Cyclic enkephalin and dermorphin analogues containing a carbonyl bridge

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Abstract: Four cyclic enkephalin analogues and four cyclic dermorphin analogues have been synthesized. Cyclization of linear peptides containing basic amino acid residues of various side chain length in position 2 and 5 (enkephalin analogues) or 2 and 4 (dermorphin analogues) 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 activity was observed, depending on the size of the ring and the location of the urea unit. The conformation of two dermorphin analogues has been studied: one of high activity ($IC_{50} = 4.15$ nm in the GPI assay) and a second of low activity ($IC_{50} = 6700$ nm in the GPI assay). The conformational space of these peptides was examined using the EDMC method. Using data from the NMR spectra, each peptide was described as an ensemble of conformers. Biological activity was discussed in light of the structural data. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

INTRODUCTION

Several cyclic enkephalin and dermorphin analogues containing a carbonyl bridge have been synthesized recently. Ring formation was achieved via a ureido group incorporating the side chain amino functions of dibasic amino acids in position 2 and 5 (enkephalin analogues) [1], or 2 and 4 (dermorphin analogues) of the peptide sequence [2]. Most of these peptides showed very high agonist potency in the GPI and MVD assays. The most active enkephalin analogue, cyclo-(N^ε, N^β-carbonyl-D-Lys^2, Dap^5)-enkephalinamide, was shown to decrease blood pressure and heart rate, and to stimulate excretion of urine, sodium, potassium and cGMP. These effects were inhibited by naloxone [3]. The peptides studied so far contained D-Lys or D-Orn in position 2, while in position 4 or 5 L-α,ω-amino acids containing shorter side chains were also incorporated.

In the present study, the synthesis, *in vitro* opioid activity profile and conformations of a new series of enkephalin and dermorphin analogues are described. These peptides (E1–E4 and D1–D4) differ from those described earlier (e1–e4 and d1–d4) in that they contain D-Dap or D-Dab in position 2.

MATERIALS AND METHODS

Synthesis of Peptides

The linear, fully protected precursor peptides, Fmoc-Tyr-D-Daa^2-Gly-Phe-Daa^5-NH$_2$ and Fmoc-Tyr-D-Daa^2-Phe-Daa^4-NH$_2$ were prepared on a 4-methylbenzhydrylamine resin as described earlier [1,2]. The crude linear peptides were then reacted with bis-(4-nitrophenyl) carbonate to form the cyclic peptides (Figure 1) and were purified using semi-preparative reversed-phase high performance liquid chromatography according to a described procedure. Molecular weights were determined by LSIMS mass spectrometry: **E1** ($C_{32}H_{44}N_8O_7$), M calcd 652.7, (M + 1) obtained 653.4; **E2** ($C_{31}H_{42}N_8O_2$), M calcd 638.7, (M + 1) obtained.639.3; **E3** ($C_{30}H_{40}N_8O_7$) M calcd 624.7, (M + 1) obtained 625; **E4** ($C_{29}H_{38}N_8O_7$), M calcd.610.7, (M + 1) obtained 611; **D1** ($C_{29}H_{39}N_7O_6$), calcd 581.3, (M + 1) obtained 582.2; **D2** ($C_{28}H_{37}N_7O_6$) M calcd 567.6, (M + 1) obtained 568.2; **D3** ($C_{28}H_{37}N_7O_6$) M calcd 567.7, (M + 1) obtained 568.6; **D4** ($C_{27}H_{35}N_7O_6$) M calcd 553.6, (M + 1) obtained 554.5.

Bioassay. 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

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Abbreviations: Abbreviations: Dab, 2,4-diaminobutyric acid; Dap, 2,3-diaminopropionic acid; EDMC, electrostatically driven monte carlo; CLUST, a program for cluster analysis; MORASS, multiple overhauser relaxation analysis and simulation.

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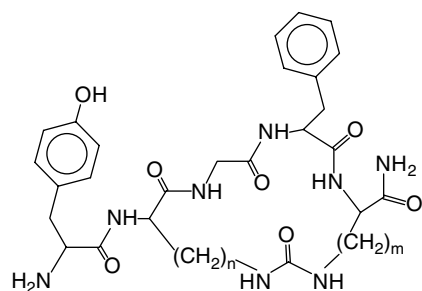
e1	Orn²/Lys⁵	(n = 3, m = 4)
E1	Lys²/Orn⁵	(n = 4, m = 3)
E2	Lys²/Dab⁵	(n = 4, m = 2)
e3	Orn²/Dab⁵	(n = 3, m = 2)
E3	Dab²/Orn⁵	(n = 2, m = 3)
e4	Orn²/Dap⁵	(n = 3, m = 1)
E4	Dap²/Orn⁵	(n = 1, m = 3)

Figure 1 Structural formulas of cyclic enkephalin analogues.

published procedure [8]. The results are presented in Tables 1 and 2.

NMR Spectroscopy and Theoretical Analysis

The NMR samples of peptides **D1** and **D4** were prepared and the spectra were recorded using the conditions and parameters already described [1,9]. The conformational space of each peptide was explored using the EDMC method proposed by Liwo *et al.* [10] (and references cited therein). The conformations were subsequently clustered into families using the program CLUST [11], using all heavy atoms and r.m.s. and energy criteria. For an estimation of the population of particular conformational families of each peptide, NOESY spectra were generated for representative conformers of each family with the program MORASS [12], using previously described parameters [1]. This permitted the determination of the statistical weights for each of the conformational families.

Table 1 GPI and MVD Assay of Cyclo-(N^{ω},N^{ω}-carbonyl-D-Daa², Daa⁵)-enkephalinamide Analogues

Compound				GPI		MVD		MVD/GPI	
	Ring size	Daa²	Daa⁵	$IC_{50}(nM)^a$	Rel. pot.	$IC_{50}(nM)^a$	Rel. pot.	IC_{50} ratio	Reference
e1	20	Orn	Lys	10.6 ± 1.2	23.2 ± 2.6	41.3 ± 10.8	0.276 ± 0.072	3.90	[1]
E1	20	Lys	Orn	2.26 ± 0.14	109 ± 7	3.52 ± 0.49	3.24 ± 0.45	1.56	
E2	19	Lys	Dab	2.32 ± 0.31	106 ± 14	2.68 ± 0.37	4.25 ± 0.59	1.16	
e3	18	Orn	Dab	2.05 ± 0.09	120 ± 5	8.70 ± 0.20	1.31 ± 0.03	4.24	[1]
E3	18	Dab	Orn	1.61 ± 0.33	153 ± 31	6.26 ± 1.07	1.82 ± 0.31	3.89	
e4	17	Orn	Dap	1.64 ± 0.46	1.50 ± 42	12.5 ± 1.0	0.912 ± 0.073	7.62	[1]
E4	17	Dap	Orn	12.1 ± 2.3	20.3 ± 3.8	46.7 ± 10.3	0.244 ± 0.054	3.86	
[Leu⁵]-enkephalin				246 ± 39	1	11.4 ± 1.1	1	0.0463	

^a Mean of 3–6 determinations $\pm$ SEM.

Table 2 GPI and MVD Assay of Cyclo-(N^{ω},N^{ω}-carbonyl-D-Daa², Daa⁴)-dermorphin-(1-4)-NH_2 Analogues

Compound				GPI		MVD		MVD/GPI	
	Ring size	Daa²	Daa⁴	$IC_{50}(nM)^a$	Rel. pot.	$IC_{50}(nM)^a$	Rel. pot.	IC_{50} ratio	LIT
d1	16	Lys	Dab	1.17 ± 0.25	210 ± 45	5.02 ± 1.39	2.27 ± 0.63	4.29	[2]
D1	16	Dab	Lys	4.15 ± 0.34	59.3 ± 4.9	8.98 ± 1.84	1.27 ± 0.26	2.16	
d2	15	Lys	Dap	4.15 ± 0.36	55.3 ± 5.1	19.5 ± 2.3	0.582 ± 0.069	4.70	[2]
D2	15	Dap	Lys	P.A. (37%)ᵇ	–	P.A. (32%)ᵇ	–		
d3	15	Om	Dab	1.63 ± 0.15	151 ± 14	1.33 ± 0.23	8.57 ± 1.48	0.816	[2]
D3	15	Dab	Orn	3360 ± 720	0.0732 ± 0.0157	1390 ± 260	0.0082 ± 0.00153	0.414	
d4	14	Orn	Dap	3.37 ± 0.09	73.0 ± 1.9	7.76 ± 1.27	1.47 ± 0.24	2.30	[2]
D4	14	Dap	Orn	6700 ± 200	0.0367 ± 0.0011	6790 ± 1250	0.00168 ± 0.00031	1.01	
[Leu⁵]-enkephalin				246 ± 39	1	11.4 ± 1.1	1	0.0463	

^a Mean of 3–6 determinations $\pm$ SEM.
^b Partial agonist (% of maximal inhibition of contractions at 1×10^{-5} M).

RESULTS AND DISCUSSION

The cyclic enkephalin and dermorphin analogues were obtained from their respective linear Fmoc-peptide amides by treatment with bis-(4-nitrophenyl) carbonate, as described earlier [1,2]. After removal of the Fmoc group, the cyclic peptides (Figures 1 and 2) were purified by semi-preparative reversed-phase HPLC. The homogeneity of the products was verified by analytical RP-HPLC. The molecular weights of the purified products were determined by LSIMS. The *in vitro* opioid activity profile was determined in the GPI and in MVD assays (Tables 1 and 2). Analogues presented in previous papers [1,2] containing cycles of the same size as those in the newly prepared peptides are also included in the Tables (compounds **e1**–**e4** and

Figure 2 Structural formulas of cyclic dermorphin analogues.
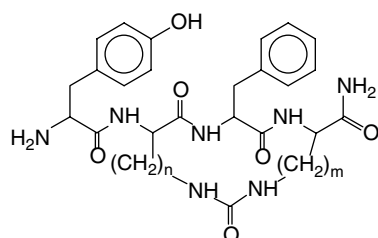
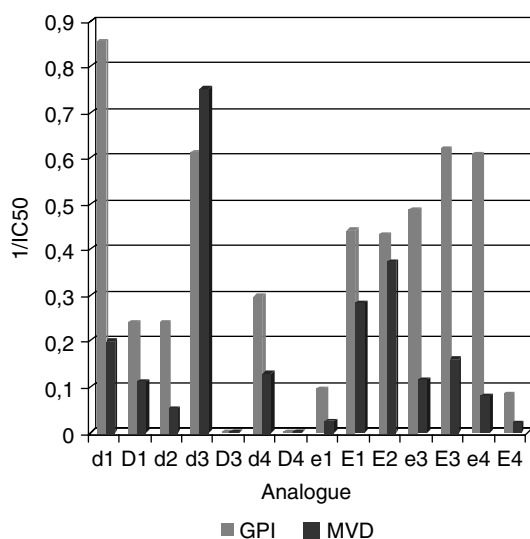|		
d1	Lys^2/Dab^4	(n = 4, m = 2)
D1	Dab^2/Lys^4	(n = 2, m = 4)
d2	Lys^2/Dap^4	(n = 4, m = 1)
D2	Dap^2/Lys^4	(n = 1, m = 4)
d3	Orn^2/Dab^4	(n = 3, m = 2)
D3	Dab^2/Orn^4	(n = 2, m = 3)
d4	Orn^2/Dap^4	(n = 3, m = 1)
D4	Dap^2/Orn^4	(n = 1, m = 3)

Figure 3 GPI and MVD assay of cyclic enkephalin and dermorphin analogues.

Table 3 Proton Chemical Shifts of Cyclo-(N^{ω},$N^{\omega'}$-carbonyl-D-Daa^2, Daa^4)-dermorphinamides in water at 25°C

Peptide	**D1** (D-Dab^2,Lys^4)		**D4** (D-Dap^2,Orn^4)
Tyr^1			
H$_\alpha$	4.18		4.18
H$_\beta$	3.02; 3.28		3.03
H$_{26}$	7.17		7.07
H$_{35}$	6.89		6.86
D-Daa^2			
H$_N$	8.53		8.25
$^3J_{H\alpha HN}$	8.5	7.8	
H$_\alpha$	4.40		4.47
H$_\beta$	1.35; 1.94		3.27; 3.58
H$_\gamma$	2.54; 2.71		—
H$_{N\ bridge}$	5.38		6.08
Phe^3			
H$_N$	7.68		8.63
$^3J_{H\alpha HN}$	7.9	8.5	
H$_\alpha$	4.64		4.18
H$_\beta$	3.00		3.02; 3.08
H$_{26}$	7.23		7.34
H$_{35}$	7.36		7.37
H$_4$	7.30		7.28
Daa^4			
H$_N$	8.35		8.21
$^3J_{H\alpha HN}$	8.7	8.0	
H$_\alpha$	4.31		4.42
H$_\beta$	1.59; 1.82		1.65; 1.89
H$_\gamma$	1.30		1.42; 1.51
H$_\delta$	1.30		2.91; 3.09
H$_\varepsilon$	3.01; 3.27		—
H$_{N\ bridge}$	5.93		6.15
NH$_2$	6.53; 6.91		6.91; 6.97

d1–**d4**). It can be seen that the activity of enkephalin analogues (Table 1) containing D-Lys in position 2 and a 20- or 19-membered ring (**E1** and **E2**) are very potent. The activity was not changed substantially when amino acids with shorter side chains and consequently smaller ring size, down to a 17-membered one, were incorporated in position 2 (**E3** and **E4**). Among the dermorphin analogues, only **D1** containing a 16-membered ring and D-Dab in position 2, was very potent. Introduction of D-Dab or D-Dap in position 2 with the formation of a 15- or 16-membered ring resulted in compounds with substantially decreased activity. Comparison of these data with those reported earlier for analogues with the same ring size (**d2**, **d3** and **d4** versus **D2**, **D3** and **D4**, respectively) clearly indicates that the location of the urea unit within the smaller rings is of crucial importance for biological activity. It is worth noting that some of the analogues showed comparable potency in the GPI and MVD assays (see Tables 1 and 2, and Figure 3).

Table 4 ^{13}C Chemical Shifts for Dermorphin Analogues in D$_2$O, 25 °C (ppm)

Residue	C$_\alpha$	C$_\beta$	C$_\gamma$	C$_\delta$	C$_\varepsilon$	C$_\zeta$
D1						
Tyr[1]	56.8	37.3		117.6	132.8	
D-Dab[2]	51.7	30.7	37.1			
Phe[3]	56.7	39.3		130.8	130.6	129.2
Lys[4]	53.5	31.7	23.5	28.5	40.5	
D4						
Tyr[1]	55.7	37.1		117.5	132.8	
D-Dap[2]	54.5	42.0				
Phe[3]	55.7	38.3		130.6	130.6	128.7
Orn[4]	53.4	29.6	25.5	40.7		

The observed large differences in activity within the dermorphin series prompted the examination of the conformations of two analogues, one very active and one of low activity: **D1** and **D4**, respectively.

Proton chemical shifts of peptides **D1** and **D4** in water were fully assigned and are listed in Table 3. Vicinal coupling constants in water were measured in 1D proton spectra. The temperature coefficients of the signals of the amide and ureido bridge protons were measured. The results obtained indicate that neither proton is involved in hydrogen bonding, nor are they

protected from exchange with solvent protons. ^{13}C chemical shifts for **D1** and **D2** are presented in Table 4.

The EDMC calculation procedure outlined in Materials and Methods yielded 13 150 conformations for **D1** and 12 734 for **D4**. 3000 of them were accepted in each case. After performing the clusterization procedure, 822 and 778 conformational families were accepted for compounds **D1** and **D4**, respectively. NOE contacts and vicinal couplings were used to estimate the statistical weights for representative conformers of the conformational families. For subsequent analysis only those conformations were chosen whose relative population was higher than 3%. Their populations sum to more than 94% in each case. The parameters that characterize the chosen conformations are listed in Table 5 and their MOLMOL drawings [13] are shown in Figure 4. As a measure of the diversity of the conformations, rmsd data are presented in Table 6. The distances between the aromatic rings of Tyr and Phe, which are known to be of great importance for the peptide activity, are shown in Figure 5. Inspection of the data presented in Table 5 (see also Figure 5) reveals a large diversity of the set of conformations between **D1** and **D4**. It should be noted that the most populated conformation of **D1** (Figure 6), in which the distance between the aromatic rings is about 11 Å, is similar to that determined for the most active analogue described in our previous paper [2], which contains D-Orn in position 2 and Dab in position 4.

Table 5 Parameters for the Most Populated Conformations of Peptides **D1** and **D4** (with Populations above 3%) Found in Water

	$\chi^1(1)$	$\psi(1)$	$\varphi(2)$	$\psi(2)$	$\varphi(3)$	$\chi^1(3)$	r	en	pop
D1 (Dab[2]/Lys[4])									
D1w-1	−68	108	150	57	148	179	11.3	3.1	28.2
D1w-2	180	158	158	59	−86	−174	7.3	3.3	20.6
D1w-3	180	149	140	55	−106	−54	5.8	3.0	16.8
D1w-4	−179	152	138	62	−95	−58	5.7	0.0	11.0
D1w-5	−179	151	131	−77	51	−47	6.4	0.5	10.3
D1w-6	−179	152	139	62	−94	−58	5.7	0.0	4.6
D1w-7	−179	152	139	62	−94	−58	5.7	0.0	3.3
D4 (Dap[2]/Orn[4])									
D4w-1	−171	149	157	−156	51	−57	9.3	2.0	13.5
D4w-2	−179	162	170	55	−111	−175	7.1	4.4	13.0
D4w-3	−174	158	155	57	−125	−55	5.9	7.6	12.0
D4w-4	−173	155	82	46	−78	−62	7.5	7.9	10.2
D4w-5	−175	109	142	−33	−92	−62	10.1	4.5	8.7
D4w-6	−175	132	156	51	−143	−57	7.7	0.0	6.9
D4w-7	177	−48	149	−93	48	−61	4.3	2.0	6.9
D4w-8	178	−48	149	−93	48	−61	4.3	2.0	6.9
D4w-9	−174	159	143	−89	56	−45	6.0	1.7	6.8
D4w-10	178	159	162	56	−154	50	4.7	3.9	6.8
D4w-11	−178	148	96	12	−147	−179	11.2	5.2	2.5

Values of selected torsional angles for the Tyr-Phe 'spacer' (in °). Distance between tyrosine and phenylalanine ring centres (r in Å), relative calculated energy (en in kcal/mol) and relative populations of conformers (pop in %).
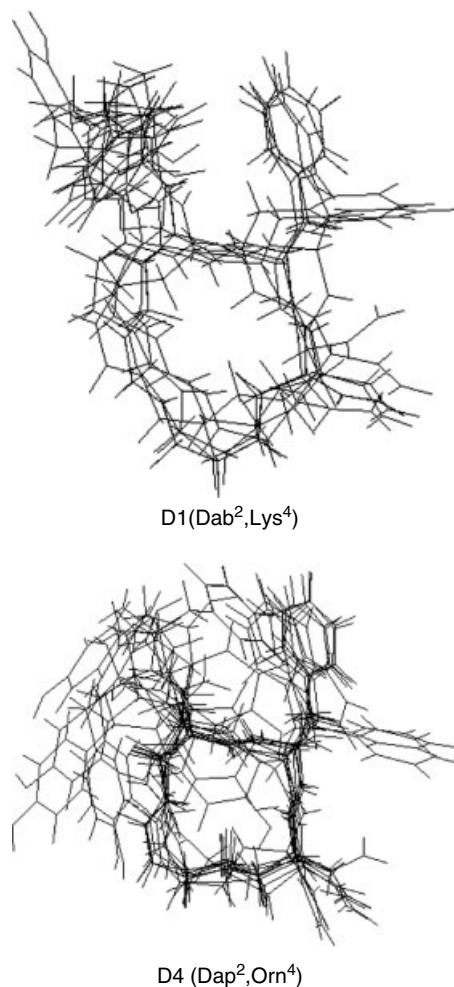
D1(Dab^2,Lys^4)

D4 (Dap^2,Orn^4)

Figure 4 MOLMOL [13] drawings of conformations of peptides **D1** and **D4** with relative populations above 3%.

Table 6 Rmsd (in Å) Calculated for the Conformations in H$_2$O with the Relative Population above 3%

	D1 (Dab^2,Lys^4)	**D4** (Dap^2,Orn^4)
a	1.354	1.812
b	0.591	0.500
c	0.029	0.025

[a] rmsd calculated using all heavy atoms.
[b] rmsd calculated using carbon and nitrogen atoms of main ring.
[c] rmsd calculated for tyrosine-phenylalanine 'spacer' only, using four heavy atoms of the backbone: carbon atoms C$_a$ and C$_O$ of the residue D-Daa^2 and N, C$_a$ of Phe^3.

CONCLUSIONS

Several cyclic enkephalin and dermorphin analogues were obtained by formation of the ring via a ureido group incorporating the side chain amino function of dibasic amino acids. The opioid activities of the new peptides containing D-Dab or D-Dap in position
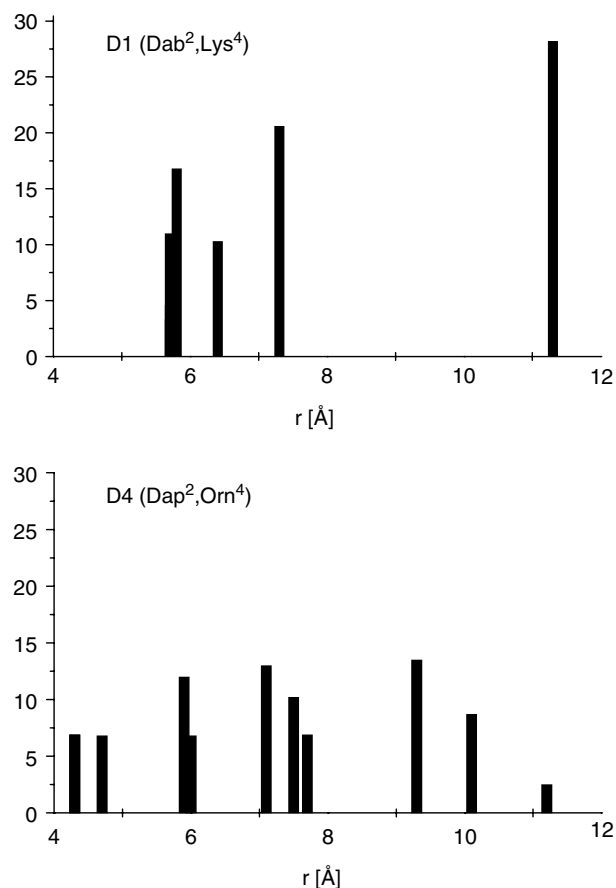
Figure 5 Relative distribution of the distance between the centres of the aromatic rings in conformations of peptides **D1** and **D4** with relative populations above 3%.
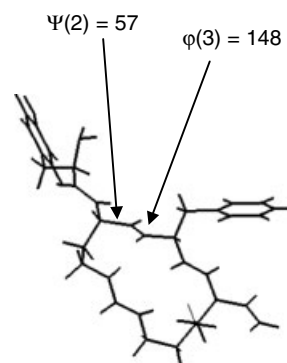

Figure 6 MOLMOL drawing [13] of the most populated conformation of **D1**. Torsion angles of a part of the main chain are specified.

2 of the peptide sequence were compared with those of previously reported analogues [1,2] having the same ring size, but Dab or Dap in position 5 (enkephalin analogues) or 4 (dermorphin analogues). In the enkephalin series, the opioid activities of all compounds having the same ring size was similar. In the dermorphin series, introduction of D-Dab or D-Dap in position 2, with simultaneous reduction of the

size of the ring resulted in a decrease of the biological activity. This result indicates that both the size of the ring and the location of the urea unit are essential for activity. NMR spectroscopy and theoretical analysis revealed that the sets of conformations obtained for a very active dermorphin analogue and for a poorly active one are different.

Acknowledgements

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