

Biological and Conformational Evaluation of Bifunctional Compounds for Opioid Receptor Agonists and Neurokinin 1 Receptor Antagonists Possessing Two Penicillamines

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Neuropathic pain states and tolerance to opioids can result from system changes in the CNS, such as up-regulation of the NK1 receptor and substance P, lead to antioioid effects in ascending or descending pain-signaling pathways. Bifunctional compounds, possessing both the NK1 antagonist pharmacophore and the opioid agonist pharmacophore with δ -selectivity, could counteract these system changes to have significant analgesic efficacy without undesirable side effects. As a result of the introduction of cyclic and topological constraints with penicillamines, **2** (Tyr-*cyclo*[D-Pen-Gly-Phe-Pen]-Pro-Leu-Trp-NH-[3',5'-(CF₃)₂-Bzl]) was found as the best bifunctional compound with effective NK1 antagonist and potent opioid agonist activities, and 1400-fold δ -selectivity over the μ -receptor. The NMR structural analysis of **2** revealed that the relative positioning of the two connected pharmacophores as well as its cyclic and topological constraints might be responsible for its excellent bifunctional activities as well as its significant δ -opioid selectivity. Together with the observed high metabolic stability, **2** could be considered as a valuable research tool and possibly a promising candidate for a novel analgesic drug.

Introduction

The clinical treatment of pain, especially prolonged and neuropathic pain, is still a major challenge, and the efficacy of current analgesic drugs, such as opioids, is often reduced by undesired dose-limiting side effects including development of tolerance and physical dependence. The mechanisms for these effects are still largely unclear, but it is clear that prolonged pain states lead to neuroplastic changes in both ascending and descending pathways in the spinal column in which there is increased release of neurotransmitters (e.g., substance P) that enhance pain and increased expression of the corresponding

receptors for those newly released pain-causing ligands.^{1–3} Current treatment of prolonged pain generally can only modulate pain and cannot counteract these induced neuroplastic changes. Thus, it is not surprising that current analgesic drugs do not work well in these pathological conditions.

To effectively address this problem, we have taken a new approach to design bifunctional ligands that act as agonists at opioid receptors and as antagonists at NK1^a receptors to block the signals mediated by substance P, which is one of these newly expressed pain-causing neurotransmitters.^{4–7} Our developing ligands have synergistic biological activities and overlapping pharmacophores (Figure 1) and would not be expected to possess the undesirable side effects of current opioids and other pain-killing drugs such as the development of tolerance. Indeed, previous reports have indicated that substance P plays a role as a mediator of signals induced by opioid stimulation.^{1–3} Combination of the agonist effects at opioid receptors together with the blocking of signals through NK1 receptors has led to enhanced antinociceptive effects in acute pain animal models and has prevented the opioid-induced tolerance in chronic trials.^{8–12} NK1 knockout mice did not show the rewarding properties of morphine.¹³ We also emphasize that designing these bifunctional compounds with multiple biological activities in a single ligand has additional advantages over a cocktail of individual drugs including easy administration, single biodistribution, no drug–drug interactions, simple pharmacokinetic profiles, and higher local concentration in the synaptic cleft, which may lead to significant synergies in potency and efficacy.¹⁴ However, obtaining a bifunctional ligand with desired bioactivity is a difficult

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^aAbbreviations: (abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977–983); Boc, *tert*-butyloxycarbonyl; Cl-HOBt, 1-hydroxy-6-chlorobenzotriazole; CHO, Chinese hamster ovary; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; DPC, dodecylphosphocholine; DPDPE, Tyr-*cyclo*[D-Pen-Gly-Phe-D-Pen]-OH; DPLPE, Tyr-*cyclo*[D-Pen-Gly-Phe-Pen]-OH; DQF-COSY, double quantum filtered correlation spectroscopy; DAMGO, [D-Ala², NMePhe⁴, Gly⁵-ol]-enkephalin; Fmoc, fluorenylmethoxycarbonyl; GPI, guinea pig isolated ileum; HBTU, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate; HCTU, 1*H*-benzotriazolium-1-[bis(dimethylamino)methylene]-5-chloro-hexafluorophosphate-(1-),3-oxide; HRMS, high-resolution mass spectroscopy; LMMP, longitudinal muscle with myenteric plexus; Mob, *p*-methoxy benzyl; MVD, mouse vas deferens; NK1, neurokinin-1; NOESY, nuclear Overhauser enhancement; rmsd, root-mean-square deviation; NMM, *N*-methyl morpholine; RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TOCSY, total correlation spectroscopy; Trp-NH-[3',5'-(CF₃)₂-Bzl], the 3',5'-(bistrifluoromethyl)-benzyl amide of tryptophan.

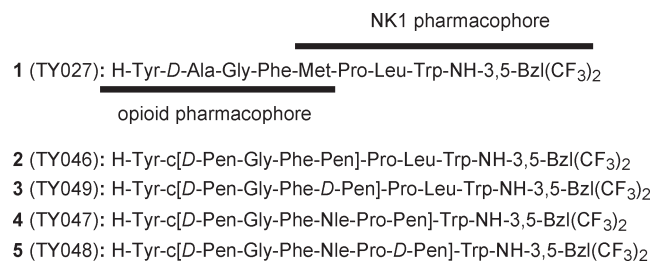


Figure 1. Sequences of opioid and NK1 receptor peptides.

challenge because connected pharmacophores interfere with each other to change or even to reduce their affinities at the corresponding receptors.

In this report, we have focused on the δ/μ activity of targeted opioid receptors for the bifunctional compounds. The μ -opioids have been well-established to show strong analgesic potency with several disadvantages such as constipation, respiratory depression, dysphoria, opioid-induced tolerance development, and addiction.^{15,16} Selective δ -opioid agonists have several potential clinical advantages over their μ -counterparts, including reduced respiratory depression¹⁷ and constipation¹⁸ as well as a decreased potential for the development of physical dependence.^{19–21} However, selective δ -opioid agonists do not attenuate the same level of pain as μ -agonists and no δ -opioid selective agonist ligand has been found to have clinical efficacy sufficient to be a useful agent for the treatment of severe pain.²² Therefore, bifunctional ligands possessing selective δ -opioid agonist activity but also some μ -opioid agonist activity together with the NK1 antagonist activity could be a promising strategy to find next-generation analgesics which have powerful pain controlling effects without development of tolerance, physical dependence, and other toxicities.^{4,5} In fact, our lead bifunctional compounds, TY005 (Tyr¹-D-Ala²-Gly³-Phe⁴-Met⁵-Pro⁶-Leu⁷-Trp⁸-O-[3',5'-(CF₃)₂-Bzl]) and TY027 (1: Tyr¹-D-Ala²-Gly³-Phe⁴-Met⁵-Pro⁶-Leu⁷-Trp⁸-NH-[3',5'-(CF₃)₂-Bzl]) possessing moderately δ -selective (up to 25-fold) δ/μ opioid agonist and potent NK1 antagonist activities (Table 1) have shown the powerful attenuation of neuropathic pain without producing the opioid-induced tolerance to effectively prove our opioid agonist/NK1 antagonist bifunctional concept in the animal models of neuropathic pain.^{23,24}

To perform further biological optimization of the bifunctional ligands, we describe here the design, synthesis, structure–activity relationships (SAR), and further biological characterizations of bifunctional compounds which have significantly improved selectivity both for affinity and agonist activity for the δ -opioid over μ -opioid receptors, together with effective NK1 antagonist activity. In fact, we previously reported the finding of a selective δ -opioid activator, possessing two cysteine residues, in the conventional MVD and GPI assays, but its δ -opioid selectivity in terms of the binding affinity was relatively lower.²⁵ While our earlier efforts led to the discovery that introduction of two topologically constrained L- or D-penicillamine (Pen or D-Pen) residues into several cyclic pentapeptide enkephalin analogues produced compounds with enhanced δ -selectivity through the reduction of opioid affinity at the μ -opioid receptor: Tyr-*cyclo*[D-Pen-Gly-Phe-D-Pen] (DPDPE) and Tyr-*cyclo*[D-Pen-Gly-Phe-Pen] (DPLPE) are the most typical δ -selective opioid agonists (Tables 1 and 2).^{5,26–28} Previous conformation–activity relationship studies suggested that the 14-membered ring structure with

Table 1. Binding Affinity, GTP Binding Assay, and E_{\max} % for Bifunctional Peptide Derivative Ligands at Opioid and Neurokinin Receptors

no.	δ			μ			radioligand binding ^{a,b}			[³⁵ S]GTP γ S binding ^{c,c'}				
	IC ₅₀	K _i (nM)	IC ₅₀	K _i (nM)	IC ₅₀	K _i (nM)	hNK1	rNK1	δ	μ	EC ₅₀	E _{max} (%)	EC ₅₀	E _{max} (%)
1 ^d	-8.84 ± 0.07	0.66	-7.44 ± 0.05	16	-10.9 ± 0.10	0.0065	-7.61 ± 0.03	7.3	-8.07 ± 0.11	8.6	60 ± 2	-8.16 ± 0.17	7.0	51 ± 3
2	-8.44 ± 0.04	1.7	-5.36 ± 0.27	2300	-10.91 ± 0.03	0.0053	-7.38 ± 0.04	10	-8.50 ± 0.39	3.2	57 ± 5	-7.54 ± 0.41	29	14 ± 2
3	-7.14 ± 0.07	38	-5.38 ± 0.08	2100	-9.38 ± 0.03	0.18	-7.74 ± 0.03	4.5	-6.94 ± 0.32	120	49 ± 3	-6.87 ± 0.22	130	28 ± 2
4	-6.50 ± 0.14	150	-5.43 ± 0.14	2000	-6.87 ± 0.06	59	-6.18 ± 0.05	160	-6.27 ± 0.11	530	63 ± 2	-5.67 ± 0.46	2900	15 ± 3
5	-5.86 ± 0.04	720	-5.69 ± 0.06	1000	-8.37 ± 0.02	1.9	-6.98 ± 0.05	26	-6.91 ± 0.29	120	42 ± 3	-6.87 ± 0.36	140	26 ± 4
Tyr-D-Ala-Gly-Phe-Met-NH ₂	-8.86 ± 0.05	0.66	-8.90 ± 0.16	0.50										
DPDPE ^e		0.5 ± 0.1												
DPDPE ^f		1.6		610										
DPDPE ^g		10		3700										
DPLPE ^g		16		2800										
biphalin		2.6 ^h		1.4 ^h										
DAMGO ⁱ		0.85 ± 0.2							-8.95 ± 0.17	1.1	83 ± 4			
L-732,138													-7.44 ± 0.19	37.0
														150

^aThe experiments were run in at least two independent experiments performed in duplicate. ^bIC₅₀ values are expressed as logarithmic mean ± standard errors. K_i values were calculated from the antilogarithmic mean IC₅₀ values using the Cheng and Prusoff equation. ^cEC₅₀ values are expressed as both logarithmic mean ± standard errors and antilogarithmic value of the mean. E_{max} values were defined as [net [³⁵S]GTP γ S bound]/basal [³⁵S]GTP γ S bound] × 100%, expressed as mean ± standard error. ^dFrom ref.⁵ ^eDissociation constant (K_D, nM) of [³H]DPDPE at human δ opioid receptor expressed as mean ± SEM. ^fFrom ref.²⁷. ^gFrom ref.²⁸. ^hFrom ref.⁵⁶. ⁱK_D value (nM) of [³H]DAMGO at rat μ opioid receptor expressed as mean ± SEM.

Table 2. Functional Assay Results for Bifunctional Peptide Ligands at Opioid and Substance P Receptors

no.	opioid agonist		SP antagonist
	MVD (δ) IC ₅₀ (nM) ^a	GPI (μ) IC ₅₀ (nM) ^a	GPI K _e (nM) ^b
1 ^c	15 ± 2	490 ± 29	10 ± 2
2	9.6 ± 3.5	26% inh at 1 μ M ^d	6.9 ± 1.8
3	130 ± 25	0.6% inh at 1 μ M ^d	8.2 ± 4.2
4	250 ± 30	6% inh at 1 μ M ^d	72 ± 7.2
5	1100 ± 250	7% inh at 1 μ M ^d	66 ± 35
DPDPE ^e	2.5 ± 0.03	2720 ± 50	
DPLPE ^e	2.2 ± 0.30	6930 ± 120	
L-732,138			250 ± 87

^a Concentration at 50% inhibition of muscle concentration at electrically stimulated isolated tissues ($n = 4$). ^b Inhibitory activity against the substance P induced muscle contraction in the presence of 1 μ M naloxone ($n = 4$). K_e: concentration of antagonist needed to inhibit Substance P to half its activity. ^c Reference 5. ^d No antagonist activity was observed at the tested concentration. ^e Reference 28.

their geminal-dimethyl group at the second position has special importance to interfere with the binding at the μ -opioid receptor so as to greatly favor binding to the δ -opioid receptor.^{29,30} We have now applied this modification to our lead bifunctional octapeptide analogue **1** in which Tyr¹, Gly³, Phe⁴, and Trp⁸ were considered as the major “message” residues of each pharmacophore⁵ and Pro⁶ had a positive effect on affinity at the NK1 receptors.³¹ Thus, the residues 2, 5, and 7 in **1** were selected as the sites to be cyclized to yield four bifunctional peptide derivatives possessing a 14- or 20-membered ring structure with two Pen or D-Pen residues (**2–5**, Figure 1). Subsequent conformational analysis was performed using NMR experiments in the presence of membrane-mimicking perdeuterated dodecylphosphocholine (DPC) micelles because understanding membrane-bound structures of ligands and ligand–membrane interactions is indispensable for further evaluation of their diverse biological behaviors,⁵ and the elucidation of the conformational motif for δ -opioid selective binding affinities could provide important information in the discovery of novel drug candidates for next-generation neuropathic pain treatment.

Results and Discussion

Peptide Synthesis. The peptide derivatives **2–5** were synthesized using a convergent peptide synthesis strategy³² because the coupling of Tyr¹ was difficult using the normal sequential method. First, the N-terminal fragment, Boc-Tyr-(tBu)-D-Pen(Mob)-Gly-OH, was synthesized with N^α-Boc chemistry using a solution peptide synthesis strategy. The C-terminal fragments, H-Phe-Pen(Trt)-Pro-Leu-Trp(Boc), H-Phe-D-Pen(Trt)-Pro-Leu-Trp(Boc), H-Phe-Nle-Pro-Pen(Trt)-Trp(Boc), and H-Phe-Nle-Pro-D-Pen(Trt)-Trp(Boc) were prepared on 2-chlorotrityl resin using N^α-Fmoc chemistry with HCTU as a coupling reagent. These two fragments were coupled on the resin in the presence of an excess amount of EDC and HOAt, followed by cleavage using 1% TFA in DCM, to afford the complete sequence of the C-terminal-free linear peptide with its protecting groups. The introduction of 3',5'-bistrifluoromethyl benzyl amide at the C-terminus was conducted by standard EDC/Cl-HOBt coupling chemistry using 2 equiv of the amine with the protected C-terminal-free peptide. All of the acid-labile protecting groups were removed with the cleavage cocktail treatment (6:1 mixture of TFA and anisole to quench the highly stabilized carbocations released from the permanent protecting

groups) at 80 °C for 30 min to give the final linear crude peptides, which were directly cyclized using the oxidizing agent K₃Fe(CN)₆.²⁵ Oxidation was performed by slowly adding a solution of the peptide into a reaction vessel containing an excess of the oxidizing agent in aqueous solution. The concentration of the reduced peptide was regulated by controlling the speed of addition of the peptide with the help of an automated syringe pump.²⁵ The crude oxidized peptides were concentrated using a solid-phase extraction technique with a C-18 reversed-phase silica gel column, and then purified with RP-HPLC followed by lyophilization ($\geq 95\%$). The final purified peptides were characterized by analytical HPLC, ¹H NMR, HRMS, and TLC.

Biological Activities. The evaluation of the synthesized bifunctional ligands **2–5** was performed as previously described.^{4–7} Binding affinities were tested on cell membranes from cells that stably express the human δ -opioid, the rat μ -opioid, the human NK1, or the rat NK1 receptors (Table 1). The [³⁵S]GTP γ S binding assays were performed to characterize the functional activities for the δ and μ opioid agonists of **2–5** (Table 1). Tissue-based functional assays also were performed to evaluate δ/μ opioid agonist as well as the substance P antagonist activities in the guinea pig isolated ileum (GPI) and mouse vas deferens (MVD) (Table 2).

At the δ -opioid receptor, the cyclic peptide possessing a 20-membered ring with D-Pen² and Pen⁷ (**4**) had a K_i of 150 nM, which was 230-fold less binding affinity than that of **1** (Table 1). The δ -opioid affinity of its D-Pen⁷ derivative (**5**) also showed a decrease in affinity of 720 nM, indicating that introduction of D-Pen or Pen at the seventh residue in the sequence of **1** decreases affinity at the δ -opioid receptor. The K_i of the D-Pen²,D-Pen⁵ cyclic analogue **3** was 38 nM for the δ receptor, demonstrating that the cyclic peptide with a smaller 14-membered ring had enhanced affinity compared to the 20-membered-ring analogues. Interestingly, the cyclic D-Pen²,L-Pen⁵ analogue **2** had the best affinity among **2–5** for the δ -opioid receptor with K_i value of 1.7 nM. This is the closest to the K_D value of DPDPE (0.5 ± 0.1 nM), which was used as a radioactive ligand of 1 nM in the competition assay on the cells membrane expressing the δ -opioid receptors (Table 1). Thus, the affinities of **2–5** for the δ -opioid receptor were different depending on the ring-size and the chirality of bridgehead penicillamine residues, and the combination of D-Pen² and Pen⁵ clearly showed the best results. Interestingly, the affinity difference at the δ -opioid receptor between pentapeptides DPDPE and DPLPE was only 1.6-fold and DPDPE showed the better affinity,²⁸ but in the case of the bifunctional compounds with the longer peptide sequences, the compound **2** (DPLPE-derivative) showed better affinity than **3** (DPDPE-derivative), and their affinity difference was 22-fold. This result apparently indicates that the extended sequence at the C-terminus (Pro-Leu-Trp-NH-3,5-Bzl(CF₃)₂) plays a critical role for the δ -opioid selectivity, and **2** has a preferable three-dimensional conformational motif for binding at the δ -opioid receptor compared to **3**. For the linear pentapeptide Tyr-D-Ala-Gly-Phe-Met-NH₂, the C-terminal elongation to the octapeptide derivative **1** resulted in a decrease in μ -opioid binding affinity with no shift in the affinity at the δ -receptor (Table 1). Consistent with the trend in the affinities at the δ -opioid receptor, the EC₅₀ values of **3–5** were more than 100 nM in the GTP γ S binding assay (120, 530, and 120 nM for **3**, **4**, and **5**, respectively), while only **2** showed significantly better efficacy with a nanomolar level EC₅₀ value (EC₅₀ = 3.2 nM, Table 1). In accord with these

results, **3–5** had relatively lower inhibitory activities in the MVD assays ($IC_{50} = 130, 250,$ and 1100 nM for **3, 4,** and **5,** respectively), whereas **2** showed good activity ($IC_{50} = 9.6$ nM) which is improved from that of **1** (Table 2).

For the μ -opioid receptor, the cyclic peptides with a 14-membered ring (**2** and **3**) showed drastically reduced affinities ($K_i = 2300$ and 2100 nM for **2** and **3,** respectively; Table 1), as expected from the results of DPDPE and DPLPE. The compounds with the larger 20-membered ring (**4** and **5**) also showed reduced affinity at the μ -opioid receptor ($K_i = 2000$ and 1000 nM for **4** and **5,** respectively), confirming that the introduction of a disulfide ring possessing D-Pen² is detrimental to the affinities at the μ -opioid receptor, regardless of the ring size of the octapeptide derivatives. In the GTP γ S binding assays, the cyclic peptides **2–5** stimulate the μ -opioid receptor poorly with the E_{max} values less than 28% (Table 1). In agreement with their low K_i and E_{max} values, **2–5** showed very weak μ -opioid-related agonist activities in the GPI assays (26, 0.6, 6, and 7% inhibition at 1 μ M for **2, 3, 4,** and **5,** respectively; Table 2). Therefore, only **2** showed significant δ -opioid selectivity over the μ -receptor (1400-fold), which is 58 times more δ -selective than **1** (24-fold) and 3.7 and 7.8 times more selective compared to DPDPE (380-fold) and DPLPE (180-fold), respectively (Table 1). The δ -selectivity of **3** was 55-fold, which is better than that of **1**.

These results suggest that three important structural factors have an influence on the opioid affinity and bioactivity for these novel bifunctional compounds: (1) the 14-membered cyclic structure with D-Pen², (2) the chirality of the fifth residue, and (3) the existence of the C-terminal NK1 pharmacophore. Apparently, introduction of a 14-membered ring with a D-Pen² resulted in a limited shift in their δ -opioid binding affinities but a more significant reduction in affinities at the μ -receptor, leading to the enhanced δ -receptor selectivity compared to DPDPE and DPLPE. Addition of the C-terminal NK1 pharmacophore to the cyclic opioid sequence in **2** resulted in improved affinity at the δ -receptor, while the combination with the N-terminus in **3** possessing a D-Pen⁵ resulted in a reduction in the δ -opioid binding affinity. These results clearly indicate that the NK1 pharmacophore works as an address region for the opioid agonist activities, and an appropriate combination of two connected pharmacophores can yield improved affinities and selectivities of the ligand. This is an important synergistic effect of our bifunctional concept.

Biological evaluations were also conducted at the NK1 receptors. Analogue **4**, possessing a D-Pen² and Pen⁷, showed reduced affinities at both the hNK1 and rNK1 receptors compared to those of **1** ($K_i = 59$ and 160 nM, respectively; Table 1). The K_i value of peptide **5** with a D-Pen⁷ was 1.9 nM at the hNK1 receptor and 26 nM at the rNK1 receptor, indicating that the D-Pen⁷ provided better affinities than L-Pen⁷ introduction. These affinities of compounds with 20-membered rings were drastically decreased from those of **1**, and their antagonist activities on the GPI tissue were consistent with the observed affinities ($K_c = 72$ and 66 nM for **4** and **5,** respectively; Table 2). However, the introduction of a ring structure between the second and seventh residues resulted in a reduction of biological activities at the NK1 receptors, suggesting an unfavorable topographical structure for the NK1R. Analogue **3**, possessing a smaller 14-membered ring with the DPDPE sequence, has 28 times lower affinity for the hNK1 receptor than that of **1** but its K_i value at the rNK1 receptor was slightly improved from that

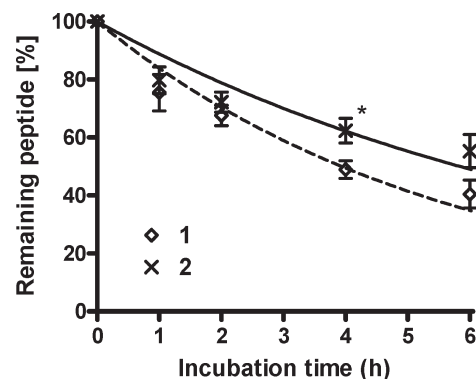


Figure 2. Comparison of the in vitro stability of peptide derivatives for **1** and **2** incubated in rat plasma at 37 °C (*, $p < 0.05$).

of **1** ($K_i = 0.18$ and 4.5 nM for hNK1 and rNK1 receptors, respectively). The antagonist activity against substance P stimulation of **3** in the GPI assay was nearly comparable to that of **1** ($K_c = 8.2$ nM). The DPLPE derivative **2** showed similar antagonist activity against substance P stimulation at the GPI, with a K_c value of 6.9 nM (Table 2). **2** also showed the best affinity at the hNK1 receptor among the synthesized cyclic peptide derivatives **2–5** with a picomolar K_i value ($K_i = 0.0053$ nM).

For further biological characterization of **2**, its metabolic stability was tested by incubation in rat plasma at 37 °C.⁶ Analogue **2** possesses significantly improved stability compared to the linear peptide derivative **1**. The calculated half-lives of the peptide derivatives ($T_{1/2}$) were 4.8 h for **1** and 5.8 h for **2** (Figure 2). Consequently, c[D-Pen², Pen⁵]TY027 (**2**) is a promising ligand with significant δ -selective opioid agonist activity, improved metabolic stability, and nearly equivalent antagonist activities for NK1 receptors compared to those of the lead compound **1**. The important conformational motif of the two pharmacophores for the bioactivities of **2** was next examined using NMR-based three-dimensional structural analysis.

Conformational Analysis of Cyclic Peptide **2** Based on Assigned ¹H NMR Resonances

The ligand binding sites for opioid and NK1 receptors have been found in their transmembrane domain,^{33,34} which is surrounded by the cell membrane, suggesting that the ligand binding event at these receptors occur near the membrane. The potential importance of membrane-bound conformations of ligands has increased recently.^{5,6} The transfer from an aqueous environment to a cell membrane environment is an important step in receptor–ligand binding and may be accompanied with a conformational change to a more biologically relevant form.^{17,38} Thus, NMR structural analysis of **2** was performed to clarify the relationship between its biological activity and its conformation in a membrane-mimicking environment.

The NMR experiments and analysis of **2** were conducted using previously reported procedures.^{5,6,35,36} The two-dimensional ¹H NMR studies included TOCSY, DQF-COSY, and NOESY and were performed in pH 4.5 buffer (45 mM CD₃CO₂Na/HCl, 1 mM NaN₃, 90% H₂O/10% D₂O) solutions in the presence of a 40-fold excess of DPC micelles, which is a commonly used membrane-like environment to determine the solution NMR structures of proteins and peptides.^{35–40} All ¹H chemical shift assignments in aqueous

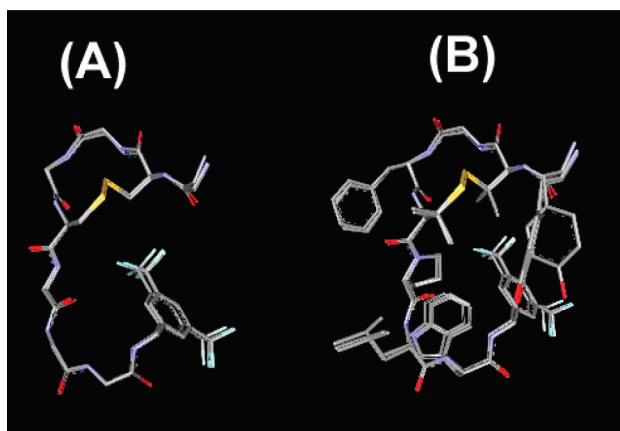


Figure 3. Ensembles of the best 10 calculated structures of **2** in 40-fold DPC micelle/pH 4.5 buffer with the lowest restraint energy, aligned on backbone atoms of residues 1–8. (A) Only backbone atoms in the aligned structures are illustrated with C-terminal benzyl moiety and disulfide bond. (B) All non-hydrogen atoms were displayed.

Table 3. Atomic rmsd Values (Å) for the Final Nine Conformers Compared to the Most Stable Conformer of Bifunctional Peptide Derivatives **2**

aligned residues	whole molecule	1–4 residues	5–8 residues and C-terminus
backbone atoms (N,C ^α ,C')	0.15 ± 0.06	0.02 ± 0.04	0.14 ± 0.05
all non-hydrogen atoms	0.52 ± 0.12	0.35 ± 0.21	0.58 ± 0.11

media and other NMR data are found in the Supporting Information.

Conformational Calculations

The total number of NOE restraints for structural calculation of **2** was 184, including 63 intraresidual NOEs, 67 sequential NOEs, 49 medium-range NOEs (2–4 residues), and 5 long-range NOEs (> 4 residues). For the $^3J_{\text{HN-H}\alpha}$ values, only those greater than 8 Hz or less than 6 Hz were used as φ dihedral angle constraints. The ^1H NMR spectra in **2** showed a negligible amount of the minor *cis/trans* rotamer at the Pro⁶ residue. For conformational calculations, the Pen⁵–Pro⁶ bond of the major rotamer was fixed in the *trans* configuration based on the observation of Pen⁵ H^α to Pro⁶ H^δ sequential NOEs, together with the absence of sequential Pen⁵ H^α–Pro⁶ H^α NOEs.

Analysis and statistics of the calculated conformation for **2** were performed on the 10 structures with the lowest total energies after restrained molecular dynamics (rMD) refinement. The number of total NOE restraint violations was 25 with a maximum NOE violation of 0.15 Å, and no φ dihedral angle violations were found. The restraints energy derived from the amber force field was only 3.9 kcal mol⁻¹ in the most stable 10 structures.

As shown in Figure 3A, the best 10 structures of **2** showed well-aligned superimposed conformations whose rmsd values were 0.15 ± 0.06 Å in an alignment with the backbone atoms (Table 3). Because these rmsd values were significantly improved from those of **1** (1.03 ± 0.57 Å for the best 10 structures; Supporting Information),⁵ the extensively structured backbone of **2** could be attributed to the cyclic and topological constraints induced by the disulfide bridge with the D-Pen² and Pen⁵ residues. It should be noted that not only

Table 4. Number of β -Turn Structural Elements and the Distance between α Carbons of *i*th and (*i* + 3)rd Residues^a

residues ^b	number of β -turns	distance (Å)
C ² α –C ⁵ α	10	5.36 ± 0.00
C ⁶ α –Bzl	10	6.43 ± 0.04

^a Out of the best 10 calculated structures. The distance is the mean distance between two α carbons ± standard deviation (SD). The sequences with less than 7 Å distance between α carbons of *i*th and (*i* + 3)rd residues without helical structure were considered as a β -turn.⁵⁷ Bzl stands for the benzyl moiety at the C-terminus. ^b Only residues possessing β -turn structural element were displayed.

the conformation of the N-terminal half, where the two penicillamines were introduced, but also the C-terminus, whose amino acid sequence is identical to **1**, showed reduced rmsd values as well. The rmsd values of **2** for the alignment with all non-hydrogen atoms were also significantly reduced (0.52 ± 0.12 Å, Table 3), indicating well-defined three-dimensional positions of the side-chains in **2** under the applied time scale in the NMR experimental (Figure 3B). Different from the observed well-defined side-chain conformation of **2**, rather poorly defined side-chains were previously observed for the linear compound **1**. Thus, the constraints in ligand **2** due to the incorporation of two penicillamines provided improved conformation stability for the backbone atoms as well as for the side-chain atoms, including the two aromatic amino acid residues Tyr¹ and Phe⁴ and the protonated N-terminal nitrogen, all of which have been reported as important pharmacophores in the binding of enkephalins to opioid receptors.⁴¹ The observed well-defined conformation of **2** suggests it has a structured conformation inside or close to the cell membrane, in close relationship with the improvement in activity and selectivity at the tested membrane-bound receptors.

As for the observed secondary structural elements, the NMR structure of **1** has two type IV β -turns at residues 2 to 5 and 6 to the C-terminus.⁵ Although the peptide sequence was different, the obtained NMR structure of ligand **2** had type IV β -turns at the same positions in all 10 of the best structures (Table 4), indicating that the introduction of constraints by D-Pen² and Pen⁵ made the two β -turns more structured without changing the secondary structural elements of ligand. DPDPE also has a β -turn at residues 2–5 in its X-ray crystal structure,⁴² in the solution NMR structure,⁴³ and in the docking study at the δ opioid receptor,⁴⁴ and thus, this turn has been hypothesized as a key structure to enhance the δ -selectivity of enkephalin analogues, through the lock-in of flexible pharmacophores at the appropriate positions (Figure 4A).

Although **1** and **2** had the same secondary structural elements, their NMR structures did not overlap completely on alignment with the backbone atoms of the whole molecule (rmsd = 2.34 Å, Figure 4B). However, the rmsd value was decreased to 1.41 Å when the alignment was made only for the N-terminal opioid pharmacophore where the common β -turn structural elements for **1** and **2** were observed (Figure 4C). It should be mentioned that when the alignment was made on the N-terminus of **1** and **2**, their corresponding C-terminal halves were directed differently and showed almost no overlap. As expected, when the alignment was made with the C-terminus (rmsd = 0.65 Å), the N-terminal halves of **1** and **2** showed almost no conformational overlap (Figure 4D). These results clearly indicated that the opioid and NK1 pharmacophores of ligands **1** and **2** independently possess nearly the same conformations in terms of their backbone atoms, but the

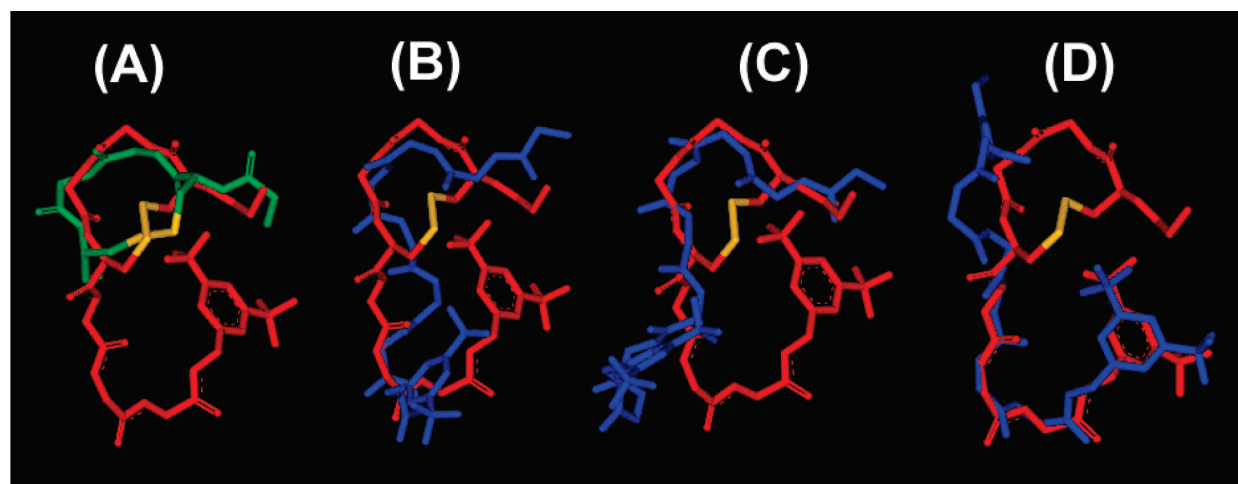


Figure 4. (A) Superimposed image of **2** (red) with the X-ray crystal structure of highly selective δ opioid agonist DPDPE (green).⁴² Superimposition was performed on all the backbone atoms of residues 1–5 (rmsd = 1.81 Å). Only backbone atoms are illustrated with C-terminal benzyl moiety and disulfide bond (yellow). Superimposed image of obtained NMR structure of **2** with the NMR structure of **1**⁵ (blue) at the lowest restraint energies. Superimposition was performed on all the backbone atoms of: (B) residues 1–8, rmsd = 2.34 Å; (C) residues 1–5, rmsd = 1.40 Å; (D) residues 5–8, rmsd = 0.65 Å.

Table 5. φ and ψ Angle Values of the Bifunctional Peptide Derivative **2** in the Observed NMR Structure^a

angle	Tyr ¹	D-Pen ²	Gly ³	Phe ⁴
φ		-154.9 ± 2.3	-85.0 ± 1.4	-105.9 ± 0.6
ψ	-70.9 ± 0.5	-173.6 ± 0.2	-73.6 ± 0.9	128.3 ± 0.3
angle	Pen ⁵	Pro ⁶	Leu ⁷	Trp ⁸
φ	-91.6 ± 0.6	-86.6 ± 0.8	-120.9 ± 4.7	-146.1 ± 1.3
ψ	118.5 ± 0.6	-102.7 ± 138.5	57.0 ± 1.1	-41.7 ± 0.9

^aAverage \pm SD values out of the best 10 calculated structures were listed. The C-terminus was considered as residue 9.

three-dimensional positionings of the NK1 pharmacophore relative to the opioid pharmacophore are different. This could have an important connection with the different address effect of the C-terminus of **1** and **2** for the observed binding affinities at δ and μ opioid receptors.

Further structural examination was made on the torsional angles of the fifth residue, where the opioid and NK1 pharmacophores were overlapped (Figure 1), to examine the relative positioning of two pharmacophores. The NMR conformer of **1** had the Met⁵ residue with positive φ and ψ angles in all 10 of the best structures.⁵ Because the positive φ angle in an L-amino acid residue is energetically unfavorable, the backbone conformation of **1** tends to bend or to twist near this “distorted” site at the connecting position of two interacting pharmacophores. While Pen⁵ in all 10 structures of **2** had negative φ angles (Table 5). This difference in torsional angles for the fifth residue leads to conformational differences near the overlapping site and modifies the positional relationship of the two connecting pharmacophores which may possibly account for the shift in biological activities at the tested receptors.

Paramagnetic Broadening Studies on ¹H NMR

To evaluate the interacting mode of **2** in a membrane-like environment, Mn²⁺ was used as a paramagnetic ion to eliminate the resonance intensities of solvent-exposed protons of **2** in the DPC micelles. The effects of the reagent were observed as an ensemble of cross-peaks belonging to the same residue spin system in TOCSY spectra measured with

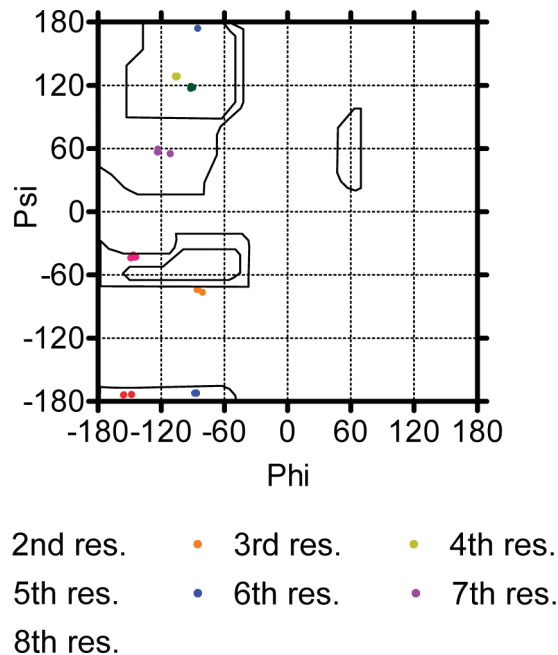


Figure 5. Ramachandran φ , ψ plots for **2** for residues 2–8 of 10 final structures.

a 62.2 ms mixing time (Figure 5).⁵ Interestingly, most of the cross-peaks derived from the backbone NH protons of **2** were eliminated with Mn²⁺ addition. The only exception was the NH proton of Pen⁵, whose cross-peak was retained in the presence of Mn²⁺ ions, suggesting that the backbone of **2** was mostly exposed to the surface of micelles and only the backbone atoms of Pen⁵ were buried inside of micelles (Figure 6). The side-chains of **2**, most of which were lipophilic groups, were unchanged after the Mn²⁺ addition, implying that these side-chain groups interact with DPC micelles very well and are buried inside of them. Therefore, these results revealed that **2** locates in DPC micelles with the amino acid side-chain groups and backbone atoms of Pen⁵ buried inside of the micelles, indicating its strong interaction with the membrane. This observed interacting mode of **2** with membrane-like micelles

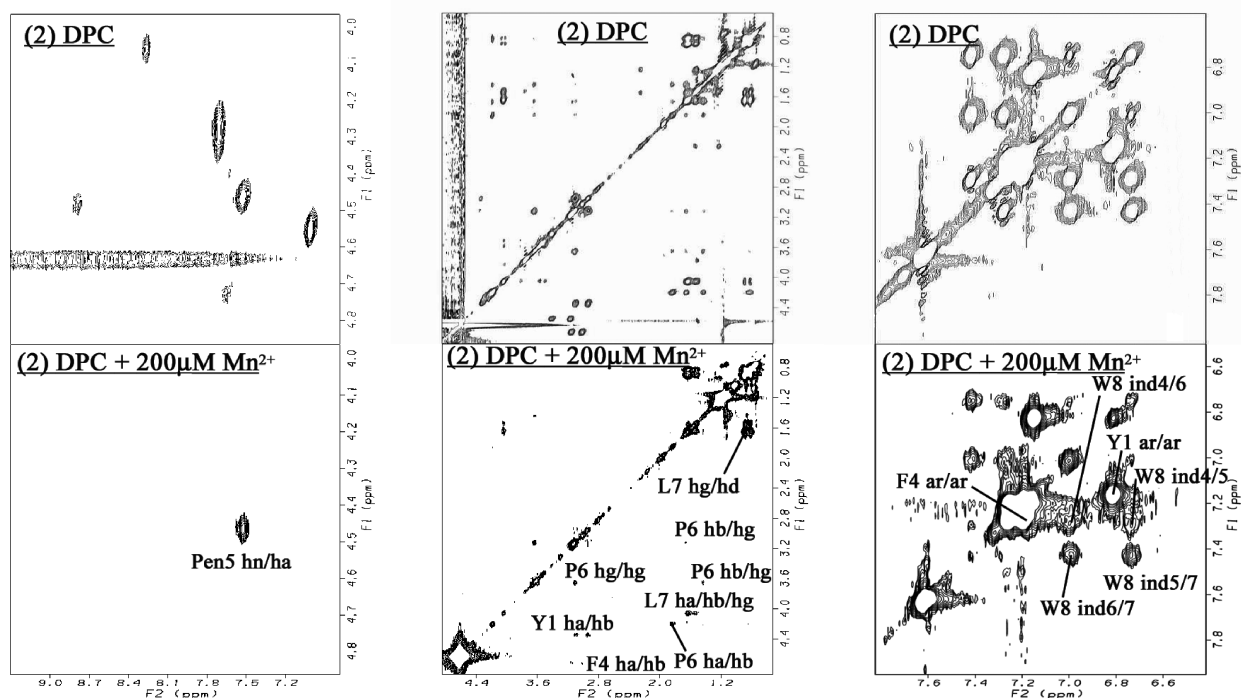


Figure 6. Typical effect of Mn^{2+} ions on TOCSY Spectra. **2** with DPC micelles (top row) and with $200 \mu\text{M Mn}^{2+}$ (bottom), for $\text{H}^{\text{N}}-\text{H}^{\alpha}$ region (left column), aliphatic side-chain region (middle), and aromatic region (right). Preserved resonances (labeled) are shown by the addition of Mn^{2+} . Spectra were compared from the same noise level.

was similar to that of **1**,⁵ suggesting that the incorporation of a 14-membered ring structure with D-Pen^2 and Pen^5 did not affect the strong interaction of these peptides with membranes but provides a more structured conformation which could modulate the affinity and selectivity of the ligand for the receptor in biological membranes.

Conclusions

Cyclic and topographical constraints with two penicillamine residues were introduced and optimized based on our developing bifunctional drug-design concept. As a result, the 14-membered ring structure with a Pen^5 and a C-terminal NK1 pharmacophore in **2** was found as the best combination to show potent antagonist actions at NK1 receptors and potent opioid agonist actions with significantly improved δ -opioid affinity selectivity over the μ -opioid (1400-fold) than those of DPDPE (380-fold), DPLPE (180-fold), and the previously reported linear peptide derivative **1** (24-fold). NMR structural analysis showed that significant δ -opioid selectivity in **2** could be attributed to its well-defined conformation with cyclic and topographical constraints and appropriate positioning of opioid and NK1 pharmacophores. The torsional angle of the fifth residue plays a key role to modulate the address effect of the C-terminal NK1 pharmacophore for the δ/μ opioid selectivity of the ligand by adjusting the three-dimensional positioning of the two connected pharmacophores. Together with its observed good metabolic stability, **2** could be considered as a valuable research tool and possibly a promising candidate for a novel analgesic drug.

Experimental Section

Materials. All amino acid derivatives and coupling reagents were purchased from EMD Biosciences (Madison, WI), Bachem (Torrance, CA), SynPep (Dublin, CA), and Chem Impex

International (Wood Dale, IL). 2-Chlorotrityl resin was acquired from Iris Biotech GmbH (Marktredwitz, Germany). Perdeuterated DPC was purchased from C/D/N Isotopes (Quebec, Canada). ACS grade organic solvents were purchased from VWR Scientific (West Chester, PA), and other reagents were obtained from Sigma-Aldrich (St. Louis, MO) and used as obtained. The polypropylene reaction vessels (syringes with frits) were purchased from Torviq (Niles, MI). Myo-[2- ^3H (N)]-inositol, [tyrosyl-3,5- ^3H (N)]-D-Ala²-Mephe⁴-glyol⁵-enkephalin (DAMGO), [tyrosyl-2,6- ^3H (N)]-(2-D-penicillamine,5-D-penicillamine)enkephalin (DPDPE), [^3H]-Substance P, and [^{35}S]-guanosine 5-(γ -thio) triphosphate ($\text{GTP}\gamma\text{S}$) were purchased from Perkin-Elmer (Wellesley, MA). Bovine serum albumin (BSA), protease inhibitors, Tris, and other buffer reagents were obtained from Sigma (St. Louis, MO). Culture medium, penicillin/streptomycin, and fetal calf serum (FCS) were purchased from Invitrogen (Carlsbad, CA).

N^{α} -Boc-Tyr(tBu)-D-Pen(Mob)-Gly-OH. Boc-D-Pen(Mob)-OH (1.50 g, 4.06 mmol) and H-Gly-OEt HCl (677 mg, 4.87 mmol) were dissolved in DMF (5 mL). HOBt (657 mg, 4.87 mmol), HBTU (1.85 g, 4.87 mmol), and NMM (904 mg, 8.95 mmol) were added to the solution at 0 °C. After stirring overnight at room temperature, saturated aqueous sodium bicarbonate was added to the solution and most of the organic solvent was removed under reduced pressure. The residue was extracted with ethyl acetate three times followed by washing with 5% aqueous citrate and saturated aqueous sodium chloride. The solution was dried over sodium sulfate. The solvent was evaporated, and the crude peptide was precipitated in cold petroleum ether, centrifuged, and dried under reduced pressure. The obtained solid was dissolved in TFA (6 mL) at 0 °C. After stirring for 3 h, the solution was concentrated. Ether (50 mL) and 4 M HCl in 1,4-dioxane (2 mL) were added to the solution to obtain 1.33 g (3.42 mmol, 84.4%) of crude H-D-Pen(Mob)-Gly-OEt HCl. The peptide was used for the next reaction without further purification. Boc-Tyr(tBu)-OH (1.38 g, 4.10 mmol) was next coupled in the presence of Cl-HOBt (694 mg, 4.10 mmol), HCTU (1.69 g, 4.10 mmol), and NMM (760 mg, 8.20 mmol) in DMF (5 mL).

The reaction mixture was stirred overnight at room temperature. Saturated aqueous sodium bicarbonate was added to the residue and extracted with ethyl acetate three times, followed by washing the combined solution with saturated aqueous sodium chloride. The solution was dried over sodium sulfate and concentrated under reduced pressure. The obtained residue was purified using silica gel chromatography (hexane:EtOAc = 10:1 to 2:1). The residue was dissolved in methanol (8 mL) and THF (8 mL), then 1 M NaOH (9 mL) was added at 0 °C. After stirring for 1 h at room temperature, 1 M HCl (10 mL) was added at 0 °C and then evaporated. The obtained residue was extracted with ethyl acetate three times, followed by washing with 5% aqueous citrate and saturated aqueous sodium chloride. The solution was dried over sodium sulfate. The solvent was evaporated, and the crude peptide was precipitated in cold ether. The precipitate was centrifuged and then dried under reduced pressure to obtain the title compound (1.53 g, 84.1%).

¹H NMR (DMSO-*d*₆) δ: 1.10–1.32 (21H, m), 1.45 (3H, s), 1.99 (2H, s), 2.28–3.10 (2H, m), 3.68–3.92 (5H, m), 4.03 (1H, q, *J* = 7.0 Hz), 4.80 (1H, d, *J* = 9.5 Hz), 6.84 (4H, d, *J* = 5.5 Hz), 7.00 (1H, d, *J* = 8.5 Hz), 7.18–7.26 (4H, m), 8.22 (1H, d, *J* = 9.5 Hz), 8.65–8.72 (1H, m). MS (ESI) 646 (MH)⁺.

Linear Peptide Synthesis. The peptide was synthesized manually by the *N*^α-Fmoc solid-phase methodology using HCTU as the coupling reagents as previously reported.^{5,7} 2-Chlorotrityl resin (500 mg, 0.78 mmol/g) was placed into a 50 mL polypropylene syringe with the frit on the bottom and swollen in DMF (10 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) and three times with DCM (15 mL) and then with DMF (3 × 15 mL). The *N*^α-Fmoc protecting group was removed by 20% piperidine in DMF (10 mL, 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). The protected amino acid (3 equiv) and HCTU (2.9 equiv) were dissolved in 30 mL of DMF, and then DIEA (6 equiv) was added. Fmoc-D-Pen(Trt)-OH, Fmoc-Pen(Trt)-OH, Fmoc-Pro-OH, Fmoc-Nle-OH, and Fmoc-Phe-OH were used for respective coupling as protected amino acids. The following coupling with BocTyr(tBu)-D-Pen(Mob)-Gly-OH (1.5 equiv) were performed using EDC (2.25 equiv), HOAt (2.25 equiv), and NMM (3 equiv) in DMF (10 mL). The coupling mixture was transferred into the syringe with the resin and then shaken for 2 h. All the other amino acids were consecutively coupled using the procedure described above, using the TNBS test or chloranil test 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 peptide was carefully washed with DMF (3 × 15 mL), DCM (3 × 15 mL), DMF (3 × 15 mL), and DCM (3 × 15 mL) and dried under reduced pressure. The peptide was cleaved off the solid support with 1% v/v TFA in DCM (10 mL) for 30 min, and most of the organic solvent was removed under reduced pressure. The obtained protected peptides with free C-terminal were precipitated out by the addition of chilled petroleum ether (30 mL) to give a white precipitate. The suspensions were centrifuged for 20 min at 7000 rpm, and then the liquid was decanted off. The crude peptides were washed with petroleum ether (2 × 50 mL), and after another centrifugation, the peptides were dried under vacuum (2 h) to obtain the title compound. The purity of the protected peptides with a free C-termini (>90%) were checked by analytical RP-HPLC using a Hewlett-Packard 1100 system (230 nm) on a reverse phase column (Waters NOVA-Pak C-18 column, 3.9 mm × 150 mm, 5 μm, 60 Å). The peptide was eluted with a linear gradient of aqueous CH₃CN/0.1% CF₃CO₂H (10–90% in 40 min) at a flow rate of 1.0 mL/min. The crude

peptide was used for the subsequent reactions without further purification.

The protected peptide with a free C-terminal (300 mg, 0.173 mmol) and Cl-HOBt (33.4 mg, 0.208 mmol) were dissolved in DMF (3 mL). 3,5-Bistrifluoromethylbenzyl amine (84.1 mg, 0.346 mmol) and EDC (39.7 mg, 0.208 mmol) were added to the solution at RT and stirred until the starting material (the protected peptide with free C-terminal) was not detected by TLC, then saturated aqueous sodium bicarbonate (100 mL) was added. The reaction mixture was extracted with ethyl acetate (100 mL) three times. The combined organic phases were washed with 5% aqueous citrate and saturated aqueous sodium chloride (100 mL each) and then dried over sodium sulfate. The solvent was evaporated off and the crude protected 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 a 6:1 mixture of TFA and anisole at 80 °C (3 mL, 30 min). The crude peptide was precipitated out by the addition of chilled petroleum ether (45 mL) to give a dark oil. 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) to give a precipitate, and after a final centrifugation, the peptides were dried under vacuum (2 h). The resulting peptides were directly used for cyclization.

Oxidative Cyclization to Disulfides.^{25,45} A solution of K₃Fe(CN)₆ was prepared as follows: 1 mmol (330 mg) of K₃Fe(CN)₆ was dissolved in a mixture of water (100 mL) and CH₃CN (100 mL). A saturated solution of ammonium acetate (20 mL) was added to it, and the pH was adjusted to 6.0 with glacial acetic acid. A solution of the linear peptide (0.173 mmol) in the mixture of CH₃CN (40 mL), DMSO (5 mL), and H₂O (5 mL) was added to the above solution dropwise overnight with the help of a syringe pump.²⁵ After the overnight reaction, glacial acetic acid was added to the reaction mixture to obtain pH 5.0, followed by 20 mL of Amberlite IRA-68 anion-exchange resin (pre-equilibrated with 1 M HCl and extensively washed with distilled water), and the suspension was stirred for 30 min until the solution turned colorless and the resin turned yellow. The resin was suction-filtered and the filtrate rotoevaporated to remove most of the organic solvent. The remaining solution was concentrated on a Sep-Pak C18 cartridge (10 g, Waters, Milford, MA) and then eluted with CH₃CN. The obtained yellow solution was concentrated under reduced pressure for the final purification by preparative RP-HPLC and then lyophilized. Preparative RP-HPLC was performed on a Waters Delta Prep 4000 with a Waters XTerra C-18 column (19 mm × 250 mm, 10 μm, a linear gradient of 33–53% or 40–60% acetonitrile/0.1% TFA at a flow rate of 15.0 mL/min).

The purified final peptides (≥95%) were characterized by HRMS, TLC, analytical HPLC, and ¹H NMR (all the data, charts, and experimental details are available in the Supporting Information).

NMR Spectroscopy in DPC Amphipathic Media and Conformational Structure Determination. All of the conformational determinations were performed as follows,^{5,6,35,36} based on the NMR spectra using a Bruker DRX600 600 MHz spectrometer.

The samples were prepared by dissolving the peptide (4.2 mM) in 0.5 mL of 45 mM sodium acetate-*d*₃ buffer (pH 4.5) containing 40 equiv of dodecylphosphocholine-*d*₃₈ and 1 mM sodium azide (90% H₂O/10% D₂O), followed by sonication for 5 min. Two-dimensional double quantum filtered correlation (DQF-COSY), nuclear Overhauser enhancement spectroscopy⁴⁶ (NOESY, mixing time = 450 ms), rotating frame Overhauser effect spectroscopy (ROESY, mixing time = 150 ms), and total correlation spectra⁴⁷ (TOCSY, MLEV-17 mixing time = 62.2 ms, spin-lock field = 8.33 kHz) were acquired using standard pulse sequences at 310 K. Coupling constants (³*J*_{NH-Hα}) were measured from 2D DQF-COSY spectra by

analysis of the fingerprint region with a curve-fitting using 5-parameter Levenberg–Marquardt nonlinear least-squares protocol to a general antiphase doublet.

For the conformational structure determination, the volumes of the assigned cross-peaks in the 2D NOESY spectrum were converted into upper distance bounds of 3.0, 3.8, 4.8, or 5.8 Å. For overlapping cross-peaks, the distance categories were increased by one or two levels, depending on the qualitative estimate of the extent of overlap. Pseudoatoms were created for nonstereospecifically assigned methylene protons with a correction of 1.0 Å applied to their upper bound distances.⁴⁸ In addition to the distance constraints, ϕ dihedral angle constraints derived from $^3J_{\text{HN-H}\alpha}$ coupling constants were set to between -90° and 40° for $^3J_{\text{HN-H}\alpha} < 6$ Hz and to between -150° and -90° for $^3J_{\text{HN-H}\alpha} > 8$ Hz. Dihedral angle constraints of $180 \pm 5^\circ$ for peptide bonds (ω) were also used to maintain the planarity of these bonds.

Structures were calculated using a hybrid distance geometry-dynamical simulated annealing protocol, followed by energy refinement using restrained molecular dynamics (rMD), using the NOE-derived distance constraints and dihedral angle (ϕ) constraints. Distance geometry calculations were performed using the DGII⁴⁹ program within the INSIGHT II package (Accelrys Inc., San Diego, CA). Solvent was not explicitly included in the calculations. The bounds for the interatomic distances were embedded in four dimensional spaces, followed by the optimization using a simulated annealing protocol with sigmoidal cooling schedule from a maximum temperature of 200 K in 10000 steps at simulation steps of 2.5 ps. The fail level was set to 2.00. All the embedded structures successfully passed the simulated annealing step and were minimized using the consistent valency force field (CVFF) (Accelrys Inc.). The entire process was repeated for 100 times to generate 100 independently optimized structures.

Of those, the 50 structures with the lowest penalty function were further refined by two rounds of rMD using the all-atom AMBER force field with additional parameters for fluorine atom,^{44,50–52} using the standalone DISCOVER version 2.98 program (Accelrys Inc.).^{35,53–55} A 12.0 Å cutoff for nonbonded interactions and a distance-dependent dielectric constant (4 r) were used. All amide bonds were constrained to trans conformation by a 100 kcal mol⁻¹ rad⁻² energy penalty. The distance constraints and dihedral angles (ϕ) constraints were applied with a force constant of 25 kcal mol⁻¹ Å⁻² and 100 kcal mol⁻¹ rad⁻², respectively. After 100 steps of steepest descents minimization and 1000 steps of conjugate gradient minimization on the initial structures, an rMD equilibration at 500 K was performed for 1.5 ps, during which a scale factor of 0.1 was applied to the experimental restraint force constants. During the next 2 ps, full values of the experimental restraint force constants were applied. A further 1 ps rMD simulation was run at 500 K, and the system was then cooled to 0 K over 3 ps. After another 1 ps at 0 K, 100 cycles of steepest descents and 2000 steps of conjugate gradient minimization were performed. The final 10 structures with the lowest energies were used for the analysis. All calculations were performed on a Silicon Graphics Octane computer.

Radioligand Labeled Binding Assay, [³⁵S]GTP- γ -S Binding Assay, GPI, and MVD in Vitro Bioassay. The methods were carried out according to that previously described.^{4–7} Briefly, the evaluation of the binding affinities of the synthesized bifunctional peptide derivatives at the human δ -opioid receptors and rat μ -opioid receptors were performed on the cell (HN9.10) membranes that stably express these corresponding receptors using [³H]-c[D-Pen²,D-Pen³]-enkephalin ([³H]DPDPE, 44 Ci/mmol) and [³H]-[D-Ala²,NMePhe⁴,Gly⁵-ol]-enkephalin ([³H]-DAMGO, 47.2 Ci/mmol) as radioligands, respectively. The affinity of [³H]DAMGO at the μ -opioid receptor and that of [³H]DPDPE for the δ -opioid receptor was defined by the dissociation constant (K_D) determined by saturation analysis as

previously described.^{4–7} For competition analysis, 10 concentrations of a test compound were each incubated with 50 μ g of membranes from δ - or μ -opioid receptors expressing cells labeled with [³H]DPDPE (1.0 nM) or [³H]DAMGO (1.0 nM), respectively. Naloxone at 10 μ M was used to define the non-specific binding of the radioligands in all assays. For the affinity at the human or rat NK1 receptors, binding assays utilized membranes from transfected CHO cells that stably express corresponding receptors, using [³H]-substance P (135 Ci/mmol, 0.3–0.4 nM) as the standard radioligand. The [³H] substance P concentration was selected based on the saturation binding experiments which showed a high affinity binding with K_D values of 0.40 ± 0.17 for hNK1 and 0.16 ± 0.03 nM for rNK1, respectively. Substance P at 10 μ M was used to define the nonspecific binding. All data were determined from a minimum of 2 independent analyses. The data were analyzed by nonlinear least-squares regression analysis using GraphPad Prism.

The [³⁵S]GTP γ S binding assays were used to estimate the functional activities of the test compounds at the δ and μ opioid receptors expressed in the transfected cells. Data were analyzed by nonlinear least-squares analysis using GraphPad Prism to determine the EC₅₀ and E_{max} values for each test compound. The isolated tissue-based functional assays also were used to evaluate opioid agonist activities in the GPI (μ) and MVD (δ). Dose effects of the test compounds against substance P stimulation were determined in isolated GPI in the presence of 1 μ M naloxone.

In Vitro Stability of Peptide Derivatives in Rat Plasma.⁶ Stock solution of compounds (50 mg/mL in DMSO) were diluted 1000-fold into rat plasma (lot 24927, Pel-Freez Biologicals, Rogers, AK) to give an incubation concentration of 50 μ g/mL. All samples were incubated at 37 °C and 200 μ L of aliquots were withdrawn at 1, 2, 4, and 6 h. Then 300 μ L of acetonitrile was added and the proteins were removed by centrifugation. The supernatant was analyzed for the amount of remaining parent compound by HPLC (Hewlett-Packard 1090 m with Vydac 218TP104 C-18 column; 4.6 mm \times 250 mm, 10 μ m, 300 Å). The samples were tested in three independent experiments ($n = 3$), and the mean values \pm SD were used for the analysis. The statistical significances were evaluated with the Student t test.

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Supporting Information Available: ¹H NMR, HPLC, and MS data of the cyclic peptide derivatives 2–5 and their intermediates. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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