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N-(ureidoethyl)amides of cyclic enkephalin analogs

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Novel *N*-(ureidoethyl)amides of cyclic enkephalin analogs have been synthesized. The *p*-nitrophenyl carbamate of 1-Boc-1,2-diaminoethane was coupled with 4-methylbenzhydrylamine (MBHA) resin. The Boc group was removed by treatment with HCl/dioxane, and the peptide chain was assembled using Boc strategy. For deprotection of amino function, HCl/dioxane was used. D-Lys or D-Orn were incorporated in position 2, and the side chains of Lys, Orn, Dab, or Dap in position 5 were protected with Fmoc group. Side chain protection was removed by treatment with 55% piperidine in DMF, and cyclization was achieved by treatment with bis-(4-nitrophenyl)carbonate to form a urea bridge. The peptide was cleaved from the resin by treatment with 45% TFA in DCM. The peptides were tested in the guinea-pig ileum (GPI) and mouse vas deferens (MVD) assays. Divers opioid activities were observed, depending on the size of the ring. In comparison with [Leu^5]enkephalin, all peptides were more active in the GPI assay (between 125 and 12 times), and some of them were also more potent in the MVD assay. The conformational propensities of each peptide were determined using the EDMC method in conjunction with NMR experiments. This approach allows treating the dynamical behavior of small peptides properly. The results were compared with those obtained previously for corresponding nonsubstituted amides and are in agreement with the biologically active conformation proposed by us earlier.

Supporting information may be found in the online version of this article

Keywords: cyclic opioid peptides; conformation; EDMC; NMR; side chain to side chain cyclization; SAR; synthesis

Introduction

Previously, we described the synthesis, biological activity, and conformation of several highly potent side-chain-to-side-chain-cyclized analogs of enkephalin amides containing a carbonyl bridge linking the two side chain amino groups of dibasic amino acid residues in position 2 and 5 to form an ureido moiety [1–5]. In this work, we designed corresponding *N*-ureidoethylamides to study the influence of this structural modification on the biological activity of the resulting peptides. For the synthesis of these peptides, we used the method elaborated recently, which is based on coupling of nitrophenyl carbamate of 1-Boc-1,2-diaminoethane with MBHA resin and assembling the peptide chain using Boc strategy. During the treatment of the peptide resin with HCl/dioxane solution, the peptide is not cleaved from the resin but is cleaved quantitatively by treatment with TFA [6].

Materials and Methods

Peptide Synthesis

General procedure

The *p*-nitrophenoxycarbonyl derivative of Boc-diaminoethane was obtained as described earlier [6]. This compound was coupled to the MBHA resin (0.25 mEq/g, 1% crosslink, 100–200 mesh, 6 Eq) in DMF at 60 °C for 48 days. Following removal of the Boc group by treatment with 15% HCl/dioxane, five Boc amino acids were successively attached using DIPCDI as coupling reagent. The side protection was as follows: D- and L-Lys, Fmoc; D- and L-Orn, Fmoc; L-Dab and L-Dap, Fmoc. Tyr side chain was not protected. Fifteen percent HCl/dioxane was used for Boc groups. The peptide resin was treated with 55% piperidine in DMF with stirring for 50 min to remove the Fmoc groups, and bis(*p*-nitrophenyl)carbonate (1 Eq) was added. The suspension in 200 ml DMF was stirred for 7 days.

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Abbreviations used: *EDMC, electrostatically driven Monte Carlo; CLUST, a program for cluster analysis; MORASS, multiple Overhauser relaxation analysis and simulation; gHSQC, gradient heteronuclear single quantum coherence spectroscopy.*

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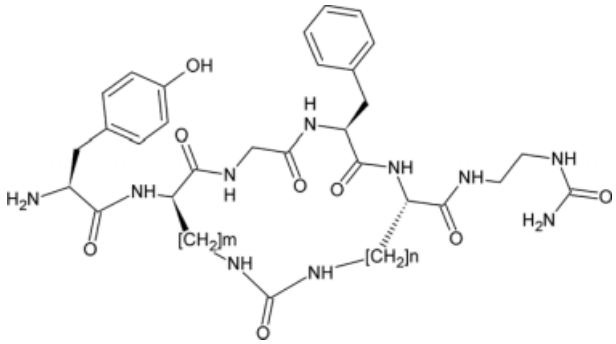

Figure 1. Structure of enkephalin analogs,
1: {[H-Tyr-D-Lys(&[1])-Gly-Phe-Dap(&[2])-$NHCH_2CH_2NHCONH_2$][&[1]CO&[2]]};
2: {[H-Tyr-D-Lys(&[1])-Gly-Phe-Dab(&[2])-$NHCH_2CH_2NHCONH_2$][&[1]CO&[2]]};
3: {[H-Tyr-D-Lys(&[1])-Gly-Phe-Orn(&[2])-$NHCH_2CH_2NHCONH_2$][&[1]CO&[2]]};
4: {[H-Tyr-D-Lys(&[1])-Gly-Phe-Lys(&[2])-$NHCH_2CH_2NHCONH_2$][&[1]CO&[2]]};
5: {[H-Tyr-D-Orn(&[1])-Gly-Phe-Dap(&[2])-$NHCH_2CH_2NHCONH_2$][&[1]CO&[2]]};
6: {[H-Tyr-D-Orn(&[1])-Gly-Phe-Dab(&[2])-$NHCH_2CH_2NHCONH_2$][&[1]CO&[2]]};
7: {[H-Tyr-D-Orn(&[1])-Gly-Phe-Orn(&[2])-$NHCH_2CH_2NHCONH_2$][&[1]CO&[2]]};
8: {[H-Tyr-D-Orn(&[1])-Gly-Phe-Lys(&[2])-$NHCH_2CH_2NHCONH_2$][&[1]CO&[2]]}.
(The nomenclature according to [7]).

One equivalent of DIPEA was gradually added during this reaction. The peptide was cleaved by treatment with 55% TFA/DCM for 1 + 20 min. TFA was evaporated under reduced pressure, and the residue was lyophilized. This procedure was used for preparation of peptides: **1, 2**, and **4–8**. Peptide **3** was prepared according to the procedure described earlier, using Fmoc-Tyr(*t*-but)-OH, Boc-D-Lys(2-Cl-Z)-OH, Boc-Orn(Z)-OH [3]. The products were purified using semipreparative RP-HPLC using solvent system: A: 0.05% TFA in water, B: 60% MeCN in A on Vydac column (Nucleosil 300, C_{18}, 5 µm, 10 × 250 mm), flow rate 2 ml/min, gradient B (15% in 15 min, 15–30% in 15 min, 30–45% in 40 min). Fractions were analyzed on a Vertex column Nucleosil-100 C_{18} (4 × 250 mm, 5 µm), flow rate 1 ml/min. For analysis, linear gradient 20–80% of B was used (1 ml/min, t = 15 min), detection at 220 nm. Homogeneous fractions containing one peak were combined and lyophilized. Structures were confirmed by ESI-MS spectra (a Finnigan MAT 95S spectrometer, Bremen, Germany). **1**: RT 12.50 min, M calcd 710.5, obtained 711.5 ($M + H^+$); **2**: RT 12.08 min, M calcd 724.6, obtained 725.6 ($M + H^+$); **3**: RT 11.90 min, M calcd 738.4, obtained 739.6 ($M + H^+$); **4**: RT 12.00 min, M calcd 752, obtained 753.4 ($M + H^+$) and 775.3 ($M + Na^+$); **5**: RT 11.77 min, calcd. 695, obtained 719.4 ($M + Na^+$); **6**: RT 11.38 min, calcd 710.5, obtained 711.7 ($M + H^+$); **7**: RT 11.39 min, calcd 724.6, fund 747.5 ($M + Na^+$); **8**: RT 13.67 min, calcd 738.4, obtained 739.4 ($M + H^+$). The structures of peptides are presented in Figure 1.

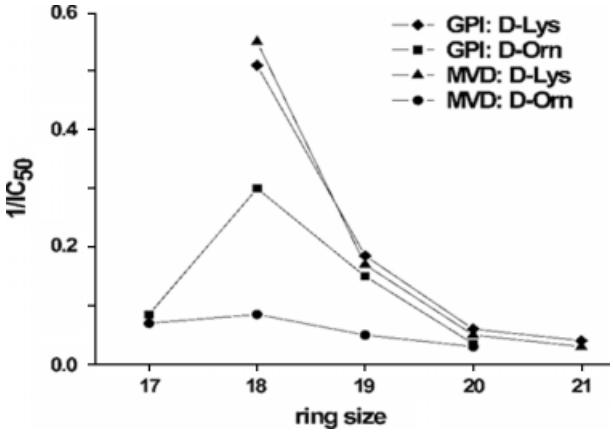

Figure 2. Relationship between size of the ring and opioid activity. Activity is expressed as $1/IC_{50}$.

Bioassays

The guinea-pig ileum (GPI) [8] and mouse vas deferens (MVD) [9] bioassays were carried out as reported in detail elsewhere [10,11]. 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 [12]. The results are presented in Table 1.

NMR Spectroscopy and Theoretical Analysis

NMR spectra of peptides **1–8** were recorded at natural abundance using the conditions and parameters described previously [2,3], and references cited therein. The aqueous solution was obtained in each case by dissolving the peptide in a mixture of H_2O doubly deionized (DDI) and D_2O (99.8% isotopic purity, Dr Glaser, Ag. Basel) at 10/1 ratio. The peptide concentration was 5 mM. NMR spectra were measured on a UNITY500plus (Varian) spectrometer using 5-mm $^1H\{^{13}C\backslash^{15}N\}$PFG triple probe. 16K points 1D proton spectra with 6 kHz spectral width were taken at 2, 10, 20, 30, 40,

Table 1. GPI and MVD assays of the peptides

	Analog	Ring size	GPI		MVD		MVD/GPI IC_{50}
			IC_{50} (nM)[a]	Rel. potency	IC_{50} (nM)[a]	Rel. potency	
1	D-Lys^2/Dap^5	18	1.97 ± 0.12	125 ± 8	1.81 ± 0.13	6.30 ± 0.45	0.919
2	D-Lys^2/Dab^5	19	5.55 ± 0.24	44.3 ± 1.9	6.00 ± 0.95	1.90 ± 0.30	1.08
3	D-Lys^2/Orn^5	20	18.1 ± 1.6	13.6 ± 1.2	22.7 ± 1.8	0.502 ± 0.040	1.25
4	D-Lys^2/Lys^5	21	20.2 ± 1.8	12.2 ± 1.1	26.6 ± 0.8	0.429 ± 0.013	1.32
5	D-Orn^2/Dap^5	17	11.3 ± 0.3	21.8 ± 0.6	14.1 ± 1.71	0.809 ± 0.096	1.25
6	D-Orn^2/Dab^5	18	3.36 ± 0.35	73.2 ± 7.6	11.6 ± 1.7	0.983 ± 0.144	3.45
7	D-Orn^2/Orn^5	19	6.87 ± 0.12	35.8 ± 0.6	16.5 ± 1.5	0.691 ± 0.063	2.40
8	D-Orn^2/Lys^5	20	16.1 ± 1.6	15.3 ± 1.5	28.2 ± 2.7	0.404 ± 0.039	1.75
	[Leu^5]enkefalin	–	246 ± 38	1	11.4 ± 1.1	1	0.0463

[a] Mean of 4–5 determinations ± SEM.

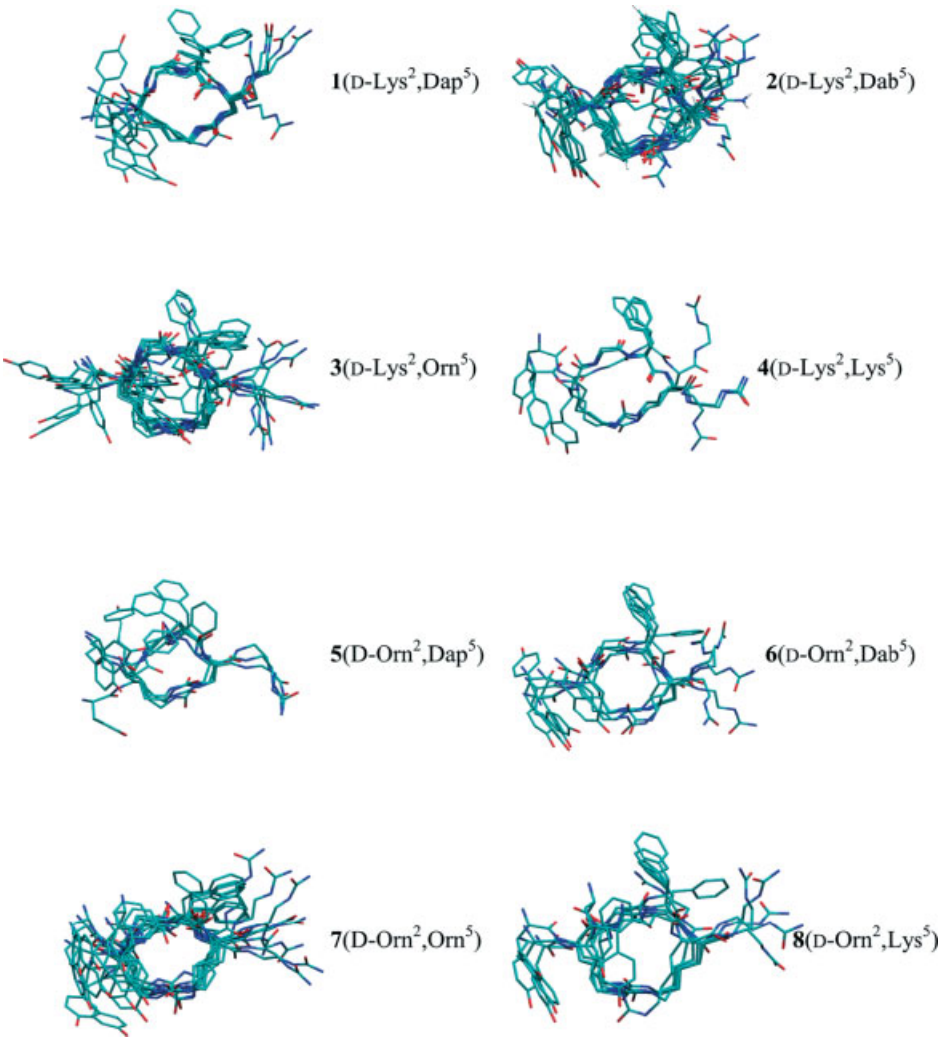

Figure 3. MVD drawings [16] of the most populated (above 3%) EDMC/NMR calculated conformations of enkephalins **1**–**8**. The structures are aligned using C and N atoms of the main ring.

Table 2. Comparison of the opioid activities of nonsubstituted [3] and substituted peptide amides (this work)

No.	Analog	Ring size	GPI amide/substituted amide IC_{50}	MVD amide/substituted amide IC_{50}
1	D-Lys²/Dap⁵	18	0.107	0.360
2	D-Lys²/Dab⁵	19	0.418	0.446
3	D-Lys²/Orn⁵	20	0.125	0.155
4	D-Lys²/Lys⁵	21	1.015	2.763
5	D-Orn²/Dap⁵	17	0.145	0.886
6	D-Orn²/Dab⁵	18	0.610	0.750
7	D-Orn²/Orn⁵	19	0.681	1.224
8	D-Orn²/Lys⁵	20	0.658	1.464

and 50 °C. 2D TOCSY and ROESY spectra (with mixing time 0.2 s) were measured using 2K points and 256 increments. {$^{1}H/^{13}C$} and {$^{1}H/^{15}N$}gHSQC experiments were measured in the proton decoupled mode with spectral width of 25K and 2K and 256 and 128 increments, respectively. Proton spectra were calibrated against water signal, and against external reference signals for carbon and nitrogen axis.

The conformational space of each peptide was explored using the EDMC method proposed by Liwo *et al*. [13] and references cited therein. This procedure yielded *ca* 3000 conformations for each peptide. The conformations were subsequently clustered into families using the program CLUST [14], taking into consideration all heavy atoms 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 [15] using the parameters described previously [3], for example, correlation time of 0.45 ns and mixing time of 0.2 s. The linear combination of generated spectra was then fitted to the experimental ones and in this way, the statistical weights for each of the conformational families were obtained.

Results and Discussion

1-*p*-Nitrophenoxycarbonycarbonyl-2-*t*-butoxycarbonyl-1,2-diaminoethane was coupled to MBHA resin. The peptide chain was assembled using DIPCDI as coupling reagent and HCl/dioxane

Table 3. Parameters for the most populated conformations of peptides **1**–**8** (with populations above 3%) found in water

	$\chi_1(1)$[a]	$\psi(1)$	$\varphi(2)$	$\psi(2)$	$\varphi(3)$	$\psi(3)$	$\varphi(4)$	$\chi_1(4)$	r[b]	En[c]	Pop[d]
1(D-Lys^2,Dap^5)											
1–1	−175	158	81	**34**	**−91**	**−13**	**−155**	57	6.9	0.0	28.6
1–2	179	153	84	**−134**	**171**	**−83**	**−144**	−180	14.5	1.4	20.8
1–3	−172	114	151	**−153**	**103**	**−100**	**−159**	−178	13.4	2.1	18.2
1–4	170	−53	74	**−94**	**76**	**−74**	**−150**	65	7.0	1.4	12.5
1–5	−178	161	81	**27**	**−134**	**−75**	**−112**	−61	11.1	2.6	12.1
1–6	−180	139	140	**58**	**−80**	**−56**	**−158**	58	8.9	0.7	3.8
2(D-Lys^2,cDab^5)											
2–1	−177	112	156	**156**	**93**	**−178**	**−69**	175	14.3	2.5	27.1
2–2	−178	146	139	**−126**	**169**	**−163**	**−106**	−56	12.4	3.0	10.3
2–3	−180	136	86	**−149**	**80**	**−88**	**−138**	−62	13.0	2.5	7.6
2–4	175	−47	158	**−162**	**87**	**−95**	**−164**	−177	14.3	1.7	6.5
2–5	−177	140	83	**−154**	**81**	**−84**	**−161**	57	12.2	1.7	6.5
2–6	−176	154	82	**−149**	**149**	**171**	**−89**	−60	12.9	1.9	6.4
2–7	−156	15	−156	**−47**	**−90**	**5**	**−101**	−72	4.8	0.6	6.2
2–8	−177	138	87	**−91**	**71**	**−111**	**−95**	−57	13.2	1.2	6.2
2–9	−176	157	82	**26**	**−85**	**−34**	**−109**	−57	9.6	0.0	4.6
2–10	177	−46	152	**−145**	**118**	**−115**	**−163**	178	14.4	0.9	4.3
3(D-Lys^2,Orn^5)											
3–1	−64	150	144	**−124**	**160**	**−164**	**−89**	−174	17.6	3.5	15.8
3–2	−57	169	150	**−36**	**−84**	**−47**	**−159**	175	8.8	4.8	13.1
3–3	−57	141	141	**−55**	**−83**	**−30**	**−117**	−177	8.3	2.5	7.4
3–4	−176	141	78	**30**	**−84**	**−40**	**−138**	−61	11.7	1.3	7.1
3–5	−66	148	155	**−160**	**147**	**−154**	**−101**	−59	15.5	4.5	6.7
3–6	−65	135	88	**−125**	**81**	**31**	**−142**	178	14.8	2.5	5.4
3–7	−64	150	145	**−125**	**161**	**−162**	**−88**	−175	17.6	4.2	5.3
3–8	−64	155	77	**35**	**−73**	**−37**	**−157**	180	14.5	2.8	5.0
3–9	−70	153	84	**28**	**−77**	**−30**	**−149**	176	14.2	2.7	4.8
3–10	−59	163	73	**31**	**−158**	**−52**	**−129**	175	9.8	4.3	4.8
3–11	−64	122	132	**−28**	**−84**	**−62**	**−156**	−178	12.3	4.2	4.7
3–12	−68	165	84	**−89**	**−71**	**−22**	**−68**	−179	7.4	0.0	4.4
4(D-Lys^2,Lys^5)											
4–1	−178	141	77	**36**	**−114**	**−73**	**−141**	−61	12.4	4.0	53.7
4–2	−177	138	84	**−153**	**175**	**−89**	**−136**	−61	13.2	5.3	23.0
4–3	−177	154	86	**−126**	**178**	**164**	**−94**	−62	11.7	0.0	10.0
4–4	−178	138	84	**−152**	**175**	**−91**	**−137**	−61	13.2	5.3	7.0
5(D-Orn^2,Dap^5)											
5–1	−175	148	81	**−156**	**157**	**−65**	**−136**	−63	13.0	0.0	72.5
5–2	−179	117	−61	**−29**	**−81**	**2**	**−67**	−178	8.2	1.8	10.3
5–3	−177	158	81	**42**	**−152**	**32**	**−141**	−57	7.5	1.8	4.8
5–4	−175	155	69	**53**	**−168**	**50**	**−146**	43	5.6	1.2	3.3
6(D-Orn^2,Dab^5)											
6–1	−173	139	156	**−170**	**168**	**−167**	**−87**	−61	13.1	1.5	40.9
6–2	−177	145	82	**−153**	**153**	**−84**	**−139**	−64	13.3	1.9	24.2
6–3	−176	155	86	**−140**	**154**	**−94**	**−157**	175	14.6	0.0	7.2
6–4	−172	156	92	**−149**	**79**	**−71**	**−141**	−61	12.2	1.8	7.0
6–5	174	−48	157	**−168**	**165**	**−164**	**−88**	−61	11.1	1.3	5.9
6–6	−173	145	84	**−97**	**78**	**−79**	**−120**	−62	12.9	1.0	5.9
7(D-Orn^2,Orn^5)											
7–1	−179	153	92	**−119**	**178**	**−97**	**−158**	172	14.9	2.5	18.9
7–2	−175	146	81	**28**	**−91**	**−56**	**−157**	59	10.4	3.0	14.2
7–3	−174	154	80	**30**	**−134**	**33**	**−87**	−180	9.3	3.6	12.7
7–4	180	136	80	**−151**	**173**	**−81**	**−148**	179	14.3	3.4	12.6
7–5	−174	142	93	**−160**	**82**	**−82**	**−163**	176	12.7	0.7	10.7
7–6	−176	143	72	**42**	**−163**	**−54**	**−122**	180	11.7	3.5	7.4
7–7	−172	147	87	**−177**	**81**	**8**	**−94**	−51	6.3	0.1	6.0

Table 3. (*Continued*)

	$\chi_1(1)$[a]	$\psi(1)$	$\varphi(2)$	$\psi(2)$	$\varphi(3)$	$\psi(3)$	$\varphi(4)$	$\chi_1(4)$	r[b]	En[c]	Pop[d]
7–8	−176	155	77	**44**	**−70**	**164**	**−69**	−178	14.6	0.0	5.0
7–9	−177	144	84	**29**	**−100**	**−62**	**−160**	176	11.8	1.5	4.4
7–10	−178	150	76	**46**	**−168**	**50**	**−94**	−52	5.6	0.9	3.8
8(D-Orn^2,Lys^5)											
8–1	179	145	143	**−138**	**164**	**−133**	**−136**	−61	12.5	3.4	43.5
8–2	−176	155	86	**−140**	**162**	**−142**	**−136**	−61	12.5	3.4	10.3
8–3	−176	143	83	**−153**	**162**	**−103**	**−154**	−178	15.0	1.5	10.1
8–4	−176	139	84	**−154**	**108**	**−1**	**−78**	−59	8.5	0.0	7.7
8–5	−177	159	78	**40**	**−161**	**−62**	**−97**	−59	10.5	2.2	6.2
8–6	−175	147	81	**29**	**−175**	**−61**	**−91**	−59	10.9	0.7	6.1

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

for deprotection of Boc group. Fmoc protection of side chain amino groups incorporated in position 2 and 5 was removed by treatment with piperidine, and cyclization was performed by treatment with bis(*p*-nitrophenyl)carbonate. The peptides were cleaved from the resin by treatment with TFA. The peptides were purified to homogeneity by HPLC. The molecular weights were confirmed by ESI-MS.

In vitro opioid activity profiles of the peptides were determined using the GPI and the MVD assays (Table 1). The peptides showed very high agonists potency both in GPI and MVD assays. A very clear relationship between the activity and the size of the ring was observed as shown in Figure 2. The most active analogs are those containing 18-membered ring. The introduction of ureaethyl group resulted in various shifts in the activity. A comparison of opioid activities of the previously reported peptide amides and substituted peptide amides is presented in Table 2. The directions of the shifts are somewhat different, but still a relatively close correlation is observed in most cases. Because the performed structural modifications may also have an impact on biological activity *in vivo*, these results suggest that some of these peptides are as good candidates as the nonsubstituted amides for further biological studies.

1D and 2D homo- and heteronuclear NMR spectra were recorded for all eight compounds in water. The full assignment of the peaks was achieved in their ^{1}H and ^{13}C NMR spectra. The proton chemical shifts along with the vicinal couplings, $^3J_{H\alpha HN}$, and the temperature coefficients, $\Delta\delta/\Delta T$, of the signals of the amide protons determined in the 1D proton spectra are presented in Supporting Information, Table S1. Carbon and nitrogen chemical shifts are listed in Supporting Information, Table S2. The observed NMR chemical shifts are very similar to those previously obtained for the nonsubstituted peptide amides.

A global conformational search was carried out for all eight compounds followed by fitting to the experimental data. NOE experimental contacts (average 10 per residue) 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 3%.

This resulted in 4–12 conformations with the sum of their statistical weights amounting to 83.9–99.8% in each case. The parameters that characterize the chosen conformations are listed in Table 3 and their drawings are shown in Figure 3. The diversity of conformations is indicated by the rmsd data presented in Table 4.

The comparison of biological activity and conformational diversity of the peptides described in this paper with those of the peptides of the same sequence in the main ring reported by us in [3] revealed that in general both groups of analogs show similar behavior. The conformational similarity is presented semiquantitatively in Table 5, where rmsd values are given for the heavy atoms of the main ring of each peptide. The data of the first and second rows are given for peptides of the [3] and this work separately, and in the third row, the main ring rmsd calculated for combined conformers of both groups of peptide are listed. It may be seen that the presence of the additional *C*-terminal ureido group does not change the rmsd value considerably.

Table 4. Calculated rmsd (in Å) for conformers of peptides **1**–**8** with the populations above 3%

Compound	**1**(Lys^2,Dap^5)	**2**(Lys^2,Dab^5)	**3**(Lys^2,Orn^5)	**4**(Lys^2,Lys^5)	**5**(Orn^2,Dap^5)	**6**(Orn^2,Dab^5)	**7**(Orn^2,Orn^5)	**8**(Orn^2,Lys^5)
[a]	1.98	2.59	1.46	1.66	2.27	1.85	1.25	2.16
[b]	0.44	0.75	0.74	0.50	0.51	0.70	0.72	0.67
[c]	0.32	0.41	0.44	0.29	0.28	0.39	0.36	0.29
[d]	96.0%	85.7%	84.5%	93.7%	90.9%	91.1%	95.7%	83.9%

[a] Calculated using all heavy atoms of all residues.
[b] Calculated using carbon and nitrogen atoms of main ring.
[c] Calculated for tyrosine–phenylalanine 'spacer', using seven heavy atoms of the backbone: C_α and C′ of D-Daa^2; N, C_α, C′ of Gly^3; N, C_α of Phe^4.
[d] The sum of relative populations of conformations above 3%.

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Table 5. Comparison of rmsd (in Å) calculated using carbon and nitrogen atoms of main ring for conformers of peptides **1**–**8** with the populations above 3% with those of [[3]]

Compound	**1**(Lys^2,Dap^5)	**4**(Lys^2,Lys^5)	**5**(Orn^2,Dap^5)	**6**(Orn^2,Dab^5)	**7**(Orn^2,Orn^5)	**8**(Orn^2,Lys^5)
[a]	0.51	0.95	0.67	0.66	0.72	0.77
[b]	0.44	0.50	0.51	0.70	0.72	0.67
[c]	0.57	0.90	0.69	0.70	0.74	0.76

[a] rmsd of conformers of the peptide taken from [[3]].
[b] rmsd of conformers of the peptide from this paper.
[c] rmsd of combined conformers of the peptide from this paper and those taken from [[3]].

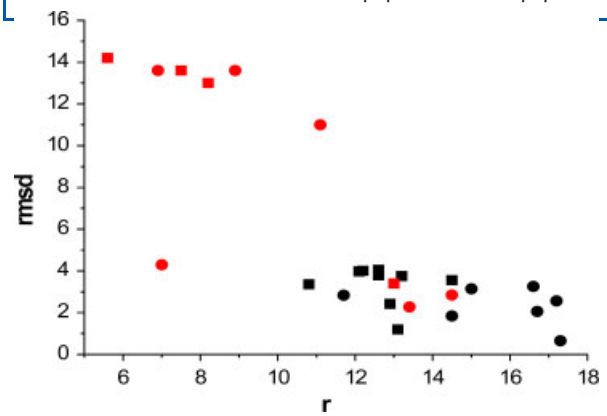

Figure 4. Diagram showing the correlation of the distances between the centers of the aromatic rings and the arbitrary calibrated rmsd values (in ´Å) calculated for torsional angles $\psi(2)$, $\varphi(3)$, $\psi(3)$, and $\varphi(4)$ in the 'spacer' segment. Data for Ref. 3 shown in black and for those of this paper shown in grey; circles, D-Lys^2/Dap^5; squares, D-Orn^2/Dap^5. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

In Ref. 3, the biologically active conformation was proposed by us on the basis of correlation between 'spacer' rmsd and distances between the aromatic rings. When the same diagram is applied to both groups of peptides (Figure 4), this also confirms the general similarity of distributions of conformations with some of them being close to this biologically active and the rest being substantially different.

However, careful inspection of the conformers in two cases, namely for D-Lys^2,Dap^5 and D-Orn^2,Dap^5, revealed significant differences in this distribution (Figure 4). Whereas for peptides of Ref. 3, only active conformers were found, and one may see that for compounds with additional ureido moiety, the conformers with large rmsd and/or small distance between aromatic rings prevailed. This finding is in reasonable correlation with the observed decrease in agonist potency of both peptides in the GPI assay. The magnitudes of the relative potencies of the two compounds of this work were lower by one order than those of corresponding peptides without the *N*-(ureidoethyl) moiety at their *C*-terminal.

This observation once again confirms that rather large distance between both aromatic rings is important for biological activity and the stiffness of the 'spacer' between both aromatic residues is also important. Probably, this reduces significantly entropic component in the peptide–protein interaction. Even though the conformation of the peptide complex with target protein remains still unknown, we believe that our results shed at least some light on the likely structural propensities of these peptide ligands.

Conclusions

The method using HCl/dioxane for removal of Boc protection and TFA for cleavage of the peptide from the resin proved to be useful for synthesis of peptide *N*-(ureidoethyl)amides. Cyclic enkephalin analogs, containing a carbonyl bridge, were obtained by using this method for construction of the peptide chain. These peptides were found to be agonists in the GPI and MVD assays. The activity depended greatly on the size of the ring. The peptides containing 18-membered ring were found to be the most potent. The activities of these *N*-substituted amides were slightly lower then their nonsubstituted counterparts described earlier. It seems, however, that the activity is sufficiently high to justify further examination *in vivo*.

Statistical weights of conformations for each peptide were obtained with global conformational search using EDMC method in combination with experimental NOE parameters. Generally, the conformational propensities of the main ring found for this group of peptides are very similar to those proposed earlier [3] for the peptides without *N*-ureidoethyl moiety. However, in two cases, namely for peptides **1** (D-Lys^2,Dap^5) and **5** (D-Orn^2,Dap^5), a significant number of conformers were found that differ to a large extent from the proposed biologically active conformation [3]. This finding is in correlation with the decrease of activity of these two peptides and corroborates the biologically active conformation proposed by us earlier [3].

Supporting information

Supporting information may be found in the online version of this article.

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