Dicarba Analogues of the Cyclic Enkephalin Peptides H-Tyr-*c*[D-Cys-Gly-Phe-D(or L)-Cys]NH_2 Retain High Opioid Activity†

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Dicarba analogues of the cyclic opioid peptides H-Tyr-*c*[D-Cys-Gly-Phe-D(or L)-Cys]NH_2 were synthesized on solid phase by substituting allylglycines for the cysteines and cyclization by ring-closing metathesis between the side chains of the allylglycine residues. Mixtures of *cis* and *trans* isomers of the resulting olefinic peptides were obtained, and catalytic hydrogenation yielded the saturated $-CH_2-CH_2-$ bridged peptides. The dicarba analogues retained high μ and δ agonist potencies. Remarkably, the *trans* isomer of H-Tyr-*c*[D-Allylgly-Gly-Phe-L-Allylgly]NH_2 was a μ agonist/δ agonist with subnanomolar potency at both receptors.

Introduction

Substitution of a bismethylene ($-CH_2CH_2-$) moiety for a disulfide bridge in cystine-containing biologically active peptides is of considerable interest because the resulting dicarba analogues may show altered biological activity profiles and conformational properties. In the past, this structural modification was synthetically demanding, as it required the replacement of cystine with diaminosuberic acid in cumbersome multistep syntheses.[1,2] Recently, the use of ring-closing metathesis (RCM[a]) has been shown to be a relatively straightforward procedure for the preparation of a dicarba analogue of oxytocin.[3]

Cystine-containing cyclic peptides can also be obtained through substitution of two Cys residues in appropriate positions of the peptide sequence of linear peptides, followed by disulfide bond formation. In comparison with the linear parent peptides, such cyclic analogues have greatly improved conformational integrity and permit more meaningful conformational studies aimed at determining the bioactive conformation. Furthermore, thermodynamic considerations predict that, in comparison with the flexible parent peptide, such a cyclic analogue constrained to assume the "correct" bioactive conformation should have higher receptor binding affinity, because the loss of internal rotational entropy upon binding is smaller.[4]

All naturally occurring opioid peptides are linear peptides. Cyclic enkephalin analogues containing a cystine bridge of the structure H-Tyr-*c*[D-Cys-Gly-Phe-D(or L)-Cys]NH_2 were synthesized independently by two groups[5,6] and were found to have high binding affinity for both μ and δ opioid receptors.[6] The corresponding peptides with a free C-terminal carboxyl group, H-Tyr-*c*[D-Cys-Gly-Phe-D(or L)-Cys-OH, showed subnanomolar δ opioid agonist potency and moderate δ receptor selectivity.[7] The compound H-Try-*c*[D-Cys-Phe-Cys]NH_2 represents an example of cystine-bridged cyclic dermorphin analogue, containing a very small (11-membered) ring structure and displaying preference for μ receptors over δ receptors.[8] Replacement of the two Cys residues in H-Tyr-*c*[D-Cys-Gly-Phe-D(or L)-Cys]-OH with penicillamine (Pen) residues resulted in compounds with high δ receptor selectivity (DPDPE and DPLPE).[9] Similarly, structural modification of the μ-selective cyclic tetrapeptide H-Tyr-*c*[D-Cys-Phe-Cys]NH_2[8] through substitution of D-Pen for Cys in the 4-position and replacement of the C-terminal carboxamide function with a free carboxyl group led to a compound, H-Tyr-*c*[D-Cys-Phe-D-Pen]OH (JOM-13), which is almost as δ-selective as DPDPE and has a 3.5-fold higher affinity for δ receptors.[10]

The dermorphin-derived cyclic tetrapeptide analogues H-Tyr-*c*[D-Cys-Phe-Cys]NH_2 and H-Tyr-*c*[D-Cys-Phe-D-Cys]NH_2 are opioid agonists at the μ and δ receptors.[8] Recently, we prepared the dicarba analogues of these peptides by solid-phase synthesis of the linear precursor peptides containing allylglycine residues in place of the Cys residues, followed by RCM.[11] In the case of the peptide with L-configuration in the 4-position, both the *cis* and the *trans* isomer of the resulting olefinic peptide were formed, whereas the *cis* isomer only was obtained with the peptide having the D-configuration in position 4. Catalytic hydrogenation yielded the saturated $-CH_2-CH_2-$ bridged peptides. In comparison with the cystine-containing parent peptides, all olefinic peptides showed significantly reduced μ and δ agonist potencies in the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays. The $-CH_2-CH_2-$ bridged peptide with L-configuration in the 4-position was nearly equipotent with its cystine-containing parent in both assays, whereas the bismethylene analogue with D-configuration in position 4 was less potent compared to its parent.

In the present paper, we describe the syntheses and in vitro opioid activity profiles of dicarba analogues of the enkephalin-derived cyclic pentapeptide analogues H-Tyr-*c*[D-Cys-Gly-Phe-D(or L)-Cys]NH_2[6] (Figure 1). The olefinic cyclic precursor peptides resulting from the RCM used in these syntheses were also pharmacologically characterized.

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[a] Symbols and abbreviations are in accordance with recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem. J.* **1984**, *219*, 345−373. Abbreviations: Allylgly, allylglycine; DAMGO, H-Tyr-D-Ala-Gly-N^{α}MePhe-Gly-ol; DIC, 1,3-diisopropylcarbodiimide; DPDPE, H-Tyr-*c*[D-Pen-Gly-Phe-D-Pen]OH; DPLPE, H-Tyr-*c*[D-Pen-Gly-Phe-L-Pen]OH; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; JOM-13, H-Tyr-*c*[D-Cys-Phe-D-Pen]OH; MVD, mouse vas deferens; Pen, penicillamine; RCM, ring-closing metathesis; RMSD, root mean standard deviation; TFA, trifluoroacetic acid; U69,593, (5α,7α,8β-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide.

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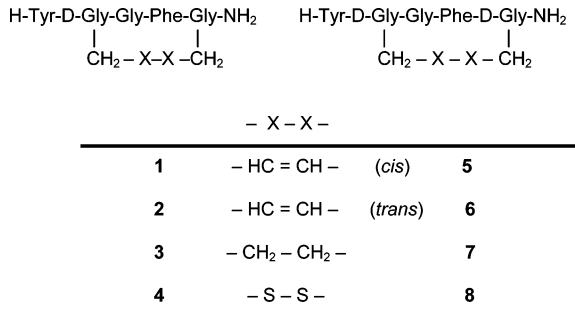

Figure 1. Dicarba analogues of H-Tyr-*c*[D-Cys-Gly-Phe-D(or L)Cys]-NH_2.

Results and Discussion

The linear precursor peptides of the dicarba analogues of H-Tyr-*c*[D-Cys-Gly-Phe-D(or L)-Cys]NH_2 with allylglycine residues substituted in positions 2 and 5 of the peptide sequence were assembled on a Rink Amide MBHA resin using Fmoc-protected amino acids and 1,3-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) as coupling agents. Cyclization between the two allylglycine residues was achieved using the second generation Grubbs catalyst benzylidene[1,3-bis-(2,4,6-trimethylphenyl)-2-imidazolidinylidene]dichloro(tricyclohexylphosphine)ruthenium.[12] The resulting cyclic olefinic peptides were cleaved from the resin by TFA treatment in the usual manner. The yield of the cyclization reaction was 75% and 73% for the D,L- and the D,D-peptide, respectively. Mixtures of *cis* and *trans* isomers were obtained in both cases. The configuration of the double bond was established by measurement of the *J* coupling constants between the olefinic protons: compound **1** ($J = 10.5$ Hz, *cis*), compound **2** ($J = 15.4$ Hz, *trans*), compound **5** ($J = 10.8$ Hz, *cis*), and compound **6** ($J = 15.6$ Hz, *trans*). *Cis*/*trans* ratios of 2:3 and 5:8 were determined for the D,L- and D,D-peptides, respectively. Catalytic hydrogenation of the cyclic olefinic peptides with 10% Pd/C in EtOH at 40 °C for 18 h ($p_{H_2} = 45$ psig) was straightforward and yielded the $-CH_2-CH_2-$ bridged peptides in 85−87% yield.

In comparison with the H-Tyr-*c*[D-Cys-Gly-Phe-L-Cys]NH_2 parent peptide (**4**), the *cis* isomer of the olefinic peptide with L-configuration in position 5 of the peptide sequence (**1**) showed about the same μ and δ opioid agonist potencies in the GPI and MVD assays, similar μ receptor binding affinity and 4−5-fold lower δ and κ receptor binding affinities (Table 1). The *trans* isomer of the olefinic peptide, H-Tyr-*c*[D-Allylgly-Gly-Phe-L-Allylgly]NH_2 (**2**), displayed about 2-fold higher μ and δ agonist potencies than parent peptide **4** in the functional bioassays. In agreement with these results, 2.8- and 1.3-fold higher μ and δ receptor binding affinities, respectively, were determined for **2**, as compared to **4**, in the receptor binding assays, whereas the κ receptor binding affinity was about the same for both peptides. The corresponding bismethylene analogue (**3**) showed about the same μ agonist potency and μ receptor binding affinity as the disulfide-bridged parent (**4**), but was a 5-fold less potent δ opioid agonist and had 4- and 8-fold lower δ and κ receptor binding affinities, respectively. Taken together, the results obtained with dicarba analogues **1**, **2**, and **3** indicate that, like their parent **4**, these compounds are balanced mixed μ agonist/δ agonists with weak κ receptor binding affinities.

In the functional assays, the *cis* isomer of the olefinic peptide with D-configuration in position 5 of the peptide sequence (compound **5**) showed 9- and 18-fold lower μ and δ agonist potency, respectively, in comparison with the cystine-containing parent peptide H-Tyr-*c*[D-Cys-Gly-Phe-D-Cys]NH_2 (**8**; Table 1). In agreement with these results, the μ and δ opioid receptor affinities determined in the receptor binding assays were also substantially lower than that of parent **8**. The corresponding olefinic peptide with *trans* configuration at the double bond (compound **6**) also displayed lower μ and δ agonist potency than **8**, even though the potency decrease was somewhat less pronounced than in the case of the *cis* isomer (**5**). This result is reflected in the receptor binding assay data, which indicate that **6** has about 2-fold lower μ and δ receptor affinities than parent **8**. The corresponding saturated dicarba analogue with D-configuration in position 5 of the peptide sequence (compound **7**) also showed a significant decrease (6−9-fold) in δ and μ agonist potency, as compared to parent **8**, in the functional assays, as well as decreased μ and δ receptor binding affinities. Thus, in general, the dicarba analogues **5**, **6**, and **7** of H-Tyr-*c*[D-Cys-Gly-Phe-D-Cys]NH_2 (**8**) turned out to be somewhat weaker μ and δ opioid agonists than their parent. While in general there is good qualitative agreement between the receptor binding data and the functional assay data of the compounds, some quantitative discrepancies were observed. Such quantitative discrepancies have often been documented and could be due to a possible differences in the structural requirements or receptor access between central and peripheral receptors.

The dicarba analogues **1**, **2**, and **3** of H-Tyr-*c*[D-Cys-Gly-Phe-L-Cys]NH_2 (**4**) showed equal or higher μ agonist potency than the cystine-containing parent (**4**), as well as high δ agonist potency. They represent the first examples of dicarba analogues of a cystine-containing cyclic peptide in which replacement of the disulfide moiety with a carbon−carbon double bond or with a bismethylene moiety did not result in a potency decrease. Corresponding dicarba analogues of the cyclic tetrapeptide agonist H-Tyr-*c*[D-Cys-Phe-D(or L)-Cys]NH_2 in general showed somewhat reduced potency as compared to their disulfide-bridged parents.[11] Even more drastic potency drops had been observed with dicarba analogues of oxytocin[1,3] and of somatostatin,[2] in which the disulfide bridge had been replaced with a bismethylene moiety.

Particularly remarkable is the *trans* isomer of H-Tyr-*c*[D-Allygly-Gly-Phe-L-Allylgly]NH_2 (compound **2**), which is a balanced mixed μ agonist/δ agonist with subnanomolar agonist potency at both receptors. Mixed μ agonist/δ agonists act synergistically at μ and δ opioid receptors to produce a potent analgesic effect and to induce less physical dependence than a pure μ agonist like morphine upon chronic administration.[13] Therefore, it will be of interest to determine in future studies whether compound **2** is an analgesic with low propensity to produce physical dependence.

There is the possibility that the disulfide bond in the cystine-containing parent peptides **4** and **8** might be reduced in tissues through the action of disulfide reductases, resulting in ring opening. Obviously, this possibility is excluded with the dicarba analogues. To examine possible differences in stability between the cystine-containing peptide **4** and dicarba analogues **1**, **2**, and **3**, a degradation study was performed by incubating these peptides in rat brain suspensions at 37 °C (for experimental details, see Supporting Information). No degradation was observed with all four peptides at incubation times up to 24 h, indicating that the disulfilde bond in peptide **4** was stable under these conditions and that none of the peptides were hydrolyzed.

A preliminary theoretical conformational analysis based on molecular mechanics calculations was performed with compounds **1**−**4**. Energy minimization of the "bare" 14-membered ring structures lacking the exocyclic Tyr^1 residue and the Phe^4 side chain resulted in 21, 11, 39, and 41 low-energy conformers

Table 1. In Vitro Opioid Activity Profiles of Cyclic Enkephalin Analogues[a]

	GPI	MVD	receptor binding			
no.	IC_{50} (nM)	IC_{50} (nM)	K_i^{μ} (nM)	K_i^{δ} (nM)	K_i^{κ} (nM)	K_i ratio $\mu/\delta/\kappa$
1	1.81 ± 0.13	0.496 ± 0.015	2.40 ± 0.11	6.55 ± 0.82	200 ± 19	1/3/83
2	0.898 ± 0.072	0.275 ± 0.017	0.616 ± 0.035	1.25 ± 0.16	57.6 ± 4.4	1/2/94
3	1.02 ± 0.16	3.19 ±0.52	2.34 ± 0.17	5.87 ± 0.82	309 ± 31	1/3/132
4	1.65 ± 0.18	0.603 ± 0.077	1.74 ± 0.09	1.61 ± 0.05	40.1 ± 0.4	1/1/23
5	11.9 ± 0.3	9.91 ± 0.52	2.27 ± 0.35	7.04 ± 0.08	498 ± 55	1/3/219
6	5.63 ± 0.70	4.38 ± 0.22	1.05 ± 0.08	1.92 ± 0.06	30.4 ± 7.7	1/2/29
7	11.2 ± 1.6	3.64 ± 0.64	1.17 ± 0.07	3.34 ± 0.49	71.5 ± 8.9	1/3/61
8	1.30 ± 0.12	0.562 ± 0.061	0.550 ± 0.052	0.822 ± 0.118	44.9 ± 11.0	1/1/82

[a] Values represent means of 3−6 determinations ± SEM.

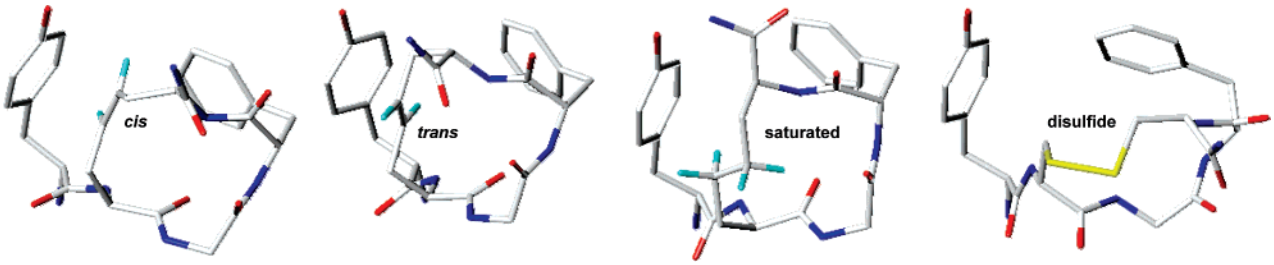

Figure 2. Low-energy conformers of compounds **1**−**4** corresponding to the model of the δ receptor-bound conformation.

(within 3 kcal/mol of the lowest-energy structure) for compounds **1**, **2**, **3**, and **4**, respectively. This result indicates that among these four peptides the *trans* isomer of the olefinic peptide (**2**) has the most rigid structure, whereas the ring structures of the bismethylene analogue (**3**) and of the disulfide-bridged parent peptide (**4**) are more flexible. After attachment of the exocyclic Tyr^1 residue and the Phe^4 side chain to these low-energy structures, further extensive energy minimization resulted in the lowest-energy conformations for compounds **1**, **2**, and **4** and the second lowest one for compound **3** (1.1 kcal/mol higher in energy than the lowest-energy structure) shown in Figure 2. The structures depicted in Figure 2 all showed good spatial overlap between their N-terminal amino group, their Tyr^1 aromatic ring, and their Phe^4 aromatic ring with the nitrogen atom, phenolic ring, and six-membered aromatic ring of the indole moiety of the δ opioid antagonist naltrindole,[14] with RMSD values of 0.620 Å, 0.595 Å, 0.551 Å, and 0.459 Å, for compounds **1**, **2**, **3**, and **4**, respectively. This result may explain why, despite some differences between the peptide ring structures of the four compounds, they all display similarly high δ opioid receptor binding affinity. Structures of compounds **1**−**4**, with only slightly higher energies than those depicted in Figure 2, showed larger intramolecular distances between the two aromatic rings (10−12 Å), similar to the corresponding distance observed in a model of the cyclic μ opioid peptide agonist JOM-6 docked to the μ opioid receptor.[15] This could be taken as an explanation for the high μ opioid agonist activity shown by these compounds.

Experimental Section

General Methods. Precoated plates (silica gel 60 F_{254}, 250 μm, Merck, Darmstadt, Germany) were used for ascending TLC in the following systems (all v/v): (I) *n*-BuOH/AcOH/H_2O (4:1:1); (II) *n*-BuOH/pyridine/AcOH/H_2O (15:10:3:12). Preparative reversed-phase HPLC was performed on a Vydac 218-TP1022 column (22 × 250 mm) with a linear gradient of 18−19% MeOH in 0.1% TFA over 35 min, at a flow rate of 13 mL/min. Analytical reversed-phase HPLC was performed on a Vydac 218-TP54 column (5 × 250 mm) with a linear gradient of 18−19% MeOH in 0.1% TFA over 35 min at a flow rate of 1.5 mL/min. The same column was also used for the determination of the capacity factors K' under the following conditions: (i) linear gradient of 18−19% MeOH in 0.1% TFA over 35 min at a flow rate of 1.5 mL/min (K'_1); (ii) linear gradient of 20−24% acetonitrile in 0.1% TFA over 30 min at a flow rate of 1.0 mL/min (K'_2). Molecular masses of compounds were determined by electrospray mass spectrometry on a Hybrid Q-Tof mass spectrometer interfaced to a MassLynx 4.0 data system.

Peptide Synthesis. The linear precursor peptides were assembled on a Rink amide Novabiochem resin according to a published protocol,[16] using Fmoc protection of the α-amino group and *t*-butyl protection of the Tyr hydroxyl group, and DIC/HOBt as coupling agents. After completion of peptide assembly, cyclization between the two allylglycine residues was achieved using the second generation Grubbs catalyst benzylidene[1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene]dichloro(tricyclohexylphosphine)-ruthenium.[12] The catalyst in 20 mol % (as compared to resin-bound peptide) was added to the peptide resin suspended in CH_2Cl_2 under a flow of argon. The reaction mixture was refluxed for 48 h, and after cooling to room temperature, DMSO (50 equiv relative to the catalyst) was injected and the mixture was stirred for another 24 h. The peptide resin was filtered and washed successively with DMSO, CH_2Cl_2, and MeOH. Fmoc protection was removed by 30% piperidine/DMF treatment, and the cyclic olefinic peptides were cleaved from the resin by TFA treatment in the usual manner. The yield of the cyclization reaction was 73 and 75% for the D,D- and D,L-peptide, respectively. In the case of the peptide with L-configuration in the 5-position of the peptide sequence, a 2:3 mixture of *cis* and *trans* isomers was obtained, whereas the *cis*/*trans* ratio was 5:8 in the case of the peptide having D-configuration in the 5-position. The crude peptides were purified by preparative HPLC and were found to be at least 98% pure, as assessed by HPLC and TLC. Catalytic hydrogenation of the cyclic olefinic peptides was performed with the mixtures of the *cis* and *trans* isomers with 10% Pd/C in EtOH at 40 °C for 18h (p_{H_2} = 45 psig). The resulting $-CH_2-CH_2-$ bridged peptides were obtained in 85−87% yield and were purified by preparative HPLC.

H-Tyr-*c*[D-Allylgly-Gly-Phe-L-Allylgly]NH_2 (*cis*; 1). HPLC K'_1 1.24; K'_2 1.27; TLC R_f 0.73 (I), R_f 0.80 (II); ES-ML *m*/*e* 551.

H-Tyr-*c*[D-Allylgly-Gly-Phe-L-Allylgly]NH_2 (*trans*; 2). HPLC K'_1 1.19, K'_2 1.24; TLC R_f 0.71 (I), R_f 0.80 (II); ES-ML *m*/*e* 551.

H-Tyr-*c*[D-Allylgly-Gly-Phe-L-Allylgly]NH_2 (reduced; 3). HPLC K'_1 1.33, K'_2 1.30; TLC R_f 0.58 (I), R_f 0.79 (II); ES-ML *m*/*e* 553.

H-Tyr-*c*[D-Allylgly-Gly-Phe-D-Allylgly]NH_2 (*cis*) (5). HPLC K'_1 1.34, K'_2 1.17; TLC R_f 0.74 (I), R_f 0.83 (II); ES-ML *m*/*e* 551.

H-Tyr-*c*[D-Allylgly-Gly-Phe-D-Allylgly]NH_2 (*trans*) (6). HPLC K'_1 1.41, K'_2 1.19; TLC R_f 0.78 (I), R_f 0.85 (II); ES-ML *m*/*e* 551.

H-Tyr-*c*[D-Allylgly-Gly-Phe-D-Allylgly]NH_2 (reduced) (7). HPLC K'_1 1.53, K'_2 1.30; TLC R_f 0.61 (I), R_f 0.79 (II); ES-ML *m*/*e* 553.

In Vitro Bioassays and Receptor Binding Assays. The GPI[17] and MVD[18] bioassays were carried out as reported in detail elsewhere.[19,20] A dose−response curve was determined with [Leu^5]-enkephalin as standard for each ileum and vas preparation, and IC_{50} values of the compounds being tested were normalized according to a published procedure.[21] Opioid receptor binding studies were performed as described in detail elsewhere.[19] Binding affinities for μ and δ receptors were determined by displacing, respectively, [^{3}H]-DAMGO (Multiple Peptide Systems, San Diego, CA) and [^{3}H]-DSLET (Multiple Peptide Systems) from rat brain membrane binding sites, and κ opioid receptor affinities were measured by displacement of [^{3}H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2 h at 0 °C with [^{3}H]DAMGO, [^{3}H]DSLET, and [^{3}H]U69,593 at respective concentrations of 0.72, 0.78, and 0.80 nM. IC_{50} values were determined from log−dose displacement curves, and K_i values were calculated from the obtained IC_{50} values by means of the equation of Cheng and Prusoff,[22] using values of 1.3, 2.6, and 2.9 nM for the dissociation constants of [^{3}H]DAMGO, [^{3}H]DSLET, and [^{3}H]U69,593, respectively.

Theoretical Conformational Analysis. All calculations were performed using the molecular modeling software SYBYL, version 7.0 (Tripos Associates, St. Louis, MO). The standard SYBYL force field was used for energy calculations, and a dielectric constant of 78 was chosen to simulate an aqueous environment. A stepwise approach was used to determine low-energy conformations of the cyclic peptides.[23] First, the bare ring structure was constructed for each peptide, containing only atoms directly attached to the ring, along with associated hydrogen atoms. After minimization, a systematic conformational grid search was performed to identify low-energy ring structures. Each rotatable bond was rotated in 30° increments over all space, and the resulting structures were minimized. All conformers having an energy >3 kcal/mol higher than that of the lowest-energy ring structure were discarded. Next, the exocyclic tyrosine residue and the phenylalanine side chain were attached to each low-energy ring structure, and a second systematic grid search was performed on the exocyclic rotatable bonds. Energies were calculated, and the resulting conformers were grouped into low-energy families. The lowest-energy member of each family was minimized, and the resulting conformations were ranked in order of increasing energy. Delta receptor-bound conformations were identified by spatial overlap of the peptide conformations with the δ opioid antagonist naltrindole. The N-terminal amino group and the two aromatic rings of the peptide studied were superimposed on the corresponding pharmacophoric moieties in naltrindole and RMSD values were calculated.[24]

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Supporting Information Available: NMR spectroscopic parameters of compounds **1**−**3** and **5**−**7** and experimental details of the enzymatic stability assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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