Cyclic Enkephalin Analogs with Exceptional Potency at Peripheral δ Opioid Receptors

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A series of super potent and δ-opioid-receptor-selective cyclic hexapeptides of the general formula H-Tyr-D-Pen-Gly-Phe(*p*-X)-Cys-Phe-OH (where X is hydrogen or halogen) has been synthesized. The unsubstituted hexapeptide H-Tyr-D-Pen-Gly-Phe-Cys-Phe-OH (HB-P2, [Phe^6]DPLCE) has extremely high potency at peripheral δ opioid receptors (IC_{50} value in the MVD assay is 0.016 nM) and in bioassays is the most selective compound in this series. The introduction of halogens in the phenyl ring of phenylalanine at position 4 led to significant changes in the selectivity and affinities at peripheral and central opioid receptors. In the binding studies, the most potent compound is the *p*-fluoro analog, whereas the most selective analog is the *p*-iodo-substituted peptide.

Introduction[1]

Since the discovery of two endogenous peptide opioids by Hughes and Kosterlitz in 1975,[2] the search for highly receptor-selective and potent opioid agonists and antagonists for opiate receptors has become an important goal. It was suggested some time ago that there are multiple opioid receptors,[3–7] and much data have been accumulated in support of this hypothesis.[8,9] On the basis of these data, it is generally accepted that there are at least three opioid receptor types—μ, δ, and κ. However, the possibility of the existence of other receptor types is not excluded.[10,11] The situation is more complicated since there have been numerous suggestions that there are subtypes of these receptors, e.g., μ1, μ2, δ1, δ2, and 3 κ subtypes.[3,4,8–12]

The physiological role of the different types of receptors is still a matter of considerable speculation.[5,6] For example, the μ receptor interacts preferably with morphine-like drugs, and its action is blocked by naloxone. δ receptors have a higher affinity for enkephalin but are relatively more weakly blocked by naloxone. The κ receptors were recognized by ketocyclazocine and related drugs and presumably have their endogenous peptide counterparts in the dynorphins,[5] but these endogenous peptides are not highly selective. In all cases, the stimulation of opioid receptors produces analgesia, generally by the reduction of central autonomic and endocrine response to a pain stimulus.

On the other hand, most commonly used opioids give many side effects such as respiratory depression, constipation, addiction, and many others. The structures of the opioid receptors are beginning to be determined,[13] but the receptors themselves are not yet available in a pure functional state, and hence, the physiological importance of receptor diversity still is an open question. During the past 12 years, special attention has been paid to the development of potent and receptor-selective peptide opioids in the hope of understanding the mechanism of pain development and its treatment. Since the δ-selective opioids do not demonstrate significant analgesic cross-tolerance with opiates acting on μ receptors, it is believed that δ-receptor-selective peptide opioids eventually could be clinically used for pain relief in long-term therapy or be substituted for high-dose treatment with μ opiates like morphine.[5,6] In this way, side effects such as respiratory depression and addition might be avoided. This, of course, will be true only if the δ receptor(s) do not mediate these side effects. In addition, many peptides are metabolized to polar end products easily eliminated from the body. The disadvantage of peptides lies in the fact that they usually do not cross the blood–brain barrier (BBB) readily, and thus, the analgesic effects of peptides administrated peripherally generally are poor.

Many efforts have led to the discovery of potent and selective peptide ligands for μ or δ receptors.[6–8] One of the most selective ligands for δ receptors is the 14-membered cyclic peptide [D-Pen^2,D-Pen^5]enkephalin (DPDPE) synthesized previously in our laboratory.[14] Introduction of two penicillamine residues gave a globally constrained molecule.[14,15] It is believed that the cyclization of the peptide chain via an S–S bridge helped this compound adopt a biologically active conformation highly preferred by the δ opioid receptor. Indeed, biological evaluation of DPDPE showed excellent δ-receptor-specific properties for this analog. The bioassay methods (based on electrically induced smooth muscle contraction of mouse vas deferens (MVD assay) and strips of guinea pig ileum longitudinal muscle–myenteric plexus (GPI assay)) have shown DPDPE to be over 1000 times more potent at δ receptors than at μ receptors. In binding studies, the selectivity ratio of DPDPE was over 100. In addition to high selectivity, DPDPE is highly potent—the IC_{50} values of DPDPE at δ receptors are 5.81 and 5.25 nM, respectively, in the MVD assay and in binding studies.[14]

Further improvement in the biological properties of DPDPE was achieved by the introduction of a *p*-halogen-substituted phenyalanine in the place of phenylalanine at position 4 of DPDPE.[18] The analogs thus obtained are more potent and δ-receptor-selective enkephalins than DPDPE itself, both in bioassays and in binding studies. The chloro-substituted analog has better antinociceptive properties in the hot-plate test after icv administration

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Table 1. Inhibitory Potency and Selectivity of Tyr-D-Pen-Gly-Phe(*p*-X)-Cys-Phe Analogs in GPI and MVD Assays (IC_{50} values with SSE)

peptide	assay: MVD IC_{50} (nM)	assay: GPI IC_{50} (nM)	selectivity ratio (GPI/MVD)
1, H-Tyr-D-Pen-Gly-Phe-Cys-Phe-OH (HB-P2)	0.016 ± 0.0026	82.7 ± 11	5100
2, H-Tyr-D-Pen-Gly-*p*-F-Phe-Cys-Phe-OH (HB-P21)	0.028 ± 0.0048	32 ± 5.50	1100
3, H-Tyr-D-Pen-Gly-*p*-Cl-Phe-Cys-Phe-OH (HB-P20)	0.076 ± 0.0023	36 ± 8.8	470
4, H-Tyr-D-Pen-Gly-*p*-Br-Phe-Cys-Phe-OH (HB-P22)	0.028 ± 0.0063	120 ± 11	4300
5, H-Tyr-D-Pen-Gly-*p*-I-Phe-Cys-Phe-OH (HB-P23)	0.30 ± 0.12	640 ± 110	2100
H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE)	4.1 ± 0.46[a]	7300 ± 1700[a]	1800
H-Tyr-D-Pen-Gly-Phe-Cys-OH (DPLCE)	0.32 ± 0.03[b]	210 ± 63[b]	660
H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 (deltorphin I)	0.16 ± 0.01[c]	>1500[c]	>9400

[a] Data from ref 28. [b] Data from ref 29. [c] Data from ref 30.

than DPDPE.[19] The antinociceptive effect of [^{3}H][*p*-Cl-Phe^4]DPDPE proved that this compound can cross the BBB, although the degree of penetration is small,[19] and its increased lipophilicity may explain the enhanced permeability of the molecule to the brain.[20]

The compounds presented in this study were designed as potential peptide–carrier conjugates. The carrier was design to be able to accommodate a large lipophilic fragment like cholesterol or lipid at the C-terminal which would enable it to conjugate to cross the BBB.[20,21] The phenylalanine at position 6 was to be part of a spacer between the [D-Pen^2,L-Cys^5]enkephalin (DPLCE) analogs and the carrier, and we have found that this analog provides a superpotent δ ligand. We report here on the synthesis and bioactivities of this analog of DPDPE and of several related para-halogenated Phe^4 analogs.

Results and Discussion

All of the peptides were synthesized by solid-phase peptide synthesis. The linear sulfhydryl peptides were oxidized by $K_3Fe(CN)_6$ in 0.05 M ammonium acetate buffer at pH = 8.5. The crude linear peptides were dissolved in 50 mL of methanol and added dropwise to a solution of the oxidant by means of a syringe pump[22] (see Experimental Section for details). The rate of addition was calculated to be approximately 10 mg of crude peptide/h/L of oxidant. However, in the case of the bromo and iodo analogs, the solubility of the peptides in methanol was poor, so the peptides were dissolved in a mixture of 1:1 methanol and DMSO. In this case, the oxidant solution was diluted with methanol to avoid precipitation of the peptide.

The structures and bioactivities of the compounds are summarized in Table 1 and 2. Table 3 contains their physicochemical data.

The new analogs were designed to increase the lipophilicity of DPDPE or its Cys^5-containing analogs to more readily cross the blood–brain barrier by adding a Phe^6 residue. We have further increased the lipophilicity by substituting a hydrogen at the para position in phenylalanine with a halogen. The second aim was to improve the pharmacological properties of the new analogs. As shown previously,[18] halogen substitution in [*p*-X-Phe^4]-DPDPE led to more selective and more potent ligands. The phenylalanine at position 6 of the peptide chain in this series of compounds also was designed as part of an enzymatically cleavable spacer between a cyclic enkephalin and a possible lipophilic carrier.

Table 2. Binding Affinities and Selectivities of Peptides Tested in Competition Experiments with [^{3}H]CTOP and [^{3}H][*p*-Cl-Phe^4]DPDPE in Rat Brain (IC_{50} values are given with SSE)

peptide	[^{3}H][*p*-Cl-Phe^4]DPDPE IC_{50} (nM)	[^{3}H]CTOP IC_{50} (nM)	ratio[a]
1, HB-P2	1.4 ± 0.2	280 ± 75	200
2, HB-P21	0.71 ± 0.06	110 ± 12	160
3, HB-P20	0.51 ± 0.11	83 ± 4.2	160
4, HB-P22	1.0 ± 0.20	530 ± 230	530
5, HB-P23	1.2 ± 0.36	1600 ± 280	1300
DPDPE	5.8 ± 0.90[b]	620 ± 280[b]	120[b]
DPLCE	12 ± 1.2[c]	180 ± 16[c]	15[c]

[a] IC_{50} (μ)/IC_{50} (δ). [b] Data from ref 28. [c] Data from ref 29.

Table 3. Analytical Properties of Synthetic Peptides of General Structure Tyr-D-Pen-Gly-Phe(*p*-X)-Cys-Phe

peptide (substituent)	TLC[a] R_f values				HPLC[b]		FAB-MS	
	I	II	III	IV	red.[c]	ox.[d]	calcd	obsd
1, HB-P2 (H)	0.88	0.86	0.82	0.61	24.29	24.04	765	765
2, HB-P21 (F)	0.84	0.88	0.83	0.63	25.7	24.41	783	783
3, HB-P20 (Cl)	0.84	0.87	0.83	0.65	26.74	25.24	799	799[e]
4, HB-P22 (Br)	0.85	0.86	0.83	0.65	27.07	25.38	843	843[e]
5, HB-P23 (I)	0.85	0.86	0.81	0.68	26.91	27.15	891	891

[a] Analtech silica gel plates, 0.25 mm, length of the solvent path, 8 cm. Solvent systems are as follows: I, *n*-butyl alcohol–acetic acid–water (4:1:1); II, *n*-butyl alcohol–acetic acid–pyridine–water (13:3:12:10); III, isopropyl alcohol–ammonia–water (3:1:1); and IV, *n*-butyl alcohol–acetic acid–ethyl acetate–water (1:1:1:1). [b] Retention times (min) for the following systems: Hewlett-Packard 1090, column C-18, 4.6 mm × 25 cm, Vydac 218TP104; buffer A, 100% acetonitrile; buffer B, 0.1% TFA in water; gradient from 0–50% A in 30 min, flow rate 1.0 mL/min, detection at 220, 254, and 280 nm. [c] Retention times (min) of linear nonoxidized peptides. [d] Retention times (min) of pure oxidized (cyclic) peptides. [e] The appropriate *P* + 2 peak for the Cl and Br isotopes were observed in the ratios expected.

Phenylalanine itself is a lipophilic amino acid, and indeed, the hexapeptide HB-P2 is more lipophilic than DPDPE. Also, as shown in Table 3, the retention times in RP-HPLC are larger the heavier the halogen atom (I > Br > Cl > F). This is good agreement with the higher lipophilicity of higher molecular weight halogens.[23] Of course other factors besides size and lipophilicity may be important, including the surface properties of the column support, the hydration of the halogens, their electronegativities, and other properties.

All the analogs have a high affinity for δ receptors and are among the most δ-potent compounds known. However, surprisingly, the most potent compound in this series is the Phe^6-containing hexapeptide 1 having an unsubstituted phenylalanine at position 4 and a Cys residue. This is in sharp contrast to the data obtained for *p*-halogen-Phe^4-substituted DPDPE analogs.[18] In the DPDPE study,[18] the most selective compound in the bioassay was the *p*-iodo analog, whereas the unsubstituted one had the poorest selectivity ratios values (17 400 vs 2000, respectively[18]).

In the present study, the compounds with para-substituted phenylalanine in position 4 of the peptide chain (**2**, **3**, **4**, and **5**) tested at the peripheral receptor assays are less δ-receptor selective as compared to the unsubstituted hexapeptide 1 (see Table 1). A dramatic loss of selectivity was observed for the chloro-substituted analog HB-P20 (selectivity ratios are 5100 and 470 for HB-P2 and HB-P20, respectively). The affinities of the substituted analogs for the peripheral δ receptor are lower than the affinity of the unsubstituted analog 1. However, all the ligands are still very potent and selective. The high selectivity of those ligands in the MVD test is caused mainly by the high affinities of those compounds for peripheral δ receptors. The relatively low selectivity of the *p*-chloro analog **3** is caused mainly by the high affinity of this peptide for both δ and μ peripheral receptors (IC$_{50}$ values are 0.028 and 32 nM at δ and μ receptors, respectively, Table 1).

The unsubstituted hexapeptide analog [D-Pen^2,L-Cys^5,-Phe^6]enkephalin (1, [Phe^6]DPLCE) (HB-P2) remains the most potent and selective ligand at peripheral δ receptors and to our knowledge is the most potent enkephalin analog in the MVD test reported to date.

In the binding studies, the selectivity generally increases with the molecular weight of the halogen, so that the most selective compound is [*p*-I-Phe^4,Phe^6]DPLCE, **5** (Table 2). This is in contrast with the results in the MVD test. However, the high selectivity of this analog is due primarily to its binding very weakly to brain μ opioid receptors (IC$_{50}$ vs [^{3}H]CTOP is 1600 nM), whereas the binding at the brain δ receptor remains almost unchanged (IC$_{50}$ value vs [^{3}H][*p*-Cl-Phe^4]DPDPE is 1.17 nM, Table 2). The *p*-chloro-substituted hexapeptide [*p*-Cl-Phe^4,Phe^6]DPLCE, **3**, has the poorest selectivity (similar to the results in the MVD study) which is in contrast to [*p*-Cl-Phe^4]DPDPE, where the chloro analog was the most selective.[18] The chloro-substituted analog **3** has a slightly improved binding potency as compared to the unsubstituted analog 1 at both μ and δ central receptors (see Tables 1 and 2).

It should be emphasized that the *p*-fluro and *p*-chloro analogs **2** and **3** (as compared to the unsubstituted hexapeptide 1) are slightly more potent at μ receptors, both peripheral and central, which suggests some preferences of μ receptors of these halogen-substituted phenylalanines.

Experimental Section

General Methods for Peptide Synthesis. All peptides were synthesized by the solid-phase method. Protected amino acids and chloromethylated polystyrene, 1% cross-linked with divinylbenzene (1 meq/g), were purchased from Bachem (Torrance, CA). D-Pen(S-*p*-MeBzl) was obtained from Peptide International (Louisville, KY). The C-terminal phenylalanine was attached to the resin via its Cs salt (substitution levels of 0.82 or 0.78 meq/g were achieved). Diisopropylcarbodiimide (DICI) was used as the coupling reagent, and 3 equiv of amino acid and DICI were used.

The para-substituted phenylalanines were coupled to the growing peptide chain by means of BOP in the presence of HOBT (see details below). TFA–DCM–anisole (48–50–2, v/v) was used to deprotect the N-terminal N^α-Boc amino protecting groups. The peptides were cleaved from resin by published methods.[24] The crude linear sulfhydryl peptides generally were obtained in purities greater than 90% and were oxidized directly to the cyclic analogs.

General Method of Oxidation. The peptides were oxidized by an improved method developed previously in this laboratory.[22] Briefly, the peptide (usually 500–600 mg) was dissolved in 50 mL of methanol and added via a syringe pump to a well-stirred solution of oxidant. The oxidant solution was prepared by dissolving potassium ferricyanide (K$_3$Fe(CN)$_6$) in 4-fold excess in 0.05 M ammonium acetate buffer, pH 8.5. The use of buffer allowed easy maintenance of pH, so pH control was unnecessary. The best results were obtained when the rate of peptide addition was calculated to be approximately 10 mg of peptide/h/L of oxidant. The formation of dimers was not observed.

However, in the synthesis of the bromo and iodo analogs **4** and **5**, the limited solubility of the peptides created some problems. In those cases, the peptide was dissolved in a 1:1 mixture of methanol and DMSO and the oxidant solution was modified by the addition of methanol (1:3) to avoid precipitation of the linear peptide before oxidation could occur. However, the yields were lower, and the formation of small amounts of polymeric materials was observed. The replacement of DMSO by dioxane did not improve the yield nor the purity.

After the addition of the peptide was completed, the reaction mixture was stirred for an additional 5–6 h and acidified by acetic acid. The ion-exchange resin Amberlite IRA-45 (Cl$^-$ form) was added to remove the ferro- and ferricyanide ions. The resin was filtered off, and the solution was concentrated in vacuo at temperatures below 40 °C and then lyophilized. The powder thus obtained was dissolved in glacial acetic acid, filtered to remove the inorganic salts formed, and then lyophilized.

The crude peptides were purified by preparative HPLC on C-18 columns (Vydac 218TP152050), 5 cm × 25 cm, using a Rainin HPXL instrument with detection at 220 and 254 nm. The purity of the peptides was checked by analytical HPLC (C-18 column, Vydac 218TP104, 4.6 mm × 25 cm) using a Hewlett-Packard 1090 instrument (detection at 220, 254, and 280 nm) with 0.1% aqueous TFA and a 0–50% acetonitrile gradient over 30 min. The chromatograms were analyzed by a computer program provided by the manufacturer of the Hewlett-Packard 1090 instrument, and the purified peptides were shown to >98% pure. TLC was done in four solvent systems on silica gel (detection by ninhydrin and iodine vapors) and indicated that the peptides were >98% pure. The amino acid analyses were performed at the University of Arizona Biotechnology Core Facility. The system used was a dedicated Applied Biosystem Model 420A amino acid analyzer with automatic hydrolysis (vapor phase at 160 °C for 1 h 40 min using 6 N HCl) and precolumn phenylthiocarbamoyl–amino acid (PTA-AA) analysis (D-Pen could not be detected). FAB-MS spectra were in agreement with the amino acid sequence and the composition of each analog. The analytical data of the compounds synthesized in this paper are given in Table 3.

[D-Pen^2,Cys^5,Phe^6]enkephalin (H-Tyr-D-Pen-Gly-Phe-Cys-Phe-OH, HB-P2, 1). The title compound was obtained by stepwise elongation of the peptide resin by the method outlined bove starting from 2 g of N^α-Boc-Phe-O-resin (substitution level 0.78). The following amino acids were added to the growing peptide chain: Boc-Cys(S-*p*-MeBzl), Boc-Phe, Boc-Gly, Boc-D-Pen(S-*p*-MeBzl), and Boc-Tyr(2,6-Cl$_2$-Bzl). After the last amino acid was coupled, the Boc group was removed by TFA and the resin was washed several times with DCM and dried overnight under vacuum; yield was 3.2 g. The peptide resin was mixed with 3.5 mL of a 1:1 mixture of cresol and *p*-thiocresol, and then, approximately 32 mL of liquid HF was added.[24] The reaction mixture was stirred for 1 h at 0 °C, and then, the HF was evaporated off in vacuo. The resin was dried, and then, 100 mL of dry ether was added. The resin and precipitated peptide were filtered off, washed four times with 50 mL of ether, and dried by passing through dry air, and the peptide was dissolved in acetic acid (4 × 30 mL). The acetic acid solutions were pooled and lyophilized; yield was 930 mg. The crude sulfhydryl peptide was oxidized as described above. The crude cyclic peptide was purified by preparative HPLC; gradient: 15 min of 0.1% TFA and then 0–60% acetonitrile in 180 min at a flow rate of 40 mL/min. The main fraction was concentrated under diminished pressure and lyophilized; yield was 98 mg of a white powder. More peptide was obtained in a less pure state (about 220 mg with purity 94–95%), and those fractions were repurified under the same conditions, yielding an additional 153 mg of the title compound. The column was washed with pure acetonitrile which removed the more lipophilic impurities. Amino acid analysis: Tyr 0.91 (1.0), Gly 1.11 (1.0), Phe 2.02 (2.0), Cys 0.69 (1.0). Analytical data are found in Table 3.

[D-Pen^2,*p*-F-Phe^4,Cys^5,Phe^6]enkephalin (H-Tyr-D-Pen-Gly-Phe(*p*-F)-Cys-Phe, HB-P21,2). This compound was synthesized in the same manner as described above starting from 2 g of resin. However, Boc-*p*-F-Phe (1.2 equiv) was coupled with BOP (1.2 equiv), HOBT (1.2 equiv), and DIPEA (4 equiv) in *N*-methylpyrrolidinone. The yield of peptide resin was 3.0 g, yield of crude sulfhydryl peptide 780 mg, and yield of pure cyclic peptide 98 mg. Amino acid analysis: Tyr 0.79 (1.0), Gly 1.09 (1.0), *p*-F-Phe 0.93 (1.0), Cys 0.62 (1.0), Phe 1.18 (1.0). The other analytical data are given in Table 3.

[D-Pen^2,*p*-Cl-Phe^4,Cys^5,Phe^6]enkephalin (H-Tyr-D-Pen-Gly-Phe(*p*-Cl)-Cys-Phe, HB-P20, 3). The peptide was synthesized and purified in a similar manner as the compound described above starting from 2 g of resin. However, Boc-*p*-Cl-Phe (1.2 equiv) was coupled with BOP (1.2 equiv), HOBT (1.2 equiv), and DIPEA (4 equiv) in *N*-methylpyrrolidinone.[25] Yield of peptide resin was 3.13 g, yield of crude linear peptide 780 mg, and yield of pure cyclic compound 132 mg. Amino acid analysis: Tyr 0.93 (1.0), Gly 1.07 (1.0), Phe(*p*-Cl) 1.11 (1.0), Cys 0.64 (1.0), Phe 1.1 (1.0). The other analytical data are given in Table 3.

[D-Pen^2,Phe(*p*-Br)^4,Cys^5,Phe^6]enkephalin (H-Tyr-D-Pen-Gly-Phe(*p*-Br)-Cys-Phe, HB-P22, 4). The title compound was synthesized as described above from 1.5 g of resin. Yield of peptide resin was 2.18 g, yield of crude linear peptide 820 mg and yield of pure peptide 78 mg. Amino acid analysis: Tyr 0.78 (1.0), Gly 1.0 (1.0), Cys 0.64 (1.0), Phe 1.0 (1.0). Additional analytical data are given in Table 3.

[D-Pen^2,Phe(*p*-I)^4,Cys^5,Phe^6]enkephalin (H-Tyr-D-Pen-Gly-Phe(*p*-I)-Cys-Phe, HB-P23, 5). This peptide was synthesized in the same manner as the analogs described above starting from 1.5 g of resin (substitution level 0.96 meq/g). Yield of peptide resin was 2.83 g; HF cleavage yielded 905 mg of crude peptide. After oxidation and purification, the yield was 112 mg. Amino acid analysis: Tyr 0.88 (1.0), Gly 1.11 (1.0), Cys 0.6 (1.0), Phe 1.0 (1.0). Additional analytical data are given in Table 3.

Radioligand Binding Methods. Membranes were prepared from whole (less cerebellum) brain taken from adult male Sprague–Dawley rats (250–300) obtained from Harlan Sprague–Dawley, Inc. Following decapitation, the brain was removed, dissected, and homogenized at 0 °C in 20 volumes of 50 mM Tris-HCl buffer adjusted to pH 7.4 using a Teflon glass homogenizer. The membrane fraction obtained by centrifugation at 48 000*g* for 15 min at 4 °C was resuspended in 20 volumes of fresh Tris buffer and incubated at 25 °C for 30 min to dissociate any receptor-bound endogenous opioid peptides. The incubated homogenate was centrifuged again and the final pellet resuspended in 20 volumes of fresh Tris buffer. Radioligand-binding inhibition assay samples were prepared in an assay buffer consisting of 50 mM Tris-HCl, 1.0 mg/mL bovine serum albumin, 30 μM bestatin, 50 μg/mL bacitracin, 10 μM captopril, and 0.1 mM toluenesulfonyl fluoride, pH 7.4. The radioligands used were [^{3}H][D-Pen^2,*p*-Cl-Phe^4,D-Pen^5]enkephalin at a concentration of 0.75 nM and [^{3}H]CTOP (New England Nuclear, Boston, MA) at a concentration of 0.5 nM. Peptide analogs were dissolved in assay buffer prior to each experiment and added to duplicate assay tubes at 10 concentrations over an 800-fold range. Control (total) binding was measured in the absence of any inhibitor while nonspecific binding was measured in the presence of 10 μM naltrexone. The final volume of the assay samples was 1.0 mL of which 10% consisted of the membrane preparation in 0.1 mL of TrisHCl buffer. Incubations were performed at 25 °C for 3 h after which the samples were filtrated through poly(ethyleneimine)-treated GF/B glass fiber filter strips. The filtrates were washed three times with 4.0 mL of ice-cold normal saline before transfer to scintillation vials. The filtrate radioactivity was measured after adding 10 mL of cocktail consisting of 16 g of Crystal Fluor (West Chemical, San Diego, CA) in 1.0 L of Triton X-100 and 2.0 L of toluene to each vial and allowing the samples to equilibrate over 8 h at 4 °C. The data were analyzed by using nonlinear least-squares regression analysis on the Apple II Plus computer. Programs were generously written by Susan Yamamura. Each assay was repeated at least three times in duplicate for an $n = 6$ or more.

GPI and MVD Bioassays. Electrically induced smooth muscle contractions of mouse vas deferens and strips of guinea pig ileum longitudal muscle–myenteric plexus were used as a bioassay.[26] Tissues came from male ICR mice weighing 25–40 g and from male Hartley guinea pigs weighing 250–500 g. The tissues were tied to a gold chain with suture silk, suspended in 20-mL baths containing 37 °C oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (magnesium free for the MVD), and allowed to equilibrate for 15 min. The tissues were then stretched to optimal length, previously determined to be 1-g tension (0.5 g for MVD), and allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum wire electrodes at 0.1 Hz, 0.4-ms pulses (2.0-ms pulses for MVD), and supramaximal voltage. Drugs were added to the baths in 14–60-μL volumes. The agonists remained in contact with the tissue for 3 min before the addition of the next cumulative doses, until maximum inhibition was reached. Percent inhibition was calculated by using the average contraction height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure to the dose of the agonist. IC_{50} values represent the mean of not less than four tissues. IC_{50} estimates, relative potency estimates, and their associated standard errors were determined by fitting the mean data to the Hill equation by using a computerized nonlinear least-squares method. Each assay was repeated at least three times in duplicate for an $n = 6$ or more.

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(1) Symbols and abbreviations are in accord with the recommendation of the IUPAC-IUB Commission on Nomenclature (*J. Biol. Chem.* 1972, *247*, 977–989). The optically active amino acids are of L-chirality unless otherwise noted. Other abbreviations included mean: BOP, benzotriazolyltris(dimethylamino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; HOBT, hydroxybenzotriazole; N^α-Boc, N^α-*tert*-butyloxycarbonyl; Pen, penicillamine; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; DPLCE, [D-Pen^2,L-Cys^5]enkephalin; DICI, diisopropylcarbodiimide; DCM, dichloromethane; DIPEA, diisopropylethylamine; and CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2.

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