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# Potent Side-Chain to Side-Chain Cyclized Dermorphin Analogues Containing a Carbonyl Bridge

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Abstract: A new family of cyclic opioid peptide analogues related to the 1–4 sequence of dermorphin/deltorphin (Tyr-D-Aaa$^2$-Phe-Aaa$^4$-$NH_2$) has been synthesized. The synthesis of the linear precursor peptides was accomplished by the solid-phase method and ring formation was achieved via a ureido group incorporating the side chain amino functions of D-Aaa$^2$ (D-Lys, D-Orn) and Aaa$^4$ (Lys, Orn, Dab, Dap). The peptides were tested in the guinea-pig ileum (GPI) and mouse vas deferens (MVD) assays. Most showed very high agonist potency in the GPI assay. The peptide containing D-Lys in position 2 and Dab in position 4 was 210 times more active than enkephalin, and that containing Orn and Dab, respectively, was 150 times more active than enkephalin. The latter peptide was also very active in the MVD assay, and showed an $IC_{50}$ MVD/GPI ratio of 0.816. NMR spectra of selected peptides were recorded, and structural parameters were determined. The conformational space of the peptides was examined using the electrostatically driven Monte Carlo method. With the help of the NMR spectra each peptide was described as an ensemble of conformations. The conformations have been interpreted with regard to the opioid activities, and comparisons have been made with a model proposed earlier for enkephalin analogues. 

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

## INTRODUCTION

The conformation of linear peptides can be effectively restricted through incorporation of cyclic structural elements. Cyclization through covalent linkage between two side chains or between a side chain and a terminal group has produced opioid peptide analogues with improved biological properties, such as metabolic stability, potency and receptor selectivity [1]. Conformational studies of highly active cyclic peptide analogues may provide indirect information about the topological requirements within the peptide–receptor complex. Cyclization can be achieved by the formation of a disulfide between half-cysteine [2] and penicillamine residues

Abbreviations: EDMC, *E*lectrostatically *D*riven *M*onte *C*arlo; ECEPPAK, a program for global conformational analysis of peptides; ECEPP/3, *E*mpirical *C*onformational *E*nergy *P*rogram for *P*eptides; 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, *M*ultiple *O*verhauser *R*elaxation *A*nalysis*S* and *S*imulation; part of ANALYZE; DSS, 2,2-dimethyl-2-silapentate-sulfonic acid.

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[3,4], amide bond formation between a side chain amino group of an $\alpha,\omega$-diamino acid residues and the *C*-terminal carboxyl group [5,6] or a side chain carboxyl group of a glutamic or aspartic acid residue [7]. Cyclic analogues of enkephalin containing a carbonyl bridge between the side chain amino functions of $\alpha,\omega$-diamino acid residues have also been reported [8]. Recently, the synthesis was reported of several enkephalin analogues of the latter type, containing various dibasic amino acid residues in positions 2 and 5 and were cyclized via the incorporation of the $\omega$-amino groups into a urea unit [9]. All these peptides were very potent in the GPI assay, and only slightly less potent in the MVD assay. The 18-membered analogue cyclo($N^{\varepsilon}$,$N^{\beta'}$-carbonyl-D-Lys$^2$,Dap$^5$)-enkephalinamide turned out to be one of the most potent μ-agonists reported so far.

In the present paper the synthesis, *in vitro* opioid activity profiles and conformations of a series of dermorphin/deltorphin analogues are described. The *N*-terminal portion of these naturally occurring opioid peptides differs from enkephalin in that one amino acid residue between Tyr and Phe is missing. As a result, the cyclic structures are smaller than those obtained by the corresponding modification of enkephalin.

## MATERIALS AND METHODS

### Synthesis of Peptides

The amino acid derivatives were purchased from Bachem (Bubendrof, Switzerland), Fluka (Buchs, Switzerland) and Chem-Impex International (Wood Dale, USA). *N*-cyclohexyl-*N'*-isopropylcarbodiimide (CIC) was used as the coupling reagent; it was synthesized as described earlier [10].

The linear, fully protected precursor peptides, Fmoc-Tyr(t-But)-D-Daa$^2$(Z)-Phe-Daa$^4$(Z)-$NH_2$, were prepared by manual solid phase peptide synthesis (SPPS) on a 4-methylbenzhydrylamine resin (0.85 meq/g; 1.176 g). The synthesis was carried out according to standard procedures: (1) deprotection with 55% TFA in DCM (1 × 1 min, 1 × 15 min), (2) neutralization with 5% DIPEA in DCM (2 × 2 min), (3) coupling with a mixture of 3 equivalents of CIC and 3 equivalents of the Boc-amino acid (or Fmoc-Tyr(*t*-But)-OH) for 2 h. Completion of the coupling step was monitored by the ninhydrin test. Side chain deprotection and cleavage of the peptides from the solid support was performed by treatment with liquid HF in the presence of anisole (10%) for 1 h at 0 °C. Fmoc protection of the terminal $\alpha$-amino group was retained under these conditions and the t-butyl protecting group of the tyrosine hydroxyl group was removed prior to cleavage with HF. After removal of HF, the resins were washed three times with ether and subsequently extracted with 50% acetic acid (4×). The extracts were combined and lyophilized.

The crude linear tetrapeptides were next reacted with bis(4-nitrophenyl)carbonate to form the cyclic peptides. Then 1 equivalent of a linear precursor was dissolved in DMF and 2 equivalents of DIPEA 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 (4–5 days). The solvent was then evaporated under reduced pressure and the residue was triturated with ether. For peptides **4** and **8** the cyclization was also performed using di(*N*-succinimidyl) carbonate and *N*,*N'*-carbonyldiimidazole, respectively. In both cases, the obtained products were contaminated with a large amount of by-products and the use of these reagents was therefore abandoned. The *N*-terminal Fmoc group was next removed by reaction of the crude, cyclic peptides with 50% piperidine in DMF. After 2 h the solvent was evaporated and the residue was washed with ether, filtered and lyophilized from acetic acid. Purification of the cyclic analogues **1**–**8** was accomplished 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. The purity of the final products was assessed by analytical HPLC on a Nucleosil column (250 × 7 mm) in a linear gradient mode (0–100% B in 30 min) at a flow rate of 1 $cm^3$/min, using the solvent system described above. The crude products were obtained as a lyophilizate (60–120 mg). After HPLC purification the final yield was 10–30 mg. Molecular weights of the cyclic peptides were determined by LSIMS mass spectrometry: **1** ($C_{31}H_{43}N_7O_6$), M calcd 609.4, (M + 1) obtained 610.4; **2** ($C_{30}H_{41}N_7O_6$), M calcd 597.4, (M + 1) obtained 598.4; **3** ($C_{29}H_{39}N_7O_6$), M calcd 581.3, (M + 1) obtained 582.3; **4** ($C_{28}H_{37}N_7O_6$), M calcd 567.4, (M + 1) obtained 568.4; **5** ($C_{30}H_{41}N_7O_6$), M calcd 597.4, (M + 1) obtained 598.4; **6** ($C_{29}H_{39}N_7O_6$), M calcd 581.3, (M + 1) obtained 582.3; **7** ($C_{28}H_{37}N_7O_6$), M calcd 567.4, (M + 1) obtained 568.4; **8** ($C_{27}H_{35}N_7O_6$), M calcd 553.3, (M + 1) obtained 554.3. The structural

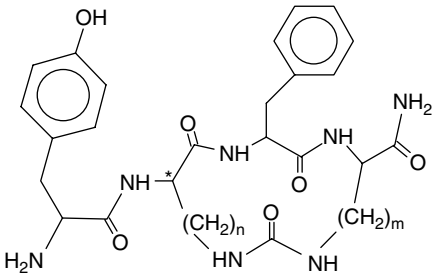
 

formulas of these cyclic dermorphin analogues are presented in Figure 1.

***Bioassay.*** The GPI [11] and MVD [12] bioassay were carried out as reported in detail elsewhere [13,14]. A log dose-response curve was determined with [Leu$^5$]-enkephalin as standard for each ileum and vas deferens preparation and the $IC_{50}$ values of the compounds being tested were normalized according to a published procedure [15].

## NMR Spectroscopy and Theoretical Analysis

NMR samples were prepared according to a previously described procedure [9]. NMR spectra were measured at 25 °C on a UNITY500plus (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. TOCSY [16–18] and ROESY [19–20] experiments were performed using the conditions and parameters reported elsewhere [21]. All spectra were analysed using VNMR 5.1A (Varian) software. All proton spectra in aqueous solution were calibrated against the water signal [22].




| | | |
|---|---|---|
| **1** | Lys$^2$/Lys$^4$ | (n = 4, m = 4) |
| **2** | Lys$^2$/Orn$^4$ | (n = 4, m = 3) |
| **3** | Lys$^2$/Dab$^4$ | (n = 4, m = 2) |
| **4** | Lys$^2$/Dap$^4$ | (n = 4, m = 1) |
| **5** | Orn$^2$/Lys$^4$ | (n = 3, m = 4) |
| **6** | Orn$^2$/Orn$^4$ | (n = 3, m = 3) |
| **7** | Orn$^2$/Dab$^4$ | (n = 3, m = 2) |
| **8** | Orn$^2$/Dap$^4$ | (n = 3, m = 1) |

Figure 1 Structural formulas of cyclic dermorphin/deltorphin analogues.

The conformational space of each peptide **3**, **4**, **7** and **8** was explored using the method proposed by Liwo *et al.* [23] which makes use of the *E*lectrostatically *D*riven *M*onte *C*arlo, EDMC method [24,25]. In our calculations the ECEPPAK program was used starting with a conformation of random geometry whose energy was minimized with the ECEPP/3 force field [26] and surface model SRFOPT [27]. The conformation with minimized energy was subsequently perturbed by changing its torsional $\psi$ and $\varphi$ angles using the Monte Carlo method [28]. The Piela algorithm [29] was also applied at this stage. The Metropolis criterion [30] was used when necessary in the acceptance procedure. The conformations were subsequently clustered into families using the program CLUST [31], using all heavy atoms and r.m.s. 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 [32,33], using a correlation time of 0.45 ns; a mixing time corresponding to that used in the experiment and a cut-off value of 6.0 Å. A linear combination of the generated spectra was fitted to the experimental

Table 1 GPI and MVD Assay of Cyclo($N^{\omega}$,$N^{\omega'}$-carbonyl-D-Daa$^2$, Daa$^4$)dermorphin(1–4)-$NH_2$ Analogues

| Compound | | | | GPI | | MVD | | MVD/GPI |
|---|---|---|---|---|---|---|---|---|
| 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 | 1150 ± 30 | 0.214 ± 0.006 | 991 ± 207 | 0.0115 ± 0.0024 | 0.861 |
| 2 | 17 | Lys | Orn | 15.6 ± 1.6 | 15.8 ± 1.6 | 47.1 ± 3.5 | 0.242 ± 0.018 | 3.01 |
| 3 | 16 | Lys | Dab | 1.17 ± 0.25 | 210 ± 45 | 5.02 ± 1.39 | 2.27 ± 0.63 | 4.29 |
| 4 | 15 | Lys | Dap | 4.15 ± 0.36 | 59.3 ± 5.1 | 19.5 ± 2.3 | 0.585 ± 0.069 | 4.70 |
| 5 | 17 | Orn | Lys | 12.2 ± 1.3 | 20.2 ± 2.1 | 15.9 ± 2.1 | 0.717 ± 0.095 | 1.30 |
| 6 | 16 | Orn | Orn | 4.77 ± 0.71 | 51.6 ± 7.7 | 11.4 ± 1.5 | 1.00 ± 0.13 | 2.39 |
| 7 | 15 | Orn | Dab | 1.63 ± 0.15 | 151 ± 14 | 1.33 ± 0.23 | 8.57 ± 1.48 | 0.816 |
| 8 | 14 | Orn | Dap | 3.37 ± 0.09 | 73.0 ± 1.9 | 7.76 ± 1.27 | 1.47 ± 0.24 | 2.30 |
| [Leu$^5$]-enkephalin | | | | 246 ± 39 | 1 | 11.4 ± 1.1 | 1 | 0.0463 |

[a] Mean of 3–6 determinations ± SEM.

spectrum using the Marquardt method [34]. A convergence criterion value of $10^{-5}$ was used. A separate term with an additional statistical weight of 0.1 was introduced to account for couplings. In this way a statistical weight for each of the conformational families was obtained.

Since the peptides expressed biological activity at a concentration lower than that of $Ca^{2+}$ ions in the tissue, NMR spectra were also measured (peptide **3**) in the presence of these ions up to a 10-fold molar excess. No chemical shift changes were observed, indicating that the ions had no effect on the conformation of these peptides.

## RESULTS AND DISCUSSION

The linear peptides Fmoc-Tyr-D-Aaa$^2$-Phe-Aaa$^4$-$NH_2$ were obtained using the method described earlier [9]. The crude peptides were cyclized by reaction with bis(4-nitrophenyl)carbonate in DMF. Also, bis(*N*-succinimidyl)carbonate and *N*,*N*′-carbonyldiimidazole were used but in these cases the purity of the crude product was not satisfactory. After cyclization, removal of the Fmoc group was achieved by treatment with 50% piperidine in DMF. The cyclic peptides (Figure 1) were purified by semi-preparative reversed-phase HPLC. Homogeneity of the product was verified by analytical HPLC. Molecular weights of purified products were determined by LSIMS. NMR spectra of the peptides were measured and all signals were assigned. The *in vitro* opioid activity profile was determined in the GPI and in the MVD assay (Table 1). To facilitate comparison Figure 2 is presented in which $1/IC_{50}$ is given for the peptides in the GPI and the MVD assay. With the exception of the 18-membered ring analogue **1** only, all peptides showed very high agonist potency in both assays. Two compounds, **5** and **7**, are agonists with dual high affinity for both μ- and δ-receptors

Table 2 Proton Chemical Shifts of Cyclo ($N^{\omega}N^{\omega'}$-carbonyl-D-Daa$^2$, Daa$^4$)dermorphinamides in Water at 25 °C

| Peptide | **3**(D-Lys$^2$,Dab$^4$) | **4**(D-Lys$^2$,Dap$^4$) | **7**(D-Orn$^2$,Dab$^4$) | **8**(D-Orn$^2$,Dap$^4$) |
|---|---|---|---|---|
| **Tyr**$^1$ | | | | |
| $H_\alpha$ | 4.13 | 4.04 | 4.13 | 4.11 |
| $H_\beta$ | 3.23; 2.95 | 3.20; 2.94 | 3.21; 2.94 | 3.19; 2.99 |
| $H_{26}$; $H_{35}$ | 7.14; 6.85 | 7.13; 6.89 | 7.14; 6.90 | 7.14; 6.89 |
| **D-Daa**$^2$ | | | | |
| $H_N$ | (not observed) | 8.17 | (not observed) | (not observed) |
| $H_\alpha$ | 4.16 | 3.99 | 4.07 | 4.05 |
| $H_\beta$ | 1.58; 1.20 | 1.52; 1.36 | 1.58; 1.38 | 1.50; 1.35 |
| $H_\chi$ | 0.84; 0.76 | 0.95 | 1.27; 1.14 | 1.21 |
| $H_\delta$ | 1.39; 1.32 | 1.37 | 2.95 | 3.03; 2.84 |
| $H_\varepsilon$ | 3.18; 3.04 | 3.01; 2.93 | — | — |
| $H_{N\ bridge}$ | 5.86 | 5.89 | 5.88 | 6.12 |
| **Phe**$^3$ | | | | |
| $H_N$ | 7.90 | 7.89 | 8.06 | 8.05 |
| $H_\alpha$ | 4.57 | 4.48 | 4.50 | 4.61 |
| $H_\beta$ | 3.06; 2.92 | 3.05 | 3.09; 2.98 | 3.00 |
| $H_{26}$; $H_{35}$ | 7.37; 7.25 | 7.37; 7.24 | 7.36; 7.25 | 7.36; 7.25 |
| $H_4$ | 7.32 | 7.31 | 7.31 | 7.31 |
| **Daa**$^4$ | | | | |
| $H_N$ | 8.37 | 8.55 | 8.43 | 8.21 |
| $H_\alpha$ | 4.35 | 4.35 | 4.22 | 4.45 |
| $H_\beta$ | 1.94; 1.70 | 3.56; 3.19 | 2.00; 1.70 | 3.48; 3.27 |
| $H_\chi$ | 3.08; 3.04 | — | 3.24; 3.17 | — |
| $H_\delta$ | — | — | — | — |
| $H_\varepsilon$ | — | — | — | — |
| $H_{N\ bridge}$ | 5.65 | 6.18 | 5.88 | 6.15 |
| $NH_2$ | 6.87; 6.16 | 6.74; 7.09 | 6.85; 6.06 | 6.97; 6.49 |

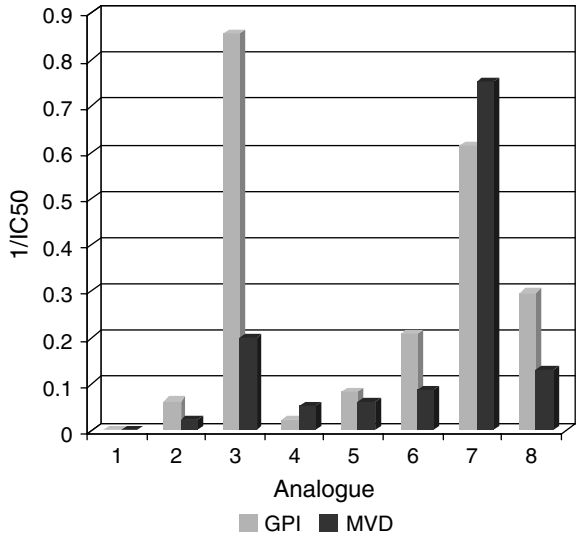



Figure 2 GPI and MVD assay of cyclic dermorphin/deltorphin analogues **1**–**8**.

showing MVD/GPI $IC_{50}$ ratios of 1.30 and 0.816, respectively. These analogues could be considered as indiscriminate ligands. This kind of analogue has the potential to yield important insight into the role of endogenous opioid peptides, the combined disposition of μ- and δ-opioid receptors and they might be useful in the management of pain [35]. It should be noticed that these potent dermorphin analogues contain (14–17)-membered rings, while earlier published potent enkephalin analogues of similar structure contained 17–20-membered rings [9]. The most potent enkephalin analogue, containing an 18-membered ring, is more active than all analogues of these two opioids. It is also worth mentioning that a highly active dermorphin analogue in which the ring was formed by an amide bond between the D-$Lys^2$ side chain amino group and the $Glu^4$ carboxyl group ($IC_{50}$ 2.93 nM in GPI and $IC_{50}$ 5.21 nM in MVD assay) contains a 15-membered ring [36] and a corresponding D-$Orn^2$, $Glu^4$ analogue ($IC_{50}$ 1.17 in GPI and $IC_{50}$ 1.11 in MVD) contains a 14-membered ring [37].

Chemical shifts of peptides **3**, **4**, **7** and **8** in water were fully assigned as described [21] and are listed in Table 2. The composition and sequence of each novel endorphin analogue was confirmed using TOCSY and ROESY spectra. The ROESY spectra used for the structure calculation were measured using a 0.15 s mixing time. Vicinal couplings within the peptide in water were measured using 1D proton spectra (the couplings are not shown). The temperature coefficients of the signals of the amide and ureido

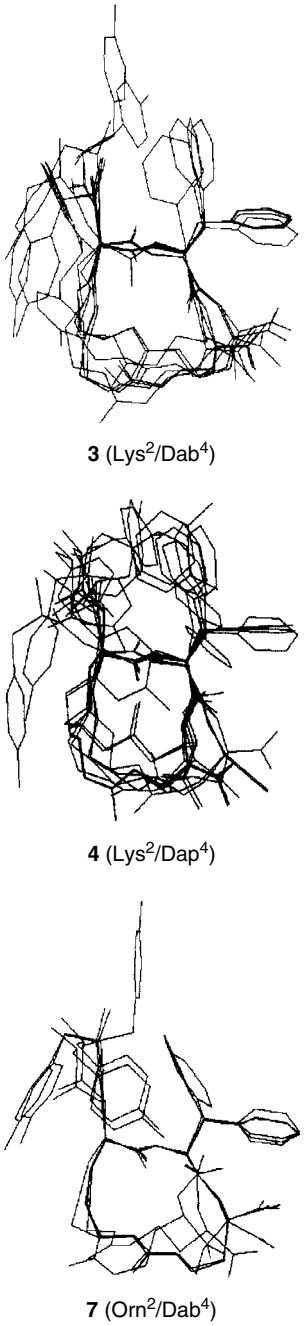



Figure 3 MOLMOL [38] drawings of conformations of peptides **3**, **4** and **7** with relative population above 3%.

bridge protons were measured. Neither proton is involved in hydrogen bonding nor is it protected from exchange with solvent protons.

It was shown [23] that EDMC calculations combined with NMR data provide a useful tool for conformational studies of cyclic peptides, since they allow an effective to be made search of the

conformational space and to describe the flexibility of a peptide in a more quantitative way. The calculation procedure outlined in Materials and Methods yielded for each peptide studied a large set of conformational families. More than 10 000 conformations were generated and 3000 of them were accepted in each case. Using the clusterization procedure, 957, 980, 407 and 529 conformational families were accepted for compounds **3**, **4**, **7** and **8**, respectively. NOE contacts (and vicinal couplings for peptides **3**, **4** and **7**) were utilized to assign statistical weights for representatives of conformational families. For subsequent analysis only those conformations whose relative population was higher than 3% were chosen. Their populations total more than 96% in each case. The chosen conformations are shown in Figure 3.

It is a common belief that the intramolecular distance between two aromatic rings plays a crucial role in opioid peptide–receptor interactions. In a number of studies, efforts were made to establish the spatial relationship between these two side chains in several opioid analogues. These studies included theoretical methods, measurements of the distance by singlet–singlet resonance energy transfer experiments and crystal structure determination [1]. The distances were between 8 and 11 Å. Inspection of the data presented in Table 3 (see also Figure 4) reveals a large diversity of conformations of the peptide chain and the aromatic rings for each conformer. Two peptides containing a 15-membered ring, **4** and **7**, present a different set of conformations, indicating that the location of the urea unit plays an important role in the spatial arrangement of the molecule. The sets of **3** and **7** are also different, but the biological activity of the compounds is similar. It is clear that in general the biologically active conformation is well set by the ureido bridge. In addition this setting may be tuned with the length of the 'spacer' between the Tyr and Phe residues and with

Table 3 Parameters for the Most Populated Conformations of Peptides **3**, **4** and **7** (with Populations above 3%) Found in Water

| | $\chi_1$(**1**) | $\psi$(**1**) | $\phi$(**2**) | $\psi$(**2**) | $\phi$(**3**) | $\chi_1$(**3**) | *r* | en | pop |
|---|---|---|---|---|---|---|---|---|---|
| **3** (Lys$^2$/Dab$^4$) | | | | | | | | | |
| **1**w-1 | −175 | 156 | 75 | **45** | **−98** | −177 | 10.9 | 1.2 | 23.0 |
| **1**w-2 | −64 | 157 | 87 | **48** | **−155** | −179 | 11.7 | 3.3 | 16.0 |
| **1**w-3 | −175 | 156 | 87 | **42** | **−149** | −179 | 10.9 | 0 | 15.9 |
| **1**w-4 | −64 | 158 | 87 | **42** | **−147** | 179 | 11.6 | 2.3 | 14.5 |
| **1**w-5 | −68 | −41 | 99 | **50** | **−91** | −61 | 5.4 | 2.0 | 12.7 |
| **1**w-6 | −177 | 152 | 139 | **−103** | **53** | −55 | 7.6 | 1.2 | 9.9 |
| **1**w-7 | −63 | 153 | 148 | **−30** | **−102** | −59 | 10.5 | 2.5 | 4.3 |
| **4** (Lys$^2$/Dap$^4$) | | | | | | | | | |
| **2**w-1 | −67 | −39 | 88 | **52** | **−151** | 177 | 8.9 | 4.3 | 18.7 |
| **2**w-2 | 175 | 156 | 166 | **65** | **−146** | 176 | 7.8 | 5.0 | 17.8 |
| **2**w-3 | −175 | 133 | 154 | **−161** | **52** | −54 | 9.6 | 5.9 | 13.9 |
| **2**w-4 | 56 | −29 | 147 | **57** | **−92** | 74 | 6.7 | 4.4 | 11.6 |
| **2**w-5 | −176 | −48 | 100 | **61** | **−83** | −65 | 4.1 | 5.7 | 9.9 |
| **2**w-6 | 167 | 145 | 163 | **59** | **−92** | −178 | 8.3 | 5.2 | 8.4 |
| **2**w-7 | −177 | −42 | 86 | **41** | **−140** | −69 | 4.7 | 7.4 | 3.7 |
| **2**w-8 | −175 | 155 | 87 | **59** | **−145** | 56 | 7.3 | 0 | 3.2 |
| **2**w-9 | 178 | 151 | 135 | **70** | **−94** | −57 | 6.0 | 6.0 | 3.2 |
| **7** (Orn$^2$/Dab$^4$) | | | | | | | | | |
| **3**w-1 | −178 | 156 | 86 | **46** | **−142** | −180 | 10.9 | 1.6 | 56.2 |
| **3**w-2 | −65 | −37 | 86 | **47** | **−139** | 180 | 8.8 | 2.5 | 19.3 |
| **3**w-3 | 179 | 159 | 167 | **56** | **−140** | 179 | 7.6 | 0.5 | 7.5 |
| **3**w-4 | −177 | 156 | 96 | **47** | **−138** | −63 | 8.3 | 1.3 | 6.0 |
| **3**w-5 | −176 | 161 | 160 | **49** | **−134** | −50 | 5.9 | 0 | 5.8 |

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.

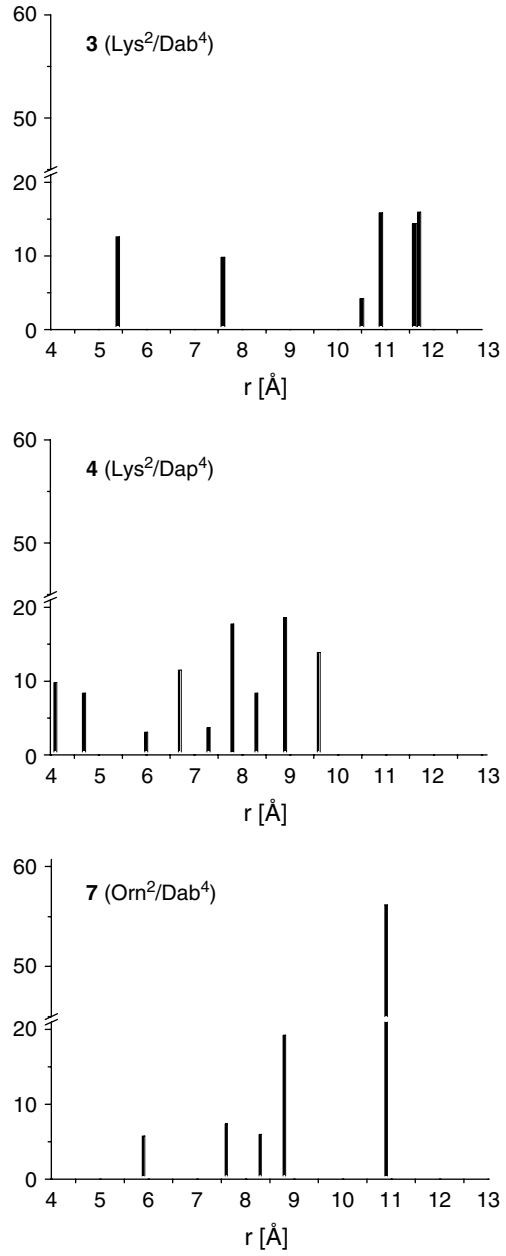



Figure 4 Relative distribution of the distances between the centres of the aromatic rings in conformations of peptides **3**, **4** and **7** with relative population above 3%.

the length of the side chains of residues at positions 2 and 4. All these changes seem to control the overall orientation of both aromatic residues. It may be seen from Figure 3 and the r.m.s.d. data given in Table 4 that the diversity of conformations decreases going from peptide **3** to **7**. However, the conformational freedom of both aromatic side chains still remains and our results suggest that the final adjustment of the biologically active conformation of the peptide is induced during its interaction with the receptor. The small differences in energies of populated conformations raise the possibility that the final conformation is not necessarily present in a high proportion in the solution, if at all. It is believed that a substantial part of the energy expense paid in the process of binding to the receptor in the case of the peptides studied was cancelled by introducing strong conformational restrictions. The energetic cost of the final adjustment of the peptide conformation is relatively small and may be easily overcome by any interaction with the receptor. This explains the high activity of the peptides studied. From this point of view the biologically active conformation may be rather understood as an ensemble of conformations closely resembling the final conformation, distinct from one another by a few and small energy local structural differences.

The conformational behaviour of compound **8** (Orn$^2$,Dap$^4$) is obviously different from that of the other three peptides. Inspection of the proton NMR spectra of this peptide shows substantial broadening of all signals at room temperature. Increasing the temperature to 40 °C causes partial narrowing of the lines, whereas spectra recorded at lower temperatures reveal a collapse of lines and then

Table 4 R.m.s.d. (in Å) Calculated for the Conformations in $H_2O$ with the Relative Population above 3%

| | **3** (Lys$^2$,Dab$^4$) | **4** (Lys$^2$,Dap$^4$) | **7** (Orn$^2$,Dab$^4$) |
|---|---|---|---|
| [a] | 1.729 | 1.915 | 1.568 |
| [b] | 0.604 | 0.586 | 0.496 |
| [c] | 0.190 | 0.199 | 0.023 |

[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 thyrosine-phenylalanine 'spacer' only, using four heavy atoms of the backbone: carbon atoms $C_\alpha$ and $C_O$ of the residue D-Daa$^2$ and N, $C_\alpha$ od Phe$^3$. The value of r.m.s.d. may be used as a measure of the relative structural differences among the conformations. The higher the value, the more geometrically different are the conformations, the smaller the value, the more similar are the conformations. In the latter case, if the number is very small, the structure may be considered as almost fixed. Inspection of the values given in the Table reveals that the 'spacer' is relatively fixed (row c), the main ring possesses some flexibility (row b) and the side chains of Tyr and Phe sample the conformational space quite freely (row a). It may also be seen that in going from compound **3** to **7**, the conformational space becomes more restricted.

their doubling at 5 °C. This temperature dependence makes it impossible to obtain ROESY spectra of good quality and does not allow the measurement of scalar couplings. This in turn makes it impossible to use the procedure applied to the other compounds of the series and it was not possible to obtain statistical weights of conformational families of this peptide. However, it may be stated that the conformational freedom of peptide **8** is heavily restricted. This peptide has the smallest number of members in the main ring (14) of the peptides studied so far. The study of the conformational barrier introduced in this compound requires separate investigations to be carried out in a non-aqueous solvent with a low temperature of melting.

## CONCLUSION

A new family of cyclic opioid peptides, analogues related to the 1–4 sequences of dermorphin and deltorphin was synthesized. The ring formation was achieved *via* a ureido group incorporating the side chain amino functions of dibasic amino acids in positions 2 and 4. Some of these peptides expressed high dual potency in the GPI and MVD assays. These peptides can provide an understanding of the differences and similarities of the structure of the binding domain of μ- and δ-receptors. With the help of NMR spectra and theoretical calculation selected peptides were presented as an ensemble of the most populated conformations. An analysis of the results reveals that the (14–18)-membered ring structures and the Phe and Tyr side chains enjoy some conformational freedom. The results also suggest that the biologically active conformation may not be present as such among the highly populated conformations of all peptides in solution but may be induced in the process of the peptide–receptor interaction.

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