Expedited Articles

Novel Ligands Lacking a Positive Charge for the δ- and μ-Opioid Receptors

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Recently we reported using minilibraries to replace Lys[9] [somatostatin (SRIF) numbering] of the potent somatostatin agonist L-363,301 (c[-Pro-Phe-D-Trp-Lys-Thr-Phe-]) to generate the potent neurokinin receptor (NK-1) antagonist c[-Pro-Phe-D-Trp-*p*-F-Phe-Thr-Phe-]. This novel cyclic hexapeptide did not bind the SRIF receptor. Thus, a single mutation converted L-363,-301, a SRIF agonist with potency ca. 2−8 times the potency of SRIF in laboratory animals,[24] into a selective NK-1 receptor antagonist with an IC_{50} of 2 nM in vitro. During the screening of the same libraries for ligands of the δ-opioid receptor, we identified four compounds (**1**−**4**) which represent a new class of δ-opioid antagonists, some of which were also NK-1 receptor antagonists. The most potent δ-opioid antagonist, c[-Pro-1-Nal-D-Trp-Tyr-Thr-Phe-] (**2**), showed a K_e value of 128 nM in the mouse vas deferens assay and a δ-receptor binding affinity constant of 152 nM in the rat brain membrane binding assay. These results are of interest because they represent a novel class of δ-opioid antagonists and, like two previously reported δ-opioid antagonists, they lack a positive charge. To examine further the requirement for a positive charge in the δ-opioid ligands, we prepared two analogues of the β-casomorphin-derived mixed μ-agonist/δ-antagonist, H-Dmt-c[-D-Orn-2-Nal-D-Pro-Gly-] (**7**), in which we eliminated the positive charge either through formylation of the primary amino group (**5**) or by the deletion of this N-terminal amino group (**6**). These latter compounds proved to be δ-opioid antagonists with K_e values in the 16−120 nM range, as well as fairly potent μ-opioid antagonists ($K_e \approx 200$ nM). These six compounds provide the most convincing evidence to date that there is no requirement for a positive charge in μ- and δ-opioid receptor antagonists. In addition, cyclic hexapeptide **4** lacks a phenolic hydroxyl group. Taken together, these data suggest that the prevailing assumptions about δ- and μ-opioid receptor binding need revision and that the receptors for these opioid ligands have much in common with the NK-1 and somatostatin receptors.

Introduction

The sequencing of diverse G-protein coupled receptors has demonstrated the presence of a common structural motif consisting of seven membrane spanning α-helices.[2,3] These helices contain several conserved residues, including two aspartates, which are believed to bind to positively charged ligands such as biogenic amines.[4] Peptide hormone and neurotransmitter receptors such as tachykinin NK-1, neuropeptide Y, somatostatin, and the enkephalins display sufficient diversity which makes it more difficult to identify specific binding sites.[4]

In a seminal 1987 paper, Strader, Sigal et al. proposed an ionic interaction between the amino group of agonist and antagonist ligands and the β_2-adrenergic receptor via the Asp[113] residue of the latter.[5] This proposition was based in part on the fact that a mutant protein, in which Asp[113] was replaced by the isosteric Asn (D113N), required 8000−40000-fold increases in the amount of agonist needed for adenylate cyclase stimulation, while the β_2-adrenergic antagonist propranolol displayed a K_i value that was increased 10000-fold.[6] In later work, Strader and co-workers[7] described another mutant of the β_2-adrenergic receptor in which the Asp[113] was replaced by Ser. This mutant similarly failed to bind conventional agonists or antagonists efficiently. Subsequent screening of catechols, in which the amine substituents on the side chain were replaced by ketones or esters, activated the D113S mutant but not the wild type receptor. The authors proposed that the agonism in the D113S mutant resulted from hydrogen bond interactions which replaced the ionic binding interaction with the wild type receptor.[7] Taken together, these

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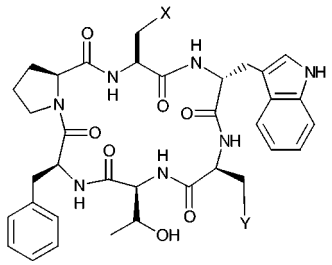
studies demonstrated convincingly that interactions via salt bridges that were thought to be an absolute requirement for the binding of catecholamines and their receptors are replaceable, in this case via hydrogen bonding.

The δ-Opioid Receptor

Similarly, it has been assumed that the binding of an *opioid* ligand to its receptor requires an ionic interaction, involving a positively charged atom, generally a basic amino nitrogen.[8] The basis of this view stems from the pioneering work of Kosterlitz[9] and Rõmer[10] which demonstrated that elimination of the positively charged nitrogen in the enkephalins, generating the corresponding desamino analogues, produces loss of agonism and binding.[9,10] Acetylation of the N-terminal amino group also produced inactive compounds.[11] On the other hand, sulfonium analogues of levorphanol and levallorphan, in which the nitrogen atom of these alkaloids has been replaced by tertiary *S*-methyl and *S*-allyl groups, were found to retain significant potency. This result was interpreted to mean that a positively charged heteroatom, other than nitrogen, can also interact with an anionic site on opioid receptors and induce agonism.[12] Ronai et al. reported in 1992 that Boc-Tyr-Pro-Gly-Phe-Leu-Thr(OtBu)-OH, an opioid peptide lacking a positive charge, was a moderately potent δ-opioid antagonist in the mouse vas deferens (MVD) assay ($K_e \approx 30$ nM).[13] However, the δ-receptor affinity in the rat brain membrane binding assay was very weak ($K_i^{\delta} = 300-1000$ nM), diminishing the impact of this report.[13,14] Conversely, the diketopiperazine c(-Dmt-Tic-), which also lacks a positive charge, was reported by Balboni et al. to be a δ-antagonist. However, the affinity in the MVD assay ($K_e = 3.8$ μM) was very low.[15]

In 1992, two reports described the cloning of the δ-opioid receptor[16,17] which subsequently permitted site-directed mutagenesis experiments. Kong et al. investigated the role of Asp[95], believed to be involved in the binding of δ-opioid ligands.[18] These authors reported that the enkephalins and enkephalin-derived agonists bind to the wild type receptor but not to the D95N mutant, consistent with conventional thinking. In contrast, δ-receptor-selective antagonists such as naltrindole, its benzofuran analogue, and 7-benzylidenaltrexone bound equally well to the wild type and mutant receptors. Furthermore, the nonselective opioid agonist bremazocine, which interacts with the wild type δ-, κ-, and μ-receptors, also interacted well with the mutant δ-receptor. The authors emphasized that "δ-selective agonists bind *differently* to the cloned δ-opioid receptor than do δ-selective antagonists or nonselective opioid agonists"; they did not comment about specific structural requirements.

Believing the requirement for a positively charged nitrogen atom to be the common property of opioid ligands,[8] Befort et al. investigated the possibility that Asp[128] of the mouse δ-opioid receptor provides the anionic component of a salt bridge.[19] However, replacement of this amino acid with alanine gave an active receptor, leading them to speculate that Asp[128] is not directly involved in the binding of the ligand but affects binding site conformation.[19,20]

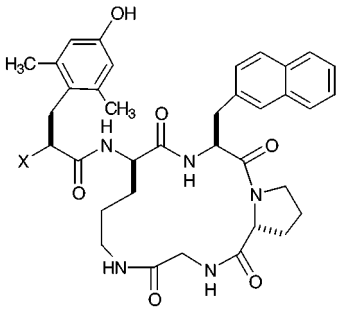


Compound	Group	X	Group	Y
1	Phe	phenyl	Tyr	phenol
2	1-Nal	α-naphthyl	Tyr	phenol
3	2-Nal	β-naphthyl	Tyr	phenol
4	Phe	phenyl	Cha	cyclohexyl

Figure 1. Cyclic hexapeptides tested at the δ-opioid receptor.

Because two neutral δ-opioid antagonists have been reported, albeit with only modest activity[13,15] (see above), we wished to examine further the requirement of a positively charged heteroatom in ligands for the opioid receptor. Believing cyclic hexapeptides to represent privileged platforms,[21] similar to such scaffolds as steroids, benzodiazepines, β-D-glucose derivatives,[22] and the so-called tricyclics, typified by amitriptyline,[23] we screened cyclic hexapeptides (Figure 1), available from the NK-1 receptor program, which had already identified a potent NK-1 antagonist ($IC_{50} = 2.0$ nM).[24] Of a total of 12 arbitrarily selected cyclic hexapeptides, *all* lacking a positive charge, two compounds, **1** and **4**, were found to bind selectively to the δ-opioid receptor and to produce δ-opioid antagonistic effects in the functional MVD assay. Compound **4** lacks both a positive charge and a phenolic hydroxyl group. Conversely, the structurally related parent SRIF agonist, L-363,301 (c[-Pro-Phe-D-Trp-Lys-Thr-Phe-]),[25] containing the free ε-amino nitrogen of Lys does not bind to these receptors. Finally, one of us (W.Y.) synthesized **2** and **3** as designed opioid receptor ligands.

Our results, therefore, establish via the MVD and rat brain membrane receptor binding assays that a positive charge is neither a necessary nor a sufficient requirement for δ-selective opioid antagonistic binding. These results encouraged us to eliminate the positive charge contained in another class of cyclic peptides, the potent β-casomorphin analogue H-Dmt-c[-D-Orn-2-Nal-D-Pro-Gly-] (**7**), a mixed μ-opioid agonist/δ-opioid antagonist (Figure 2).[26] Formylation or deletion of the N-terminal amino group resulted in compounds **5** and **6**, respectively, which retained δ-opioid antagonist activity in the



Compound	X	Group
5	NHCHO	NH-formyl
6	H	hydrogen
7	NH_2	amino

Figure 2. β-Casomorphin analogues tested.

MVD assay. Interestingly, these transformations converted agonists at the μ-receptor into moderately potent μ-opioid antagonists.

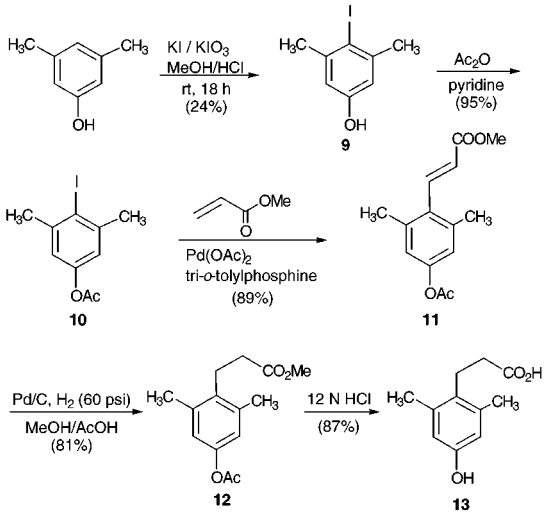
Chemistry

Cyclic Hexapeptide Synthesis. Cyclic hexapeptides **1**−**4** were prepared from protected, linear precursors, the latter prepared via solid-phase peptide synthesis.[26,27] The peptides were removed from the resin with 0.4% TFA in CH_2Cl_2 and cyclized with DPPA via the method previously reported from these laboratories.[28] The protecting groups were then removed from the cyclic peptides using our standard conditions (TFA:DCM:EDT:H_2O, 50:45:3:2; 1 h at room temperature)[24] and purified by reversed-phase HPLC. The resultant cyclic peptides **1**−**4** were characterized by ^{1}H and ^{13}C NMR spectroscopy, high-resolution mass spectrometry, and analytical HPLC using two different solvent systems to confirm structure and purity.

***β*-Casomorphin Analogue Synthesis.** Synthesis of the formyl analogue CHO-Dmt-c[-D-Orn-2-Nal-D-Pro-Gly-] (**5**) proceeded with the addition of *p*-nitrophenyl formate and DIPEA to **7**. Purification was performed by HPLC to furnish **5** in 92% yield.

For the synthesis of the desamino analogue Dhp-c[-D-Orn-2-Nal-D-Pro-Gly-] (**6**), 3-(2′,6′-dimethyl-4′-hydroxyphenyl)propionic acid (Dhp, **13**) was required (Scheme 1). To this end, 3,5-dimethylphenol was iodinated, using the procedure of Hünig and Schwarz[29] to afford **9** in 24% yield. Subsequent acetylation (95%) and Heck coupling with methyl acrylate yielded **11** in 89% yield. Catalytic hydrogenation (H_2/Pd−C, 60 psi, 60 °C) followed by hydrolysis with 12 N HCl then afforded **13** in 87% yield.

Scheme 1



The linear peptide precursor Dhp-D-Orn-2-Nal-D-Pro-Gly-OH (**15**) was prepared by standard solid-phase peptide synthesis techniques using Boc chemistry.[30] The protecting groups of Orn(Fmoc) and Dhp(Boc) were removed with 20% (v/v) piperidine in DMF and 50% (v/v) TFA in CH_2Cl_2, respectively. Cyclization between the δ-amino group of D-Orn and the C-terminal carboxyl group in dilute solution with DPPA, followed by preparative HPLC, yielded Dhp-c[-D-Orn-2-Nal-2-Pro-Gly-] (**6**).

Opioid Receptor Binding Assays and in Vitro Bioassays. Binding affinities of compounds for μ- and δ-opioid receptors were determined by displacing [^{3}H]-DAMGO and [^{3}H]DSLET, respectively, from rat brain membrane binding sites; κ-opioid receptor affinities were measured by displacement of [^{3}H]U69,593 from guinea pig brain membrane binding sites. For the determination of their in vitro opioid activities, compounds were tested in bioassays based on inhibition of electrically evoked contractions of the guinea pig ileum (GPI) and MVD. The GPI assay is usually considered as being representative for μ-receptor interactions, even though the ileum also contains κ-receptors. In the MVD assay, opioid effects are primarily mediated by δ-receptors; however μ- and κ-receptors also exist in this tissue. K_e values for δ-antagonists were determined in the MVD assay against the δ-agonist DPDPE, while the highly selective μ-agonist TAPP (H-Tyr-D-Ala-Phe-Phe-NH_2)[30] was used for the determination of K_e values of μ-receptor antagonists in the GPI assay.

Results

Replacement of the Lys^9 residue of the potent somatostatin receptor agonist L-363,301, c[-Pro-Phe-D-Trp-Lys-Thr-Phe-], by tyrosine generated **1**, c[-Pro-Phe-D-Trp-Tyr-Thr-Phe-],[24] which showed significant δ-antagonist activity (K_e = 238 ± 26 nM) against the selective δ-agonist DPDPE in the MVD assay (Table 1).

Further structural modification through replacement of the Phe between Pro and D-Trp with 3-(1-naphthyl)-alanine (1-Nal) led to compound **2**, c[-Pro-1-Nal-D-Trp-Tyr-Thr-Phe-], with a 2-fold increase in δ-antagonist potency (K_e = 128 ± 18 nM). Interestingly, the corresponding analogue **3** with 2-Nal in place of 1-Nal was 20 times less potent as a δ-opioid antagonist (Table 1). Finally, replacement of the Lys residue in L-363,301 with cyclohexylalanine (Cha)[24] produced compound **4**, which also demonstrated δ-antagonist activity (K_e = 202 ± 38 nM). Unlike the compounds by Rónai[14] and by Balboni,[15] the δ-opioid receptor affinities of compounds **1**−**4**, measured in the rat brain membrane binding assay (Table 2), were in good agreement with their respective δ-antagonist potencies determined in the MVD assay (Table 1). None of these compounds showed antagonist or agonist activity in the μ-receptor-selective GPI assay, and none of them bound to μ- or κ-receptors at concentrations up to 10 μM. Various other amino acid replacements at Phe^2, D-Trp^3, or Lys^4 of c[-Pro-Phe-D-Trp-Lys-Thr-Phe-] led to compounds that either had weak δ-antagonist potency (K_e > 2 μM) or failed to bind altogether and showed neither μ-agonist nor μ-antagonist activity in the GPI assay.

To examine the requirement for a positive charge in opioid receptor ligands further, we prepared analogues of the cyclic β-casomorphin peptide H-Dmt-c[-D-Orn-2-Nal-D-Pro-Gly-] **(7)** in which the positive charge on the N-terminal amino group was eliminated by either formylation or deamination. Compound **7** is a potent δ-antagonist (K_e = 2.13 ± 0.51 nM, MVD assay) and a potent μ-agonist (IC_{50} = 7.88 ± 0.94 nM, GPI assay) (Table 1).[26] It has subnanomolar binding affinity for both μ- and δ-receptors (Table 2). Formylated analogue **5** retained high δ-opioid antagonist potency in the MVD

Table 1. K_e Values of Opioid Antagonists Determined in the MVD and GPI Assays Compared with NK-1 Results

	compd	NK-1 IC_{50} (nM)[a]	MVD K_e (nM)[b,c]	GPI K_e (nM)[b,d]
1	c[-Pro-Phe-D-Trp-Tyr-Thr-Phe-]	1045 ± 230	238 ± 26	inactive[e]
2	c[-Pro-1-Nal-D-Trp-Tyr-Thr-Phe-]	165	128 ± 18	inactive[e]
3	c[-Pro-2-Nal-D-Trp-Tyr-Thr-Phe-]	N/A	2460 ± 240	inactive[e]
4	c[-Pro-Phe-D-Trp-Cha-Thr-Phe-]	216 ± 141	202 ± 38	inactive[e]
5	CHO-Dmt-c[-D-Orn-2-Nal-D-Pro-Gly-]	N/A	16.3 ± 5.3	216 ± 28
6	Dhp-c[-D-Orn-2-Nal-D-Pro-Gly-]	N/A	121 ± 16	237 ± 19, agonist
7	H-Dmt-c[-D-Orn-2-Nal-D-Pro-Gly-][31]	N/A	2.13 ± 0.51	(IC_{50} = 7.88 ± 0.94 nM)

[a] As reported in ref 24. [b] Mean of three to six determinations ± SEM. [c] Determined against DPDPE. [d] Determined against TAPP. [e] Inactive at concentrations up to 10 μM. N/A = not available.

Table 2. Binding Affinities of Peptide Analogues at μ- and δ-Receptors in Rat Brain Membrane Homogenates

	compd	K_i^{δ} (nM)[a,b]	K_i^{μ} (nM)[a,c]	K_i^{μ}/K_i^{δ}
1	c[-Pro-Phe-D-Trp-Tyr-Thr-Phe-]	759 ± 140	>10000	>13.2
2	c[-Pro-1-Nal-D-Trp-Tyr-Thr-Phe-]	152 ± 16	>10000	>65.8
3	c[-Pro-2-Nal-D-Trp-Tyr-Thr-Phe-]	1070 ± 270	>10000	>9.35
4	c[-Pro-Phe-D-Trp-Cha-Thr-Phe-]	486 ± 65	>10000	>20.6
5	CHO-Dmt-c[-D-Orn-2-Nal-D-Pro-Gly-]	32.8 ± 1.6	218 ± 28	6.65
6	Dhp-c[-D-Orn-2-Nal-D-Pro-Gly-]	109 ± 12	450 ± 77	4.13
7	H-Dmt-c[-D-Orn-2-Nal-D-Pro-Gly-][31]	0.467 ± 0.066	0.476 ± 0.015	1.02
8	[Leu^5]enkephalin	2.53 ± 0.35	9.43 ± 2.07	3.73

[a] Mean of three to six determinations ± SEM. [b] Displacement of [^{3}H]DSLET. [c] Displacement of [^{3}H]DAMGO.

assay (K_e = 16.3 ± 5.3 nM) although it was about 8 times less potent than the parent peptide (Table 1). In agreement with this result, compound **5** also showed marked δ-receptor affinity (K_i^{δ} = 32.8 ± 1.6 nM) in the rat brain membrane binding assay. In the GPI assay, **5** was found to be a μ-antagonist (K_e = 216 ± 28 nM) against the selective μ-agonist TAPP in good agreement with the relatively low μ-receptor affinity (K_i^{μ} = 218 ± 28 nM). Desamino analogue **6** was a δ-opioid antagonist in the MVD assay (K_e = 121 ± 16 nM) with a 7-fold lower potency than analogue **5** and was a μ-antagonist about equipotent with analogue **5** in the GPI assay. The μ- and δ-receptor affinities of **6** in the rat brain membrane binding assays were in good agreement with the K_e values obtained in the functional assays.

Neither **5** nor **6** bound to κ opioid receptors at concentrations up to 10 μM. These results indicate that compounds **5** and **6** retain significant δ-antagonist potency. These analogues also show significant μ-opioid receptor affinity and, in contrast to the μ-agonist peptide **7**, are μ-opioid antagonists. They represent the first compounds lacking a positive charge with significant μ-opioid antagonist activity. Here again, our results show good agreement between binding affinities and potency in functional assays.

Discussion

Ionic bond formation is thought to play a key role in the binding of G-protein coupled receptors to their ligands.[2,3] When the δ-opioid receptor was first cloned in 1992, it became apparent that it was a member of the G-protein coupled receptor family of membrane proteins.[16,17] Extensive literature reports have supported the widely accepted conclusion that all ligands for opioid receptors carry a positively charged heteroatom (nitrogen or sulfur) which interacts with the receptor, presumably via a salt bridge.[8,32] This view remained unchallenged until 1992 when Ronai and collaborators[13] reported that the linear hexapeptide Boc-Tyr-Pro-Gly-Phe-Leu-Thr(OtBu)-OH, which lacks a positive charge, was a moderately potent δ-opioid antagonist in the MVD assay (K_e = 30 nM). Because the δ-receptor binding affinity (K_d = 300−1000 nM) was too weak to be consistent with the results of the MVD assay, this report did not significantly change the view that a positive charge is needed in the ligand.[14] Similarly, Balboni et al.[15] stated that the neutral diketopiperazine c(-Dmt-Tic-) is a δ-opioid antagonist. Again, this paper did not significantly challenge the conventional thinking because the diketopiperazine had very low affinity in the MVD assay (K_e = 3.8 μM).

The successful cloning of the δ-opioid receptor[16,17] enabled Kong et al.[18] to replace Asp^{95} by Asn via site-directed mutagenesis. The enkephalins and related compounds bound only to the wild type receptor, but not to the D95N mutant, suggesting that Asp95 represents the anion that forms a salt bridge with the positively charged ligand. Their results are consistent with the conventional assumption that a salt bridge between an aspartyl residue of the receptor and a positive charge on the ligand is required for binding. However, Kong et al. reported also that the nonselective bremazocine binds to both the wild type and mutant δ-receptors as an agonist. The authors concluded that δ-selective agonists bind to the cloned δ-opioid receptor differently than do δ-selective antagonists or nonselective agonists, but the requirement for a positive charge in opioid ligands was not addressed.

Different binding modes of chemically closely related compounds with similar biological profiles are frequently observed in medicinal chemistry.[33,34] In the steroid field, it is well-precedented that the oxygen atom at C3, long thought to be an absolute requirement for binding, can be replaced without loss of activity.[35] Thus, a wide array of experiments supported the view that a C3-ketone, as in cortisol, is an absolute requirement for antiinflammatory activity. This view was later shown to be erroneous.[36] The work of Strader and co-workers is also relevant.[5−7] Their work showed that while Asp^{113} of the β_2-adrenergic receptor binds catecholamines via a salt bridge, the mutant D113N binds catechols lacking a basic nitrogen as agonists.[6] Last, recent work by Schwartz and co-workers demonstrated that by changing Asp^{113} of the β_2-adrenergic receptor to a His residue,

signal transduction can be induced with zinc or copper ions in the absence of catecholamines.[37] Taken together such reports demonstrate, convincingly, that a given binding mode (i.e., a salt bridge) can be modified by substitutions, for example, hydrogen bonding, metal chelation, or hydrophobic interactions.

The experiments described herein do not involve replacement of receptor aspartate residues by asparagine but, like the studies of Ronai and of Balboni, involve the generation of small molecules lacking a positive charge. The one common feature of the peptides reported herein (especially **1**−**4**) is their large content of aromatic amino acids. Balboni et al. pointed out that aromaticity and hydrophobicity are important factors in the binding of the δ-antagonist c(-Dmt-Tic-) to δ-receptors.[15] Such aromatic/aromatic interactions are very common.[38] We also show that in the cyclic β-casomorphin analogues **5** and **6** removal of the positive charge through formylation or elimination of the N-terminal amino group resulted in a 70−230-fold reduction in δ-receptor binding affinity which corresponds to a 2.6−3.3 kcal/mol loss in binding energy (Table 2). For the ligands described above, this loss in binding energy may be replaced in part via energy gained from the interaction with aromatic side chains of the δ-opioid receptor.[38]

A recent report suggested that the high-affinity binding site of δ-selective peptide agonists may be located in the transmembrane (TM) region of helices V−VII, as well as part of TM III.[39] Site-directed mutagenesis of several aromatic residues in TMs IV−VII revealed varying degrees of importance of individual residues.[40] These aromatic residues, conserved across the opioid receptor subtypes, are believed to form a general binding domain for opioids and, possibly, signal transduction.[39,40]

The phenolic hydroxyl group of the N-terminal Tyr residue in opioid peptides is an important binding element in the interaction with opioid receptors.[8] Replacement of the Tyr[1] hydroxyl group in linear and cyclic opioid peptides, by Phe, has been shown to result in a potency decrease by 1 or 2 orders of magnitude.[41] In marked constrast, the replacement in Tyr of **1** by Cha (affording **4**) had little or no effect on potency. This result suggests that the hydroxyl group of the Tyr residue in the cyclic hexapeptide need not be required for binding to the δ-receptor and that the resulting compounds have a receptor binding mode that is different from opioid peptides which contain an N-terminal Tyr residue.

Finally, the route which this work has taken is also of interest. It was originally discovered by Terenius that SRIF has weak affinity for the μ-opioid receptor.[42] Utilizing this fact, Hruby designed and synthesized somatostatin analogues that have high potency at the μ-opioid receptor and low affinity at the SRIF receptor, thus changing the biological profile.[43] Our previous work has demonstrated that through design and synthesis, we converted a SRIF agonist into a NK-1 antagonist, changing ligand−receptor affinity.[24] This paper demonstrates that an SRIF analogue can be modified also to produce an opioid ligand; however, this time we targeted the δ-opioid receptor. Finally this paper also demonstrates that the SRIF, NK-1, and δ-opioid receptors have more features in common than had heretofore been appreciated.

Conclusions

The cyclic hexapeptides described in this paper represent a new class of δ-opioid antagonists. The β-casomorphin analogues **5** and **6** displayed both δ- and μ-antagonist properties and represent the first neutral compounds with significiant μ-antagonist activity. The results reported herein strengthen the evidence that an electrostatic interaction is not an absolute requirement for δ- and μ-opioid receptor−ligand interactions. Our results also support the suggestion that neutral molecules can interact with δ- and μ-opioid receptors as antagonists via aromatic and/or aliphatic side chains. It is noteworthy that **4**, in which Tyr has been replaced by Cha, lacks a phenolic hydroxyl group but, nevertheless, retains the ability to act as an antagonist at the δ-opioid receptor. Taken together, the results reported herein show that δ-opioid receptors can bind ligands via diverse binding modes and that cyclic hexapeptides are privileged platforms.[44] Further work is underway, both in Montréal and in Philadelphia, to explore these findings more fully.

Experimental Section

General Methods. All solvents and reagents were obtained from commercial sources and used without further purification. Assembly of linear peptides was carried out on a model 431A Applied Biosystems peptide synthesizer using L-Pro-2-chlorotrityl polystyrene resin (Advanced Chem Tech or Anaspec). N-α-FMOC amino acids (Bachem Bioscience or Advanced Chem Tech) were employed throughout. Precoated plates (silica gel F_{254}, 250 μm; Merck, Darmstadt, FRG) were used for TLC in the following solvent systems (all v/v): (I) $CHCl_3$/MeOH/AcOH (85:10:5), (II) hexane/EtOAc (4:1), (III) toluene/ethyl acetate (2:3), (IV) *n*-BuOH/AcOH/H_2O (4:1:5, organic phase), and (V) *n*-BuOH/pyridine/AcOH/H_2O (15:10:3:12).

Proton magnetic resonance spectra were recorded at 25 °C on either a Varian VXR-400S spectrometer or a Bruker AM 500 MHz spectrometer using tetramethylsilane or residual solvent as the internal standards. Molecular weights of compounds were determined by either FAB mass spectrometry on an MS-50 HMTCTA mass spectrometer interfaced to a DS-90 data system (Dr. M. Evans, Department of Chemistry, University of Montreal) or by ESI mass spectrometry on a VG 70/70H micromass spectrometer. Optical rotations were measured on a Perkin-Elmer model 241 polarimeter.

HPLC Analysis. 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 purity determination of the β-casomorphin peptides. Analytical reversed-phase HPLC chromatography was carried out on a Vydac 218-TP column (4.6 × 250 mm) under isocratic conditions with 50% MeOH in 0.1% TFA at a flow rate of 1.0 mL/min. Preparative reversed-phase HPLC was performed using a Vydac 218-TP column (22 × 250 mm) with a linear gradient of 20−45% acetonitrile in 0.1% TFA at a flow rate of 7 mL/min, absorption being measured at 216 and 280 nm. Analytical HPLC for the cyclic hexapeptides was performed on a Waters 600E multisolvent delivery system equipped with a 996 photodiode array detector. Reversed-phase HPLC chromatography was performed with a Vydac 238TP54 column (4.6 × 250 mm) with a 0−100% gradient of aqueous 0.1% TFA against either acetonitrile or methanol at a flow rate of 1.0 mL/min. Preparative HPLC was performed on a Rainin solvent delivery system equipped with a Dynamax detector and utilizing a Dynamax C18 (300 Å, 21.4 × 250 mm) column with a 30−70% gradient of aqueous 0.1% TFA to MeCN.

Generalized Cyclic Hexapeptide Synthesis. Assembly of the peptides started with either 0.1 or 0.25 mmol of Fmoc-L-Pro-2-chlorotrityl polystyrene resin and sequential addition of the approriately protected N-α-Fmoc amino acids.[27] Peptides were removed from the resin using a solution of 0.40% TFA in CH_2Cl_2. After 30 min, the slurry was filtered and washed with additional TFA solution. The filtrate was concentrated, azeotroped with benzene, and dried. Cyclization occurred by suspending the peptide (1 equiv) in anhydrous DMF, along with $NaHCO_3$ (15 equiv) and DPPA (1.5 equiv).[28] This solution was stirred for at least 18 h at 0 °C and monitored by HPLC. Once complete, the DMF was removed and the peptide was subjected to silica gel chromatography. The protecting groups were removed from the cyclic peptides using TFA:CH_2Cl_2:EDT:H_2O (50:45:3:2), followed by precipitation with ethyl ether:hexane. The crude peptide was filtered, dried, subjected to HPLC purification, and lyophilized as an amorphous solid.

Cyclo(1-Nal-D-Trp-Tyr-Thr-Phe-Pro) (2): $[\alpha]^{25}_D$ −0.70° (*c* 0.19, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 0.78 (m, 2H), 0.86, (m, 1H), 1.15 (d, 3H, *J* = 6.4 Hz), 1.37 (m, 1H), 1.73 (m, 1H), 2.49 (dd, 1H, *J* = 5.4, 13.9 Hz), 2.56 (dd, 1H, *J* = 5.0, 14.2 Hz), 2.78 (m, 2H), 2.88 (m, 1H), 3.03 (m, 2H), 3.18 (m, 1H), 3.41 (dd, 1H, *J* = 7.7, 15.6 Hz), 3.52 (dd, 1H, *J* = 7.4, 13.6 Hz), 3.61 (d, 1H), 4.11 (dd, 1H, *J* = 4.7, 6.4 Hz), 4.15 (dd, 1H, *J* = 5.1, 8.0 Hz), 4.30 (dd, 1H, *J* = 5.6, 10.8 Hz), 4.38 (d, 1H, *J* = 4.6 Hz), 4.44 (dd, 1H, *J* = 5.5, 9.9 Hz), 4.82 (d, 1H, *J* = 7.6 Hz), 6.56 (s, 4H), 6.80 (s, 1H), 7.06 (t, 1H, *J* = 7.5), 7.14 (t, 1H, *J* = 10.0 Hz), 7.18 (m, 2H), 7.25 (m, 6H), 7.37 (d, 1H, *J* = 8.2 Hz), 7.42 (d, 1H, *J* = 7.9 Hz), 7.44 (m, 1H), 7.73 (d, 1H, *J* = 8.1 Hz), 7.83 (d, 1H, *J* = 7.8 Hz), 8.22 (d, 1H, *J* = 8.4 Hz); ^{13}C NMR (125 MHz, CD_3OD) δ 18.9, 22.2, 28.4, 31.6, 36.4, 36.6, 38.6, 47.3, 55.3, 55.8, 56.2, 57.3, 57.9, 62.5, 68.6, 110.3, 112.6, 116.4, 119.3, 119.9, 122.6, 124.4, 124.9, 126.4, 126.8, 128.1, 128.4, 128.6, 128.7, 128.9, 129.8, 130.0, 130.6, 130.9, 133.6, 134.3, 135.4, 136.8, 138.0, 157.2, 171.4, 171.9, 172.6, 173.1, 173.7, 174.2; HRMS (ESI) *m*/*z* calcd for $C_{51}H_{53}N_7O_8$ + Na 914.3853, found 914.3830 [(M + Na)$^+$].

Cyclo(2-Nal-D-Trp-Tyr-Thr-Phe-Pro) (3): $[\alpha]^{25}_D$ −2.01° (*c* 0.17, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 0.80 (m, 2H), 1.12 (d, 3H, *J* = 6.4 Hz), 1.25 (m, 1H), 1.68 (m, 1H), 2.65 (dd, 1H, *J* = 5.0, 14.2 Hz), 2.70 (dd, 1H, *J* = 6.4, 14.0 Hz), 2.78 (dd, 1H, *J* = 8.3, 14.2 Hz), 2.90 (m, 2H), 3.06 (m, 3H), 3.20 (m, 2H), 3.60 (d, 1H, *J* = 7.6 Hz), 4.09 (dd, 1H, *J* = 4.5, 6.4 Hz), 4.20 (dd, 1H, *J* = 5.0, 8.3 Hz), 4.37 (m, 1H), 4.44 (dd, 1H, *J* = 6.5, 9.0 Hz), 4.76 (dd, 1H, *J* = 6.7, 7.7 Hz), 6.57 (d, 2H, *J* = 6.0 Hz), 6.64 (d, 2H, *J* = 6.5 Hz), 6.89 (s, 1H), 7.05 (t, 1H, *J* = 7.5 Hz), 7.13 (m, 1H), 7.25 (m, 6H), 7.37 (m, 4H), 7.44 (s, 1H), 7.64 (m, 2H), 7.74 (m, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 17.5, 20.6, 26.9, 30.1, 35.3, 37.3, 38.3, 45.9, 53.9, 54.5, 54.6, 55.9, 56.4, 61.1, 67.1, 109.1, 111.2, 115.0, 117.9, 118.5, 121.2, 123.1, 125.3, 125.7, 126.9, 127.0, 127.1, 127.2, 127.3, 127.6, 127.7, 128.6, 129.2, 129.5, 132.5, 133.4, 134.1, 135.5, 136.7, 155.9, 170.0, 170.6, 171.1, 171.8, 172.3, 173.0; HRMS (ESI) *m*/*z* calcd for $C_{51}H_{53}N_7O_8$ + Na 914.3853, found 914.3853 [(M + Na)$^+$].

CHO-Dmt-cyclo(D-Orn-2-Nal-D-Pro-Gly) (5). To a solution of peptide **7** (15 mg, 0.02 mmol) at 0 °C were added DIPEA (0.005 mL, 0.029 mmol) and *p*-nitrophenyl formate (0.304 mL, 0.029 mmol). The reaction was stirred for 2 h and allowed to warm to room temperature. Solvent was removed and the peptide was directly purified by HPLC (12.2 mg, 92.4%): $[\alpha]^{25}_D$ −1.02° (*c* 0.26, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 1.03 (m, 1H), 1.28 (m 3H), 1.54 (m, 3H), 1.67 (m, 1H), 2.26 (s, 6H), 2.30 (m, 1H), 2.82 (d, 1H, *J* = 13.2 Hz), 2.96 (dd, 1H, *J* = 4.8, 13.8 Hz), 3.10 (m, 2H), 3.43 (m, 3H), 3.53 (m, 1H), 4.10 (dd, 1H, *J* = 4.5, 8.0 Hz), 4.28 (m, 2H), 4.32 (dd, 1H, *J* = 5.4, 11.0 Hz), 4.43 (dd, 1H, 4.3, 10.9 Hz), 6.50 (s, 2H), 7.36 (dd, 1H, *J* = 1.7, 8.4 Hz), 7.48 (m, 2H), 7.69 (s, 1H), 7.83 (m, 3H), 8.09 (d, 1H, *J* = 1.0 Hz); ^{13}C NMR (125 MHz, CD_3OD) δ 20.3, 23.5, 25.0, 27.2, 29.9, 31.9, 37.9, 38.8, 43.2, 48.3, 54.2, 54.7, 56.2, 62.9, 116.2, 125.2, 127.2, 127.6, 128.6, 128.7, 129.1, 129.4, 134.1, 134.4, 134.9, 139.9, 157.1, 163.6, 171.8, 174.0, 174.1, 174.3, 174.5; HRMS (ESI) *m*/*z* calcd for $C_{37}H_{44}N_6O_7$ + Na 707.3169, found 707.3169 [(M + Na)$^+$].

3,5-Dimethyl-4-iodophenol (9).[29] To a solution of 3,5-dimethylphenol (24.4 g, 0.20 mol) in MeOH (406 mL) and concd HCl (162 mL) was added a mixture of KI (22.4 g, 0.14 mol) and KIO_3 (13.8 g, 4.5 mmol). The mixture was initially cooled in an ice bath and allowed to warm to room temperature overnight. The precipitated product was collected by filtration and washed with MeOH/H_2O (1:1). The crude product was dissolved in toluene, filtered through Celite, and precipitated with pentane to give **9** (12.2 g, 24%): mp 133−135 °C (lit.[29] 131 °C); TLC R_f 0.85 (I).

3,5-Dimethyl-4-iodophenyl Acetate (10).[45] To a solution of **9** (12.0 g, 48.8 mmol), in pyridine (12.0 mL) was added acetic anhydride (6.9 mL, 72.6 mmol). The reaction was heated to 50 °C for 30 min, cooled to room temperature and diluted with 0.5 N HCl (200 mL) to effect crystallization. The product was washed with 120 mL 0.5 N HCl followed by 60 mL portions of H_2O until the final wash reached a pH of 5. Vacuum drying yielded 13.4 g (95%) of crystalline **10**: mp 46−47 °C (lit.[45] 51 °C); TLC R_f 0.9 (II).

Methyl 3-(2′,6′-Dimethyl-4′-acetoxyphenyl)acrylate (11). To a solution of **10** (5.0 g, 17.24 mmol), methylacrylate (1.63 mL, 18.08 mmol), tri-*o*-tolylphosphine (0.27 g, 0.91 mmol), and NEt_3 (4.77 mL, 34.0 mmol) was added $Pd(AcO)_2$ (71.6 mg, 0.32 mmol) in MeCN (24.3 mL). The solution was heated to reflux for 28 h prior to cooling to room temperature. The catalyst was filtered through Celite, and the solvent removed in vacuo. The crude product was dissolved in H_2O (30 mL) and extracted with EtOAc (3 × 40 mL). The combined EtOAc extracts were washed with brine (3 × 30-mL), treated with activated carbon and dried over $MgSO_4$. Solvent removal in vacuo yielded a red oil which crystallized upon standing at 5 °C for 18 h. Recrystallization from ethyl acetate/hexane afforded 3.80 g (89%) of **11**: mp 59−60 °C; TLC R_f 0.48 (II); ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.26 (s, 3H, $COCH_3$), 2.36 (s, 6H, Ar−CH_3), 3.80 (s, $COOCH_3$), 6.03 (d, 1H, *J* = 16.4 Hz), 6.78 (s, 2H, Ar), 7.76 (d, 1H, *J* = 16.4 Hz).

Methyl 3-(2′,6′-Dimethyl-4′-acetoxyphenyl)propionate (12). To a solution of **11** (3.72 g, 15 mmol) in MeOH/AcOH (1:1) (50 mL) was added 10% Pd−C (2.42 g). The reaction vessel was purged with argon and pressurized to 60 psi with H_2. The reaction mixture was stirred vigorously and the temperature raised to 60 °C. When the pressure dropped to 0 psi, the mixture was cooled to 10 °C, vented with argon and repressurized to 60 psi with H_2. This procedure was repeated 10 times until TLC analysis (III) indicated complete reduction. The mixture was filtered through Celite and the solvents removed in vacuo to yield 3.05 g (81.3%) of **12** as an oil: TLC R_f 0.36 (II), R_f 0.76 (III); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.28 (s 3H, OCH_3), 2.32 (s, 6H, CH_3 Ar), 2.44 (t, 2H, CH_2), 2.94 (t, 2H, CH_2), 3.71 (s, 3H, $COOCH_3$), 6.74 (s, 2H, Ar).

3-(2′,6′-Dimethyl-4′-hydroxyphenyl)propionic Acid (Dhp) (13). A mixture of **12** (3.05 g, 12.2 mmol) and 12 N HCl (14 mL) was heated to reflux for 7.5 h, followed by a cooling to 4 °C overnight. The solid product was collected and recrystallized from boiling water to give **13** (2.06 g, 87%): mp 123−124 °C; TLC R_f 0.18 (II), R_f 0.77 (III); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.32 (s, 6H, CH_3 Ar), 2.44 (t, 2H, CH_2), 2.94 (t, 2H, CH_2), 6.81 (s, 2H, ar); FAB-MS *m*/*e* 195.

O-Boc Derivative of 3-(2′,6′-Dimethyl-4′-hydroxyphenyl)propionic Acid [Dhp(OBoc)] (14). To a solution of **13** (2.06 g, 10.61 mmol) in THF/H_2O (1:1) (24 mL) at 0 °C were added DMAP (0.288 g, 2.35 mmol), NEt_3 (3.86 mL, 27.56 mmol) and Boc_2O (2.67 g, 11.67 mmol) in the THF/H_2O (1:1) (10 mL). The reaction stirred for 1.5 h before the THF was removed in vacuo. Ethyl acetate (30 mL) was added, followed by addition of a 5% aqueous solution of $KHSO_4$ until a pH of 6 was reached. The organic phase was separated, dried over $MgSO_4$, and evaporated to yield the crude product as an oil. Crystallization from ethyl acetate/hexane afforded crystalline **14** (2.35 g, 80%): mp 108−110 °C; TLC R_f 0.87 (I); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.55 (s, 9H, $C(CH_3)_3$), 2.31 (s, 6H, CH_3 Ar), 2.45 (t, 2H, CH_2), 2.95 (t, 2H, CH_2), 6.82 (s, 2H, Ar); FAB-MS *m*/*e* 295.

Dhp-D-Orn-2-Nal-D-Pro-Gly-OH (15). Peptide synthesis was performed by the manual solid-phase technique using a Merrifield Boc-Gly-OH resin (1% cross-linked, 100−200 mesh,

0.76 mmol; Bachem Bioscience, King of Prussia, PA). The peptide was assembled using Boc-protected amino acids (Bachem) and DIC/HOBt coupling according to a published protocol.[26,31] The protecting groups of Orn (Fmoc) and Dhp (Boc) were removed with 20% (v/v) piperidine in DMF and 50% (v/v) TFA in CH_2Cl_2, respectively. The peptide was removed from the resin by treatment with HF (20 mL) and anisole (1.0 mL) for 60 min at 0 °C. After evaporation of the HF, the resin was extracted three times with Et_2O, followed by extraction with glacial AcOH. The linear peptide **15** was obtained in solid form through lyophilization of the acetic acid extract and purification by preparative HPLC: TLC R_f 0.51 (IV), R_f 0.59 (V); FAB-MS *m/e* 659 (M^+).

Dhp-c[-D-Orn-2-Nal-D-Pro-Gly-] (6). To a solution of **15** (100 mg, 0.151 mmol) in DMF (20 mL) were added DPPA (65 μL, 0.302 mmol) and NMM (16.5 μL, 0.151 mmol) in DMF (138 mL). The reaction was cooled to −25 °C and the cyclization was monitored by HPLC analysis. Additional DPPA (32 μL, 0.151 mmol) and NMM (16.5 μL, 0.151 mmol) were added to the reaction mixture until HPLC analysis indicated a complete reaction (48 h). Evaporation of the DMF and trituration of the residue with petroleum ether resulted in a crude oil. The product was dissolved in EtOH, precipitated with 5% aq $KHSO_4$, filtered, and washed with H_2O. The product was redissolved in EtOAc, precipitated with diisopropyl ether, and filtered. The crude product was purified by preparative HPLC and was >98% pure by analytical HPLC: TLC R_f 0.65 (IV), R_f 0.89 (V); $[\alpha]^{25}_D$ +7.33 ° (*c* 0.15, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 1.26 (m, 1H), 1.68 (m, 2H), 1.70 (t, 2H, *J* = 7.3 Hz), 1.75 (d, 2H, *J* = 8.0 Hz), 2.22 (s, 6 H), 2.25 (dd, 1H, *J* = 7.6, 14.3 Hz), 2.34 (dd, 1H, *J* = 6.0, 14.3 Hz), 2.66 (m, 1H), 2.78 (t, 2H, *J* = 7.8 Hz), 2.89 (d, 1H, *J* = 12.9 Hz), 3.22 (m, 2H), 3.48 (dd, 1H, *J* = 3.4, 14.0 Hz), 3.55 (dd, 1H, *J* = 11.5, 11.5 Hz), 3.70 (m, 1H), 4.16 (dd, 1H, *J* = 5.6, 6.9 Hz), 4.28 (d, 1H, *J* = 9.0 Hz), 4.32 (br s, 1H), 4.62 (m, 1H), 6.45 (s, 2H), 6.80 (d, 1H, *J* = 9.6 Hz), 7.39 (d, 1H, *J* = 8.4 Hz), 7.44 (m, 2H), 7.57 (d, 1H, *J* = 5.4 Hz), 7.71 (br s, 2H), 7.80 (m, 5H), 7.94 (br s, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 20.1, 23.6, 25.3, 25.6, 27.5, 30.0, 36.6, 37.4, 38.9, 43.2, 55.6, 55.8, 63.1, 116.0, 127.2, 127.6, 128.4, 128.6, 128.7, 129.0, 129.5, 129.7, 134.1, 134.4, 134.9, 138.6, 156.2, 171.9, 174.3, 174.7, 175.0, 176.2; FAB-MS *m/e* 641 (M^+); HRMS (ESI) *m/z* calcd for $C_{36}H_{44}N_5O_6$ + Na 665.3189, found 665.3218 [$(M + Na)^+$].

In Vitro Bioassays and Receptor Binding Assays. The GPI[46] and MVD[47] bioassays were carried out as reported in detail elsewhere.[47,48] K_e values for antagonists were determined from the ratio (*DR*) of IC_{50} values obtained with an agonist in the presence and absence of a fixed antagonist concentration (*a*), using the equation: $K_e = a/(DR - 1)$.[49] δ-Antagonist K_e values of all compounds were determined in the MVD assay against the δ-agonist DPDPE using antagonist concentrations ranging from 50 to 4000 nM. The μ-antagonist K_e values of the cyclic β-casomorphin analogues were determined in the GPI assay against the μ-agonist TAPP with an antagonist concentration of 500 nM.

Opioid receptor binding studies were performed as described in detail elsewhere.[47] Due to their poor water solubility, the cyclic hexapeptides (~2 mg) were first dissolved in 150 μL of DMSO and then 9.85 mL of buffer was added. Since some precipitation occurred after addition of the buffer, the precipitated peptide was removed by filtration and the actual peptide concentration of the clear solution was determined by optical density measurement. To prevent peptide degradation, an enzyme inhibitor cocktail[50] consisting of bestatin (100 μM), captopril (1.0 μM), thiorphan (1.0 μM), and L-leucyl-L-leucine (100 μM) was added to the buffer. Binding affinities for μ- and δ-opioid receptors were determined by displacing, respectively, [^{3}H]DAMGO (Multiple Peptide Systems, San Diego, CA) and [^{3}H]DSLET (Multiple Peptide Systems) from rat brain membrane binding sites, and κ opioid receptor affinities were measured by displacement of [^{3}H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2 h at 25 °C with [^{3}H]DAMGO, [^{3}H]DSLET, and [^{3}H]U69,593 at respective concentrations of 0.72, 0.78, and 0.80 nM. IC_{50} values were determined from log dose−displacement curves, and K_i values were calculated from the obtained IC_{50} values by means of the equation of Cheng and Prusoff,[51] 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]U69,593, respectively.

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Supporting Information Available: Analytical HPLC traces of compounds **1**−**4** and **6** and Table 3 of low-activity compounds tested in the MVD assay. This information is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC−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: Boc, *tert*-butoxycarbonyl; Cha, cyclohexylalanine; CTOP, H-D-Phe-c[-Cys-Tyr-D-Trp-Orn-Thr-Pen-]-Thr-NH_2; DAMGO, H-Tyr-D-Ala-Gly-N$^{\alpha}$MePhe-Gly-ol; Dhp, 3-(2′,6′-dimethyl-4′-hydroxyphenyl)propionic acid; DIC, 1,3-diisopropylcarbodiimide; Dmt, 2′,6′-dimethyltyrosine; DPDPE, H-Tyr-c[-D-Pen-Gly-Phe-D-Pen-]; DIPEA, diisopropylethylamine; DPPA, diphenyl phosphorazidate; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; DIC, 1,3-diisopropylcarbodiimide; MVD, mouse vas deferens; 1-Nal, 3-(1-naphthyl)alanine; 2-Nal, 3-(2-naphthyl)alanine; NMM, *N*-methylmorpholine; DMAP, 4-(dimethylamino)pyridine, TAPP, H-Tyr-D-Ala-Phe-Phe-NH_2; TFA, trifluoroacetic acid; THF, tetrahydrofuran; U69,593, (5α,7α,8β)-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide.

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