown on Figure 7 correlates the distances between the aromatic rings calculated for each analysed conformation with the r.m.s.d. calculated for torsional angles of the 'spacer', using angles of the 'ideal' conformation as a reference. One may see that two families of conformations may be distinguished on this basis. The reference conformers of peptide **6** all fall in the family with small 'spacer' angles r.m.s.d.. This is also true for conformers of the peptide **5**. The conformers of peptide **4**, **3** and **2** share families with both small and large r.m.s.d. values, with an increasingly larger proportion of families with large r.m.s.d. values. This trend is accompanied by a decrease of the distance between the aromatic rings. From that picture, one may conclude that the distance between rings and the conformation of the 'spacer', both affect the activity of the peptide. The conformational and behaviour of peptide **1** is different, and may be explained in terms of a much larger structural flexibility of its 'spacer'. Its r.m.s.d. value is much higher than that observed with the other peptides in water. Therefore, one may assume that, in the case of this peptide, the entropic effect may already be significant, and may be the reason for its relatively low biological activity.

Table 4 Parameters for the Most Populated Conformations (with Populations above 3%) Found in Water

	χ_1(**1**)	ψ(**1**)	φ(**2**)	ψ(**2**)	φ(**3**)	ψ(**3**)	φ(**4**)	χ_1(**4**)	r	en	pop
1 (Lys^2,Lys^5)											
1w-1	−180	153	76	**25**	**180**	**60**	**−86**	−63	10.8	0.0	17.3
1w-2	−176	155	75	**41**	**179**	**59**	**−112**	57	16.8	5.8	16.0
1w-3	−66	−37	147	**−136**	**156**	**−141**	**−154**	−179	16.2	3.0	10.7
1w-4	−174	155	76	**46**	**−146**	**33**	**−115**	−50	13.1	4.3	9.7
1w-5	−64	150	148	**−104**	**173**	**−150**	**−146**	179	13.0	3.3	9.7
1w-6	−66	−38	147	**−119**	**151**	**−39**	**−89**	−59	14.2	5.9	7.9
1w-7	−71	−42	72	**52**	**−157**	**−54**	**−78**	−59	13.7	5.5	5.3
1w-8	58	11	156	**57**	**171**	**60**	**−100**	−49	12.4	7.0	4.9
1w-9	−60	−39	152	**−148**	**85**	**46**	**−160**	174	13.1	5.7	4.2
1w-10	−63	−37	136	**−31**	**−149**	77	**−140**	64	15.2	4.4	4.0
Iw-11	−63	−36	79	**28**	**−179**	**−59**	**−77**	−176	13.2	6.3	3.3
2 (Orn^2,Lys^5)											
2w-1	−65	154	77	**39**	**81**	**−74**	**−162**	62	12.6	3.8	24.7
2w-2	−173	141	77	**35**	**73**	**−38**	**−160**	180	12.7	3.9	13.8
2w-3	−169	131	117	**−32**	**−82**	**−39**	**−158**	179	10.3	2.2	10.8
2w-4	179	136	82	**−141**	**81**	**19**	**−92**	−179	11.1	9.8	10.6
2w-5	−176	139	81	**−159**	**167**	**−91**	**−161**	−179	14.9	4.4	9.1
2w-6	−176	145	83	**35**	**−162**	**−55**	**−141**	179	11.4	1.0	5.2
2w-7	−179	138	80	**−141**	**81**	**21**	**−95**	−179	11.2	5.7	5.1
2w-8	179	118	−56	**−34**	**−73**	**−13**	**−68**	−174	8.0	3.7	4.7
2w-9	−178	109	157	**54**	**158**	**58**	**−119**	54	5.1	0.0	4.4
2w-10	−66	154	78	**36**	**−138**	**27**	**−94**	−52	6.5	3.0	4.0
3 (Orn^2,Orn^5)											
3w-1	−169	163	84	**20**	**−174**	**−54**	**−123**	−63	11.0	2.9	18.0
3w-2	−68	103	154	**−138**	**161**	**−49**	**−94**	−56	14.5	4.8	15.6
3w-3	−178	167	77	**37**	**180**	**50**	**−158**	177	9.6	1.8	13.4
3w-4	−179	141	79	**25**	**−152**	**31**	**−102**	−177	9.6	2.4	9.8
3w-5	−62	141	84	**32**	**−140**	**−58**	**−146**	−60	6.1	2.5	8.6
3w-6	−174	143	78	**28**	**−95**	**−66**	**−159**	178	11.5	2.4	4.7
3w-7	−177	158	73	**24**	**−179**	**−52**	**−135**	57	11.2	9.0	4.5
3w-8	−176	136	86	**−139**	**90**	**−70**	**−139**	180	13.6	3.5	4.3
3w-9	−177	138	85	**36**	**−149**	**−61**	**−146**	−64	11.4	0.0	3.6
3w-10	−174	−36	152	**61**	**−159**	**44**	**−97**	178	11.9	1.9	3.4
4 (Orn^2,Dab^5)											
4w-1	−176	155	76	**42**	**−142**	**37**	**−91**	−175	9.7	2.2	22.0
4w-2	−64	154	78	**37**	**−92**	**−52**	**−160**	53	10.6	2.8	14.2
4w-3	−176	139	82	**−155**	**87**	**−75**	**−132**	−63	12.9	3.3	13.0
4w-4	−177	154	88	**−138**	**155**	**−153**	**−84**	−60	13.6	3.9	10.0
4w-5	−172	143	94	**−162**	**77**	**−78**	**−119**	−63	12.5	6.9	8.8
4w-6	−174	138	85	**−159**	**87**	**−80**	**−137**	−63	12.9	3.1	8.6
4w-7	−178	154	77	**43**	**−155**	**50**	**−108**	−50	5.5	3.6	3.8
4w-8	−175	154	77	**37**	**−92**	**−52**	**−161**	53	9.9	0.0	3.3
4w-9	−178	141	75	**32**	**−94**	**−64**	**−138**	−62	12.4	3.9	3.3
4w-10	−177	141	78	**34**	**−97**	**−76**	**−141**	−62	12.5	3.4	3.1
5 (Orn^2,Dap^5)											
5w-1	−177	115	154	**−154**	**98**	**−98**	**−141**	−64	12.9	9.5	25.7
5w-2	−179	154	84	**−134**	**179**	**−74**	**−154**	179	14.5	1.9	22.6
5w-3	−178	154	84	**−132**	**140**	**−93**	**−139**	−64	13.1	2.4	11.5
5w-4	−175	159	79	**−107**	**170**	**−64**	**−137**	−55	12.6	2.8	10.3

Table 4 (Continued)

	χ_1(**1**)	ψ(**1**)	φ(**2**)	ψ(**2**)	φ(**3**)	ψ(**3**)	φ(**4**)	χ_1(**4**)	r	en	pop
5w-5	−179	154	85	**−77**	**94**	**−83**	**−135**	−64	12.6	1.2	5.9
5w-6	178	139	154	**−112**	**170**	**−84**	**−98**	−56	12.2	1.8	5.6
5w-7	−173	135	156	**−162**	**170**	**−89**	**−127**	55	10.8	2.5	4.6
5w-8	−177	125	155	**−169**	**158**	**−143**	**−116**	−56	13.2	0.0	4.5
5w-9	−176	155	86	**−147**	**79**	**−69**	**−158**	179	12.1	5.1	3.4
6 (Lys^2,Dap^5)											
6w-1	−68	136	146	**−101**	**87**	**−92**	**−158**	179	16.6	6.8	24.1
6w-2	63	163	80	**−155**	**92**	**−81**	**−153**	178	15	2.0	17.0
6w-3	−64	152	83	**−136**	**172**	**−104**	**−157**	176	17.2	1.3	16.2
6w-4	−64	152	83	**−136**	**100**	**−98**	**−159**	177	16.7	2.7	11.8
6w-5	68	170	80	**−138**	**167**	**−137**	**−129**	−62	11.7	2.5	10.9
6w-6	−66	142	146	**−134**	**126**	**−106**	**−154**	178	17.3	1.8	7.8
6w-7	−175	154	83	**−143**	**144**	**−86**	**−154**	−179	14.5	0.0	3.1

Values of selected torsional angles for the Tyr-Phe 'spacer', distance between tyrosine and phenylalanine ring centres (r in Å), relative calculated energy (en in kcal/mol) and relative populations of conformers (pop in %). Torsional angles of the 'spacer' are given in bold.

Table 5 R.m.s.d. (in Å) Calculated for the Conformations with the Relative Population above 3%

	1 (Lys^2,Lys^5)	**2** (Orn^2,Lys^5)	**3** (Orn^2,Orn^5)	**4** (Orn^2,Dab^5)	**5** (Orn^2,Dap^5)	**6** (Lys^2,Dap^5)
DMSO-d_6[a]	2.23	2.33	2.51	2.04	1.65	2.10
H_2O[a]	1.93	2.16	1.93	1.65	1.50	1.45
DMSO-d_6[b]	0.97	0.87	0.76	0.81	0.65	0.76
H_2O[b]	0.99	0.83	0.79	0.71	0.82	0.61
DMSO-d_6[c]	0.33	0.33	0.27	0.39	0.27	0.23
H_2O[c]	0.47	0.33	0.23	0.32	0.23	0.22

[a] r.m.s.d. calculated using all heavy atoms.
[b] r.m.s.d. calculated using carbon and nitrogen atoms of main ring.
[c] r.m.s.d. calculated for tyrosine-phenylalanine 'spacer' only, using seven heavy atoms of the backbone: carbon atoms C_α and C_O of the residue D-Daa$^2_1$; N, C_α, C_O of Gly^3; and N, C_α of Phe^4.

CONCLUSION

Six novel cyclic enkephalin analogues have been synthesized. The cyclization was accomplished by incorporation of the side-chain amino group of various basic amino acids in position 2 (D-Lys, D-Orn) and 5 (Lys, Orn, Dab, Dap) into a urea residue. All analogues showed higher opioid activity than the previously prepared 21-membered cyclo(N^ε,$N^{\varepsilon\prime}$-carbonyl-D-Lys^2, Lys^5)enkephalinamide. Compound **6**, containing an 18-membered ring, showed 1160 times higher μ-agonist potency than [Leu^5]-enkephalin. It is one of the most potent μ-agonist reported to date. The distribution of conformations arrived at from NMR measurements, and theoretical calculations indicate that the conformational freedom is clearly more restricted in aqueous solution than in DMSO solution. This is especially the case for the most active peptide **6**. The highly populated conformation of **6**, in which the two aromatic side-chains are oriented in opposite directions from one another with a 14–18 Å separation is likely to closely resemble the bioactive conformation at the μ-receptor (Figure 6).

Additional tables are available on request from JW (jacekw@ibb.waw.pl).

(A) Number of conformations generated (a) and accepted (b) for each cyclo(N^ω,$N^{\omega\prime}$-carbonyl-D-Daa^2,Daa^5)enkephalinamide.

(B) Parameters used in clustering procedure and numbers of families of conformations found for each cyclo(N^ω,$N^{\omega\prime}$-carbonyl-D-Daa^2,Daa^5)-enkephalinamide.

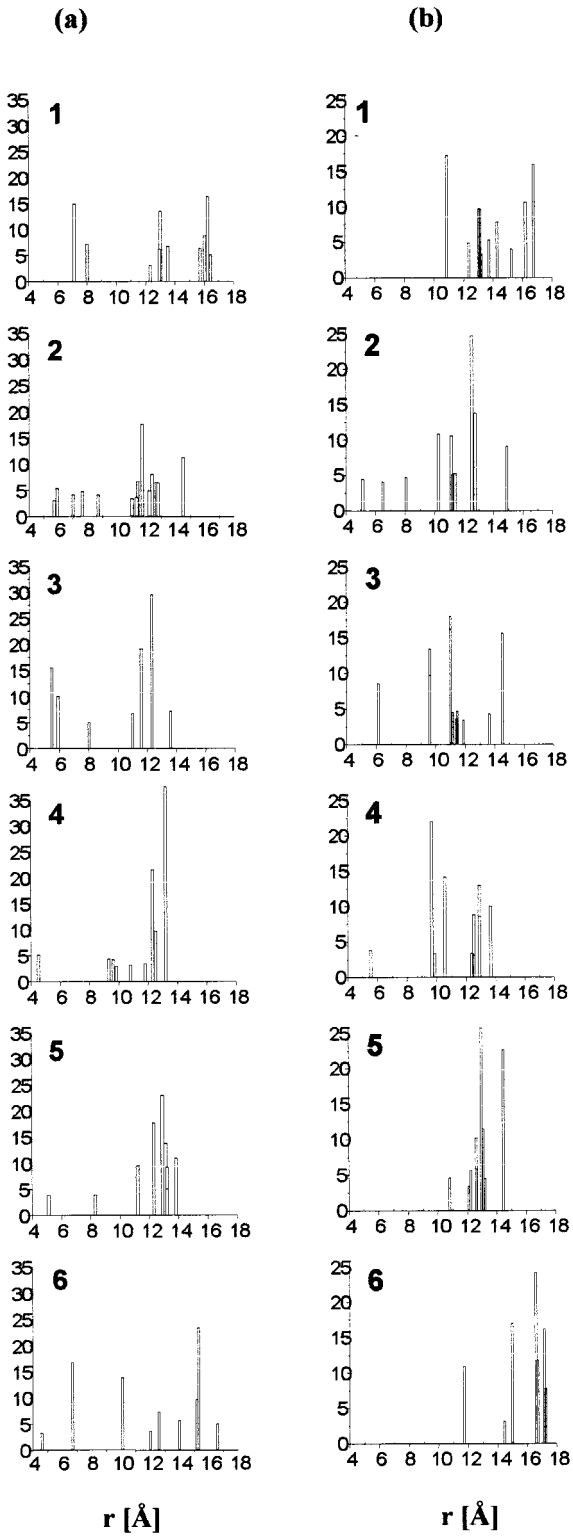


Figure 5 Distribution of the distances between the centres of the tyrosine and phenylalanine aromatic rings in conformations with a relative population above 3%. The height of the bars reflects the relative population. (a) in DMSO-d_6, (b) in water.

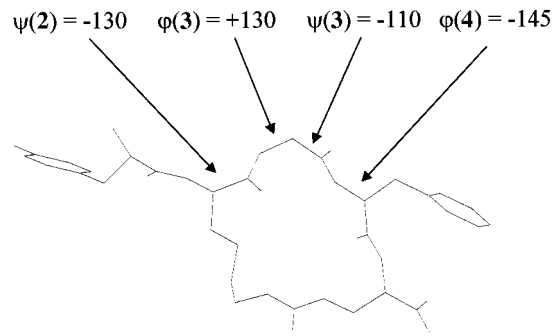


Figure 6 MOLMOL [36] drawing of the conformation which is likely to be the biologically active one. Mean torsional angles of part of the main chain are specified.

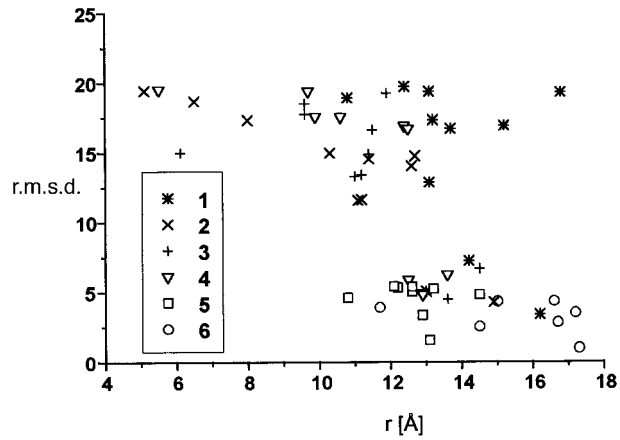


Figure 7 Diagram showing the correlation of the distances between the centres of the aromatic rings and the arbitrary calibrated r.m.s.d. values calculated for torsional angles ψ(**2**), φ(**3**), ψ(**3**) and φ(**4**) in the 'spacer' segment.

(C) Number of NOE contacts found in the spectra of cyclo (N^{ω},$N^{\omega'}$-carbonyl-D-Daa^2,Daa^5)-enkephalinamides measured in DMSO-d_6 (a) and water (b) at 20°C.

(D) Parameters for the most populated conformations (with populations above 3%) found in DMSO. Values of selected torsional angles for the Tyr,Phe 'spacer', distance between tyrosine and phenylalanine ring centres (r in Å), relative calculated energy (en in kcal/mol) and relative populations of conformers (pop in %). Torsional angles of the 'spacer' are given in bold.

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