A Structure–Activity Relationship Study and Combinatorial Synthetic Approach of C-Terminal Modified Bifunctional Peptides That Are δ/μ Opioid Receptor Agonists and Neurokinin 1 Receptor Antagonists

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A series of bifunctional peptides with opioid agonist and substance P antagonist bioactivities were designed with the concept of overlapping pharmacophores. In this concept, the bifunctional peptides were expected to interact with each receptor separately in the spinal dorsal horn where both the opioid receptors and the NK1 receptors were found to be expressed, to show an enhanced analgesic effect, no opioid-induced tolerance, and to provide better compliance than coadministration of two drugs. Compounds were synthesized using a two-step combinatorial method for C-terminal modification. In the method, the protected C-terminal-free carboxyl peptide, Boc-Tyr(*t*Bu)-D-Ala-Gly Phe-Pro-Leu-Trp(Boc)-OH, was synthesized as a shared intermediate using Fmoc solid phase chemistry on a 2-chlorotrityl resin. This intermediate was esterified or amidated in solution phase. The structure–activity relationships (SAR) showed that the C-terminus acted as not only a critical pharmacophore for the substance P antagonist activities, but as an address region for the opioid agonist pharmacophore that is structurally distant from the C-terminal. Among the peptides, H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NH-Bzl (**3**) demonstrated high binding affinities at both δ and μ receptors (K_i = 10 and 0.65 nM, respectively) with efficient agonist functional activity in the mouse isolated vas deferens (MVD) and guinea pig isolated ileum (GPI) assays (IC_{50} = 50 and 13 nM, respectively). Compound **3** also showed a good antagonist activity in the GPI assay with substance P stimulation (K_e = 26 nM) and good affinity for the hNK1 receptor (K_i = 14 nM). Consequently, compound **3** is expected to be a promising and novel type of analgesic with bifunctional activities.

Introduction

Opioid analgesics are the mainstay for treatment of moderate to severe pain. Research on opioids and their receptors has been active over the past decades.[1] However, the clinical use of opioid drugs for the treatment of chronic pain states still encounters many obstacles, including side effects and analgesic tolerance, which is explained as a reduction of pain relief during prolonged administration of opioids.[2] Repeated morphine exposure resulted in enhanced levels of substance P, a transmitter of pain signals, both in vitro and in vivo.[3,4] This induces increased pain that could require a higher dose of pain-relief and thus be manifested as "antinociceptive tolerance".[5] Supporting this concept, NK1 knockout mice do not show opioid-induced antinociceptive tolerance, and coadministration of opioid agonist and substance P antagonist showed enhanced antinociceptive potency in acute pain states and in prevention of opioid-induced tolerance.[6–11] Moreover, studies have shown that the distributions of opioids and substance P containing neurons overlap, and NK1 and opioid receptors exist nearby in a synaptic cleft.[12–14] We hypothesized that bifunctional peptides with opioid agonist and substance P antagonist pharmacophores in one molecule will interact with each receptor separately. Such interaction can lead to a potent antinociceptive activity without development of tolerance.[15,16] In fact, several bifunctional compounds have showed concerted efficacy due to successful combination of the functions.[17–19] Our design is based on the overlapping pharmacophore concept that has been developed in our group mainly for bifunctional peptides with opioid agonist and cholecystokinin (CCK) antagonist activities.[20–22] The enkephalin-based tetrapeptide Tyr-D-Ala-Gly-Phe was used as an opioid agonist pharmacophore.[23,24] As for a pharmacophore for a substance P antagonist, the 3′,5′-bis(trifluoromethyl)-benzyl ester of the tetrapeptide Phe-Pro-Leu-Trp was chosen and was placed at the C-terminal (Figure 1).[15,16,25,26] Because both pharmacophores share a Phe, an initial sequence for the bifunctional peptide was designed as a 3′,5′-bis(trifluoromethyl)-benzyl ester of H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp with the Phe overlapped (Figure 1). However, because the enzymatic hydrolysis of 3′,5′-bis(trifluoromethyl)-benzyl ester has been reported, and the two highly lipophilic trifluoromethyl groups might lead to low solubility in aqueous solutions, our design approach has focused on modifications of the 3′,5′-bis(trifluoromethyl)-benzyl ester at the C-terminal to replace its ester by an amide or by complete removal of the trifluoromethyl groups from the C-terminal.[27,28]

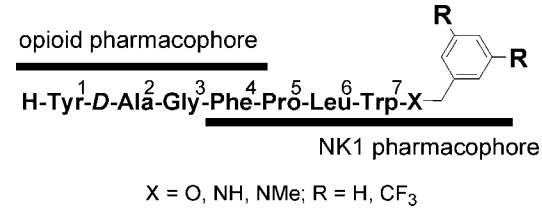

Figure 1. Design of bifunctional peptides possessing a δ/μ opioid pharmacophore and neurokinin-1 pharmacophore.

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Table 1. Sequence and Analytical Data of Bifunctional Peptide Ligands

No.	sequence	*m/z* (M + H)$^{+a}$ obs (ESI)	*m/z* (M + H)$^{+a}$ calc	HPLC log k'^{b} A[c]	HPLC log k'^{b} B[d]	TLC (R_f)[e] I	TLC (R_f)[e] II	TLC (R_f)[e] III	purity (%)[d,f]	yield (%)[f]	log $D_{7.4}$[g]
1	H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-O-3,5-Bzl$(CF_3)_2$	1079.4466	1079.4495	19.03	7.36	0.13	0.71	0.77	85.3	57.0	>4
2	H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-O-Bzl	943.4719	943.4764	15.82	3.42	0.18	0.71	0.77	84.2	57.0	3.8
3	H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NH-Bzl	942.4879	942.4942	13.80	4.28	0.07	0.57	0.78	93.3	57.0	3.3
4	H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NMe-Bzl	956.5035	956.5188	14.97	4.97	0.11	0.58	0.81	66.9	56.0	3.5
5	H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NH-3,5-Bzl$(CF_3)_2$	1078.4626	1078.4616	16.83	6.27	0.11	0.67	0.81	80.3	55.9	>4
6	H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NMe-3,5-Bzl$(CF_3)_2$	1092.4783	1092.4806	17.98	6.76	0.13	0.68	0.81	74.6	57.0	>4

[a] High-resolution mass spectroscopy using electrospray ionization method. [b] HPLC log k' = log [(peptide retention time − solvent retention time)/solvent retention time]. All the obtained final peptides showed >99% purity. [c] 10–90% of acetonitrile containing 0.1% TFA within 40 min and up to 95% within additional 5 min, 1 mL/min, 230 nm, Waters NOVA-Pak C-18 column (3.9 × 150 mm, 5 μm, 60 Å). [d] 30–70% of acetonitrile containing 0.1% TFA within 40 min and up to 95% within an additional 5 min, 1 mL/min, 230 nm, Vydac 218TP104 C-18 column (4.6 × 250 mm, 10 μm, 300 Å). [e] (I) $CHCl_3$/MeOH/AcOH = 90:10:3, (II) EtOAc/*n*-BuOH/water/AcOH = 5:3:1:1, (III) *n*-BuOH/water/AcOH = 4:1:1. [f] Determined of the crude product. [g] Logarithm of octanol/saline distribution coefficient in 0.05 N N-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid (HEPES) buffer in 0.1 N NaCl solution.

Figure 2. Synthesis of bifunctional peptides on 2-chlorotrityl resin. (a) Fmoc-Trp(Boc)-OH, diisopropylethylamine (DIEA), dichloromethane (DCM), 2 h. (b) Stepwise chain elongation using 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU)/20% piperidine chemistry. (c) 1% Trifluoroacetic acid (TFA) in DCM, 30 min. (d) benzyl bromide or 3,5-bis(trifluoromethyl)benzyl bromide, Cs_2CO_3, dimethylformamide (DMF), 2 h. (e) benzyl amine or 3,5-bis(trifluoromethyl)benzyl amine or (3,5-Bis-trifluoromethyl-benzyl)-methyl-amine, 6-Chloro-1-Hydroxybenzotriazole (Cl-HOBt), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), DMF, 1 h. (f) 82.5% v/v TFA, 5% water, 5% thioanisol, 2.5% 1,2-ethanedithiol, 5% phenol, 1 h.

To date, many classes of C-terminal modified peptides have attracted our interest, and a number of approaches to modifying the C-terminal have been reported.[29–31] These approaches can be classified into many categories, including nucleophilic cleavage of protected peptides bound from appropriate resins, attachment with a C-terminal functional group, side chain anchoring followed by normal solid phase N-to-C peptide synthesis, backbone amide attachment onto a solid support, inverse C-to-N solid phase peptide synthesis, and conventional solution phase synthesis (see ref 30 for a review). However, it is difficult to synthesize C-terminal esters or tertiary amides by the first two methods, and our designed peptides did not have a suitable side chain moiety to anchor on a resin. Repeated inverse C-to-N coupling leads to severe racemization, and conventional Boc solution phase peptide synthesis is very labor intensive for large amounts of longer peptides. Therefore, we combined solid phase and solution phase chemistry to develop a two-step combinatorial approach. In this method, a side chain-protected peptide with a free C-terminal carboxylate was synthesized using a N^{α}-Fmoc solid phase peptide synthesis (SPPS) on 2-chlorotrityl resin, followed by esterification or amidation in solution phase without any detectable racemization (Figure 2). Here, we report on the design, synthesis, and biological activities of novel bifunctional peptides with opioid agonist and substance P antagonist activities.

Results and Discussion

Peptide Synthesis. A series of peptides was obtained through a two-step synthetic approach. The first step was the synthesis of Boc-Tyr(*t*Bu)-D-Ala-Gly Phe-Pro-Leu-Trp(Boc)-OH as a shared intermediate using N^{α}-Fmoc chemistry on a 2-chlorotrityl resin, which is a common support for batch SPPS of protected peptides with a free C-terminal.[28] First, Fmoc-Trp(Boc)-OH was introduced on a resin in the presence of diisopropylethylamine (DIEA) in dimethylformamide (DMF). Resin-bound Fmoc-Trp(Boc) was treated with 20% piperidine to remove a N^{α}-Fmoc protecting group. Couplings of the following amino acids were carried out with standard in situ activating reagents used in routine Fmoc SPPS with 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), in the presence of DIEA, to generate 6-Chloro-1-Hydroxyben-

Table 2. Binding Affinities of Bifunctional Peptides at δ/μ Opioid Receptors and NK1 Receptors

	hDOR[a], [^{3}H]DPDPE[b]		rMOR[a], [^{3}H]DAMGO[c]			hNK1[d], [^{3}H]substance P[e]		rNK1[d], [^{3}H]substance P[f]		
No.	log IC_{50}[g]	K_i (nM)	log IC_{50}[g]	K_i (nM)	$K_i(\mu)/K_i(\delta)$	log IC_{50}[g]	K_i (nM)	log IC_{50}[g]	K_i (nM)	K_i(rNK1)/K_i(hNK1)
1	-7.0 ± 0.10	50	-6.4 ± 0.20	180	3.6	-10.0 ± 0.14	0.046	-8.3 ± 0.06	1.6	35
2	-7.2 ± 0.14	31	-7.2 ± 0.17	29	0.94	-6.7 ± 0.07	100	-6.1 ± 0.04	270	2.7
3	-7.7 ± 0.09	10	-8.9 ± 0.09	0.65	0.065	-7.5 ± 0.03	14	$30 \pm 9.9\%$[h]		
4	-6.8 ± 0.24	77	-7.0 ± 0.33	46	0.60	-8.5 ± 0.04	1.6	$33 \pm 7.6\%$[h]		
5	-6.8 ± 0.08	72	-7.7 ± 0.21	9.5	0.13	-8.9 ± 0.06	0.61	-7.0 ± 0.06	33	54
6	-6.5 ± 0.09	31	-7.8 ± 0.13	6.8	0.22	-8.5 ± 0.06	1.4	-7.7 ± 0.03	6.1	4.4
Biphalin[i]		2.6		1.4	0.54					
L-732,138						-8.8 ± 0.02	0.73	-6.4 ± 0.12	130	180

[a] Competition analyses were carried out using membrane preparations from transfected HN9.10 cells that constitutively expressed the δ and μ opioid receptors, respectively. [b] $K_d = 0.45 \pm 0.1$ nM. [c] $K_d = 0.50 \pm 0.1$ nM. [d] Competition analyses were carried out using membrane preparations from transfected CHO cells that constitutively expressed the human or rat NK1 receptors. [e] $K_d = 0.40 \pm 0.17$ nM. [f] $K_d = 0.16 \pm 0.03$ nM. [g] The $IC_{50} \pm$ standard errors are expressed as logarithmic values determined from the nonlinear regression analysis of data collected from two independent experiments (four independent experimental values per drug concentration). The K_i values are calculated using the Cheng and Prusoff equation to correct for the concentration of the radioligand used in the assay. [h] Inhibition % at 1μM. [i] Reference 49.

Table 3. Opioid Agonist Functional Activities in [^{35}S]GTP-γ-S Binding Assays

	hDOR[a]			rMOR[a]		
No.	log EC_{50}[b]	EC_{50} (nM)[c]	E_{max} (%)[d]	log EC_{50}[b]	EC_{50} (nM)[c]	E_{max} (%)[d]
1	-7.5 ± 0.28	35	16 ± 0.7	-6.8 ± 0.24	140	26 ± 2
2	-7.1 ± 0.14	85	131 ± 6	-7.4 ± 0.21	36	66 ± 4
3	-7.8 ± 0.28	17	56 ± 6	-8.2 ± 0.44	0.71	100 ± 16
4	-6.8 ± 0.16	150	70 ± 4	-7.5 ± 0.36	29	120 ± 16
5	-7.1 ± 0.13	80	161 ± 7	-7.2 ± 0.26	57	61 ± 4
6	-6.9 ± 0.21	120	137 ± 7	-7.1 ± 0.17	72	72 ± 3
Biphalin	-9.0 ± 0.17	1.1	83			
DPDPE	-8.8 ± 0.25	1.6	69			
DAMGO				-7.4 ± 0.19	37	150

[a] Expressed from HN9.10 cell. [b] The log $EC_{50} \pm$ standard error are logarithmic values determined from the nonlinear regression analysis of data collected from two independent experiments performed in duplicates. [c] Antilogarithmic value of the respective EC_{50}. [d] The E_{max} value is the saturable, maximum level of [^{35}S]GTPγS binding in the cell membranes upon incubation with the peptide, expressed as [Net [^{35}S]GTPγS bound/basal [^{35}S]GTPγS bound] $\times$ 100 $\pm$ standard error.

zotriazole (Cl-HOBt) esters. The obtained resin-bound Boc-Tyr(*t*Bu)-D-Ala-Gly-Phe-Pro-Leu-Trp(Boc) was cleaved off the resin with 1% TFA in dichloromethane (DCM) in 30 min. The protected peptide was obtained after evaporation, followed by precipitation with chilled petroleum ether. The resulting white solid was washed twice with chilled petroleum ether, then dried under vacuum to give the protected peptide with good purity (98.8%) and moderate yield (57.0%: based on the substitution of the resin).

The second step was esterification or amidation of the protected intermediate, followed by side-chain deprotection in the solution phase. The esterification was performed employing cesium carbonate to form the cesium salt of the protected peptide to react with benzyl bromide or 3′,5′-bis(trifluoromethyl)-benzyl bromide.[25,26] The crude esters, H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-O-3,5-Bzl(CF_3)$_2$ (**1**) and H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-O-Bzl (**2**), were obtained quantitatively by one hour treatment with the cleavage cocktail (82.5% v/v TFA, 5% water, 5% thioanisole, 2.5% 1,2-ethanedithiol, and 5% phenol) to quench the highly stabilized carbocations released from permanent protecting groups. Some acid-mediated hydrolysis of C-terminal benzyl ester occurred during the final cleavage, but the purities of the final crude peptides were still moderate to good (84% for **1** and 67% for **2**). For the amidation, standard 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/Cl-HOBt coupling chemistry with two equivalents of reactant amine was used. The crude amides **3**-**6** were obtained with 98% quantitative yield and good purity (75∼93%). The crude peptides **1**-**6** were purified by RP-HPLC (>99% purity) and were characterized by analytical HPLC, ^{1}H NMR, HRMS, and TLC (Table 1). ^{1}H NMR studies showed cis/trans isomerization at the Pro^5 residue. The ratios of two amide rotamers and their assignments are available in the Supporting Information. Peptides **1**-**6** were also prepared using N$^\alpha$-Boc solution phase chemistry with a previously reported protocol.[15] The two-step approach described in this publication showed the same ^{1}H NMR spectra with the ones through N$^\alpha$-Boc solution chemistry, implying that the reported approach led to the undetectable amounts of racemization.

Table 4. Functional Assay Result for Bifunctional Peptide Ligands at Opioid and Substance P Receptors

	opioid agonist[a,b]		substance P antagonist[b]
No.	MVD (δ), IC_{50} (nM)[c]	GPI (μ), IC_{50} (nM)[c]	GPI, K_e (nM)[d]
1	400 ± 23	520 ± 40	3.6 ± 1.1
2	40 ± 4.4	74 ± 25	150 ± 17
3	50 ± 10	13 ± 3.3	26 ± 3.9
4	41 ± 8.6	9.0 ± 0.5	59 ± 18
5	45 ± 6.3	350 ± 91	8.5 ± 2.1
6	150 ± 26	52 ± 9.4	6.9 ± 1.1
Biphalin	2.7 ± 1.5	8.8 ± 0.3	
L-732,138			250 ± 87

[a] All the tested compounds were full agonists compared to the standards DPDPE and PL-017 for MVD and GPI assays, respectively. [b] Calculated from four isolated tissues ($n = 4$). [c] Concentration at 50% inhibition of muscle concentration at electrically stimulated isolated tissues. [d] Inhibitory activity against the substance P-induced muscle contraction in the presence of 1 μM naloxone, K_e: concentration of antagonist needed to inhibit substance P to half of its activity. The K_e values were obtained from full concentration−effect curve for substance P in the absence and presence of the test compounds in the presence of 1 μM naloxone to block opioid effects on the tissue.

Structure–Activity Relationships. All receptor binding affinities and functional activities of peptides **1**–**6** were evaluated as previously described (Tables 2–4).[15] For evaluating lipophilicity of peptides, the logarithm of distribution coefficient between 1-octanol and saline (log $D_{7.4}$) was measured (Table 1).[32]

In evaluating the test peptides' antagonist activity at the NK1 receptor, radioligand analysis indicated that these peptides showed moderate selectivity between the NK1 receptors from rat and human. A number of NK1 antagonists have been previously reported to exhibit selectivity among NK1 receptors from different species. For example, CP-96,345 and RP-67,580, both of which are well-defined NK1 antagonists, showed significant selectivity between the human and rat NK1 receptor, and their affinity at the guinea pig NK1 receptor was similar to that for the human NK1 receptor. The selectivity of these compounds among species homologues has been proposed to be due to structural differences identified in the primary sequences of the receptors. The rat NK1 receptor is known to have 95% homology with the human NK1. Twenty-one out of 407 amino acid residues differ between the two receptor homologues, including 6 amino acid residues in the transmembrane domains, whereas the guinea pig NK1 shares 97% homology with the human NK1 homologue with 100% identical transmembrane domains. These structural differences, especially in the critical transmembrane segments, have been proposed to account for differences in the inhibition constants of NK-1 antagonists for the rat NK1 when compared with that for human NK1 or guinea pig NK1[4,33–36] Consistent with the earlier observations, L-732,138 (Ac-Trp-3,5-O-Bzl(CF_3)$_2$), which was a part of substance P antagonist pharmacophore of **1** and shared the same binding pocket with CP-96,345 and RP-67,580, showed high affinity at the human NK1 receptor when compared with that for the rat NK1 receptor (Table 2).[37] As shown in Table 2, peptide **1,** with a 3′,5′-bis(trifluoromethyl)-benzyl ester at the C-terminal, had good binding affinity for both human and rat NK1 receptors and exhibited functional antagonism against substance P stimulation in guinea pig ileum (Ke = 3.6 nM, GPI). This peptide had a longer peptide sequence than L-732,138. Compared with L732,138, peptide **1** showed much less selectivity between the human and rat NK1 receptor homologues (35-fold selectivity for the human NK1 receptor vs 200-fold selectivity in the case of L732,138). Peptide **1** also had moderate binding affinities at the DOR and MOR (K_i = 50 and 180 nM, respectively). In the MVD, GPI, and [^{35}S]GTPγS binding assays, the opioid agonist activities of **1** were consistent with the binding assays. As expected, **1** showed a lipophilic character with its log $D_{7.4}$ value being above the detectable limit (greater than 4.0). Interestingly, the removal of the two trifluoromethyl groups of **1** (**2**) resulted in increasing opioid affinities especially at the MOR (K_i = 31 nM, DOR; 29 nM, MOR) (Table 2). In the functional tissue assays, both the activities in the MVD and GPI assays also were increased from those of **1** (IC_{50} = 40 nM, MVD; 74 nM, GPI). However, the removal of trifluoromethyl groups at the C-terminal ester resulted in reduced antagonist activities for the NK1 receptors, indicating a much more critical role for the trifluoromethyl groups for the substance P antagonist pharmacophore. For the derivative without trifluoromethyl groups (**2**), the affinities at the NK1 receptors were much decreased from those of **1**, and these results were consistent with the Ke value in the GPI assay (K_i = 270 nM, rNK1; K_i = 100 nM, hNK1; K_e = 150 nM, GPI).

In contrast to the large decreased activities of the C-terminal benzyl ester derivative without trifluoromethyl groups (**2**) for the substance P antagonist activity in the GPI assay, the K_e value of its benzyl amide derivative (**3**) was 26 nM, which was 6 and 10 times more active than those of **2** and L-732,138, respectively (Table 4). Because **3** had only low affinity for the rNK1 receptor (30% inhibition at 1.0 μM), the species difference between the guinea pig and the rat should be enhanced from that of **2** (more than 40-fold). As expected from the result of the GPI assay, **3** showed a good affinity at hNK1 (K_i = 14 nM). Surprisingly, the opioid activities of **3** showed large increases from those of both **1** and **2**, especially at the MOR. Its affinity at the DOR was 10 nM, and subnanomolar-level affinity was found at the MOR (K_i = 0.65 nM), which were consistent with the results in the [^{35}S]GTPγS binding (EC_{50} = 17 nM, DOR; 0.71 nM, MOR) and tissue assays (IC_{50} = 50 nM, MVD; 13 nM, GPI) (Tables 3 and 4). These results indicated that **3** is expected to work as a potent bifunctional peptide with μ-preferable opioid agonist and substance P antagonist activities in humans, but not in rats. However, a simple N-methylation in the C-terminal amide of **3** (**4**) decreased affinities at both the opioid receptors (K_i = 77 nM, DOR; 46 nM, MOR) from those of **3**, and this biological trend was maintained in the [^{35}S]GTPγS binding assay. Contrary to the results from both the radioligands and the [^{35}S]GTPγS binding assays, **4** showed good potencies in the MVD and GPI assays (IC_{50} = 41 nM, MVD; 9.0 nM, GPI) (Table 4). The functional activity of **4** as a substance P antagonist was more than two times lower than **3** (K_e = 59 nM, GPI), although its K_i value at hNK1 was improved (1.6 nM). These biological results of the compounds without trifluoromethyl groups (**2**-**4**) proved that the C-terminus may act as an address region for opioid activities, although it is structurally apart from the opioid pharmacophore. In fact, simple ester-to-amide modifications at the C-terminus changed the opioid selectivity into μ-preference. The log $D_{7.4}$ values of the peptides without trifluoromethyl groups (**2**-**4**) showed moderately lipophilic values (3.8, **2**; 3.3, **3**; 3.5, **4**), which were decreased from that of **1** (Table 1).

Because the introduction of amide at the C-terminus was successful in the benzyl amide derivative **3**, we next prepared the 3′,5′-bis(trifluoromethyl)-benzyl amide derivative (**5**). It also showed a μ-selective binding affinities with nanomolar-level affinity at the MOR and a moderate affinity at the DOR (K_i = 72 nM, DOR; 9.5 nM, MOR) (Table 2). However, in the tissue assays, the IC_{50} value in the MVD assay (45 nM) was better than that in the GPI assay (350 nM). Therefore, **5** could strongly bind at the MOR, but its functional activity to regulate a μ opioid signal in the tissue was apparently decreased. As for its substance P antagonist activity, ligand **5** had decreased affinities at both the hNK1 and rNK1 receptors from those of the C-terminal ester **1** (K_i = 0.61 and 33 nM, respectively) (Table 2). In the GPI assay, the substitution of a benzyl ester to amide with trifluoromethyl groups showed less influence on substance P antagonist activity compared to the results of **2** and **3** (K_e = 3.6 nM for **1** and 8.5 nM for **5**) (Table 4). Finally, the N-methylation in the C-terminal amide (**6**) was also tested. Relatively small differences (less than 2.5-fold) were found in the binding affinities at rMOR, hDOR, and hNK1 receptors from those of **5**. For opioids, **6** showed moderate binding at the DOR and good affinity for the MOR (K_i = 31 nM, DOR; 6.8 nM, MOR), and this trend was maintained in the IC_{50} values in MVD and GPI assays (150 and 52 nM, respectively), although its functional activities were relatively low in the [^{35}S]GTPγS binding (EC_{50} = 120 nM, DOR; 72 nM, MOR). The binding affinity of **6** at the hNK1 receptor was two times less than that of **5** (K_i = 1.4 nM), but its K_e value in the GPI assay with substance P stimulation was 6.9 nM, which was almost equipotent to **5** (Tables 2 and 4). Consequently, **6** was also found as a potent bifunctional peptide derivative with opioid agonist

and substance P antagonist activities that are expected to work in both human and rat.

Conclusions

A series of linear peptides was designed based on the concept of overlapping pharmacophores with direct connection of the enkephalin-derived pharmacophore for opioid agonist (Tyr-D-Ala-Gly-Phe) and the pharmacophore for substance P antagonist (Phe-Pro-Leu-Trp-O-3,5-Bzl(CF_3)$_2$). The modifications in this study focused on the C-terminus of the peptides, and our two-step combinatorial approach was applied to the synthesis with good yields and with no detectable racemization. These modifications at the C-terminus, which are a part of the pharmacophore for substance P antagonist, resulted in different affinities as well as bioactivities not only for the NK1 receptors, but also for the opioid receptors. Among the obtained peptides, the benzyl amide derivative **3** (H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NH-Bzl) exhibited potent and μ-preferable opioid agonist activities together with good substance P antagonist activity in the GPI assay. Although **3** showed low affinity for the rNK1 receptor, which implies that **3** has limited substance P antagonist activity in rat, **3** is expected to be a potent and novel type of analgesic in humans due to its bifunctional activities. The N-methyl benzyl amide derivative with trifluoromethyl groups in the C-terminus (**6**) showed efficient potency in both opioid agonist and substance P antagonist activities together with the nanomolar affinities at both rNK1 and hNK1 receptors, indicating the importance of trifluoromethyl groups especially for the rNK1 affinity. It should be noted that the C-terminus of peptide derivatives acted as not only a critical pharmacophore for the substance P antagonist, but also as an address region for the opioid agonist pharmacophore, and its modification could lead to potent μ opioid selective bifunctional compounds from the δ-selective enkephalin-based pharmacophore.

Experimental Section

Materials. All amino acid derivatives and HCTU were purchased from EMD Biosciences (Madison, Wisconsin), Bachem (Torrance, California), SynPep (Dublin, California) and Chem Impex International (Wood Dale, Illinois). 2-Chlorotrityl resin was acquired from Iris Biotech GmbH (Marktredwitz, Germany). ACS grade organic solvents were purchased from VWR Scientific (West Chester, Pennsylvania), and other reagents were obtained from Sigma-Aldrich (St. Louis, Missouri) and were used as obtained. The polypropylene reaction vessels (syringes with frits) were purchased from Torviq (Niles, Michigan). Myo-[2-^{3}H(N)]-inositol; [tyrosyl-3,5-^{3}H(N)]-D-Ala^2-Mephe^4-glyol^5-enkephalin (DAMGO); [tyrosyl-2,6-^{3}H(N)]-(2-D-penicillamine, 5-D-penicillamine) enkephalin (DPDPE); [^{3}H]-substance P; and [^{35}S]-guanosine-5′-(γ-thio) triphosphate were purchased from Perkin-Elmer (Wellesley, Massachusetts). Bovine serum albumin (BSA), protease inhibitors, Tris, and other buffer reagents were obtained from Sigma (St. Louis, Missouri). Culture medium (MEM, DMEM, and IMDM), penicillin/streptomycin, and fetal calf serum (FCS) were purchased from Invitrogen (Carlsbad, California).

Boc-Tyr(*t*Bu)-D-Ala-Gly-Phe-Pro-Leu-Trp(Boc)-OH (7). The peptide was synthesized manually by the N$^{\alpha}$-Fmoc solid-phase methodology using HCTU as the coupling reagents. 2-Chlorotrityl resin (2.0 g, 1.56 mmol/g) was placed into a 50 mL polypropylene syringe with the frit on the bottom and swollen in DMF (20 mL) for 1 h. The resin was washed with DMF (3 × 15 mL) and then with DCM (3 × 15 mL). Fmoc-Trp(Boc)-OH (1.2 equiv) was dissolved in 30 mL of DCM, and then DIEA (5 equiv) was added. The reaction mixture was transferred into the syringe with the resin and then shaken for 2 h. The resin was washed three times with DMF (15 mL), three times with DCM (15 mL), and then with DMF (3 × 15 mL). The Fmoc protecting group was removed by 20% piperidine in DMF (1 × 2 min and 1 × 20 min). The deprotected resin was washed with DMF (3 × 15 mL) DCM (3 × 15 mL) and then with DMF (3 × 15 mL). Fmoc-Leu-OH (3 equiv) and HCTU (2.9 equiv) were dissolved in 30 mL of DMF, then DIEA (6 equiv) was added. The coupling mixture was transferred into the syringe with the resin, and then the mixture was shaken for 2 h. All the other amino acids, Pro, Phe, Gly, D-Ala, and Tyr were consecutively coupled using the procedure described above, using the TNBS test (all amino acids except for Phe) or chloranil test (only for Phe) to check the extent of coupling. In case of a positive test result, the coupling was repeated until a negative test result was obtained. The resulting batch of the resin-bound protected Boc-Tyr(*t*Bu)-D-Ala-Gly-Phe-Pro-Leu-Trp(Boc) was carefully washed with DMF (3 × 15 mL), DCM (3 × 15 mL), DMF (3 × 15 mL), and DCM (3 × 15 mL) and then dried under vacuum. The dry resin was placed in 10 mL fritted polypropylene syringes and was swollen with DCM for 1 h. The peptide was cleaved off the solid support with 1% v/v TFA in DCM (30 mL) for 30 min, and most of the organic solvent was removed under vacuum. The obtained crude peptide was precipitated out by the addition of chilled petroleum ether (45 mL) to give a white precipitate. The suspension was centrifuged for 20 min at 7000 rpm, and then the liquid was decanted off. The crude peptide was washed with petroleum ether (2 × 50 mL), and after the final centrifugation, the intermediate peptide was dried under vacuum (2 h) to obtain the title compound (1.99 g, 57.0%). The purity of the final products (99.8%) was checked by analytical RP-HPLC using a Hewlett-Packard 1090m system (230 nm) on a reverse phase column (Vydac 218TP104, C-18, 4.6 × 75 mm, 5 μm). The peptide was eluted with a linear gradient of aqueous CH_3CN/0.1% CF_3CO_2H (30–100% in 40 min) at a flow rate of 1.0 mL/min. The crude peptide was used for subsequent reactions without further purification. MS (ESI) *m/z* 1109 $(MH)^+$.

H-Tyr-D-Ala-Gly Phe-Pro-Leu-Trp-O-3,5-Bzl(CF_3)$_2$·TFA (1). Boc-Tyr(tBu)-D-Ala-Gly-Phe-Pro-Leu-Trp(Boc)-OH (150 mg, 0.135 mmol) and 3,5-bis(trifluoromethyl)benzyl bromide (104 mg, 0.338 mmol) were dissolved in DMF (2 mL). Cesium carbonate (220 mg, 0.676mmol) was added to the solution at 0 °C. After being stirred for 2 h at room temperature (rt), saturated aqueous sodium bicarbonate (50 mL) was added to the solution, and the product was extracted with ethyl acetate (30 mL) three times. The combined organic phases were washed with 5% aqueous citrate and saturated aqueous sodium chloride (50 mL each) and were dried over sodium sulfate. The solvent was evaporated, and the protected peptide was precipitated in cold petroleum ether (PE, 45 mL). The PE suspension was centrifuged for 20 min at 7000 rpm, and then the liquid was decanted off. The crude peptide was washed with petroleum ether (2 × 50 mL), and after the final centrifugation, the intermediate peptide was dried under vacuum. The obtained protected peptide was treated with 82.5% v/v TFA, 5% water, 5% thioanisole, 2.5% 1,2-ethanedithiol, and 5% phenol (1.5 mL, 1 h). The crude peptide was precipitated out by the addition of chilled diethyl ether (45 mL) to give white precipitates. The suspension was centrifuged for 20 min at 7000 rpm, and then the liquid was decanted. The crude peptides were washed with diethyl ether (2 × 45 mL), and after the final centrifugation, the peptides were dried under vacuum (2 h). The resulting white residues (116 mg, quantitative) were dissolved in a 3:1 mixture of acetonitrile and distilled water (5 mL), and the insoluble impurities were removed by passing the solutions through syringe filters (Gelman Laboratory, Ann Arbor, Michigan, Acrodisc 13 mm syringe filter with 0.45 μM PTFE membrane). Final purification was accomplished by preparative RP-HPLC, followed by lyophilization.

H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-O-Bzl·TFA (2). The title peptide was prepared using the same method as described for H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-O-3,5-Bzl(CF_3)$_2$·TFA (**1**). The yield of obtained crude peptide was 98.3%.

H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NH-Bzl·TFA (3). Boc-Tyr(*t*Bu)-D-Ala-Gly-Phe-Pro-Leu-Trp(Boc)-OH (50 mg, 0.045 mmol) and Cl-HOBt (8.7 mg, 0.054 mmol) were dissolved in DMF (1 mL). Benzyl amine (5.8 mg, 0.090 mmol) and EDC (10.4 mg, 0.054 mmol) were added to the solution at room temperature and were

stirred until the starting material disappeared in TLC; then, saturated aqueous sodium bicarbonate (50 mL) was added to the mixture. The reaction mixture was extracted three times with ethyl acetate (30 mL). The combined organic phases were washed with 5% aqueous citrate and saturated aqueous sodium chloride (50 mL each) and then dried over sodium sulfate. The solvent was evaporated, and the crude peptide was precipitated in cold petroleum ether (45 mL). The product was twice dispersed in cold petroleum ether, centrifuged, and decanted, and then dried under reduced pressure. The obtained protected peptide was treated with 82.5% v/v TFA, 5% water, 5% thioanisole, 2.5% 1,2-ethanedithiol, and 5% phenol (1.5 mL, 1 h). The crude peptide was precipitated out by the addition of chilled diethyl ether (45 mL) to give a white precipitate. The resulting peptide suspensions were centrifuged for 20 min at 7000 rpm, and the liquid was decanted. The crude peptides were washed with diethyl ether (2 × 45 mL), and after a final centrifugation, the peptides were dried under vacuum (2 h). The resulting white residues (53 mg, quantitative) were dissolved in a 3:1 mixture of acetonitrile and distilled water (1 mL), and the insoluble impurities were removed by passing the solutions through syringe filters (Gelman Laboratory, Acrodisc 13 mm syringe filter with 0.45 μM PTFE membrane). Final purification was accomplished by preparative RP-HPLC. The pure title compound was obtained after lyophilization.

H-Tyr-*D*-Ala-Gly-Phe-Pro-Leu-Trp-NMe-Bzl·TFA (4). The title peptide was prepared using same method as described for H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NH-3,5-Bzl·TFA (**2**). The crude peptide was obtained quantitatively.

H-Tyr-*D*-Ala-Gly-Phe-Pro-Leu-Trp-NH-3,5-Bzl(CF_3)$_2$·TFA (5). The title peptide was prepared using same method as described for H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NH-3,5-Bzl·TFA (**2**). The yield of obtained crude peptides was 98.1%.

H-Tyr-*D*-Ala-Gly-Phe-Pro-Leu-Trp-NMe-3,5-Bzl(CF_3)$_2$·TFA (6). The title peptide was prepared using same method as described for H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NH-3,5-Bzl·TFA (**2**). The crude peptide was obtained quantitatively.

Characterization of Peptides. Preparative RP-HPLC was performed on Waters Delta Prep 4000 with a Waters XTerra C-18 column (19 × 250 mm, 10 μm, a linear gradient of 33–53% or 40–60% acedtonitrile/0.1% TFA at a flow rate of 15.0 mL/min). The purified peptides were characterized by HRMS, TLC, analytical HPLC, and ^{1}H-1D-NMR. Sequential assignment of proton resonances was achieved by 2D-TOCSY NMR experiments.[38–42] High-resolution MS were taken in the positive ion mode using ESI methods at the University of Arizona Mass Spectrometry Facility. TLC was performed on aluminum sheets coated with a 0.2 mm layer of silica gel 60 F_{254} Merck using the following solvent systems: (1) $CHCl_3$/MeOH/AcOH = 90:10:3; (2) EtOAc/*n*-BuOH/water/AcOH = 5:3:1:1; and (3) *n*-BuOH/water/AcOH = 4:1:1. TLC chromatograms were visualized by UV light and by ninhydrin spray followed by heating (hot plate). Analytical HPLC was performed on a Hewlett-Packard 1100 or Hewlett-Packard 1090m with a Waters NOVA-Pak C-18 column (3.9 × 150 mm, 5 μm, 60Å) or a Vydac 218TP104 C-18 column (4.6 × 250 mm, 10 μm, 300 Å). ^{1}H-1D-NMR spectra were recorded on Bruker DRX-500 or DRX-600 spectrometer. 2D-TOCSY NMR spectra were performed on a Bruker DRX-600 spectrometer equipped with 5 mm Nalorac triple-resonance single-axis gradient probe. The NMR experiments were conducted in DMSO-d_6 solution at 298 K. Spectra were referenced to residual solvent protons as 2.49 ppm. The processing of NMR data was performed with the XwinNmr software (Bruker BioSpin, Fremont, California) and the Felix2000 package (Accelrys Inc., San Diego, California). In TOCSY experiments, the TPPI mode[43] with MLEV-17 Mixing Sequence[44] was used with a mixing time of 62.2 ms, at a spin-lock field of 8.33 kHz. TOCSY spectra were acquired with 2k complex pairs in t_2 and 750 FIDs using a 90°-shifted sine-squared window function in both dimensions.

Octanol/Saline Distribution (log $D_{7.4}$). HEPES buffer (0.05 M HEPES buffer in 0.1 M NaCl, pH 7.4, 500 μL) was added to 2 mg of peptide and was mixed with 500 μL of 1-octanol. The sample was shaken at rt for 12 h to allow equilibrating. The sample was centrifuged at 6500 rpm in a VanGuard V6500 (GlaxoSmithKline, Research Triangle Park, North Carolina) for 15 min. The layers were separated, and each layer was once again centrifuged. The peptide concentrations of the obtained layers were analyzed by HPLC (30–70% of acetonitrile containing 0.1% TFA within 20 min and up to 95% within additional 5 min, 1 mL/min, 230 nm, Vydac 218TP104 C-18 column). The logarithm of 1-octanol/saline distribution (log $D_{7.4}$) was calculated as the ratio of peptide concentration in the 1-octanol and saline phases.

Cell Lines.[15] For opioid receptors, the cDNA for the human δ opioid receptor (DOR) was a gift from Dr. Brigitte Kieffer (IGBMC, Illkirch, France). The cDNA for the rat μ opioid receptor (MOR) was a gift from Dr. Huda Akil (University of Michigan, Michigan). Stable expression of the rat MOR (pCDNA3) and the human DOR (pCDNA3) in the neuroblastoma cell line, HN9.10 were achieved with the respective cDNAs by calcium phosphate precipitation followed by clonal selection in neomycin. Expression of the respective receptors was initially verified, and the level of expression was periodically monitored by radioligand saturation analysis (see below). All cells were maintained at 37 °C, with a 95% air/5% CO_2 humidified atmosphere in a Forma Scientific incubator in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 100 U mL penicillin/100 μg mL streptomycin.

For the NK1 receptor, the hNK1/CHO and rNK1/CHO cell lines were obtained from Dr. James Krause (University of Washington Medical School, St. Louis, Missouri). Expression of the receptor was verified as previously described by Krause et al.[45] All cells were maintained at a 37 °C, 95% air and 5% CO_2, humidified atmosphere, in a Forma Scientific incubator in Ham's F12 with 2.5 mM HEPES, 10% fetal bovine serum, and 100 U mL penicillin/100 μg mL streptomycin/ 500 μg mL Geneticin.

Radioligand Labeled Binding Assays. For the opioid receptors, crude membranes were prepared as previously described[46] from the transfected cells that express either the rat MOR (rMOR) or the human DOR (hDOR). The protein concentration of the membrane preparations was determined by the Lowry method, and the membranes were stored at −80 °C until use. Membranes were resuspended in assay buffer (50 mM Tris, pH 7.4, containing 50 μg/mL bacitracin, 30 μM bestatin, 10 μM captopril, 100 μM phenylmethylsulfonylfluoride (PMSF), and 1 mg/mL BSA). The dissociation constant (K_d) of tritiated [D-Ala^2, NMePhe^4, Gly^5-ol]-enkephalin ([^{3}H]DAMGO) at the rMOR and that of tritiated c[D-Pen^2, D-Pen^5]-enkephalin ([^{3}H]DPDPE) at the hDOR were as previously described.[15] For competition analysis, 10 concentrations of a test compound were each incubated, in duplicates, with 50 μg of membranes from MOR or DOR expressing cells and the K_d concentration of [^{3}H]DAMGO (1.0 nM, 50 Ci/mmol), or of [^{3}H]DPDPE (1.0 nM, 44 Ci/mmol), respectively. Naloxone at 10 μM was used to define the nonspecific binding of the radioligands in all assays. All assays were carried out in duplicates and were repeated. The samples were incubated in a shaking water bath at 25 °C for 3 h, followed by rapid filtration through Whatman grade GF/B filter paper (Gaithersburg, MD) presoaked in 1% polyethyleneimine, washed 4 times each with 2 mL of cold saline, and then radioactivity determined by liquid scintillation counting (Beckman LS5000 TD).

For the NK1 receptor, competition binding assays for the human and rat NK1 receptors were carried out on crude membranes prepared from transfected CHO cells expressing the corresponding NK1 receptor. Ten concentrations of a test compound were each incubated, in duplicates, with 50–100 μg of membrane homogenate and 0.3−0.4 nM [^{3}H] substance P (135 Ci/mmol, Perkin-Elmer) in 1 mL final volume of assay buffer (50 mM Tris, pH 7.4, containing 5 mM $MgCl_2$, 50 μg/mL bacitracin, 30 μM bestatin, 10 μM captopril, and 100 μM phenylmethylsulfonylfluoride (PMSF)) at 25 °C for 20 min. Substance P at 10 μM was used to define the nonspecific binding. Membrane concentrations used in the assay were within the tissue linearity range. The [^{3}H] substance P concentration was selected based on the saturation binding experiments that showed a high affinity binding with $K_d = 0.40 \pm 0.17$ nM or $K_d = 0.16 \pm 0.03$ nM for the hNK1 and rNK1 receptors,

respectively. The incubation times correspond to the binding equilibrium as determined from the kinetics experiments. The reaction was terminated by rapid filtration and was washed as described above. The filter-bound radioactivity was measured by liquid scintillation counting (Beckman LS 6000SC). Log IC_{50} values for each test compound were determined from nonlinear regression analysis of data collected from two independent experiments performed in duplicates (40 independent experimental values) using GraphPad Prizm 4 software (GraphPad, San Diego, California). The inhibition constant (K_i) was calculated from the antilogarithmic IC_{50} value by the Cheng and Prusoff equation.

[^{35}S]GTPγS Binding Assay. The method was carried out as previously described.[15,46] Membrane from transfected rMOR or hDOR cells (10 μg) in a final volume of 1 mL reaction mix (50 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 30 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 0.1 mM PMSF, 0.1% BSA, and 0.1 nM [^{35}S]GTPγS) was incubated with various concentrations of the test drug ranging from 1×10^{-12} to 1×10^{-5} M, in duplicates, for 90 min at 30 °C in a shaking water bath. Reactions were terminated by rapid filtration through Whatman GF/B filters (presoaked in water), followed by four washes with 4 mL of ice-cold wash buffer (50 mM Tris, 5 mM $MgCl_2$, 100 mM NaCl, and pH 7.4). The radioactivity was determined by liquid scintillation counting as above. Basal level of [^{35}S]GTPγS binding was defined as the amount bound in the absence of any test drug. Nonspecific binding was determined in the presence of 10 μM unlabeled GTPγS. Total binding was defined as the amount of radioactivity bound in the presence of test drug. The effect of the drug at each concentration on [^{35}S]GTPγS binding was calculated as a percentage by the following equation: [Total bound − Basal]/[Basal − Nonspecific] × 100. Data were analyzed by nonlinear least-squares regression analysis using GraphPad Prism4 and using data collected from two independent experiments in duplicates. The EC_{50} for a test drug is converted from the logarithmic EC_{50} value derived from the best fit curve, and the maximum stimulatory effect is expressed as E_{max} ± standard error.

For both radioligand and [^{35}S]GTPγS binding, we adopted two independent experiments as our standard procedure for the initial evaluation of all compounds using cell lines because of the large number of compounds we routinely screen on multiple receptor types to determine both affinity and biological activity. These initial analyses were designed to identify trends of structure–activity relationship as rapidly as possible and were not intended for determining geometric mean values of K_i or EC_{50}. The data pooled from two independent experiments indicated that the data are reproducible.

Guinea Pig Isolated Ileum Assay. The in vitro tissue bioassays were performed as previously described.[15] Male Hartley guinea pigs under ether anesthesia were sacrificed by decapitation, a nonterminal portion of the ileum was removed, and the longitudinal muscle with myenteric plexus (LMMP) was carefully separated from the circular muscle as previously described.[47] These tissues were tied to gold chains with suture silk, mounted between platinum wire electrodes in 20 mL organ baths at a tension of 1 g, bathed in oxygenated (95:5 O_2:CO_2) Kreb's bicarbonate buffer at 37 °C, stimulated electrically (0.1 Hz, 0.4 msec duration) at supramaximal voltage. Following an equilibration period, compounds were added cumulatively to the bath in volumes of 14–60 μL until maximum inhibition was reached. A baseline for PL-017 was constructed to determine tissue integrity and to allow calculation of antagonist activity before opioid analogue testing began. If no agonist activity was observed at 1 μM, a repeat PL-017 dose–response curve was constructed to test for antagonist qualities.

All substance P antagonist compounds testing was performed in the presence of 1 μM naloxone to block opioid effects on the tissue. Two minutes after naloxone was added to the bath, the test compound was added. Four minutes after naloxone was added, the test dose of substance P was added to the bath, the peak height was noted, and the tissues were washed. Agonist activity of the analog was also observed during this period. Testing stopped at 1 mM concentrations of the test compound.

Mouse Isolated Vas Deferens (MVD) Assay. The in vitro tissue bioassay was performed as described previously.[15] Male ICR mice under ether anesthesia were sacrificed by cervical dislocation, and the vasa deferentia were removed. Tissues were tied to gold chains with suture silk, mounted between platinum wire electrodes in 20 mL of organ baths at a tension of 0.5 g, bathed in oxygenated (95:5 O_2:CO_2) magnesium free Kreb's buffer at 37 °C, and stimulated electrically (0.1 Hz, single pulses, 2.0 msec duration) at supramaximal voltage as previously described.[48] Following an equilibration period, compounds were added to the bath cumulatively in volumes of 14–60 μL until maximum inhibition was reached. Response to an IC_{50} dose of DPDPE (10 nM) was measured to determine tissue integrity before a compound testing.

Analysis of the GPI and MVD Assays. For opioid data analysis, percentage inhibition was calculated using the average tissue contraction height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure to the dose of agonist. IC_{50} values represent the mean of not less than four tissues. IC_{50} and E_{max} estimates were determined by computerized linear regression analysis over a typical drug concentration range of 100-fold (2 logarthmic dose range) (the pharmacological statistics package FlashCalc: Dr. Michael Ossipov, University of Arizona, Tucson, Arizona).

For substance P data analysis, the height of the maximum peak produced during the control substance P dose–response curve was used as a 100% response, and other values were calculated as a percentage. K_e values represent the mean of not less than four tissues. K_e estimates were determined by computerized linear regression analysis as above (FlashCalc).

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Supporting Information Available: ^{1}H NMR data and in vivo result presented in reference 16. This material is available free of charge via the Internet at http://pubs.acs.org.

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