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Highly Potent Side Chain–Main Chain Cyclized Dermorphin–Deltorphin Analogues: An Integrated Approach Including Synthesis, Bioassays, NMR Spectroscopy and Molecular Modelling

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Abstract: Our continuing efforts to study structure–activity relationships of peptide opioids have resulted in the synthesis of a series of cyclic opioids related to dermorphins and deltorphins. The biological activities of the compounds have been determined and the conformational analyses carried out using ^{1}H-NMR spectroscopy and molecular modelling. The three compounds in the series Tyr-c[D-Orn-Phe-Ala], Tyr-c[D-Lys-Phe-Ala], and Tyr-c[A_2bu-Phe-Ala-Leu] are cyclized *via* a lactam bridge from the side-chain of the residue at the second position with the carboxyl terminus of each compound. The molecules incorporate 12-, 13- and 14-membered rings, respectively. They include a phenylalanine at the third position which is a distinguishing characteristic of dermorphins and deltorphins. The guinea pig ileum and mouse vas deferens assays show that the compounds are highly active at both μ- and δ-opioid receptors. The compounds are all highly effective antinociceptive agents as measured by the intrathecal rat hot plate test. Conformational analyses of the molecules indicate that they can adopt topochemical arrays required for bioactivity at both μ- and δ-receptors which explains their high activity in both guinea pig ileum and mouse vas deferens *in vitro* assays. The results support our models for μ- and δ-receptor activity for constrained peptide opioids.

Keywords: Opioid bioactivities; bioactive conformations; NMR studies; molecular modelling; synthesis of cyclic opioids

Dermorphin(Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) [1] and deltorphins (deltorphin or dermenkephalin: Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2; deltorphin I, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2; and deltorphin II, Tyr- D-Ala-Phe-Glu-Val-Val-Gly-NH_2)[2, 3] are families of naturally occurring peptide opioids isolated from the skin of a South American frog (*Phyllomedusa*). These peptides contain an N-terminal Tyr residue and a Phe residue which are connected by a D-amino acid. Structure–bioactivity

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studies of these peptides indicated that the free amino and phenolic groups of Tyr and the phenyl ring of Phe were important for opioid activity [3–5]. These pharmacophoric groups are arranged in a specific array when they bind to an opioid receptor. Thus, to understand the molecular basis of opioid bioactivity, it is critical to explore the molecular topology and bioactivity relationships.

We have employed an integrated approach including synthesis, bioassays and conformational analysis of constrained analogues to study the bioactivity of dermorphin analogues. A series of linear compounds in which N-methylated amino acids are systematically incorporated into the N-terminal tetrapeptide of dermorphin (e.g. Tyr-D-[NMe]Ala-Phe-D-Pro-NH_2) was studied [6]. The N-methylation affects the torsion angles which define the three-dimensional array of the pharmacophoric groups of dermorphin. Correlation of these values and bioactivities of the corresponding analogues provided torsion angles which could be utilized to establish the conformations responsible for the bioactivity of dermorphin at the μ-receptor. The resulting conformation is similar to those proposed for the μ-receptor bioactivities of morphiceptin [7] and enkephalin [8] analogues.

We also studied side chain–mainchain and side chain–side chain cyclized analogues of dermorphin (Tyr-c[D-Orn-Phe-Gly] [9] and Tyr-c[D-Orn-Phe-Asp]-NH_2 [10]). In *in vitro* bioassays, the 12-membered Tyr-c[D-Orn-Phe-Gly] molecule is highly μ-receptor selective but shows some activity at the δ-receptor. The 13-membered Tyr-c[D-Orn-Phe-Asp]-NH_2 compound shows even higher preference for μ- receptors over δ-receptors. Conformational analyses of these analogues resulted in two preferred conformations for each analogue [11, 12]. One of the two preferred conformations for the 12-membered analogue and both preferred conformations for the 13-membered analogue are topologically the same as the conformations for μ-receptor recognition from the studies of linear dermorphins [6], morphiceptins [7], and cyclic enkephalins [8]. Thus, we proposed these conformations as a general topology responsible for the bioactivity of dermorphin at the μ-receptor.

Another preferred conformation of the 12-membered analogue is topochemically similar to the conformations responsible for δ-receptor recognition of enkephalin opioids [8, 13]. This type of pharmacophoric array was not observed among the preferred conformations of δ-receptor inactive opioid analogues of our previous studies. However, it is difficult to propose this conformation as the topology responsible for the bioactivity of dermorphin analogues at the δ-receptor because the 12-membered analogue shows a low level of activity at the δ-receptor. To explore the conformation responsible for the bioactivity of dermorphin–deltorphin family at the δ-receptor, we set out to study highly δ-receptor active analogues with a molecular structure similar to that of Tyr-c[D-Orn-Phe-Gly].

To obtain analogues which are highly potent at the δ-receptor, we attempted to stabilize the proposed backbone conformation of Tyr-c[D-Orn-Phe-Gly] by incorporating local constraints. The Gly of this analogue adopted a conformation where $\phi = -70°$ and $\psi = 80°$ in both preferred conformations [14]. Since these angles are those of the most probable conformations for Ala [15], we synthesized the 12-membered analogue Tyr-c[D-Orn-Phe-Ala]. We also synthesized an Ala^4 containing 14-membered analogue, Tyr-c[D-A_2bu-Phe-Ala-Leu] which contains the sequential characteristics of the dermorphin-deltorphin family. However, its molecular structure is based on that of the 14-membered cyclic enkephalin analogue Tyr-c[D-A_2bu-Gly-Phe-Leu] originally reported by Schiller and DiMaio [16]. Since this type of molecular structure was extensively studied in our laboratories using cyclic opioid peptide analogues (e.g. Tyr-c[D-A_2bu-Gly-βNal(1)-Leu] and Tyr-c[D-A_2bu-Phe-Phe-Leu] [8,17], we can use our accumulated conformational information. In addition, our study includes the 13-membered analogue Tyr-c[D-Lys-Phe-Ala] which was originally reported by Spatola and coworkers [18]. This analogue differs from Tyr-c[D-Orn-Phe-Ala] only in its ring size and is one of the most potent opioid analogues reported to date at both μ- and δ-receptors. These three analogues contain the common pharmacophoric residues of the dermorphin-deltorphin family and are cyclized by lactam formation between the side chain amine of the second residue and the carboxyl terminus. Thus, we denote these analogues as sidechain–mainchain-cyclized dermorphin–deltorphin analogues.

Synthesis of Analogues

To synthesize the 12-membered analogue Tyr-c[D-Orn-Phe-Ala], the fully protected tetrapeptide, Boc-Tyr(*t*Bu)-D-Orn(Cbz)-Phe-Ala-OBzl (Boc, *t*-butyloxycarbonyl; *t*Bu, *t*-butyl; Cbz, benzyloxycarbonyl; and Bzl, benzyl) was assembled from the carboxy terminus to the amino terminus. The Boc protecting strategy and the coupling method using 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) and *N*-hydroxybenzotriazole (HOBt) were applied. After removal of the Cbz and Bzl protecting groups from

the side chain amine of D-Orn and the carboxy terminus of Ala, respectively, cyclization of the resulting tetrapeptide was carried out in dilute *N,N*-dimethylformamide (DMF) solution. As a coupling reagent for cyclization, the BOP-reagent [benzotriazolyloxytri(dimethylamino)phosphoniumhexafluorophosphate] was utilized in the presence of the insoluble base sodium bicarbonate ($NaHCO_3$). The Boc and *t*Bu protecting groups of the protected cyclic peptide were removed by acidolytic deprotection using trifluoroacetic acid (TFA) in the presence of thioanisole. The resulting crude product was purified by flash chromatography and gel filtration (Sephadex LH-20).

The 13-membered analogue Tyr-c[D-Lys-Phe-Ala] was synthesized using a method combining solid-phase peptide synthesis and peptide synthesis in solution. The partially protected linear precursor Fmoc-Tyr-D-Lys-Phe-Ala-OH was assembled on a standard Merrifield resin and cleaved using the hydrogen fluoride protocol. Side chain to main chain cyclization was carried out in solution using diphenylphosphoryl azide (DPPA) and HOBt, resulting in Fmoc-Tyr-c[D-Lys-Phe-Ala]. After removal of the Fmoc protecting group with piperidine, the crude product was purified using gel filtration (Sephadex LH-20) and medium pressure chromatography on a Vydac C_{18} reversed-phase column.

The linear precursor of the 14-membered cyclic analogue, Boc-Tyr(*t*Bu)-D-A_2bu-Phe-Ala-Leu-OBzl, was synthesized using a similar procedure employed for the synthesis of the 12-membered cyclic analogue. The Boc-D-A_2bu(Cbz)-OH was obtained from Boc-D-Gln-OH. The side chain amide of Boc-D-Gln-OH was converted to an amine by treatment with bromine and an NaOH solution according to the mechanism of the Hofmann rearrangement. The resulting amine was protected with a Cbz group. The cyclization of this 14-membered analogue was carried out in a solution of 10% di-isopropylethylamine (DIEA) in DMF. The BOP reagent was used as a coupling agent. After acidolytic deprotection of the resulting protected cyclic peptides, the desired products were purified by MCI GEL CHP-20P and Sephadex LH-20 chromatography.

GPI and MVD Assays and Spinal Antinociceptive Effects

The *in vitro* biological potencies of the analogues were determined by measuring the inhibition of stimulated contractions of isolated muscle preparations. Agonist activities of analogues at the μ and δ opioid receptors were determined using the guinea pig ileum (GPI) and the mouse vas deferens (MVD) assay, respectively. For the *in vivo* assay, the potency of producing spinal antinociception was examined [19]. After injection of our compounds into the rat spinal cord, antinociceptive end points were assessed using a thermally evoked escape response as the measured end point. The observed bioactivities are summarized in Table I.

The cyclic dermorphin–deltorphin analogues Tyr-c[D-Orn-Phe-Ala], Tyr-c[D-Lys-Phe-Ala) and (Tyr-c[D-A_2bu-Phe-Ala-Leu) are superactive μ- and δ-agonists. The replacement of the Gly residue in Tyr-c[D-Orn-Phe-Gly] with Ala drastically increases the δ-receptor activity (two orders of magnitude difference). On the contrary, the μ-agonist potency was increased only by a factor of five. Increasing the ring size from 12 to 13 atoms through incorporation of Lys in place of Orn enhanced the bioactivity at both μ- and δ-receptors. Both the 13- and the 14-membered analogues are considerably more active when compared with the enkephalin analogues with similar

Table I Biological Activities of Side chain–Main chain Cyclized Dermorphin–Deltorphin Analogues

Analogues	GPI IC_{50} [nM]	MVD IC_{50} [nM]	MVD/GPI IC_{50}-ratio	*in vivo* ED_{50} [nmol]
Tyr-c[D-Orn-Phe-Ala]	1.72 ± 0.42	3.51 ± 0.3 5	2.04	0.016
Tyr-c[D-Lys-Phe-Ala]	0.19[a]	0.51[a]	2.7[a]	0.0 30
Tyr-c[D-A_2bu-Phe-Ala-Leu]	0.63 ± 0.049	1.72 ± 0.2 7	2.71	0.025
Tyr-D-Ala-Phe-Gly-NH_2	45.2 ± 3.19[b]	510 ± 31.82[b]	11.3	–
Tyr-c[D-Orn-Phe-Gly]	8.60 ± 0.78	145 ± 15	16.9	0.13
Morphine	58.8[c]	644 [c]	11[c]	15

[a][18].
[b]S. Salvadori *et al.* (1982). *Int. J. Peptide Protein Res. 19*, 536.
[c]S. Salvadori *et al.* (1984). *Hoppe-Seyler's Z. Physiol. Chem., 364*, 1199.

molecular structure; Tyr-c[D-Lys-Gly/Ala-Phe] [18] and Tyr-c[D-A$_2$bu-Gly/Ala-Phe-Leu] [9,16].

In the *in vivo* assays, these 12-, 13- and 14-membered analogues showed the highest activities among the opioid analogues tested under the same conditions. The degree of spinal antinociception was determined by the ED$_{50}$ value obtained from the analysis of the rat hot plate test data. The 12-membered analogue Tyr-c[D-Orn-Phe-Ala] is the most potent. The ED$_{50}$ of the 12-membered analogue indicates that it is almost 1000 times more potent than morphine. The 13- and 14-membered analogues exhibit activities 500–600 times higher than that of morphine.

NMR Spectroscopy

The 12-Membered Cyclic Analogue Tyr-c[D-Orn-Phe-Ala]. The ^{1}H-NMR spectra for Tyr-c[D-Orn-Phe-Ala] were obtained in DMSO-d$_6$ at 27°C. To assign the proton resonances, two-dimensional experiments including homonuclear Hartmann–Hahn (HOHAHA) [20], correlated spectroscopy (COSY) [21], and rotating frame nuclear Overhauser spectroscopy (ROESY) [22, 23] were carried out. The NOEs were also obtained from the ROESY experiments and were qualitatively classified according to their intensities, i.e., s, strong; m, medium; and w, weak. The NOE data for this analogue are summarized in Table II.

The vicinal ^{1}H–^{1}H coupling constants between protons of H–N–C$^\alpha$–H ($J_{NH-C^\alpha H}$) and H–C$^\alpha$–C$^\beta$–H ($J_{\alpha-\beta}$) were determined by analysing the NH, α- and β-proton signals from one-dimensional spectra. The $J_{NH-C^\alpha H}$ is related to the torsion angle ϕ, which represents the rotational state around the skeletal single bond NH–C$^\alpha$H. The ϕ angles for each residue of Tyr-c[D-Orn-Phe-Ala] were obtained using a Karplus-type equation [24]:

$$J_{NH-C^\alpha H} = A\cos^2|\phi \pm 60°| - B\cos|\phi \pm 60°| + C \quad (1)$$

[(+) for a D-configuration and (−) for an L-configuration, respectively]. As values for the A, B and C coefficients, we used (i) (8.6, 2.9, 0.0) (Cung *et al.* [25]) and (ii) (8.6, 1.0, 0.4) (Bystrov *et al.* [26]). The two sets of ϕ angles estimated are summarized in Table III. Using the $J_{N^\gamma H-C^\gamma H}$ value and the same procedure, the torsion angle χ_4 around the C$^\delta$–N$^\delta$ bond of the D-Orn residue was also estimated. In this determination, another type of Karplus equation, suitable for an achiral residue, was utilized:

$$J_{NH-C^\alpha H} = A\cos^2\phi - B\cos\phi + C\sin^2\phi \quad (2)$$

(A, B, C) = (6.0, 1.5, 12.5) [27]. The calculated χ_4 values of the D-Orn residue are also included in Table III.

The three possible rotational states of the C$^\alpha$–C$^\beta$ bond (χ_1) are illustrated by the Newman projections

Table II Summary of the Important NOEs

Tyr-c[D-Orn-Phe-Ala]		Tyr-c[D-Lys-Phe-Ala]		Tyr-c[D-A$_2$bu-Phe-Ala-Leu]	
NOE	Intensity	NOE	Intensity	NOE	Intensity
Tyr^{1}H$_\alpha$-D-Orn^{2}H$_N$	Strong	Tyr^{1}H$_\alpha$-D-Lys^2-H$_N$	Strong	Tyr^{1}H$_\alpha$-D-A$_2$bu^{2}H$_N$	Strong
Tyr^{1}H$_\alpha$-Tyr^{1}H$_\beta$	Medium	Tyr^{1}H$_\alpha$-Tyr^{1}H$_{\beta l}$	Strong	Tyr^{1}H$_\alpha$-Tyr^{1}H$_{\beta l}$	Medium
		Tyr^{1}H$_\alpha$-Tyr^{1}H$_{\beta h}$	Medium	Tyr^{1}H$_\alpha$-Tyr^{1}H$_{\beta h}$	Medium
T yr^{1}H$_{\phi 2,6}$-Ala^{4}H$_\beta$	Weak	Tyr^{1}H$_{\phi 2,6}$-Ala^{4}H$_\beta$	Weak	Tyr^{1}H$_{\phi 2,6}$-Ala^{4}H$_\beta$	Weak
Tyr^{1}H $_{\phi 3,4}$-Ala^{4}H$_\beta$	Weak	Tyr^{1}H$_{\phi 3,4}$-Ala^{4}H$_\beta$	Weak	Tyr^{1}H$_{\phi 3,4}$-Ala^{4}H$_\beta$	Weak
D-Orn^{2}H$_N$-D-Orn^{2}H$_\alpha$	Weak	D-Lys^{2}H$_N$-D-Lys^{2}H$_\alpha$	Weak	D-A$_2$bu^{2}H$_N$-D-A$_2$bu^{2}H$_\alpha$	Medium
D-Orn^{2}H$_\alpha$-Phe^{3}H$_N$	Weak	D-Lys^{2}H$_\alpha$-Phe^{3}H$_N$	Weak	D-A$_2$bu^{2}H$_\alpha$-Phe^{3}H$_N$	Strong
D-Orn^{2}H$_{N\delta}$-Ala^{4}H$_\alpha$	Medium	D-Lys^{2}H$_{N\varepsilon}$-Ala^{4}H$_\alpha$	Strong	D-A$_2$bu^{2}H$_{N\gamma}$-Leu^{5}H$_\alpha$	Medium
D-Orn^{2}H$_\alpha$-D-OrnH$_{\beta l}$	Medium	D-Lys^{2}H$_\alpha$-D-Lys^{2}H$_{\beta l}$	Medium	D-A$_2$bu^{2}H$_\alpha$-D-A$_2$bu^{2}H$_{\beta l}$	Strong
D-Orn^{2}H$_\alpha$-D-OrnH$_{\beta h}$	Weak	D-Lys^{2}H$_\alpha$-D-Lys^{2}H$_{\beta h}$	Weak	D-A$_2$bu^{2}H$_\alpha$-D-A$_2$bu^{2}H$_{\beta h}$	Weak
				D-A$_2$bu^{2}H$_{N\gamma}$-Leu^{5}H$_N$	Medium
Phe^{3}H $_N$-Phe^{3}H$_\alpha$	Weak	Phe^{3}H$_N$-Phe^{3}H$_\alpha$	Weak	Phe^{3}H$_N$-Phe^{3}H$_\alpha$	Medium
Phe^{3}H< SIα-Ala^{4}H$_N$	Medium	Phe^{3}H$_\alpha$-Ala^{4}H$_N$	Medium	Phe^{3}H$_\alpha$-Ala^{4}H$_N$	Medium
Phe^{3}H< SIα-Phe^{3}H$_{\beta l}$	Medium	Phe^{3}H$_\alpha$-Phe^{3}H$_{\beta l}$	Medium	Phe^{3}H$_\alpha$-Phe^{3}H$_{\beta l}$	Weak
Phe ^{3}H$_\alpha$-Phe^{3}H$_{\beta h}$	Medium	Phe^{3}H$_\alpha$-Phe^{3}H$_{\beta h}$	Medium	Phe^{3}H$_\alpha$-Phe^{3}H$_{\beta h}$	Medium
Ala^{4}H$_N$-Ala^{4}H$_\alpha$	Medium	Ala^{4}H$_N$-Ala^{4}H$_\alpha$	Medium	Ala^{4}H$_N$-Ala^{4}H$_\alpha$	Weak
				Ala^{4}H$_\alpha$-Leu^{5}H$_N$	Weak
				Leu^{5}H$_N$-Leu^{5}H$_\alpha$	Medium
				Leu^{5}H$_\alpha$-Leu^{5}H$_{\beta l}$	Strong
				Leu^{5}H$_\alpha$-Leu^{5}H$_{\beta h}$	Weak

Table III NMR Parameters and Calculated Conformational Preferences for Tyr-c[D-Orn-Phe-Ala]

Residue	$J_{NH-H\alpha}$ (Hz)	ϕ (deg)[a] (i)	(ii)	$d\delta/dT$ (p.p.b./deg)	$J_{H\alpha-H\beta}$ $J_{H\alpha-H\beta\ l}$	$J_{H\alpha-H\beta\ h}$	Side Chain Populations[c] g^-	t	g^+
D-Orn[2]	7.24	−60	−42	−4.2	3.33	7.17	0.52	0.42	0.07
		80	−78						
		160	87						
			153						
D-Orn[2]	<line width	± 45[b]	−3.3	–	–	–	–	–	–
(Sidechain)	6.23	± 160	–	–	–	–	–	–	–
Phe[3]	6.65	60	36	−3.3	9.37	7.16	0.57	0.35	0.08
		−77	84						
		−163	−83						
			−157						
Ala[4]	8.09	60	−93	−5.7	–	–	–	–	–
		−85	−147						
		−155							
Tyr[1]	–	–	–	–	7.72	7.17	0.40	0.35	0.25
							(0.35)	(0.40)	

[a]Values were calculated using $J_{NH\text{-}H\alpha} = A\cos^2\phi - B\cos\phi + C$, where (+) is for the D-configuration and (−) is for the L-configuration.
(i) (A, B, C) = (8.6, 2.9, 0.0) as proposed by Cung *et al.* [25], (ii) (A, B, C) = (8.6, 1.0, 0.4) as proposed by Bystrov *et al.* [26] for a chiral residue.
[b] Values were calculated using $\Sigma J_{NH\text{-}H\alpha} = 6.0\cos^2\phi + 12.5\sin^2\phi$ as proposed by Kopple *et al.* [27] for an achiral residue.
[c] Values were calculated using J_τ = 13.56 and J_G = 2.60 Hz for a nonaromatic side chain [28], J_τ = 13.85 and J_G = 3.55 Hz for an aromatic sidechain [29].

in Figure 1. The two β-protons are distinguished by the subscripts a and b. Fractions of the individual conformers $f_1(\chi_1)$ for an L-residue may be estimated by using a conventional expression such as

$$\begin{aligned} f_1(g^-) &= (J_{\alpha-\beta a} - J_G)/(J_T - J_G) \\ f_1(t) &= (J_{\alpha-\beta b} - J_G)/(J_T - J_G) \\ f_1(g^+) &= 1 - [f_1(g^+) + f_1(t)] \end{aligned} \quad (3)$$

In the case of a D-residue, $f_1(g^+)$ and $f_1(t)$ are estimated from the vicinal coupling constants $J_{\alpha-\beta b}$, respectively. The Pachler values of J_T = 13.56 Hz and J_G = 2.60 Hz [28] were used for the *trans* and *gauche* couplings, respectively. For an aromatic amino acids Tyr and Phe, the values J_T = 13.85 Hz and J_G = 3.55 Hz [29] were employed.

The two geminal β-protons of each amino acid were assigned by employing the same methods as used in our previous studies [17]. The two protons of $C^\beta H_2$ groups are distinguished by appending a subscript 'h' for the proton at higher field of NMR spectra and 'l' for that at lower field. Since no informative NOEs permitting the assignment of the two β-protons of Tyr were observed, the identity of $H^{\beta a} = H^{\beta l}$ and $H^{\beta b} = H^{\beta h}$ was assumed according to the previous results. The $H^{\beta l}$ and $H^{\beta h}$ of the Phe[3] residue were assigned as $H^{\beta a}$ and $H^{\beta b}$, respectively and for the D-Orn residue, $H^{\beta l}$ and $H^{\beta h}$ were assigned as $H^{\beta a}$ and $H^{\beta b}$ (Figure 1), respectively, considering NOEs ($H^{\beta h}$–H^α, $H^{\beta l}$–H^α, $H^{\beta l}$–NH and $H\gamma l$–H^α), $J_{\alpha-\beta l}$ (3.33 Hz), $J_{\alpha-\beta h}$ (7.17 Hz) and the estimated ϕ^2 angles from $J_{NH\text{-}C\alpha H}$.

The temperature coefficients of the NH proton chemical shifts $d\delta/dT$ for the 12-membered analogue are presented in Table III. Temperature coefficients measure solvent shielding of an amide proton. Thus, these data can provide indications of the presence of intramolecular hydrogen bonding. It is generally accepted that the magnitude of $d\delta/dT$ is small for amide protons involved in intramolecular hydrogen bonding and large for protons engaged in intermolecular hydrogen bonding with another solute or a solvent molecule. The temperature coefficients ($d\delta/dT$) of all amide protons of Tyr-c[D-Orn-Phe-Ala] suggest that no intramolecular hydrogen bonding exists. These results are additionally supported by strong and medium sequential NOEs (between the NH and α-proton of the preceding residues), indicating a cyclic peptide backbone conformation in which the amide planes are oriented nearly perpendicular to the 12-membered backbone ring.

The 13-membered Cyclic Analogue Tyr-c[D-Lys-Phe-Ala]. The NMR characterizations for this analogue

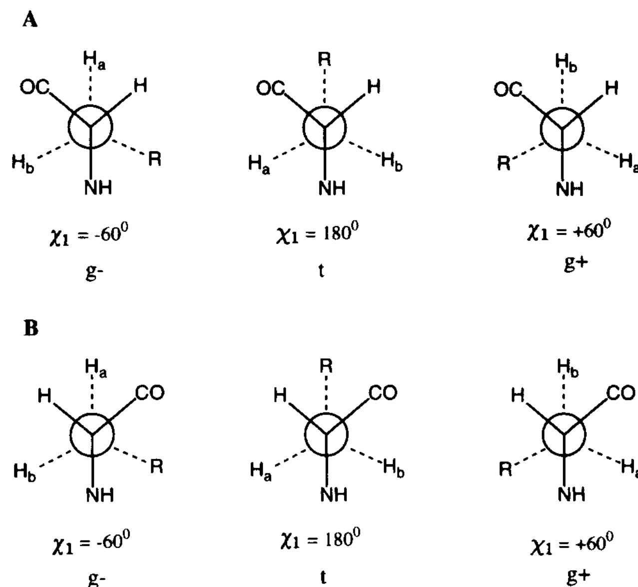

Figure 1. Newman projection about the C^{α}-C^{β} bonds in the sidechain: A for L-residue and B for D-residue.

were performed by using the same procedures as in the analysis of the 12-membered analogue. The results are summarized in Tables II and IV. Interestingly, the ϕ angles of this analogue estimated from $J_{NH-C\alpha H}$ are essentially the same as those of the 12-membered analogue. The assignment of the prochiral protons of the β-carbons was also attempted. No informative NOEs were observed regarding the assignment of the C^{β}-protons of Tyr and Phe residues. The C^{β}-protons of Tyr were assigned in a similar manner as that used in the analysis of Tyr-c[D-Orn-Phe-Ala]. For the Phe residue, such an assignment is unimportant since the $J_{\alpha-\beta l}$ and $J_{\alpha-\beta h}$ are similar and thus, the fractions of g- and t-conformers are essentially equal. The $H^{\beta h}$ and $H^{\beta l}$ of D-Lys were assigned as $H^{\beta b}$ and $H^{\beta a}$, respectively (Figure 1).

Among the absolute values of the temperature coefficients of the amide protons (Table IV), only the value of the Phe amide proton is smaller than 3.0 p.p.b./K. The sequential NOE between Phe^3 and D-Lys^2 has an intensity between weak and medium while all the others are strong. These results may indicate that this amide proton is involved in a weak intramolecular hydrogen bond.

The 14-membered Cyclic Analogue Tyr-c(D-A₂bu-Phe-Ala-Leu).

The NMR parameters obtained for this analogue are summarized in Tables II and V. The prochiral protons on the β-carbon of each residue were assigned. As in the above two cases, no informative NOEs were observed regarding the assignment of β protons for the Tyr residues. The prochiral β protons of the other residues were assigned in manner similar to that used in previous studies [17]. In the case of the D-A_2bu residue, $H^{\beta l}$ is $H^{\beta a}$ and $H^{\beta h}$ is $H^{\beta b}$ and for the Phe residue, the identities $H^{\beta l} = H^{\beta a}$ and $H^{\beta h} = H^{\beta b}$ were obtained (Figure 1). The $H^{\beta l}$ and $H^{\beta h}$ of Leu were assigned as $H^{\beta b}$ and $H^{\beta a}$, respectively (Figure 1). These assignments are in agreement with those of Tyr-c[D-A_2bu-Phe-Phe-Leu] which has a similar molecular structure [17].

The temperature coefficients $d\delta/dT$ indicate the existence of two intramolecular hydrogen bonds in this analogue: one involving the Leu NH ($d\delta/dT = 2.5$ p.p.b./K) and the other involving the D-A_2bu side chain $N^{\gamma}H$ ($d\delta/dT = -0.7$ p.p.b./K). The hydrogen bond acceptor can be deduced from the NOEs and coupling constants. The NOEs between Ala NH–Leu NH (weak) and the weak sequential NOE between Ala $C^{\alpha}H$–Leu NH are evidence for a β-type turn about Phe^3–Ala^4. In addition, the estimated ϕ angles of Phe^3 and Ala^4 agree with such a turn structure. A medium NOE between D-A_2bu$N^{\gamma}H$ and Leu NH indicates that the amide proton of the D-A_2bu side chain is directed toward the cyclic backbone, forming a seven-membered ring with a hydrogen bond to the D-A_2bu C(O). (A very weak NOE was observed between the D-A_2bu $N^{\gamma}H$ and the Leu $C^{\alpha}H$. This provides further evidence for the direction of the side chain amide bond of D-A_2bu toward the backbone ring.) Thus, the common acceptor for the hydrogen bonds emanating from Leu NH and

Table IV NMR Parameters and Calculated Conformational Preferences for Tyr-c[D-Lys-Phe-Ala]

Residue	$J_{NH-H\alpha}$ (Hz)	ϕ (deg)[a] (i)	(ii)	$d\delta/dT$ (p.p.b./deg)	$J_{H\alpha-H\beta}$ $J_{H\alpha-H\beta\ l}$	$J_{H\alpha-H\beta\ h}$	Side Chain Populations[c] g^-	t	g^+
D- Lys[2]	7.51	−60	−46	−3.6	3.60	7.50	0.46	0.45	0.09
		81	−74						
	159	89							
			151						
D- Lys[2]	3.38	± 73[b]	−4.2	–	–	–	–	–	
(Side chain)	8.12	± 122	–	–	–	–	–	–	
Phe[3]	7.47	60	46	−2.5	7.89	7.14	0.35	0.42	0.23
		−81	74						
		−159	−89						
			−151						
Ala[4]	7.54	60	47	− 5.4	–	–	–	–	–
		−82	72						
		−158	−89						
			−151						
Tyr[1]	–	–	–	–	6.96	7.97	0.33	0.43	0.24
							(0.43)	(0.33)	

[a] Values were calculated using $J_{NH-H_\alpha} = A\cos^2|\phi \pm 60| - B\cos|\phi \pm 60| + C$ where (+) is for the D-configuration and (−) is for the L-configuration.
(i) (A, B, C) = (8.6, 2.9, 0.0) as proposed by Cung *et al.* [25], (ii) (A, B, C) = (8.6, 1.0, 0.4) as proposed by Bystrov *et al.* [26] for a chiral residue.
[b] Values were calculated using $\Sigma J_{NH-H\alpha} = 6.0\cos^2\phi - 1.5\cos\phi + 12.5\sin^2\phi$ as proposed by Kopple *et al.* [27] for an achiral residue.
[c] Values were calculated using J_τ = 13.56 and J_G = 2.60 Hz for a nonaromatic side chain [28], J_τ = 13.85 and J_G = 3.55 Hz for aromatic side chain [29].

Table V NMR Parameters and Calculated Conformational Preferences for Tyr-c[D-A$_2$bu-Phe-Ala-Leu]

Residue	$J_{NH-H\alpha}$ (Hz)	ϕ (deg)[a] (i)	(ii)	$d\delta/dT$ (p.p.b./deg)	$J_{H\alpha-H\beta}$ $J_{H\alpha-H\beta\ l}$	$J_{H\alpha-H\beta\ h}$	Side Chain Populations[c] g^-	t	g^+
D-A$_2$bu[2]	7.40	−60	−49	− 5.9	3.18	7.97	0.46	0.49	0.05
		81	−71						
		159	90						
			150						
D-A$_2$bu[2]	3.39	± 69[b]		− 0.7	–	–	–	–	–
(Sidechain)	7.71	± 126	–	–	–	–	–	–	
Phe[3]	7.09	60	41	−6.2	10.23	4.47	0.65	0.09	0.26
		−79	79						
		−161	−86						
			−154						
Ala[4]	5.24	45	24	− 5.3	–	–	–	–	–
		75	96						
		−69	−74						
	−171	−166							
Leu[5]	9.25	− 92	− 103	+2.5	3.70	10.18	0.69	0.10	0.21
		−148	−137						
Tyr[1]	–	–	–	− 6.01	8.01	0.24	0.43	0.33	

[a] Values were calculated using $J_{NH-H\alpha} = A\cos^2\phi - B\cos\phi + C$ where (+) is for the D-configuration and (−) is for the L- configuration.
(i) (A, B, C) = (8.6, 2.9, 0.0) as proposed by Cung *et al.* [25], (ii) (A, B, C) = (8.6, 1.0, 0.4) as proposed by Bystrov *et al.* [26] for a chiral residue.
[b] Values were calculated using $\Sigma J_{NH-H\alpha} = 6.0\cos^2\phi - 1.5\cos\phi + 12.5\sin^2\phi$ as proposed by Kopple *et al.* [27] for an achiral residue
[c] Values were calculated using J_τ = 13.56 and J_G = 2.60 Hz for a nonaromatic side chain [28], J_τ = 13.85 and J_G = 3.55 Hz for an aromatic side chain [29].

D-A_2bu N^γ H is D-A_2bu C(O). The same hydrogen-bonding pattern was observed in our studies of other 14-membered analogues, such as Tyr-c[D-A_2bu-Gly-βNal(1)-Leu] [8] and Tyr-c[D-A_2bu-Phe-Phe-Leu].[16]

Conformational Calculations

The search for preferred conformations consistent with the above NMR data started with distance geometry calculations to find all possible conformations consistent with the NOE data. Information about distances between two protons derived from observed NOEs was utilized. Ascribing a particular interproton distance to a strong, medium or weak NOE is based on the assumption that the cross-peak intensity for a particular pair of protons depends only on the distance separating these protons. We used ranges of 2.5 Å or less for strong, 2.5–3.0 Å for medium and 3.0–4.0 Å for weak NOEs. For each analogue, we generated 500 conformations from these calculations.

The structures obtained from the distance geometry calculations were minimized without NOE constraints for 100 steps to release steric constraints using the steepest descent algorithm. Cluster analysis was carried out for the 500 minimized conformations of each analogue to divide large numbers of structures into families of conformations. These calculations were based on similarity of backbone torsion angles. To obtain the conformations that comply with NMR data, the resulting conformational families of each analogue were examined. The NMR data included the estimated ϕ angles and the distance from the amide protons with low temperature coefficients to the potential hydrogen- bonding acceptors. From these processes, we obtained nine conformational families for the 12-membered Tyr-c[D-Orn-Phe-Ala] analogue, four for the Tyr-c[D-Lys-Phe-Ala] analogue and five for the Tyr-c[D-A_2bu-Phe-Ala-Leu] analogue.

For each of the above conformational families, molecular dynamics calculations were carried out using only NOE constraints related to backbone conformation. Structures appearing at each picosecond (ps) out of a total 100 ps for each conformation were fully minimized. To obtain preferred backbone conformations of each analogue, all the resulting minimized structures were compared with NMR parameters including the estimated ϕ angles, NOE data and hydrogen-bonding information. Four conformational families were obtained for the 12-membered analogue and four families were found for the 13-membered analogue. Calculations of the 14-membered analogue provided five different conformational families. The lowest energy conformation of each resulting conformational families is indicated in Table VI. The table includes torsion angles, total energy and distances between Tyr C^β–Phe C^β (d1), Tyr N^α–Tyr C^β (d2) and Tyr N^α–Phe C^β (d3).

Sidechain conformations of each residues were also examined. For each of the preferred backbone conformations of the 12- and 13-membered analogues, the favoured χ_1 of the second residue were g^- and t. In all families of preferred conformations, the structure containing the g^--conformation has lower energy than the structure containing the

Table VI Preferred Backbone Conformations of Cyclic Dermorphine–Deltrophin Analalogues

Conformation	ψ^1	ϕ^2	ψ^2	ϕ^3	ψ^3	ϕ^4	ψ^4	ϕ^5	ψ^5	energy	d1	d2	d3
1. Tyr-c[D-Orn-Phe-Ala]													
12a	143	180	55	−92	−75	−103	88			116.391	6.83	2.48	8.09
12b	151	172	31	−114	88	51	64			123.690	6.95	2.47	7.93
12c	115	150	53	−100	94	75	−75			122.324	8.27	2.48	7.69
12d	116	154	58	−90	−77	−105	90			116.377	8.15	2.48	7.79
2. Tyr-c[D-Lys-Phe-Ala]													
13a	130	159	44	−129	106	66	88			115.833	7.99	2.48	7.95
13b	173	64	54	−134	−74	−98	89			115.580	6.60	2.46	8.22
13c	123	153	47	−130	−73	−92	91			115.135	7.92	2.47	7.51
13d	114	76	41	−124	103	64	87			116.127	7.69	2.52	5.79
3. Tyr-c[D-A_2bu-Phe-Ala-Leu]													
14a	152	176	−117	78	−89	−99	−59	−81	−70	123.434	7.25	2.48	7.80
14b	129	144	−96	75	−92	−82	−64	−84	−57	122.815	7.68	2.48	8.25
14c	119	152	−128	72	−92	−87	−63	−89	−62	124.505	8.48	2.48	7.38
14d	129	103	−96	76	−91	−82	−64	−85	−57	125.332	7.65	2.48	6.09
14e	139	106	−127	77	−89	−88	−64	−88	−67	123.833	7.96	2.48	6.48

t-conformation. (Since the torsion angles in Table VI are from the lowest energy structure of each conformational family, only the g^--conformation appeared). For the 14-membered analogue, two different sets of $\chi(\chi_1, \chi_2)$ were observed in the preferred backbone conformations. One is (g^-, g^-) and the other is (t, g^+). These results are consistent with our previous studies of the cyclic opioid peptides with similar molecular structure: Tyr-c[D-A_2bu-Gly-βNal(1)-Leu] [8] and Tyr-c[D-A_2bu-Phe-Phe-Leu]. [17]

Conformational preferences of the side chains of Tyr, Phe and Leu were examined by rotation of the χ_1 angle by 120° increments. If serious overlaps with other functional groups were found at a specific angle, the conformation containing this angle was not considered as a preferred conformation. After minimization of the conformations with different rotational states of the side chains, the energy of the resulting conformations were compared. If a conformation possessed an energy 10 kcal/mol above that of the lowest energy conformer, this conformation was not considered as a preferred conformation. For the Tyr residue of the analogues, all three conformers (g^-, t and g^+) can exist. These results are consistent with the NMR studies of these analogues. For all three analogues, the side chain rotamer populations of Tyr estimated from $J_{\alpha-\beta}$ are not much different from each other. The side chain of Phe of all three analogues prefers $\chi_1^1 = g^-$ and t. Between the g^-- and t-conformations, the g^--conformation is energetically more favourable than the t-conformation. These results agree with the observation that the fraction of the g^--conformer obtained from $J_{\alpha-\beta}$ is higher than those of the other conformers (t and g^+).

General Considerations

On the basis of the results of the biological assays and of the conformational studies, we proposed models of the conformations required for the biological activities of the side chain–mainchain cyclized dermorphin–deltorphin analogues at the μ- and δ-receptors. We believe that the topology responsible for the bioactivity at a receptor should be observed among the preferred conformations of all analogues which are active at that receptor. Thus, the preferred conformations obtained from the studies of these analogues and from previous studies of other dermorphin or deltorphin analogues were compared and correlated with their biological activities. We extracted the characteristics which were common to the active molecules and were not found in the inactive molecules. The resulting conformations were then proposed as the topologies responsible for the bioactivities of the analogues. In addition, we recognize that the solvent employed in the NMR experiments to obtain preferred conformations differs from the conditions in the biodomain. However, the conformations adopted by the cyclic dermorphin–deltorphin analogues used in this study are limited since they are partially constrained. Thus, the environment could affect the populations among the individual preferred conformers but does not drastically change the accessible conformations.

As mentioned, the bioactivities of opioid analogues are primarily determined by the spatial array of the pharmacophoric groups (free amine and the phenolic group of Tyr and phenyl group of Phe). Thus, we only considered the three-dimensional arrangements of the pharmacophoric groups in the comparison of preferred conformations. The array of these pharmacophoric groups for opioid acitivity can be presented by a combination of torsion angles of the bonds linking these groups. In the case of dermorphin-deltorphin, the relative spatial arrangements of pharmacophoric groups can be defined by eight torsional angles: ψ^1, χ_1^1 and ω^1 of Tyr; ϕ^2, ψ^2 and ω^2 of the second residue; and ϕ^3 and χ_1^3 of Phe (Figure 2). Among these eight torsional angles, ω^1 and ω^2 must be 180°. The NMR studies of these three cyclic dermorphin-deltorphin analogues showed that all the amide bonds adopt the *trans* conformation. The side chain conformations of Tyr and Phe are flexible enough to adopt three possible rotameric states (g^-, t and g^+). Thus, the four backbone angles (ψ^1, ϕ^2, ψ^2 and ϕ^3) play critical roles in arranging the pharmacophoric groups into an array required for recognition by opioid receptors. The common pharmacophoric backbone topologies were examined by comparing the four torsion angles and the three

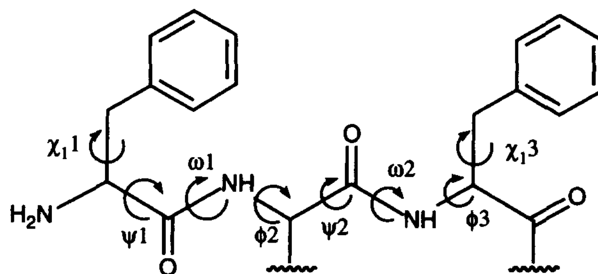

Figure 2. A schematic representation of the dermorphin structure. Torsion angles given in this figure define spatial orientations of opioid pharmacophores, the amino and phenolic groups of Tyr and the aromatic ring of Phe, required for receptor recognition.

distances between Tyr C^{β}–Phe C^{β}, Tyr NH_2–Tyr C^{β} and Tyr NH_2–Phe C^{β}.

Two distinct families of common topologies were obtained from these comparisons. The conformations 12a, 12b, 13b and 14a represent one family of topologies (topology A) and the conformations 12c, 12d, 13c and 14c represent another family (topology B). The four important torsion angles of the common structures selected from the preferred conformations of 12- and 13-membered analogues are similar to each other in both topologies. The ψ^1s of these structures (12a, 12b, 12c, 12d, 13b and 13c) are approximately 160° and 120° for topologies A and B, respectively. The second residue adopts a conformation in which $\phi = 150°$ and $\psi = 50°$. The ϕ^3s are approximately −110° with variations in both structures. The ψ^1 and ϕ^2 of the selected common conformations (14a and 14c) from the preferred conformations of the 14-membered analogues are essentially the same as the other three in both topologies. However, the ψ^2 and ϕ^3 are approximately −120° and 70° respectively. These angles are also different by 180° from the corresponding angles of the 12- and 13-membered analogues in each common topology set. Since the bonds represented by ψ^2 and ϕ^3 are connected by an amide plane, the simultaneous 180° rotation of these two angles represents only a change in the direction of the amide bond. This rotation does not affect the overall topology of the pharmacophoric groups.

In the NMR studies of the three analogues (discussed earlier in this paper), the side chain conformation of Tyr ($\chi_1{}^1$) is flexible and displays no clear preference for one of the three possible rotamers (g^-, t and g^+). The three rotomer populations of the Tyr side chain are similar to each other for all three analogues (Tables III, IV and V). Thus, to determine the side chain conformation of Tyr responsible for bioactivity, we employed the results obtained from the studies of other cyclic dermorphin–deltorphin analogues carried out by Schiller and coworkers [30]. In these studies, they incorporated constrained Tyr mimetics such as Hat (2-amino-6-hydroxytetralin-2-carboxylic acid) and Htc (or HO-Tic: 7-hydroxy-1,2,3,4,-tetrahydroisoquinoline carboxylic acid), into the side chain–side chain cyclized demorphin–deltorphin analogue, Tyr-c[D-Orn-Phe-Glu]. Both the D- and the L-Hat-containing analogue were active at μ- and δ-receptors. The side chain of D-Hat can adopt only the g^+- and t-conformations and that of L-Hat can assume only the g^-- and t-conformations. Thus, it can be deduced that the t-conformation which is common to the D- and L-Hat residues is needed for bioactivity. In addition, both of the analogues containing Htc, (D and L)-Htc-c[D-Orn-Phe-Glu], are essentially inactive at both μ- and δ-receptors. Since the D- and L-Htc residues cannot adopt the t-conformation, these inactivities also support the importance of the t-conformation of the Tyr side chain for the bioactivity of the dermorphin–deltorphin families.

From the side chain variation, we observed that the Phe side chain for the common backbone conformations preferentially adopted g^-- and t-conformations. The g^+-conformations generated show serious overlap between the phenyl ring and the cyclic backbone structure leading to energetically unfavorable structures. These results indicate that either the g^-- or the configuration t should be considered as required for the bioactivity of the cyclic dermorphin–deltorphin analogues. No *direct evidence permitted us unequivocally to* select one of these two conformations as the required side chain conformation of Phe for bioactivity.

However, we have already proposed a topology for μ-receptor recognition of dermorphin from studies of the following highly μ-receptor selective analogues: Tyr-D-(NMe)Ala-Phe-D-Pro-NH_2,[6] Tyr-c[D-Orn-Phe-Asp]-NH_2 and Tyr-c[D-Orn-Phe-Gly] [11, 12, 14]. In this proposed structure, the two aromatic side chains are on opposite sides of the cyclic backbone ring and the side chains of the Tyr and Phe residues adopt the t-conformation. For the compounds in this study, we found that the conformation which adopt the backbone topology B (12c, 12d, 13c and 14c in Table VI) with the t-conformation for the side chain of Tyr and Phe (Figure 3) are essentially the same as our previously suggested topology. Since the analogues studied are highly active at the μ-receptor, we reconfirm the topology responsible for the bioactivity of the dermorphin–deltorphin family at the μ-receptor.

Figures 4 and 5 depict the conformations which contain the backbone topology A (12a, 12b, 13b and 14a in Table VI). Figure 4 depicts the conformation with t-conformation for the Phe side chain while Figure 5 depicts the g^--conformation for the Phe side chain. These conformations are characterized by the Tyr side chain above the peptide backbone ring with a relatively short distance between the phenyl ring of Phe and the side chain of Ala. This structure is supported by the observation of a weak NOE between the phenolic ring proton of Tyr and the C^{β}-protons of Ala in the ROESY spectra of all three analogues. The examination of the energy terms of these conforma-

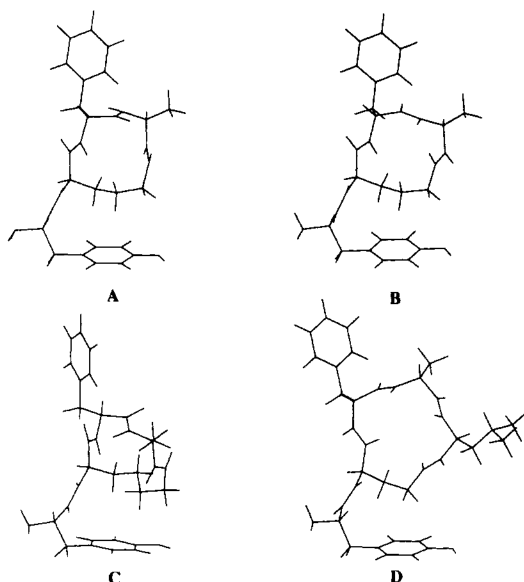

Figure 3. The topologies responsible for the bioactivity of cyclic dermorphin–deltorphin analogs at the μ-receptor. The backbone conformations are from: A the conformation 12c; B the conformation 12d; C the conformation 13c; and D the conformation 14c of Table VI. The side chain conformations (χ_1) of Tyr and Phe are t and t, respectively.

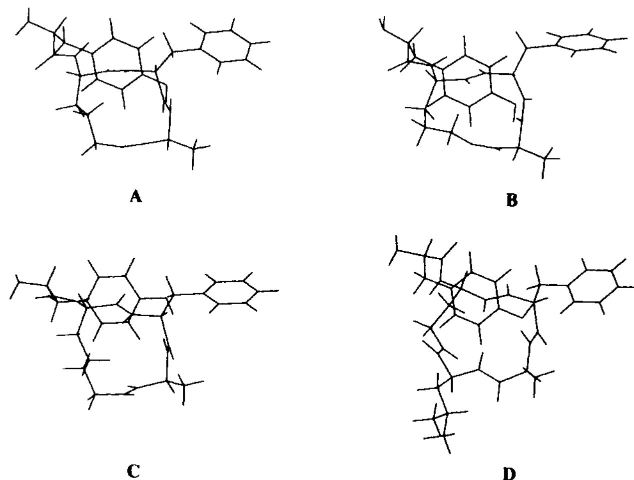

Figure 4. The topologies responsible for the bioactivity of cyclic dermorphin-deltorphin analogues at the δ-receptor. The backbone conformations are from: A the conformation 12a; B the conformation 12b; C the conformation 13b; and D the conformations 14a of Table VI. The sidechain conformations (χ_1) of Tyr and Phe are t and t, respectively.

tions indicated that these structures are stabilized by non-bonded hydrophobic clustering.

The conformations in Figures 4 and 5 were observed as the preferred conformations of Tyr-c[D-

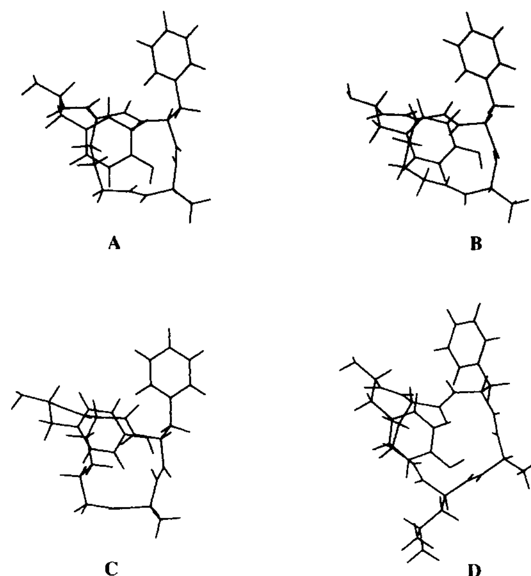

Figure 5. The topologies responsible for the bioactivity of cyclic dermorphin- -deltorphin analogues at the δ-receptor. The backbone conformations are from: A the conformation 12a; B the conformation 12b; C the conformation 13b; and D the conformation 14a of Table VI. The sidechain conformation (χ_1) of Tyr and Phe are t and g, respectively.

Orn-Phe-Gly] which shows low activity at the δ-receptor. However, these conformations were not observed among the preferred conformations of the dermorphin analogues Tyr-c[D-Orn-Phe-Asp]-NH_2 and Tyr-D-(NMe)Ala-Phe-D-Pro-NH_2, which are only active at the μ-receptor [6, 11, 12, 14]. In addition, the topologies in Figure 4 are essentially the same as those suggested for the δ-receptor recognition of cyclic enkephalins by Hruby *et al.* [13] and our laboratories [8]. On the contrary, the topologies in Figure 5 are similar to the conformation responsible for the δ-receptor activity of DPDPE suggested by Mosberg *et al.* [31]. In our studies of cyclic enkephalins, we also found a similar topology in which χ_1^4 adopts a g-conformation. In those studies, the energies of the conformations where $\chi_1^4 = g^-$ were 3–5 kcal/mol higher than the suggested conformations where $\chi_1^4 = t$.

Thus, we propose that these conformations (Figures 4 and 5) can explain the superactivity of the side chain-main chain cyclized dermorphin-deltorphin analogues at the δ-receptor. Deltorphins have essentially the same pharmacophoric sequences as these analogues and are only active at the δ-receptor. Thus, either one or both of the conformations in Figures 4 and 5 represent the conformation responsible for the bioactivities of deltorphins.

Conclusions

The side chain–mainchain cyclized dermorphin-deltorphin analogues reported here are highly potent both *in vitro* and *in vivo*. From conformational studies, specific conformations responsible for the bioactivities of dermorphin–deltorphin analogues at the δ- and μ-receptors are suggested. The topology for δ activity is defined by the placement of the phenolic ring over the backbone cyclic structure with a distance less than 9 Å between the two aromatic rings. The structure for μ activity is characterized by the placement of the two aromatic rings on opposite sides of the cyclic backbone with a relatively large distance between them (> 11 Å). Since deltorphin is only active at the δ-receptor, we propose either one or both of the conformations suggested for recognition of the δ-receptor (Figures 4 and 5) as the conformation(s) required for the bioactivity of deltorphins.

The conformations emerging from these studies are topologically equivalent to the conformations proposed for the bioactivities of enkephalin at the μ- and δ-receptors and the bioactivity of morphiceptin at the μ-receptor. Thus, these proposed topologies represent the conformations required for the bioactivities of peptide opioids at the δ- and μ-receptors. We now use these topologies as general models to design novel opioids.

EXPERIMENTAL

Amino acid derivatives were purchased from Bachem Inc. All other chemicals and solvents were of reagent grade and were used with or without further purification after purchase from Fisher Scientific Co., Aldrich Chem. Co. and Sigma Chem. Co. The resin for solid-phase synthesis was purchased from BioRad Laboratories, Inc. Reaction progress and product purity were routinely monitored by thin-layer chromatography (TLC) and are designated using the following abbreviations: CMA for $CHCl_3$/MeOH/AcOH,CM for $CHCl_3$/MeOH and BWA for n-BuOH/H_2O/AcOH. Sephadex LH-20 and MCI GEL CHP-20P were purchased from Sigma Chem. Co. and Mitsubishi Chemical Industries. A Waters gradient high-performance liquid chromatography (HPLC) system was employed for checking the purity of products using a Vydac 'Protein & Peptide C18' 4 mm × 25 cm column (Cat. No. 218TP54 # 36). The ^{1}H-NMR spectra were obtained with a General Electric GN-500 spectrometer operating at 500 MHz and/or a 360 MHz Fourier Transform built and operated at the University of California, San Diego. Chemical shifts (δ) are reported in p.p.m. downfield from TMS as the internal standard. Amino acid analyses were carried out in the laboratories of Dr T. Hugli at Scripps Clinic, La Jolla, California, on a Biotronic amino acid analyser LC 6001. The FAB-MS were measured by Dr R. Kondrat at the Mass Spectrometry Facility at the University of California, Riverside.

N_α-*tert*-Butyloxycarbonyl-phenylalanyl-alanine Benzyl Ester (Boc)Phe-Ala-OBzl) (1)

In DMF, Boc-Phe-OH (1.33 g, 5.01 mmol) and HCl*-Ala·OBzl (1.19 g, 5.52 mmol) were dissolved and HOBt (0.811 g, 6.00 mmol) was added. After cooling the reaction mixture down to −10°C, EDC (1.15 g, 6.0 mmol) was added. The pH of the reaction mixture was adjusted to 7 and stirring was continued at 0°C for 30 min and at room temperature for 15 h. After checking the completion of the reaction by TLC, water was added to the reaction mixture. The product was extracted with ethyl acetate (EtOAc). The combined organic layers were washed with saturated sodium bicarbonate ($NaHCO_3$), brine, 0.5 N HCl and brine until the pH of the brine layers were approximately 7. After the washed organic layer was dried over magnesium sulphate ($MgSO_4$), the solvent was removed under reduced pressure to yield an oil. Recrystallization of the resulting oil from EtOAc/hexane provided **1** as a white solid (1.90 g, 89.1%); R_f CMA (95 : 5 : 3) 0.60; ^{1}H-NMR (360 MHz, DMSO-d_6, 25°C) δ 1.265 (s, 9H, Boc), 1.320 (d, 3H, Ala $C^\beta H_3$), 2.885, 2.932 (mm, 2H, Phe $C^\beta H_2$), 4.197 (m, 1H, Ala $C^\alpha H$), 4.346 (m, 1H, Phe $C^\alpha H$), 5.109 (s, 2H, Bzl CH_2), 6.863 (d, 1H, Phe NH), 7.170, 7.237 (mm, 5H, Phe ϕ), 7.344 (m, 5H, Bzl ϕ), 8.422 (d, 1H, Ala NH).

N_α-*tert*-Butyloxycarbonyl-N_γ-benzyloxycarbonyl-D-ornithyl-phenylalanyl-alanine benzyl ester (Boc-D-Orn(Z)-Phe-Ala-OBzl) (2)

The compound **1** (Boc-Phe-Ala-OBzl: 0.853 g, 2.00 mmol) was dissolved in a CH_2Cl_2 and TFA mixture (1 : 1) at 0°C. After 10 min, the reaction was warmed to room temperature. The ice bath was removed. Stirring was continued at room temperature until all the starting material was consumed. After removal of CH_2Cl_2 and TFA *in vacuo*, the resulting dipeptide salt was coupled to Boc-Orn(Cbz)-OH (0.806 g, 2.20 mmol). For this coupling, the same method used in the synthesis of

compound **1** was employed. Recrystallization of the resulting crude product from EtOAc/hexane provided **2** as a white solid (1.23 g, 91.5%); R_F CM (90:10) 0.31; FAB-MS(MH)$^+$ 675; ^{1}H-NMR (360 MHz, DMSO-d$_6$, 25°C) δ 1.1 (m, 2H, D-Orn C$^\gamma$H$_2$), 1.244 (m, 2H, D-Orn C$^\beta$H$_2$), 1.315 (s, 9H, Boc), 1.347 (m, 3H, Ala C$^\beta$H$_3$), 2.637 (m, 1H, Phe C$^\beta$H), 2.818 (m, 2H D-Orn C$^\delta$H$_2$), 3.003 (m, 1H, Phe C$^\beta$H), 3.805 (m, 1H, Ala C$^\alpha$H), 4.345 (m, 1H, D-Orn-C$^\alpha$H), 4.530 (m, 1H, Phe C$^\alpha$H, 4.999 (s, 2H, Z CH$_2$), 5.101 (s, 2H, Bzl CH$_2$), 6.728 (m, 1H, D-Orn N$^\alpha$H), 7.098 (m, 1H, D-Orn N$^\gamma$H), 7.171, 7.334 (mm, 15H, Z, Bzl, and Phe ϕ), 8.120 (m, 1H, Ala NH), 8.397 (m, 1H, Phe NH).

N_α-*tert*-Butyloxycarbonyl-O-tert-butyl-tyrosyl-N_γ-benzyloxycarbonyl-D-ornithyl-phenylalanyl-alanine benzyl ester (Boc-Tyr(*t*Bu)-D-Orn(Z)-Phe-Ala-OBzl) (3)

The Boc group of compound **2** (Boc-D-Orn(Z)- Phe-Ala-OBzl: 0.674 g, 1.00 mmol) was cleaved and the resulting peptide was coupled to Boc-Tyr(*t*Bu)-OH (0.374 g, 1.10 mmol) employing the same methods used in the synthesis of compound **2**. Recrystallization of the resulting residue from EtOAc/hexane produced **3** as a white solid (0.809 g, 90.6%); R_F CM (90 : 10) 0.39; FAB-MS(MH)$^+$ 894; ^{1}H-NMR (360 MHz, DMSO-d$_6$, 25°C) δ 1.153 (m, 2H, D-Orn C$^\gamma$H$_2$), 1.226 (s, 9H, Boc), 1.243 (s, 9H, *t*Bu), 1.2 (m, 2H, D-Orn C$^\beta$H$_2$), 1.323 (d, 3H, Ala C$^\beta$H$_3$), 2.628, 2.816, 2.967 (m, 5H, Phe and Tyr C$^\beta$ and D-Orn C$^\delta$H), 3.006 (m, 1H, D-Orn C$^\delta$H), 4.202 (m, 2H, D-Orn and Ala C$^\alpha$H), 4.347 (m, 1H, Phe C$^\alpha$H), 4.560 (m, 1H, Tyr C$^\alpha$H), 4.988 (s, 2H, Z CH$_2$), 5.112 (s, 2H, Bzl CH$_2$), 6.79 (m, 1H, Tyr NH), 6.816 (d, 2H, Tyr $\phi^{3,5}$H), 7.00 (m, 1H, D-Orn N$^\delta$H), 7.115 (d, 2H, Tyr $\phi^{2,6}$H), 7.197 (mm, 5H, Phe ϕ), 7.335 (m, 10H, Z and Bzl ϕ), 7.921 (m, 1H, D-Orn N$^\alpha$H), 8.315 (m, 1H, Ala NH), 8.544 (m, 1H, Phe NH).

N_α-*tert*-Butyloxycarbonyl-O-tert-butyl-tyrosyl-D-ornithyl-phenylalanyl-alanine(Boc-Tyr(*t*Bu)-D-Orn-Phe-Ala-OH) (4)

Compound **3** (Boc-Tyr(*t*Bu)-D-Orn(Z)-Phe-Ala-OBzl: 0.43 g, 0.48 mmol) was dissolved in MeOH (20 ml) and 10% Pd/C (0.03 g) was added. Hydrogenolysis was continued until the consumption of hydrogen ceased. After checking by TLC, the catalyst was removed by filtration through Celite 545. The filtrate was evaporated to give the crude product. Flash column chromatography yielded the desired product as a white solid (0.2 g, 62%); R_F CMA (85 : 10 : 5) 0.18, BWA (4 : 1 : 1) 0.45; FAB-MS(MH)$^+$ 670; ^{1}H-NMR (360 MHz, DMSO-d$_6$, 25°C) δ 1.204 (m, 3H, Ala C$^\beta$H$_3$), 1.258 (s, 9H, Boc), 1.288 (s, 9H, *t*Bu), 1.400 (m, 2H, D-Orn C$^\delta$H$_2$), 1.601 (m, 2H, D-Orn C$^\beta$H$_2$), 2.702, 2.887, 3.061 (m, 6H, Phe and Tyr C$^{\beta H_2}$), 3.766 (m, 1H, Ala C$^\alpha$H), 4.187 (m, 3H, D-Orn and Tyr and Phe C$^\alpha$H), 6.868 (d, 2H, Tyr $\phi^{3,5}$H), 7.086 (m, 1H, Tyr NH), 7.146 (d, 2H, Tyr $\phi^{2,6}$H), 7.206 (m, 5H, Phe ϕ), 7.521 (m, 1H, D-Orn N$^\alpha$H), 8.246 (m, 2H, Phe and Ala NH).

N_α-*tert*-Butyloxycarbonyl-*O*-*tert*-butyl-tyrosyl-c(D-ornithyl-phenylalanyl-alanine) (Boc-Tyr(*t*Bu)-c(D-Orn-Phe-Ala)) (5)

Compound **4** (Boc-Tyr(*t*Bu)-D-Orn-Phe-Ala-OH: 0.14 g, 0.21 mmol) was dissolved in DMF (250 ml) and BOP reagent (0.57 g, 1.3 mmol), HOBt (0.18 g, 1.3 mmol), and NaHCO$_3$ (0.1 g, 1.3 mmol) were added at 0°C. Stirring was continued overnight at room temperature. The solvent was removed *in vacuo* and the resulting oil was purified by flash column chromatography and preparative TLC to give **5** as a white powder (55 mg, 40%). R_F CMA (95 : 5 : 3) 0.40, CMA (80 : 20 : 3) 0.93; FAB-MS(MH)$^+$ 652; ^{1}H-NMR (360 MHz), DMSO-d$_6$, 25°C) δ 1.150 (d, 3H, Ala C$^\beta$H$_3$), 1.248 (s, 9H, Boc), 1.299 (s, 9H, *t*Bu), 1.483 (m, 2H, D-Orn C$^\delta$H$_2$), 1.660 (m, 2H, D-Orn C$^\beta$H$_2$), 2.691, 2.793, 2.955 (m, 6H, Phe and Tyr C$^\beta$H and D-Orn C$^\delta$H), 3.812 (m, 1H, Ala C$^\alpha$H), 3.950 (m, 1H, D-Orn C$^\alpha$H), 4.087 (m, 2H, Phe and Tyr C$^\alpha$H), 6.497 (m, 1H, Tyr NH), 7.052 (m, 1H, D-Orn-N$^\gamma$H), 6.855 (d, 2H, Tyr $\phi^{3,5}$H), 7.110 (d, 2H, Tyr $\phi^{2,6}$H), 7.164 (m, 5H, Phe ϕ), 7.722 (m, 1H, D-Orn N$^\alpha$H), 7.819 (m, 1H, Phe NH), 8.074 (m, 1H, Ala NH).

Tyrosyl-c(D-ornithyl-phenylalanyl-alanine) acetic acid salt (AcOH*Tyr-c(D-Orn-Phe-Ala) (6)

The protected cyclic peptide **5** (Boc-Tyr(*t*Bu)-c[D-Orn-Phe-Ala]: 40 mg, 61 μmol) was dissolved in CH$_2$Cl$_2$ (1 ml). After adding thioanisole (0.2 ml), the reaction mixture was cooled to 0°C. TFA (0.8 ml) was slowly added to the reaction mixture. After stirring for 10 min, the ice bath was removed. Stirring was continued at room temperature until all the starting material had reacted. Thioanisole was removed by evaporation with toluene and extraction with hexane/ethylether/toluene (7/5/2) from dilute acetic acid solution. The resulting crude product was purified using Sephadex LH20 and MCI GEL HP20P. After lyophilization, **6** was obtained as a white powder (23 mg, 62%); R_FBWA (4 : 1 : 1) 0.63;

FAB-MS(MH)$^+$ 496 (without AcOH); ^{1}H-NMR (500 MHz, DMSO-d$_6$, 25°C) δ 1.192 (d, 3H, Ala $C^{\beta}H_3$), 1.504, 1.579 (mm, 2H, D-Orn $C^{\beta}H_2$), 1.348, 1.484 (m, 2H, D-Orn $C^{\gamma}H_2$), 2.682, 3.176 (mm, 2H, D-Orn $C^{\delta}H_2$), 2.981, 3.065 (mm, 2H, Phe $C^{\beta}H_2$), 2.895 (m, 2H, Tyr $C^{\beta}H_2$), 3.853 (mm, 2H, Ala $C^{\alpha}H$), 3.975, 3.935 (m, 2H, D-Orn and Tyr $C^{\alpha}H$), 4.097 (m, 1H, Phe $C^{\alpha}H$), 6.630 (m, 1H, D-Orn $N^{\delta}H$), 6.722 (d, 2H, Tyr $\phi^{3,5}H$), 7.019 (d, 2H, Tyr $\phi^{2,6}H$), 7.189, 7.268 (mm, 5H, Phe ϕ), 8.155 (m, 3H, Tyr NH_3), 8.118 (d, 1H, Phe NH), 8.312 (d, 1H, D-Orn $N^{\alpha}H$), 8.253 (m, 1H, Ala NH).

N_{α}-Fluorenylmethyloxycarbonyl-*O-t*-butyl-tyrosyl-D-*N_{ε}-benzyloxycarbonyl*-D-*lysyl-phenylalanyl-alanyl-resin* (*Fmoc-Tyr(tBu)*-D-Lys(Z)-Phe-Ala-OCH$_2$-resine) (7)

The first amino acid (Boc-Ala-OH) was attached to the chloromethylated polystyrene (BioRad SX-1), giving a substitution level of 0.71 mmol/g. The Boc-Ala-OCH$_2$-polymer (1.4 g, 1 mmol) was subjected to three cycles of solid phase synthesis using the following protocol: CH_2Cl_2 wash (three times, 1 min), 40% TFA in CH_2Cl_2 (two times, 2 + 25 min), CH_2Cl_2 (six times, 1 min), 10% TEA in CH_2Cl_2 (two times, 2 + 5 min), CH_2Cl_2 (two times, 2 + 5 min), CH_2Cl_2 (three times, 1 min), DMF (three times, 1 min), coupling *N*-protected amino acid (4 mmol), BOP (4 mmol), HOBt (4 mmol), DIEA (8 mmol) for 2 h, DMF (three times, 1 min), CH_2Cl_2 (three times, 1 min). Completion of each coupling was monitored using the ninhydrin test. After coupling the last amino acid [Fmoc-Tyr(tBu)-OH], the peptide resin was extensively washed and dried to yield 2.38 g.

N_{α}-Fluorenylmethyloxycarbonyl-tyrosyl-D-lysyl-phenylalanyl-alanine (Fmoc-Tyr-D-Lys-Phe-Ala-OH) (8)

The assembled tetrapeptide on the resin (2.0 g) was treated with hydrogen fluoride (20 ml) in the presence of anisole (1 ml) and methylethyl sulphide (1 ml) at 0°C for 30 min. Then HF and scavengers were evaporated under reduced pressure. The remaining solid residue was washed three times with diethyl ether and the peptide was extracted with 50% acetic acid. A yellowish powder (758 mg) was obtained after lyophilization. Amino acid analysis: Ala 1.00; Tyr 0.89; Phe 0.99; Lys 0.99.

Tyrosyl-cyclo(D-lysyl-phenylalanyl-alanine) (H-Tyr-c(D-Lys-Phe-Ala) (9)

Partially protected tetrapeptide (Fmoc-Tyr-D-Lys-Phe-Ala-OH, 354 mg, 0.47 mmol) and *N*-hydroxybenzotriazole (71 mg, 0.47 mmol) were dissolved in 200 ml of dimethylformamide. The reaction mixture was cooled to −20°C, and diphenylphosphonyl azide (83 µl, 0.47 mmol) and triethylamine (65 µl, 0.47 mmol) were added. The reaction mixture was stirred for 2 h at −20°C and placed in a freezer (−20–25°C) for 48 h. During that period the pH was checked and maintained between 7.5 and 8 with addition of triethylamine. The progress of the cyclization was followed by TLC and HPLC. After completion of cyclization, the reaction mixture was acidified to pH 3–4 with glacial acetic acid and mixed bed ion exchange resin was added in 25 ml of water. The slurry was stirred for 30 min and then the resin was removed by filtration and the solvent removed *in vacuo*. To the oily residue, a 20% solution of piperidine in DMF (50 ml) was added and the reaction mixture was stirred for 30 min. The solvent was removed *in vacuo* and the remaining product was dissolved in 10 ml of 50% AcOH and applied on a Sephadex LH-20 column. Further purification by HPLC afforded 23 mg of the desired product; R_F BWA (4 : 1 : 1) 0.62, R_F CMA (15 : 4 : 1) 0.55;FAB-MS(MH)$^+$ 509; amino acid analysis Tyr (1.02) Lys (0.99) Phe (1.00) Ala (1.09).

N_{α}-*tert*-Butyloxycarbonyl-N_{γ}-benzyloxycarbonyl-diaminoisobutyric acid (Boc-D-A$_2$bu(Z)-OH) (10)

Boc-D-Gln-OH (4.0 g, 16.2 mmol) was dissolved in a 10% NaOH solution at −5°C. In a separate flask, bromine (1.08 ml, 21.1 mmol) and NaOH (4.70 g, 117.5 mmol) were dissolved in water (40 ml) to give a second solution, which was then added to the first solution. Stirring was continued for 2 h maintaining the temperature of the reaction mixture at 45°C. After checking by TLC (R_F BWA [4 : 1 : 1] 0.4), the reaction mixture was washed with EtOAc (2 × 30 ml). The aqueous layer was acidified using 6N HCl at 0°C and washed with chilled EtOAc until the yellow colour was removed. To protect the γ-amino group, the pH of the aqueous layer was adjusted to 10 with 4N NaOH and tetrahydrofuran (THF) (100 ml) was added. After cooling this solution to 0°C, benzylchloroformate (2.54 ml, 17.8 mmol) and 4N NaOH (2.48 ml, 9.92 mmol) were added. Stirring was continued overnight at room tempera-

ture. THF was removed *in vacuo* and the remaining aqueous layer was washed with a mixture of 50% ether/hexane (3 × 60 ml). The aqueous solution was acidified to pH 2 at 0°C using 6N HCl and the desired product was extracted with ether. After washing this ether layer with brine, removal of the solvent resulted in an oily product (2.70 g, 51%); R_FCMA (85:10:5) 0.53; FAB-MS(MH)$^+$ 353; ^{1}H-NMR (360 MHz, $CDCl_3$, 25°C) δ 1.442 (s, 9H, Boc), 1.830, 2.051 (mm, 2H, $C^{\beta}H_2$), 3.120, 3.502 (mm, 2H, $C^{\gamma}H_2$), 4.390 (m, 1H, $C^{\alpha}H$), 5.110 (d, 2H, Z CH_2), 5.370 (m, 1H, $N^{\gamma}H$), 5.53 (m, 1H, NH), 7.358 (m, 5H, Z ϕ).

N_{α}-*tert*-Butyloxycarbonyl-alanyl-leucine benzyl ester (Boc-Ala-Leu-OBzl) (11)

Using the coupling method used in the synthesis of compound **1**, Boc-Ala-OH (1.89 g, 10.0 mmol) and TsOH*LeuOBzl were coupled using EDC (2.16 g, 13.0 mmol) and HOBt (1.52 g, 13.0 mmol). The desired product was obtained quantitatively as an oil; R_F CMA (85:10:5) 0.61; FAB-MS(MH)$^+$ 393.

N_{α}-*tert*-Butyloxycarbonyl-phenylalanyl-alanyl-leucine benzyl ester (Boc-Phe-Ala-Leu-OBzl) (12)

After removing the Boc group from compound **11** (Boc-Ala-Leu-OBzl: 3.71 g, 9.47 mmol) by the standard procedure, the resulting dipeptide salt was coupled to Boc-Phe-OH (2.5 g, 9.47 mmol). For this coupling, the procedure used in the synthesis of compound **1** was applied using EDC (1.9 g, 9.9 mmol) and HOBt (1.4 g, 10.4 mmol). Recrystallization of the product from EtOAc/hexane provided **12** as a white powder (4.36 g, 85.3%); R_F CMA (85:10:5) 0.84; FAB-MS(MH)$^+$ 540; ^{1}H-NMR (360 MHz, $CDCl_3$, 25°C) δ 0.878 (m, 6H, Leu $C^{\delta}H_3$), 1.266 (m, 1H, Leu $C^{\gamma}H$), 1.290 (d, 3H, Ala $C^{\beta}H_3$), 1.348 (s, 9H, Boc), 1.621 (m, 2H, Leu $C^{\beta}H_2$), 2.860, 3.121 (mm, 2H, Phe $C^{\beta}H_2$), 4.314 (m, 1H, Phe $C^{\alpha}H$), 4.484 (m, 2H, Ala and Leu $C^{\alpha}H$), 5.118 (s, 2H, Bzl CH_2), 6.265 (m, 1H, Phe NH), 7.229 (m, 5H, Phe ϕ), 7.335 (m, 5H, Bzl ϕ), 7.792 (m, 1H, Leu NH), 7.923 (m, 1H, Ala NH).

N_{α}-*tert*-Butyloxycarbonyl-N_{γ}-Benzyloxycarbonyl-D-diaminobutyryl-phenylalanyl-alanyl-leucine benzyl ester (Boc-D-A_2bu(Z)-Phe-Ala-Leu-OBzl) (13)

The Boc group of compound **12** (Boc-Phe-Ala-Leu-OBzl: 2.7 g, 5.0 mmol) was cleaved, and the resulting peptide was then coupled to Boc-D-A_2bu(Z)-OH (1.76 g, 5.0 mmol). All the procedures were the same as those used for the synthesis of compound **2**. Recrystallization of the resulting residue produced **13**, a white solid (3.1 g, 80.0%); R_F CMA (85:10:5) 0.56; FAB-MS(MH)$^+$ 774; ^{1}H-NMR (360 MHz, DMSO, 25°C) 0.844, 0.888 (dd, 6H, Leu $C^{\delta}H_3$), 1.205 (d, 3H, Ala $C^{\beta}H_3$), 1.295 (m, 1H, Leu $C^{\gamma}H$), 1.347 (s, 9, Boc), 1.549, 1.664 (mm, 4H, Leu and D-A_2bu $C^{\beta}H_2$), 2.708, 2.800, 3.030, 3.070 (m, s, 4H, Phe $C^{\beta}H_2$ and D-A_2bu $C^{\gamma}H_2$), 3.900 (m, 1H, D-A_2bu $C^{\alpha}H$), 4.326 (m, 2H, Ala and Leu $C^{\alpha}H$), 4.532 (m, 1H, Phe $C^{\alpha}H$), 5.011 (s, 2H, Z CH_2), 5.107 (s, 2H, Bzl CH_2), 6.883, 7.050 (mm, 1, D-A_2bu NH), 7.138 (m, 1H, D-A_2bu $N^{\gamma}H$), 7.355 (m, 10H, Z and Bzl CH_2), 8.050 (m, 1H, Leu NH), 8.163 (m, 1H, Ala NH), 8.231 (m, 1H, Phe NH).

N_{α}-*tert*-Butyloxycarbonyl-*O*-*tert*-butyl-tyrosyl-D-N_{γ}-benzyloxycarbonyl-diamino-butyryl-phenylalanyl-alanyl-leucine benzyl ester (Boc-Tyr(*t*Bu)-D-A_2bu(Z)-Phe-Ala-Leu-OBzl) (14)

After removal of the Boc group of compound **13** (Boc-D-A_2bu(Z)-Phe-Ala-Leu-OBzl: 2.32 g, 3.0 mmol), the resulting tetrapeptide salt was attached to Boc-Tyr(*t*Bu)-OH (1.01 g, 3.0 mmol). All the procedures are the same as those for the synthesis of compound **2**. After flash column chromatography, the desired product was obtained as a white powder (2.7 g, 90.6%); R_F CMA (85:10:5) 0.76; FAB-MS(MH)$^+$ 993; ^{1}H-NMR (360 MHz, DMSO, 25°C) δ 0.842, 0.888 (dd, 6H, Leu $C^{\delta}H_3$), 1.211 (d, 3H, Ala $C^{\beta}H_3$), 1.240 (m, 1, Leu $C^{\gamma}H$), 1.269 (s, 9H, Boc), 1.314 (s, 9H, *t*Bu), 1.558 (m, 4H, Leu $C^{\gamma}H_2$ and D-A_2bu $C^{\beta}H_2$), 2.680, 3.100 (mm, 6H, Tyr and Phe $C^{\beta}H_2$ and D-A_2bu CH_2), 4.180 (m, 1H, Tyr $C^{\alpha}H$), 4.320 (m, 3H, Leu, Ala and D- A_2bu $C^{\alpha}H$), 4.590 (m, 1H, Phe $C^{\alpha}H$), 6.826 (d, 2H, Tyr $\phi^{3,5}$H) 6.970 (m, 1H, Tyr NH), 7.153 (d, 2H, Tyr $\phi^{2,6}$H), 7.188 (m, 1H, D-A_2bu $N^{\gamma}H$), 7.251 (m, 5H, Phe ϕ), 7.369 (m, 10H, Z and Bzl ϕ), 8.050 (m, 1H, Leu NH), 8.140 (m, 1H, D-A_2bu NH), 8.257 (m, 2H, Phe and Ala NH).

N_{α}-*tert*-Butyloxycarbonyl-*O*-*tert*-butyl-tyrosyl-D-diaminobutyryl-phenylalanyl-alanyl-leucine (Boc-Tyr(*t*Bu)-D-A_2bu-Phe-Ala-Leu-OH) (15)

The Cbz and Bzl groups of the compound **14** (Boc-Tyr(*t*Bu)-D-A_2bu(Z)-Phe-Ala-Leu-OBzl: 2.0 g, 1.3 mmol) were removed by hydrogenolysis using the same procedure described in the synthesis of compound **5**. The desired product was obtained by flash column chromatography (1.5 g, 90%); R_F CMA (85:10:5) 0.28; FAB-MS(MH)$^+$ 769; ^{1}H-NMR (360 MHz, 50% DMSO/$CDCl_3$, 25°C) δ 0.950 (m, 6H, Leu

$C^{\delta}H_3$), 1.251 (m, 1H, Leu $C^{\gamma}H$), 1.284 (m, 9H, Boc), 1.331 (m, 3H, Ala $C^{\beta}H_3$), 1.379 (s, 9H, tBu), 1.472 (m, 2H, Leu $C^{\beta}H_2$), 1.641, 1.722 (mm, 2H, D- A_2bu $C^{\beta}H_2$), 2.822, 2.993, 3.105 (mmm, 6H, Tyr and Phe $C^{\beta}H_2$ and D-A_2bu $C^{\gamma}H_2$), 4.213 (m, 1H, Tyr $C^{\alpha}H$), 4.313 (m, 1H, Leu $C^{\alpha}H$), 4.788 (m, 3H, Phe, D-A_2bu and Ala $C^{\alpha}H$), 6.793 (d, 2H, Tyr $\phi^{3,5}$), 6.989 (m, 1H, Tyr NH), 7.137 (d, 2H, Tyr $\phi^{2,6}H$), 7.184 (m, 5H, Phe ϕ).

N_{α}-tert-Butyloxycarbonyl-*O-tert*-butyl-tyrosyl-c(D-*diamino-butyryl-phenylalanyl-alanyl-leucine*) (Boc-Tyr(tBu)-c(D-A_2bu-Phe-Ala-Leu)) (16)

Compound **15** (Boc-Tyr(tBu)-D-A_2bu-Phe-Ala-Leu-OH: 0.3 g, 0.39 mmol) was dissolved in 10% DIEA/DMF (400 ml). After the reaction bath was cooled to the 0°C, BOP reagent (0.52 g, 1.17 mmol) and HOBt (0.06 g, 0.44 mmol) were added. Stirring was continued overnight. The solvent was removed *in vacuo* and the resulting oil was triturated in DMF/water (220 mg, 75.1%). Further purification was carried out using flash column chromatography and preparative TLC; R_F CM (9:1) 0.61, CM (95:5) 0.31, EtOAc 0.21; FAB-MS$(MH)^+$ 751; ^{1}H-NMR (360 MHz, $CDCl_3$, 25°C) δ 0.913 (m, 6H, Leu$^{\delta}H_3$), 1.268 (m, 3H, Ala $C^{\beta}H_3$), 1.322 (s, 9, Boc), 1.429 (s, 9, tBu), 1.464 (m, 1, Leu $C^{\gamma}H$), 1.547 (m, 2H, Leu $C^{\beta}H_2$), 2.100 (m, 2H, D-A_2bu $C^{\beta}H_2$), 2.961, 3.180 (mm, 6H, Tyr and Phe $C^{\beta}H_2$ and D-A_2bu $C^{\gamma}H_2$), 3.973 (m, 1H, Tyr $C^{\alpha}H$), 4.211 (m, 3H, Ala, Leu and D-A_2bu $C^{\alpha}H$), 4.700 (m, 1H, Phe $C^{\alpha}H$), 5.136 (m, 1H, Tyr NH), 6.923 (d, 2H, Tyr $\phi^{3,5}H$), 7.142 (d, 2H, Tyr $\phi^{2,6}H$), 7.159, 7.289 (mm, 5H, Phe ϕ).

Tyrosyl-c(D-diaminobutyryl-phenylalanyl-alanyl-leucine) acetic acid salt (AcOH*Tyr-c(D-A_2bu-Phe-Ala-Leu)) (17)

The Boc and tBu groups were removed from compound **16** (Boc-Tyr(tBu)-c[D-A_2bu-Phe-Ala-Leu], 68 mg, 61 μmol) and the resulting peptide was purified employing the same method used in the synthesis of compound **6**. After lyophilization, a white powder was obtained (32 mg, 52%); R_F BWA (4:1:1) 0.76; FAB-MS$(MH)^+$ 595 (without AcOH); amino acid analysis Tyr (1.00) Phe (1.01) Ala (1.06) Leu (1.05); ^{1}H-NMR (500 MHz, DMSO-d_6, 25°C) δ 0.829, 0.848 (mm, 6H, Leu $C^{\delta}H_3$), 1.346 (d, 3H, Ala $C^{\beta}H_3$), 1.559 (m, 1H, Leu $C^{\gamma}H$), 1.706 (m, 2H, Leu $C^{\beta}H_2$), 1.905 (m, 2H, D-A_2bu $C^{\beta}H_2$), 1.915 (s, 3H, AcOH CH_3), 2.495, 2.885 (mm, 2H, Tyr $C^{\beta}H_2$), 2.743, 3.329 (mm, 2H, D-A_2bu $C^{\gamma}H_2$), 3.166, 3.268 (mm, 2H, Phe $C^{\beta}H_2$), 3.385 (m, 1H, Tyr $C^{\alpha}H$), 3873 (m, 1H, Phe $C^{\alpha}H$), 4.010 (m, 1H, Ala $^{\alpha}H$), 4.241 (m, 1H, Leu $C^{\alpha}H$), 4.271 (m, 1H, D-A_2bu $C^{\alpha}H$), 6.639 (d, 2H, Tyr $\phi^{3,5}$), 6.810 (m, 1H, D-A_2bu $N^{\gamma}H$), 7.020 (d, 2H, Tyr $\phi^{2,6}$), 7.085, 7.186 (mm, 5H, Phe ϕ), 7.186 (m, 1H, Leu NH), 7.875 (m, 1H, D-A_2bu $N^{\gamma}H$), 8.627 (m, 1H, Ala NH), 8.736 (m, 1H, Phe NH).

***In Vitro* Assays.** The *in vitro* bioactivities of the compounds were tested in the GPI [32] and MVD [33]* assays as reported elsewhere [34, 35]. A log dose–response curve was determined with [Leu^5]-enkephalin as standard for each *ileum* and *vas* preparation. The IC_{50} values of the compounds tested were normalized according to a published procedure [36].

***In Vivo* Assays.** Male Sprague-Dawley rats (280–320 g; Harlan Industries) were implanted with chronic lumbar intrathecal (IT) catheters under halothane anesthesia according to modification of the method described by Yaksh and Rudy [37]. After recovery, the compounds which were dissolved in 20% 2-hydroxy-β-cyclodextrin (Research Biochemical Inc.) were administered intrathecally [19]. The antinociceptive potencies (ED_{50}) of the analogues were assessed by the hot plate test (52.5°C) according to the procedure published [19, 38].

Spectroscopy. The ^{1}H-NMR spectra were recorded at 500 MHz on a Bruker AMX spectrometer. Samples were prepared in DMSO-d_6 at concentrations between 10–15 mM. The resonance of DMSO-d_6 (δ = 2.49 p.p.m.) was used as an internal standard. The one-dimensional spectra were collected with 16k data points and with spectral widths of ± 3000. The two-dimensional homonuclear 16k data points and with spectral widths of ± 3000. The two-dimensional homonuclear Hartmann–Hahn(HOHAHA) experiments [20], were performed using the MLEV-17 sequence and the time-proportional phase increment. Mixing times of 75 ms with a spin-lock field of 10 kHz were employed. The rotating frame nuclear Overhauser (ROESY) experiments [22] were carried out with a mixing time of 150 ms and a spin locking field of 2.5 kHz. All the two-dimensional spectra were obtained using 2k data points in the t2 domain and 512 points in the t1 domain. Zero-filling was applied twice in the t1 domain. Multiplications with a phase-shifted sine function were employed.

Computer Simulations. All calculations were performed on a personal Iris 40–25 work station and an IRIS 40–340 computer. The distance geometry program DGEOM [39], was used to generate structures compatible with NOE constraints. Energy minimizations and molecular dynamic simulations were carried out with the DISCOVER force field program [40]. A dielectric constant of 45 was used for all calculations to represent the solvent environment of DMSO. Structures generated from DGEOM were minimized by the steepest descent algorithm for 100 steps. Molecular dynamics simulations were carried out for 100 ps preceded by 3 ps heating and equilibrating at 300 K. Structures were exported every 1 ps and minimized without constraints by using the VA09A algorithm until all the derivatives were smaller than 0.001 kcal/mol.

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