Cyclic β-Casomorphin Analogues with Mixed μ Agonist/δ Antagonist Properties: Synthesis, Pharmacological Characterization, and Conformational Aspects

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Analogues of the potent and moderately μ-opioid-receptor-selective cyclic β-casomorphin-5 derivative H-Tyr-c[-D-Orn-Phe-D-Pro-Gly-] (2) were prepared by conventional solution synthesis. Replacement of the Phe^3 residue by 2-naphthylalanine (2-Nal) led to a peptide (4) with high affinity for both μ and δ opioid receptors. This compound turned out to be an agonist in the μ-receptor-representative guinea pig ileum (GPI) assay but a moderately potent antagonist against various δ agonists in the δ-receptor-representative mouse vas deferens (MVD) assay. It thus represents the first known cyclic opioid peptide analogue with mixed μ agonist/δ antagonist properties. Interestingly, replacement of 2-Nal^3 in compound 4 with 1-naphthylalanine (1-Nal) resulted in an analogue (5) showing high affinity for μ receptors and a full agonist effect in the MVD assay that was mediated by both μ and δ receptors. Substitution of Trp for Phe^3 in 2 (compound 8) was well tolerated at both receptors and led to an analogue with agonist activity in both the GPI and MVD assays. Variation of the peptide ring size in 4 was achieved by substitution of D-Orn^2 with D-Lys (compound 6) or D-2,4-diaminobutyric acid (compound 7). Analogue 6 was also a mixed μ agonist/δ antagonist with somewhat lower potency than 4, whereas compound 7 displayed μ agonist and partial δ agonist properties. Further reduction of the peptide ring size, as achieved by deletion of the Gly^5 residue, produced a compound (9) which was a full agonist in both bioassays. Conformational analysis of analogues 2, 4, and 5 by ^{1}H NMR spectroscopy and molecular mechanics studies suggested that the overall conformation of parent compound 2 and the 2-Nal-containing peptide 4 was similar, while the side-chain orientation of 1-Nal in peptide 5 was different. These results suggest that the δ antagonist properties of analogue 4 may not be due to a difference in its overall conformation as compared to the agonist 2 but may be a direct effect of the 2-naphthyl moiety *per se* preventing proper alignment of the peptide for receptor activation.

The development of potent opioid agonists and antagonists with high specificity for each of the three major opioid receptor classes (μ, δ, κ) continues to be of major concern in opioid pharmacology. Furthermore, special attention is presently focused on the search for mixed opioid agonist/antagonists that produce an agonist effect at one receptor type and act as an antagonist at another receptor class. Recently, it was demonstrated that pretreatment of mice with the nonpeptide δ antagonist naltrindole prevented the development of morphine tolerance and dependence.[2] This interesting observation suggested that the development of a single compound with mixed μ agonist/δ antagonist properties may have considerable therapeutic potential. The first known example of a mixed μ agonist/δ antagonist is the recently reported opioid tetrapeptide analogue H-Tyr-Tic-Phe-Phe-NH_2 (TIPP-NH_2).[1,3]

The fact that μ and δ opioid receptors differ in their conformational requirements was first established through comparison of the receptor-binding profiles of the μ-selective cyclic enkephalin analogue H-Tyr-c[-D-A_2bu-Gly-Phe-Leu-] and its nonselective linear correlate.[4] This observation led to the development of numerous cyclic analogues of enkephalin and dermorphin with excellent opioid activity *in vitro* and high selectivity for either μ or δ receptors (for reviews, see refs 5 and 6). In subsequent studies, the Phe residue in the 3- or 4-position of these cyclic opioid peptide analogues was replaced with amino acid residues that contain more extended aromatic ring systems in their side chains, such as 1- or 2-naphthylalanine (1-Nal or 2-Nal) or Trp. In the case of the μ-selective cyclic enkephalin analogue H-Tyr-c[-D-A_2bu-Gly-Phe-Leu-], substitution of Phe^4 by 1-Nal resulted in a compound with improved potency in both the μ-receptor-representative guinea pig ileum (GPI) assay and the δ-receptor-representative mouse vas deferens (MVD) assay.[7] Replacement of Phe^3 by 1-Nal in the potent but nonselective cyclic dermorphin analogue H-Tyr-D-Orn-Phe-Glu-NH_2 did not greatly affect μ receptor affinity but significantly reduced the affinity for δ receptors.[8] Interestingly, substitution of 2-Nal for 1-Nal in that same compound produced a 2-fold increase in δ receptor affinity but a 40-fold potency decrease in the MVD assay, suggesting that signal transduction in the 2-Nal analogue was somewhat impaired (unpublished results). Finally, replacement of Phe^3 with 1-Nal or 2-Nal in the δ-selective cyclic dermorphin analogue H-Tyr-D-Cys-Phe-D-Pen-OH (JOM-13) resulted in only slightly diminished affinities for both the μ and δ receptors.[9]

Recently, cyclic β-casomorphin analogues structurally related to the cyclic prototype enkephalin analogue H-Tyr-c[-D-Orn-Gly-Phe-Leu-][10] were prepared and pharmacologically tested. In particular, the cyclic peptides H-Tyr-

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Table 1. Analytical Data of Cyclic β-Casomorphin-5 Analogues[a]

no.	compound	amino acid analysis					TLC[b] R_f values			HPLC[c] (k')		FAB-MS (MH+)
		Tyr	Xaa[2]	Yaa[3]	Pro	Gly	D	E	F	I	II	
1	Tyr-c[-D-Orn-Phe-Pro-Gly-]	0.99	1.03	1.0	1.0	1.11	0.57	0.69	0.72	2.44	2.10	579
2	Tyr-c[-D-Orn-Phe-D-Pro-Gly-]	0.97	1.0	1.0	1.0	1.08	0.62	0.73	0.73	3.53	2.40	579
3	Tyr-c[-D-Orn-2-Nal-Pro-Gly-]	1.0	0.96	nd	1.0	1.14	0.48	0.74	0.72	6.25	11.13	629
4	Tyr-c[-D-Orn-2-Nal-D-Pro-Gly-]	1.0	0.95	nd	1.0	1.13	0.53	0.79	0.73	6.38	11.32	629
5	Tyr-c[-D-Orn-1-Nal-D-Pro-Gly-]	1.0	0.86	nd	0.88	1.02	0.53	0.80	0.73	5.92	10.10	629
6	Tyr-c[-D-Lys-2-Nal-D-Pro-Gly-]	0.90	1.06	nd	0.91	1.0	0.50	0.76	0.72	5.96	9.51	643
7	Tyr-c[-D-A_2bu-2-Nal-D-Pro-Gly-]	0.94	1.09	nd	0.95	1.0	0.52	0.81	0.75	6.54	12.55	615
8	Tyr-c[-D-Orn-Trp-D-Pro-Gly-]	0.95	0.95	1.11	1.0	1.07	0.54	0.71	0.70	2.27	3.70	618
9	Tyr-c[-D-Orn-2-Nal-D-Pro-]	1.02	1.0	nd	0.95		0.59	0.83	0.75	7.67	20.69	572

[a] Structures of all peptides were confirmed by one- and two-dimensional ^{1}H NMR. [b] See General Methods. [c] LiChrospher 100 RP-18e, 250 × 4 mm; I, gradient 20–50% B in 25 min (A—0.1% TFA in H_2O, B—0.1% TFA in acetonitrile); II, isocratic 78% A/22% B; flow rate 1.5 mL/min; detection 215 and 280 nm.

c[-D-Orn-Phe-Pro-Gly-] (1) and H-Tyr-c[-D-Orn-Phe-D-Pro-Gly-] (2) displayed high potency in the μ- and δ-receptor-representative-binding assays and *in vitro* bioassays[11] and, furthermore, were shown to have very high antinociceptive activity in rats.[12] Since the replacement of the Phe^3/Phe^4 residue in the cyclic dermorphin and enkephalin analogues described above had interesting, divergent effects on the opioid activity profile, it was of interest to prepare correspondingly substituted cyclic β-casomorphin analogues as well. In the present paper, we describe the syntheses, opioid activity profiles, and conformational aspects of a number of analogues of cyclic peptides 1 and 2 that contain 1-Nal, 2-Nal, or Trp in place of Phe^3. Two of these compounds were shown to possess mixed μ agonist/δ antagonist properties.

Synthesis

The cyclic tetra- and pentapeptides (Table 1) were synthesized in solution by stepwise elongation, using N^α-Boc, N^ω-Z, and carboxy terminal ONb (or Bzl) protection and mixed anhydrides for the couplings, as described in detail elsewhere.[13,14] D-A_2bu was prepared from D-Glu[15] and converted to the N^α-Boc-, N^γ-Z-protected derivative by usual procedures. The N$^\omega$-amino group of the diaminocarboxylic acid and the C-terminal COOH function were deprotected simultaneously by catalytic hydrogenation, and cyclization was then carried out under optimized conditions (high dilution, −25 °C) with diphenyl phosphorazidate as coupling agent.[10,14] Progress of the cyclization reaction was followed by HPLC, and the reaction was generally carried out for 3–5 days until peptidic starting material was no longer present. The cyclization yield was, in general, higher than 80%, and the purity of the crude cyclic peptides determined by RP-HPLC was at least 90%. In nearly all cyclizations, the cyclic monomer only was obtained, but cyclodimerization did occur to some extent in the case of the cyclic peptides Boc-Tyr(tBu)-c[-D-Orn-2-Nal-Pro-Gly-] (20) and Boc-Tyr(tBu)-c[-D-Orn-2-Nal-D-Pro-] (46). The crude peptides were deprotected by TFA treatment and purified to homogeneity by preparative reversed-phase liquid chromatography. The purity of the cyclic peptides was assessed by TLC and HPLC in different solvent systems, and the structural identity was established by amino acid analysis, FAB mass spectrometry, and one- and two-dimensional ^{1}H NMR spectroscopy (Table 1).

Receptor-Binding Assays and *in Vitro* Bioassays

Binding affinities for μ and δ opioid receptors were determined by displacing respectively tritiated H-Tyr-D-Ala-Gly-MePhe-Gly-ol ([^{3}H]DAMGO) and tritiated H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH ([^{3}H]DSLET) from rat-brain-membrane-binding sites, and κ opioid receptor affinities were measured by displacement of tritiated (5α,7α,8β)-(−)-*N*-methyl-*N*-(7-pyrrolidinyl-1-oxaspiro[4.5]-dec-8-yl)benzeneacetamide ([^{3}H]U69,593) from guinea-pig-brain-membrane-binding sites. For the determination of their *in vitro* opioid activities, the cyclic β-casomorphin analogues were tested in bioassays based on inhibition of electrically evoked contractions of the guinea pig ileum and the mouse vas deferens. The GPI assay is usually considered as being representative for μ receptor interactions, even though the ileum also contains κ receptors. κ receptor interactions in the GPI assay are indicated by relatively high K_e values for naloxone as antagonist (20–30 nM),[16] in contrast to the low K_e values (1–3 nM) observed with μ receptor ligands.[17] In the MVD assay, opioid effects are primarily mediated by δ receptors; however, μ and κ receptors also exist in this tissue. Both δ and κ interactions in the MVD are characterized by relatively high K_e values for naloxone as antagonist (~20 nM),[18] whereas low K_e values (<3 nM) are observed with μ agonists in this preparation.[19] The K_e values for the compounds with δ antagonist properties were determined from the ratio of IC_{50} values obtained with the δ agonists [Leu^5]enkephalin, [D-Ala^2]deltorphin I, and DPDPE in the presence and absence of a fixed antagonist concentration.

Results and Discussion

Structure–Activity Relationships. As previously reported,[11] the cyclic parent peptide H-Tyr-c[-D-Orn-Phe-D-Pro-Gly-] (2) displays high affinity for μ opioid receptors but also binds quite well to δ receptors and, therefore, is only moderately μ selective. In comparison with 2, the corresponding L-Pro^4 analogue (compound 1) shows 4-fold decreased μ affinity and 100-fold weaker δ affinity, thus exhibiting considerable preference for μ over δ opioid receptors (Table 2). Substitution of the Phe^3 moiety with 1-Nal or 2-Nal obviously increases the steric bulk and the hydrophobicity at the crucial 3-position residue, as reflected by the dramatically increased k' values (RP-HPLC) determined for compounds 3, 4, and 5 in comparison with those for parent peptides 1 and 2 (Table 1).

Substitution of the Phe^3 residue in compound 1 with 2-Nal (compound 3) produced a 20-fold decrease in μ receptor affinity and a somewhat less pronounced loss in δ affinity (Table 2). In qualitative agreement with its reduced μ receptor affinity, compound 3 showed a 100-fold potency drop in the GPI assay as compared to 1 (Table 3). In the MVD assay, analogue 3 was a weak partial

Table 2. Receptor-Binding Assay of Cyclic β-Casomorphin-5 Analogues

		[^{3}H]DAMGO		[^{3}H]DSLET		
no.	compound	K_i^μ, nM[a]	rel potency[b]	K_i^δ, nM[a]	rel potency[b]	K_i^δ/K_i^μ ratio
1	Tyr-c[-D-Orn-Phe-Pro-Gly-]	3.99 ± 0.34	2.36 ± 0.20	1280 ± 170	0.00198 ± 0.0003	321
2	Tyr-c[-D-Orn-Phe-D-Pro-Gly-]	0.881 ± 0.076	10.7 ± 0.9	13.2 ± 0.5	0.192 ± 0.0072	15.0
3	Tyr-c[-D-Orn-2-Nal-Pro-Gly-]	81.3 ± 14.4	0.116 ± 0.021	2140 ± 800	0.00118 ± 0.00044	26.3
4	Tyr-c[-D-Orn-2-Nal-D-Pro-Gly-]	5.89 ± 0.11	1.60 ± 0.03	17.2 ± 4.9	0.147 ± 0.042	2.92
5	Tyr-c[-D-Orn-1-Nal-D-Pro-Gly-]	3.42 ± 0.03	2.76 ± 0.02	23.6 ± 2.2	0.107 ± 0.010	6.90
6	Tyr-c[-D-Lys-2-Nal-D-Pro-Gly-]	17.1 ± 2.6	0.551 ± 0.084	62.6 ± 13.2	0.0404 ± 0.0085	3.66
7	Tyr-c[-D-A$_2$bu-2-Nal-D-Pro-Gly-]	6.41 ± 0.36	1.47 ± 0.08	70.8 ± 25.8	0.0357 ± 0.0130	11.0
8	Tyr-c[-D-Orn-Trp-D-Pro-Gly-]	2.09 ± 0.08	4.1 ± 0.17	10.3 ± 0.5	0.246 ± 0.012	4.93
9	Tyr-c[-D-Orn-2-Nal-D-Pro-]	2.74 ± 0.31	3.44 ± 0.39	28.3 ± 6.0	0.0894 ± 0.0190	10.3
	Tyr-Tic-Phe-Phe-NH$_2$ (TIPP-NH$_2$)[c]	78.8 ± 7.1	0.120 ± 0.011	3.00 ± 0.15	0.843 ± 0.043	0.0381
	[Leu^5]enkephalin	9.43 ± 2.07	1	2.53 ± 0.35	1	0.268

[a] Mean of three to six determinations ± SEM. [b] Potencies relative to [Leu^5]enkephalin. [c] Reference 3.

Table 3. Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assays of Cyclic β-Casomorphin-5 Analogues

		GPI		MVD		
no.	compound	IC_{50}, nM[a]	rel potency[b]	IC_{50}, nM[a]	rel potency[b]	MVD/GPI IC_{50} ratio
1	Tyr-c[-D-Orn-Phe-Pro-Gly-][c]	13.4 ± 2.4	18.3 ± 3.3	69.9 ± 18.0	0.163 ± 0.042	5.22
2	Tyr-c[-D-Orn-Phe-D-Pro-Gly-][c]	2.14 ± 0.33	115 ± 18	4.89 ± 1.28	2.33 ± 0.61	2.29
3	Tyr-c[-D-Orn-2-Nal-Pro-Gly-]	1220 ± 230	0.202 ± 0.038	10000 (42%)[d]		
4	Tyr-c[-D-Orn-2-Nal-D-Pro-Gly-]	384 ± 52	0.641 ± 0.087	antagonist		
5	Tyr-c[-D-Orn-1-Nal-D-Pro-Gly-]	14.9 ± 0.3	16.5 ± 0.3	29.9 ± 3.6	0.381 ± 0.046	2.01
6	Tyr-c[-D-Lys-2-Nal-D-Pro-Gly-]	609 ± 194	0.404 ± 0.129	antagonist		
7	Tyr-c[-D-A$_2$bu-2-Nal-D-Pro-Gly-]	353 ± 68	0.697 ± 0.134	1000 (21%)[d]		
8	Tyr-c[-D-Orn-Trp-D-Pro-Gly-]	27.5 ± 2.9	8.95 ± 0.94	16.1 ± 1.1	0.708 ± 0.048	0.585
9	Tyr-c[-D-Orn-2-Nal-D-Pro-]	11.8 ± 1.4	20.8 ± 2.5	32.2 ± 9.55	0.354 ± 0.105	2.73
	Tyr-Tic-Phe-Phe-NH$_2$ (TIPP-NH$_2$)[e]	1700 ± 220	0.145 ± 0.019	antagonist		
	[Leu^5]enkephalin	246 ± 39	1	11.4 ± 1.1	1	0.0463

[a] Mean of three to six determinations ± SEM. [b] Potencies relative to [Leu^5]enkephalin. [c] Reference 11. [d] Partial agonist; value in parentheses indicates maximal inhibition of the contraction (%) obtained at high concentrations. Agonist effect was TIPP reversible. [e] Reference 3.

Table 4. K_e Values Determined for δ Antagonists against Various δ Agonists in the MVD Bioassay

		K_e[nM][a]		
no.	compound	[Leu^5]enkephalin	[D-Ala^2]deltorphin I	DPDPE
4	Tyr-c[-D-Orn-2-Nal-D-Pro-Gly-]	268 ± 22	202 ± 24	233 ± 28
6	Tyr-c[-D-Lys-2-Nal-D-Pro-Gly-]	603 ± 174	677 ± 125	305 ± 52
	Tyr-Tic-Phe-Phe-NH$_2$ (TIPP-NH$_2$)[b]	5.86 ± 0.33	2.96 ± 0.02	4.80 ± 0.20

[a] Data are the mean of 4–10 determinations ± SEM. [b] Reference 3.

agonist and its effect was completely reversed by the highly selective δ antagonist H-Tyr-Tic-Phe-Phe-OH (TIPP)[3] (50 nM), indicating that it was mediated *via* interaction with δ receptors.

The corresponding D-Pro-containing peptide H-Tyr-c[-D-Orn-2-Nal-D-Pro-Gly-] (4) displayed over 6 times lower μ receptor affinity than its parent peptide (2) but, interestingly, was 180 times less potent in the GPI bioassay. This discrepancy may be due to impaired signal transduction at the μ receptor in the GPI or to a difference in μ receptor subtype populations between rat brain and ileum. Compound 4 retained good δ affinity (K_i^δ = 17 nM), similar to that of 2. Surprisingly, this compound showed no agonist effect in the MVD assay at concentrations up to 50 μM. Analogue 4 turned out to be a moderately potent antagonist against the δ-selective agonists [Leu^5]enkephalin, [D-Ala^2]deltorphin I, and DPDPE in the MVD assay with K_e values of 268, 202, and 233 nM, respectively (Table 4). Thus, like the recently reported tetrapeptide analogue TIPP-NH$_2$,[3] this cyclic casomorphin analogue displays mixed μ agonist/δ antagonist properties. In comparison with the linear tetrapeptide δ antagonist TIPP-NH$_2$, cyclic peptide 4 displayed about 1/20 of the δ antagonist potency but was a 4 times more potent μ agonist in the GPI assay. The relatively moderate δ antagonist potency observed with 4 in the MVD assay as compared to its high δ receptor affinity determined in the binding assay may be due to the existence of different δ receptor subtypes in vas deferens and rat brain.

Replacement of 2-Nal with 1-Nal in analogue 4 resulted in a compound, H-Tyr-c[-D-Orn-1-Nal-D-Pro-Gly-] (5), with about 2 times higher μ receptor affinity and 25 times higher potency in the GPI assay. The potency of 5 in the latter assay was only 7 times lower than that of parent peptide 2. Similar increases in μ receptor affinity had been observed upon substitution of 1-Nal for 2-Nal in H-Tyr-D-Cys-2-Nal-D-Pen-OH[9] and H-Tyr-D-Orn-2-Nal-Glu-NH$_2$ (unpublished results). Analogue 5 showed δ receptor affinity comparable to that of compounds 2 and 4 and, most interestingly, turned out to be a full agonist in the MVD assay with about 1/6 of the potency of parent peptide 2. The agonist effect of 5 in the MVD assay was fully naloxone reversible and was partially reversed by TIPP (100 nM) to an extent of 28%, indicating that it was mediated by both μ and δ receptors. A μ-receptor-mediated agonist effect is not observed with compound 4 in the MVD assay. Since 4 and 5 showed very similar μ receptor affinities in the receptor-binding assay, these results could be interpreted to indicate that compound 4 has a lower intrinsic activity ("efficacy") than analogue 5 at the μ receptor. However, such an efficacy difference could not be detected in the GPI assay because of the very large μ

receptor reserve known to exist in the ileum.[20] Obviously, it is not possible to determine in the MVD assay whether analogue 5 is a full or partial δ agonist because of its μ agonist component in this assay system. This compound needs to be further examined in the assay system based on inhibition of adenylate cyclase in NG108-15 neuroblastoma × glioma hybrid cells which contain δ receptors only.[21] Peptide analogues 4 and 5 differ only in the position of the attachment of the aromatic naphthyl moiety to the β-carbon in the 3-position residue, resulting in a different orientation of the aromatic function relative to the rest of the peptide molecule. The recently proposed "broad" and "deep" binding pocket of the δ receptor[9] seems to tolerate the binding of the bulky naphthyl moiety in either orientation.

Three analogues of compound 4, characterized by replacement of D-Orn^2 with D-Lys or D-A_2bu or by deletion of the Gly^5 residue, were synthesized in order to study the effect of peptide ring size variation on the opioid activity profile. Expansion of the 15-membered ring in 4 to a 16-membered one (D-Lys^2 substitution) resulted in compound 6 which had 3 times lower μ receptor affinity and about half the potency in the GPI assay. In the MVD assay, 6 was a 1.5–3.5 times less potent δ antagonist than 4 (Table 4), in good correlation with its 3.5 times lower δ receptor affinity as compared to that of the latter compound. Thus, H-Tyr-c[-D-Lys-2-Nal-D-Pro-Gly-] is a weaker mixed μ agonist/δ antagonist than 4 with regard to both μ agonist and δ antagonist potency. Contraction to a 14-membered peptide ring structure, as achieved through substitution of D-A_2bu in the 2-position, resulted in an analogue (7) which showed about the same potency as 4 in the μ-receptor-binding assay and GPI assay. Compound 7 had about the same δ receptor affinity as 6 but turned out to be a partial agonist in the MVD assay (maximal inhibition of the contractions = 21% at 1 μM). The latter effect was again fully TIPP reversible. Deletion of the Gly^5 residue in 4 resulted in the cyclic tetrapeptide analogue 9 containing a 12-membered ring structure. In comparison with parent peptide 2, the latter compound showed 3 times lower μ receptor affinity and 5 times lower potency in the GPI assay. This compound retained δ receptor affinity comparable to that of analogue 4 and displayed a full agonist effect in the MVD assay which, as in the case of compound 5, was due to interaction with both μ and δ receptors. These results suggest that the relative spatial disposition of the Tyr^1 moiety and the 2-Nal^3 side chain plays a crucial role for signal transduction.

In comparison with parent peptide 2, the cyclic casomorphin analogue with Trp substituted in the 3-position (compound 8) showed about half the affinity at the μ receptor and equal δ affinity. Previously, it had been shown that substitution of Phe^3 in H-Tyr-D-Cys-Phe-D-Pen-OH with Trp was also well tolerated at both the μ and δ receptors.[9] The μ and δ receptor affinities of analogue 8 are slightly higher than those of the 2-Nal^3- and 1-Nal^3-containing peptides 4 and 5. In the GPI assay, 8 was a potent μ agonist, and its full agonist effect in the MVD assay was again characterized by a μ and δ component.

None of the cyclic casomorphin analogues modified in the 3-position showed increased μ receptor selectivity in comparison with the corresponding parent peptides 1 and 2. Furthermore, our results suggest that alteration of the electronic, steric, and lipophilic properties in the 3-position, as achieved by replacement of the Phe residue with 1-Nal, 2-Nal, or Trp, is tolerated at both receptors with regard to recognition and binding. However, substitution of 2-Nal in the 3-position of the cyclic pentapeptides affected signal transduction, resulting in two mixed μ agonist/δ antagonists (4 and 6) and two compounds with partial δ agonist properties (3 and 7).

In the GPI assay, all analogues showed K_e values for naloxone as antagonist in the range 1–3 nM (data not shown). Such low K_e values are typical for μ receptor interactions and rule out an additional interaction with κ receptors, since κ receptor interactions would be characterized by much higher K_e values.[16] In agreement with these results, none of the compounds displayed significant affinity for κ receptors in the guinea-pig-brain-membrane-binding assay at concentrations up to 20 μM.

Conformational Analysis by ^{1}H NMR Spectroscopy and Molecular Modeling. The conformations of analogues 2, 4, and 5 were studied in DMSO-d_6 by 1- and 2-dimensional NMR techniques and theoretical conformational analysis (detailed manuscript in preparation). Complete assignment of all proton resonances was achieved using COSY, TOCSY, and ROESY experiments. None of the three peptides showed NOEs between C$^\alpha$H protons as evidence for the existence of a *cis* peptide bond, and no chemical exchange cross peaks were observed in the TOCSY and ROESY spectra. These results indicated that the average preferred solution conformation of all three peptides is characterized by *all-trans* peptide bonds. The results of a molecular mechanics study revealed that in all D-Pro^4 peptides, the *trans* Xaa-D-Pro peptide bond was energetically favored.

Temperature-dependence studies of the amide proton chemical shifts indicated the existence of solvent-inaccessible amide protons. In the case of small peptides, a low temperature coefficient ($-\Delta\delta/\Delta T < 2.5$ ppb/K) is usually taken as evidence for participation in an intramolecular hydrogen bond.[22] For analogue 2, NH temperature gradients indicative of hydrogen bonds were obtained for the D-Orn (N$^\alpha$H), D-Orn (N$^\delta$H), Phe, and Gly residues, whereas the Tyr amino protons showed a nonlinear dependence. In the case of analogue 4, the NH_2 proton signals of Tyr and the amide protons of D-Orn (N$^\alpha$H), D-Orn (N$^\delta$H), and 2-Nal showed low temperature coefficients, whereas the Gly-NH exhibited an intermediate value (2.9 ppb/K). With compound 5, low NH temperature gradients were observed for the D-Orn (N$^\delta$H) and Gly residues, whereas Tyr, D-Orn (N$^\alpha$H), and 1-Nal showed gradients in the medium range. Analysis of these results by comparison with low-energy conformers obtained in a molecular mechanics study (see below) suggested that analogues 2 and 4 had similar backbone conformations, both being stabilized by Tyr^1-CO···NH-Phe^3 (or 2-Nal^3) and D-Orn^2-CO···NH$^\delta$-D-Orn^2 hydrogen bonds. The same type of analysis indicated that peptide 5 was characterized by Tyr^1-CO···NH-1-Nal^3 and D-Pro^4-CO···NH$^\delta$-D-Orn^2 hydrogen bonds. ROESY experiments revealed NOEs between the Phe-2H and the D-Pro-C$^\alpha$H/D-Pro-C$^\delta$H in 2 and between the 1H, 3H, and 7H of 2-Nal and the C$^\alpha$H, C$^\beta$H, and C$^\delta$H of Pro in 4. These NOEs were indicative of a close proximity between the aromatic moiety of the 3-position residue and the pyrrolidine ring of the D-Pro^4 residue in these two compounds. Interestingly, no NOEs between the aromatic protons of 1-Nal^3 and the pyrrolidine ring protons of D-Pro were observed with analogue 5. These results indicate that the side-chain orientation of the 1-Nal^3

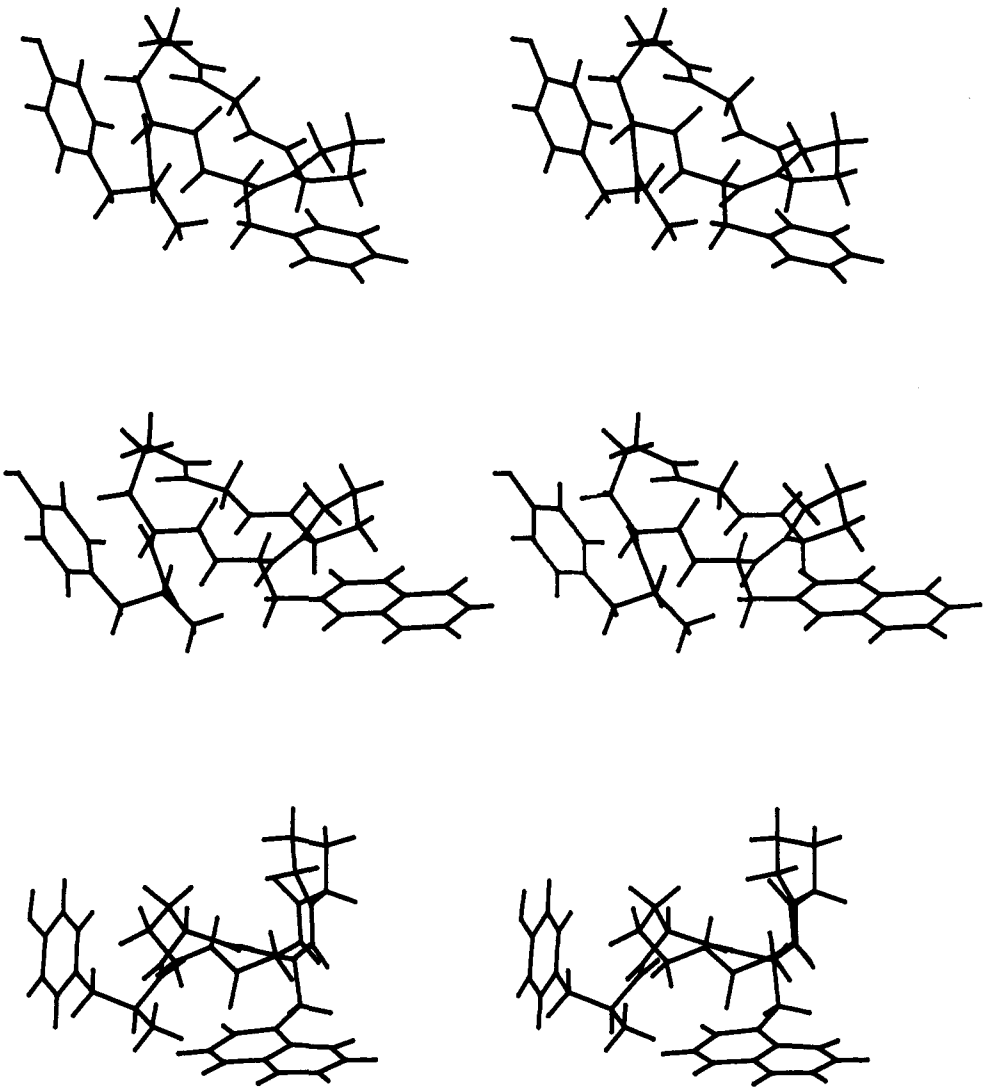

Figure 1. Stereoviews of the proposed solution conformations of cyclic casomorphin analogues **2** (top), **4** (middle), and **5** (bottom).

residue in **5** differs from that of the aromatic 3-position residue in compounds **2** and **4**.

About 500 stable conformations within an energy range of 7 kcal/mol were obtained for each peptide, based on theoretical calculations using force field methods. The comparison of all calculated low-energy conformations with various ^{1}H NMR parameters, such as NOE distance constraints, hydrogen bonds, and torsion angles, led to proposals for the solution conformation of analogues **2**, **4**, and **5** (Figure 1). Although none of these structures was the energetically most stable one, they were all found to be within 5 kcal/mol above the energy minimum. In correspondence with the experimental NMR conditions, molecular dynamics simulations were performed at 300 K for the structures consistent with the NMR data in a box of 486 DMSO molecules using periodic boundary conditions. During the simulation time of 40 ps, the overall conformation of all compounds remained nearly constant except for small periodic fluctuations of the flexible tyrosine and phenylalanine or naphthylalanine side chains.

Conclusions

Structure–activity studies based on replacement of Phe^3 in the cyclic casomorphin analogue H-Tyr-c[-D-Orn-Phe-D(or L)-Pro-Gly-] with amino acid residues containing more extended aromatic ring systems in the side chain resulted in the discovery of a new class of mixed μ agonist/δ antagonists. The key compound turned out to be the 2-Nal^3, D-Pro^4 analogue **4** which was a potent μ agonist in the GPI assay and a moderately potent δ antagonist in the MVD assay. The first reported and only other known example of a mixed μ agonist/δ antagonist is the tetrapeptide analogue TIPP-NH_2[3] which, in comparison with H-Tyr-c[-D-Orn-2-Nal-D-Pro-Gly-], is a 4 times less potent μ agonist and a 20 times more potent δ antagonist. On the basis of the results of a recent study,[2] mixed μ agonist/δ antagonists are thought to have considerable potential as analgesics that do not produce tolerance and dependence. Further efforts are required to improve the μ agonist component of TIPP-NH_2 and the δ antagonist component of H-Tyr-c[-D-Orn-2-Nal-D-Pro-Gly-] through structural modifications.

Within the series of the 2-Nal^3-containing cyclic analogues, a 15-membered peptide ring structure was found to be optimal for the manifestation of δ antagonism. Expansion of the peptide ring (compound **6**) produced a decrease in δ antagonist potency, and ring size reduction (compounds **7**) led to a partial restoration of δ agonism. Conformational analysis by NMR spectroscopy in conjunction with molecular mechanics studies indicated that parent peptide **2** and the 2-Nal^3 analogue **4** had a similar backbone conformation and the same side-chain orien-

tation at the 3-position. These results suggest that the δ antagonist properties of 4 may not be due to a difference in its overall conformation as compared to 2 but rather may be the result of a direct interference of the 2-naphthyl moiety *per se* at the receptor-binding site preventing proper alignment of the peptide such as required for signal transduction. The GPI and MVD data obtained with the peptide H-Tyr-c[-D-Orn-1-Nal-D-Pro-Gly-] (5) can be interpreted to indicate that this compound may have a higher intrinsic activity at the μ receptor than analogue 4. In comparison with 4, the naphthyl moiety of 5 is attached differently to the β-carbon of the 3-position side chain and was shown by ^{1}H NMR spectroscopy to assume a different orientation relative to the rest of the peptide molecule. These results confirm the crucial importance of aromatic moieties in receptor ligands for signal transduction.

Experimental Section

General Methods. Melting points were determined on a micro hot plate according to BOETIUS and are uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. Boc-amino acids were purchased from Bachem Bioscience. Solvents for the synthesis were of analytical grade and used without further purification with the exception of DMF, which was distilled from ninhydrin and stored under N_2. TLC was performed on precoated silica gel plates 60F-254 (E. Merck, Darmstadt, FRG) in the following solvent systems (all v/v): (A) chloroform/MeOH (9/1), (B) benzene/acetone/AcOH (25/10/0.5), (C) ethyl acetate/pyridine/AcOH/H_2O (90/15/4.5/8.3), (D) 2-BuOH/HCOOH/H_2O (75/15/20), (E) 1-BuOH/AcOH/ethyl acetate/H_2O (1/1/1/1), and (F) 1-BuOH/pyridine/AcOH/H_2O (15/10/3/12). Peptides were visualized with UV, the ninhydrin spray reagent, and KI/starch. The HPLC system GOLD (Beckman) consisting of a programmable solvent module 126 and a diode array detector module 168 was used for the purification and the purity control of the peptides. Recording and quantification were accomplished using the GOLD software. For all analytical applications, a LiChrospher 100 RP-18e column (250 × 4 mm, 5-μm particle size) from E. Merck, Darmstadt, FRG, was used. The solvents were of HPLC grade and were filtered and degassed prior to use. HPLC was carried out using a gradient made up from two solvents: (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile. The analytical determinations were performed with a linear gradient of 20–50% B over a period of 25 min as well as under isocratic conditions with 22% B at a flow rate of 1.5 mL/min, absorptions being measured at both 216 and 280 nm (Table 1, HPLC systems I and II). Preparative reversed-phase HPLC was carried out on a LiChrospher 100 RP-18e column (250 × 25 mm, 10-μm particle size) with a linear gradient of 20–60% B over 60 min at a flow rate of 12 mL/min. The cyclization reaction was monitored by using the following HPLC conditions (HPLC solvent system III): LiChrospher 100 RP-18e column, linear gradient 30–80% B over 25 min, flow rate 1.5 mL/min, detection at 216 nm. Peptides (0.2 mg) were hydrolyzed for amino acid analysis in 6 N HCl containing a small amount of phenol for 24 h at 110 °C in deaerated tubes. The samples were analyzed after precolumn derivatization as PITC amino acids on a Supelco LC-18DB column (250 × 4 mm, 5 μm) at 245 nm (M. Boni, BioChem ImmunoSystems, Montreal, Quebec). Molecular weights of the peptides were determined by FAB mass spectrometry on an MS-50 HMTCTA mass spectrometer interfaced with a DS-90 data system (Drs. M. Evans and M. Bertrand, Department of Chemistry, University of Montreal).

Peptide Synthesis. Mixed Anhydride Method. Method A. NMM (1 equiv) was added to a stirred solution of 0.2–3 mmol of Boc-protected amino acid in THF. The mixture was cooled to −15 °C, treated with IBCF (1 equiv), and allowed to react for 3–4 min. Subsequently, the amino component in the form of the peptide hydrochloride (1 equiv) was added followed by NMM (1 equiv). Stirring proceeded for 30 min at −15 °C, and then the mixture was allowed to reach room temperature. The solvent was removed *in vacuo*, and the residual oil was dissolved in 150 mL of EtOAc. The resulting solution was extracted consecutively with brine, 5% $KHSO_4$, brine, saturated $NaHCO_3$, and brine. The organic phase was dried ($MgSO_4$), filtered, and evaporated to dryness. The residue was crystallized from appropriate solvents.

Cyclization Reaction. Method B. The Boc-protected cyclization precursor (0.1–0.3 mmol) was dissolved in 20 mL of DMF and added to a cold solution (−25 °C) of DMF (final peptide concentration 1 mM) containing NMM (1 equiv) and DPPA (2 equiv). The solution was continuously stirred at −25 °C, and progress of the reaction was monitored by HPLC (see General Methods). Every 24 h, additional NMM and DPPA (1 equiv) were added and the reaction was allowed to continue until peptidic starting material could no longer be detected. The solvent was then removed under reduced pressure (bath temperature 25 °C), and the obtained residue was triturated 3 times with PE. The precipitate was dissolved in EtOAc, and the resulting solution was extracted with brine, dried over $MgSO_4$, finally filtered, and evaporated to dryness. The protected cyclic peptides were crystallized from EtOAc/DIPE.

Deprotection Procedures. Method C. The Boc-protected peptide (0.3–3 mmol) was treated with 1.1 N HCl/AcOH (3 equiv) for 30 min at room temperature. The solvent was evaporated *in vacuo* at 20 °C, and the residue was precipitated with dry ethyl ether. Crude products were crystallized from EtOH/ether or EtOH/DIPE.

Method D. Hydrogenations were carried out in aqueous MeOH under atmospheric pressure and at room temperature in the presence of Pd black using a peptide to catalyst ratio of 3:1. After complete removal of the benzyl-type protecting groups as monitored by TLC, the solution was filtered, the filtrate was concentrated *in vacuo*, and the residue was crystallized from EtOH/ether.

Method E. The Boc-protected cyclic peptides were deprotected using aqueous 95% TFA containing thioanisole (3%) under stirring and cooling with ice. After evaporation *in vacuo*, the peptide TFA salts were obtained by crystallization from EtOH/ether.

Boc-2-Nal-D-Pro-Gly-ONb (11). According to method A, Boc-2-Nal-OH (3 mmol) was reacted with H-D-Pro-Gly-ONb*HCl[13] (3 mmol). Crude 11 was crystallized from EtOAc/DIPE (75%): mp 93–95 °C; $[\alpha]^{20}_D$ +36.8° (c 1.0, MeOH); TLC R_fA 0.65, R_fB 0.35, R_fC 0.84.

Boc-D-Orn(Z)-2-Nal-D-Pro-Gly-ONb (13). As described in method A, Boc-D-Orn(Z)-OH (0.93 mmol) was reacted with H-2-Nal-D-Pro-Gly-ONb*HCl (obtained from 11 by using deprotection procedure C), yielding 13 after crystallization from DIPE (80%): mp 74–76 °C; $[\alpha]^{20}_D$ +31.9° (c 1.0, MeOH); TLC R_fA 0.50, R_fB 0.26, R_fC 0.91.

Boc-Tyr(tBu)-D-Orn(Z)-2-Nal-D-Pro-Gly-ONb (15). Boc-Tyr(tBu)-OH (0.59 mmol) and H-D-Orn(Z)-2Nal-D-Pro-Gly-ONb*HCl, obtained from 13 by deprotection using method C, were coupled (method A) to yield 15 after crystallization from DIPE (85%): mp 96–99 °C; $[\alpha]^{20}_D$ +28.4° (c 1.0, MeOH); TLC R_fA 0.72, R_fB 0.30, R_fC 0.93.

Boc-Tyr(tBu)-D-Orn-2-Nal-D-Pro-Gly-OH (17). Hydrogenation of 15 (0.48 mmol) was carried out by using method D. Compound 17 was obtained after precipitation from a methanolic solution with dry ether (77%): mp 163–166 °C; $[\alpha]^{20}_D$ +47.8° (c 1.0, MeOH); TLC R_fE 0.67, R_fF 0.64; k' (HPLC system III) 12.05.

Boc-Tyr(tBu)-c[-D-Orn-2-Nal-D-Pro-Gly-] (19). Compound 17 (0.36 mmol) was treated for 96 h as described in method B, yielding the cyclic peptide 19 after crystallization from EtOAc (84%): k' (HPLC system III) 14.55 (98% purity); FAB-MH$^+$ 785.

Boc-2-Nal-Pro-Gly-ONb (12). According to method A, Boc-2-Nal-OH (3 mmol) was coupled with H-Pro-Gly-ONb*HCl[13] (3 mmol). Crude 12 was obtained from EtOAc/DIPE/hexane (74%) as an amorphous product: mp 58–65 °C; $[\alpha]^{20}_D$ −17.7° (c 1.0, MeOH); TLC R_fA 0.65, R_fB 0.35, R_fC 0.83.

Boc-D-Orn(Z)-2-Nal-Pro-Gly-ONb (14). As described in method A, Boc-D-Orn(Z)-OH (0.9 mmol) was reacted with H-2-Nal-Pro-Gly-ONb*HCl (prepared from 12 using deprotection method C), yielding 14 after crystallization from DIPE (78%): mp 101–103 °C; $[\alpha]^{20}_D$ −16.4° (c 1.0, MeOH); TLC R_fA 0.45, R_fB 0.24, R_fC 0.89.

Boc-Tyr(tBu)-D-Orn(Z)-2-Nal-Pro-Gly-ONb (16). Boc-Tyr(tBu)-OH (0.58 mmol) and H-D-Orn(Z)-2-Nal-Pro-Gly-ONb*HCl (from 14 by deprotection with method C) were coupled using method A to yield 16 after crystallization from EtOH (72%): mp 150–152 °C; $[\alpha]^{20}_D$ −8.2° (c 1.0, MeOH); TLC R_fA 0.72, R_fB 0.24, R_fC 0.92.

Boc-Tyr(tBu)-D-Orn-2-Nal-Pro-Gly-OH (18). Compound 16 (0.48 mmol) was deprotected (method D) to yield 18 after precipitation from MeOH with dry ether (82%): mp 166–169 °C; $[\alpha]^{20}_D$ −5.4° (c 1.0, MeOH); TLC R_fE 0.66, R_fF 0.68; k' (HPLC system III) 10.15.

Boc-Tyr(tBu)-c[-D-Orn-2-Nal-Pro-Gly-] (20). Compound 18 (0.27 mmol) was treated for 110 h according to method B, yielding the cyclic peptide 20 and its cyclic dimer (monomer/dimer ratio 3:1) after crystallization from EtOAc (92%): k' (HPLC system III) 12.72 (monomer), 21.2 (dimer).

Boc-1-Nal-D-Pro-Gly-ONb (21). According to method A, Boc-1-Nal-OH (1.45 mmol) was reacted with H-D-Pro-Gly-ONb*HCl[13] (1.45 mmol). Crude 21 was crystallized from CH_2Cl_2/ether/PE (70%): mp 56–60 °C; $[\alpha]^{20}_D$ +79.0° (c 1.0, MeOH); TLC R_fA 0.68, R_fB 0.33.

Boc-D-Orn(Z)-1-Nal-D-Pro-Gly-ONb (23). Using method A, Boc-D-Orn(Z)-OH (0.71 mmol) was coupled with H-1-Nal-D-Pro-Gly-ONb*HCl (obtained from 21 according to deprotection procedure C), yielding 23 after crystallization from EtOAc/DIPE (82%): mp 76–79 °C; $[\alpha]^{20}_D$ +60.5° (c 1.0, MeOH); TLC R_fA 0.69, R_fB 0.38.

Boc-Tyr(tBu)-D-Orn(Z)-1-Nal-D-Pro-Gly-ONb (25). Boc-Tyr(tBu)-OH (0.5 mmol) and H-D-Orn(Z)-1-Nal-D-Pro-Gly-ONb*HCl, obtained from 23 by deprotection method C, were coupled (method A) to yield 25 after crystallization from DIPE (71%): mp 87–90 °C; $[\alpha]^{20}_D$ +60.0° (c 1.0, MeOH); TLC R_fA 0.70, R_fB 0.34.

Boc-Tyr(tBu)-D-Orn-1-Nal-D-Pro-Gly-OH (27). Hydrogenation of 25 (0.36 mmol) (method D) and crystallization from EtOH/ether afforded compound 27 (81%): mp 181–185 °C; $[\alpha]^{20}_D$ +92.2° (c 1.0, MeOH); TLC R_fD 0.43, R_fF 0.55; k' (HPLC system III) 11.93.

Boc-Tyr(tBu)-c[-D-Orn-1-Nal-D-Pro-Gly-] (28). Compound 27 (0.28 mmol) was treated according to method B (reaction time 100 h), yielding cyclic peptide 28 after crystallization from EtOAc/DIPE (96%): k' (HPLC system III) 13.81 (94% purity); FAB-MH$^+$ 785.

Boc-D-Lys(Z)-2-Nal-D-Pro-Gly-ONb (29). Using method A, Boc-D-Lys(Z)-OH (0.71 mmol) was coupled with H-2-Nal-D-Pro-Gly-ONb*HCl (obtained from 11 according to deprotection procedure C), yielding 29 after crystallization from EtOAc/DIPE (92%): mp 62–64 °C; $[\alpha]^{20}_D$ +67.4° (c 1.0, MeOH); TLC R_fA 0.66, R_fB 0.35.

Boc-Tyr(tBu)-D-Lys(Z)-2-Nal-D-Pro-Gly-ONb (30). Boc-Tyr(tBu)-OH (0.55 mmol) and H-D-Lys(Z)-2-Nal-D-Pro-Gly-ONb*HCl, obtained from 29 by using deprotection method C, were coupled (method A). Compound 30 was secured by crystallization from DIPE (74%): mp 98–100 °C; $[\alpha]^{20}_D$ +58.2° (c 1.0, MeOH); TLC R_fA 0.83, R_fB 0.31.

Boc-Tyr(tBu)-D-Lys-2-Nal-D-Pro-Gly-OH (31). Hydrogenation of 30 (0.40 mmol) was carried out by using method D. Compound 31 was obtained after crystallization from EtOH/ether (81%): mp 172–174 °C; $[\alpha]^{20}_D$ +62.3° (c 1.0, MeOH); TLC R_fD 0.39, R_fF 0.53; k' (HPLC system III) 11.88.

Boc-Tyr(tBu)-c[-D-Lys-2-Nal-D-Pro-Gly-] (32). Compound 31 (0.31 mmol) was treated for 96 h according to method B, yielding cyclic peptide 31 after crystallization from EtOAc/DIPE (90%): k' (HPLC system III) 13.20 (97% purity); FAB-MH$^+$ 799.

Boc-D-A$_2$bu(Z)-2-Nal-D-Pro-Gly-ONb (33). Using method A, Boc-A$_2$bu(Z)-OH (0.58 mmol) was reacted with H-2-Nal-D-Pro-Gly-ONb*HCl (obtained from 11 according to deprotection procedure C), yielding 33 after crystallization from EtOAc/DIPE/PE (80%): mp 89–92 °C; $[\alpha]^{20}_D$ +62.2° (c 1.0, MeOH); TLC R_fA 0.67, R_fB 0.48.

Boc-Tyr(tBu)-D-A$_2$bu(Z)-2-Nal-D-Pro-Gly-ONb (34). Applying method A, Boc-Tyr(tBu)-OH (0.33 mmol) and H-D-A$_2$bu(Z)-2-Nal-D-Pro-Gly-ONb*HCl, obtained from 33 by deprotection method C, were coupled to yield 34 after crystallization from CH_2Cl_2/DIPE/PE (82%): mp 113–115 °C; $[\alpha]^{20}_D$ +59.5° (c 1.0, MeOH); TLC R_fA 0.67, R_fB 0.43.

Boc-Tyr(tBu)-D-A$_2$bu-2-Nal-D-Pro-Gly-OH (35). Hydrogenation of 34 (0.23 mmol) (method D) afforded compound 35 after crystallization from EtOH/ether/PE (88%): mp 182–185 °C; $[\alpha]^{20}_D$ +62.9° (c 1.0, MeOH); TLC R_fE 0.73, R_fF 0.51; k' (HPLC system III) 12.76.

Boc-Tyr(tBu)-c[-D-A$_2$bu-2-Nal-D-Pro-Gly-] (36). Compound 35 (0.19 mmol) was treated for 100 h according to method B. Cyclic peptide 36 was isolated after crystallization from EtOAc/DIPE/hexane (87%): k' (HPLC system III) 14.93 (92% purity); FAB-MH$^+$ 771.

Boc-Trp-D-Pro-Gly-ONb (37). Using method A, Boc-Trp-OH (1.0 mmol) was reacted with H-D-Pro-Gly-ONb*HCl[13] (1.0 mmol). Crude 37 was crystallized from EtOAc (66%): mp 176–177 °C; $[\alpha]^{20}_D$ +54.7° (c 1.0, MeOH); TLC R_fA 0.44, R_fB 0.29.

Boc-D-Orn(Z)-Trp-D-Pro-Gly-ONb (38). According to method A, Boc-D-Orn(Z)-OH (0.55 mmol) was coupled with H-Trp-D-Pro-Gly-ONb*HCl (obtained from 37 according to deprotection procedure C), yielding 38 after crystallization from EtOAc/DIPE/hexane (60%): mp 96–98 °C; $[\alpha]^{20}_D$ +42.7° (c 1.0, MeOH); TLC R_fA 0.55, R_fB 0.21.

Boc-Tyr(tBu)-D-Orn(Z)-Trp-D-Pro-Gly-ONb (39). Using method A, Boc-Tyr(tBu)-OH (0.27 mmol) and H-D-Orn(Z)-Trp-D-Pro-Gly-ONb*HCl, obtained from 38 applying method C, were coupled to yield 39 after crystallization from DIPE (80%): mp 97–99 °C; $[\alpha]^{20}_D$ +25.7° (c 1.0, MeOH); TLC R_fA 0.63, R_fB 0.26.

Boc-Tyr(tBu)-D-Orn-Trp-D-Pro-Gly-OH (40). Hydrogenation of 39 (0.20 mmol) (method D) and crystallization from EtOH/ether afforded compound 40 (80%): mp 208–211 °C; $[\alpha]^{20}_D$ +53.3° (c 1.0, DMF); TLC R_fD 0.43, R_fF 0.55; k' (HPLC system III) 10.17.

Boc-Tyr(tBu)-c[-D-Orn-Trp-D-Pro-Gly-] (41). Compound 40 (0.28 mmol) was treated according to method B (cyclization time 48 h), yielding cyclic peptide 41 after crystallization from EtOAc/DIPE (85%): k' (HPLC system III) 11.44 (93% purity); FAB-MH$^+$ 774.

Boc-2-Nal-D-Pro-OBzl (42). According to method A, Boc-2-Nal-OH (1.20 mmol) was reacted with H-D-Pro-OBzl*HCl (Bachem) (1.20 mmol). Crude 42 was obtained after precipitation from CH_2Cl_2 with DIPE as an amorphous product (77%): $[\alpha]^{20}_D$ +35.7° (c 1.0, MeOH); TLC R_fA 0.71, R_fB 0.68.

Boc-D-Orn(Z)-2-Nal-D-Pro-OBzl (43). Using method A, Boc-D-Orn(Z)-OH (0.50 mmol) was coupled with H-2-Nal-D-Pro-OBzl*HCl (obtained from 42 according to deprotection procedure C), yielding 43 after crystallization from $CHCl_3$/DIPE/PE (80%): mp 65–68 °C; $[\alpha]^{20}_D$ +46.5° (c 1.0, MeOH); TLC R_fA 0.56, R_fB 0.29.

Boc-Tyr(tBu)-D-Orn(Z)-2-Nal-D-Pro-OBzl (44). According to method A, Boc-Tyr(tBu)-OH (0.34 mmol) and H-D-Orn(Z)-2-Nal-D-Pro-OBzl*HCl, obtained from 43 by using method C, were coupled to yield 44 after crystallization from CH_2Cl_2/DIPE/PE (82%): mp 126–129 °C; $[\alpha]^{20}_D$ +45.0° (c 1.0, MeOH); TLC R_fA 0.71, R_fB 0.50.

Boc-Tyr(tBu)-D-Orn-2-Nal-D-Pro-OH (45). Hydrogenation of 44 (0.27 mmol) (method D) and crystallization from EtOH/ether yielded compound 45 (82%): mp 188–190 °C; $[\alpha]^{20}_D$ +40.4° (c 1.0, MeOH); TLC R_fD 0.52, R_fF 0.61; k' (HPLC system III) 14.12.

Boc-Tyr(tBu)-c[-D-Orn-2-Nal-D-Pro-] (46). Compound 45 (0.20 mmol) was treated for 60 h according to method B. A mixture of the cyclic monomer 46 and of the cyclodimer (monomer/dimer ratio 85:15) was obtained after crystallization from EtOAc/DIPE (87%): k' (HPLC system III) 17.25 (monomer), 26.15 (dimer).

Compounds 3–9. The final products 3–9 were obtained after deprotection of the appropriate precursor molecules according to method E. Crude peptides were purified by preparative RP-HPLC as described in General Methods. Analytical data are summarized in Table 1.

Receptor-Binding Assays and Bioassays. The opioid-receptor-binding assays were performed as reported in detail elsewhere.[23] Binding affinities for the μ and δ opioid receptors were determined by displacing respectively [^{3}H]DAMGO (Amersham) and [^{3}H]DSLET (New England Nuclear) from rat brain membrane preparations, and κ opioid receptor affinities were

measured by displacement of [^{3}H]U-69,593 (New England Nuclear) from guinea pig brain membranes. Incubations were performed for 2 h at 0 °C with [^{3}H]DAMGO, [^{3}H]DSLET, and [^{3}H]U-69,593 at respective concentrations of 0.72, 0.78, and 0.80 nM. IC_{50} values were obtained from the dose–displacement curves, and the K_i values were calculated from the IC_{50} values by means of the equation of Cheng and Prusoff,[24] using values of 1.3, 2.6, and 2.9 nM for the dissociation constants of [^{3}H]-DAMGO, [^{3}H]DSLET, and [^{3}H]U-69,593, respectively.

In vitro opioid activities of the compounds were tested in the GPI[25] and MVD[26] bioassays as reported in detail elsewhere.[10,23] A log dose–response curve was determined with [Leu^5]enkephalin as standard for each ileum and vas deferens preparation, and IC_{50} values of the compounds being tested were normalized according to a published procedure.[27] K_e values for the antagonists were determined from the ratio of IC_{50} values obtained in the presence and absence of a fixed antagonist concentration, as described in the literature.[28] Antagonist concentrations used were as follows: naloxone, 5 nM; compound 4, 500 nM; compound 6, 1000 nM; and TIPP, 30 nM.

NMR Experiments. NMR samples were prepared by dissolving around 5 mg of the appropriate peptide (concentration ~10 mM) in H_2O and adjusting the pH of the solution to 4.0. After lyophilization and drying, the samples were dissolved in DMSO-d_6 (>99.9% D, CIL, MSD), filtered into 5-mm tubes, and degassed by four freeze–pump–thaw cycles. ^{1}H NMR spectra were measured on a Varian VXR-400S spectrometer operating at 400 MHz and equipped with a Sun 3-160 computer. All experiments were carried out at 308 K. Temperature coefficients ($-\Delta\delta/\Delta T$) were determined from spectra collected at temperatures ranging from 20 to 70 °C. Resonance assignments were made by analysis of the 2D H,H-COSY,[29] 2D TOCSY,[30] and 2D ROESY[31] spectra. The TOCSY and ROESY spectra were recorded in the phase-sensitive mode.[32,33]

The TOCSY experiments employed the MLEV-17 sequence[34] for the spin–lock with a mixing time of 80 ms; 512 FID's of 2K data points, 32 scans each, were accumulated. In ω_2, the data were processed using the $\pi/2$-shifted sine-bell squared window. In ω_1, the resulting FID's were zero filled to 1K and multiplied with a $\pi/2$-shifted squared sine bell.

The ROESY experiments were carried out using mixing times of 300–400 ms. Typically, 512 FID's of 4K data points, 32 scans each, were accumulated. In ω_2, the data were processed using a $\pi/2$-shifted squared sine-bell window. $\pi/2$-shifted squared sine-bell multiplication and zero filling to 2K in ω_1 were applied.

Molecular Modeling. All theoretical conformational analyses were performed by using the molecular modeling program SYBYL 5.4 and 5.5 (Tripos Associates, St. Louis, MO) on an Evans and Sutherland workstation 10 and a VAXstation 3500 connected to an Evans and Sutherland PS330 computer graphics display terminal. The Tripos force field was used for energy calculations.[35] Partial charges were calculated according to Gasteiger and Marsili,[36] and a distance-dependent dielectric constant of $\epsilon = 4D$ was employed. All molecules were studied in the N-protonated form.

A stepwise procedure was used to determine low-energy conformations of the peptides studied. In the first step, the "bare" ring structures, containing only the atoms directly attached to the ring, and their associated hydrogen atoms were constructed.[37] An extensive systematic search was performed on all rotatable bonds, allowing for both *trans* and *cis* peptide bonds around the proline residue. Ring closure was obtained by allowing a bond-length variation of 0.3 Å and a valence-angle variation of 15° around the ring-closure bond. To each low-energy ring structure obtained, the Tyr^1 residue and the 3-position side chain were added, and a systematic search was performed on all rotatable exocyclic bonds using a 30° grid over all space. Conformations that were within 7 kcal/mol of the minimum-energy structure were grouped into families based on similarity of their dihedral angles, and the lowest energy member of each conformational family was extensively minimized.

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Supplementary Material Available: Complete tabulation of chemical shifts, temperature dependencies of NH chemical shifts, and proton–proton distances (calculated from ROESY spectra) of compounds **2**, **4**, and **5** and tabulation of torsional angles, intramolecular distances, and relative energies of the proposed conformations of analogues **2**, **4**, and **5** (11 pages). Ordering information is given on any current masthead page.

References

(1) Symbols and abbreviations are in accordance with recommendations of the IPUAC-IUB Joint Commission on Biochemical Nomenclature: Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem. J.* **1984**, *219*, 345–373. The other abbreviations are as follows: A_2bu, 2,4-diaminobutyric acid; Boc, *tert*-butoxycarbonyl; [D-Ala^2]deltorphin, I, H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2; DAMGO, H-Tyr-D-Ala-Gly-MePhe-Gly-ol; DIPE, diisopropyl ether; DPDPE, H-Tyr-D-Pen-Gly-Phe-D-Pen-OH; DPPA, diphenyl phosphorazidate; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; GPI, guinea pig ileum; IBCF, isobutyl chloroformate; MVD, mouse vas deferens; Nal, naphthylalanine; NMM, *N*-methylmorpholine; OBzl, benzyl ester; ONb, *p*-nitrobenzyl ester; Orn, ornithine; PE, petroleum ether; Pen, penicillamine; PITC, phenyl-isothiocyanate; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIPP, H-Tyr-Tic-Phe-Phe-OH; tBu, *tert*-butyl; U69,593, (5α,7α,8β)-(−)-*N*-methyl-*N*-(7-pyrrolidinyl-1-oxaspiro-[4.5]dec-8-yl)benzeneacetamide; Z, benzyloxycarbonyl.

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