Design of Cyclic Deltorphins and Dermenkephalins with a Disulfide Bridge Leads to Analogues with High Selectivity for δ-Opioid Receptors[1,2]

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We earlier suggested that the low receptor selectivity observed for previously synthesized constrained analogues of deltorphin I (DT I) was the result of a reduction in the lipophilic surface of the C-terminal of the peptide. To confirm this prediction and to further test a previously proposed conformational model for bioactivity at delta opioid receptors, we have synthesized several new cyclic analogues with the general structure [D-Xaa^2,Yaa^5]deltorphin I and II in which Xaa^2 is D-cysteine or D-penicillamine (D-Pen), and Yaa^5 is an L- or D-penicillamine residue. Additional substitutions at positions 4, 6, and 7 also were examined. The analogues were tested for binding to μ- and δ-opioid receptors and in mouse vas deferens and guinea pig ileum biological assays. The introduction of a lipophilic L-Pen in position 5 and D-Cys or D-Pen in position 2 resulted in a highly δ-selective series of analogues, which fully confirmed our prediction. The cyclic analogues [D-Pen^2,Pen^5]DT I and [D-Pen^2,Pen^5,Nle^6]DT I are among the most δ-selective analogues described thus far.

Introduction

The deltorphins are the linear heptapeptides isolated from the skin of the Phyllomedusa bicolor frog.[3] These peptides express high affinity for the δ receptor[4] and possess potent antinociceptive effects.[5] The presence of an aspartic acid residue in position 4 of the peptide chain of deltorphin I (DT I) and of a glutamic acid residue in the same position of deltorphin II (DT II) is the only difference between these peptides. A third deltorphin, called deltorphin A[6] or dermenkephalin,[7] has similar δ-opioid receptor selectivity but a significantly different sequence than deltorphins I and II. Nevertheless it has been suggested that the deltorphins and dermenkephalin possess common topographical features.[8] It has been suggested previously[7,9-13] that the C-terminal hydrophobic tripeptide is necessary for the high δ-opioid receptor selectivity of deltorphin and dermenkephalin analogues. NMR studies by Balboni et al.[14] suggested that the Asp residue is important when it is placed in the fourth position, since it can stabilize a β-turn conformation of the putative message domain by means of an electrostatic interaction with the N-terminal α-amino group, a feature that is beneficial to both μ and δ activities. Structure studies of deltorphin analogues by Lazarus et al.[6] pointed out that the location of the charged groups relative to the hydrophobic residues in the address domain of these peptides might be critical determinants for both δ affinity and selectivity. In addition, our recent studies have suggested that δ selectivity of all three deltorphins requires a well defined lipophilic surface, in the C-terminal region, and a hydrophilic surface, which we referred to as "hot dog"-like, in its three dimensional structure.[8]

The deltorphins are relatively small, conformationally flexible peptides, and the question arises as to which of the conformations are biologically relevant.[15] Complementary methods such as the quantum and molecular mechanics, spectroscopic and structural modeling analysis, on the one hand, and biological analysis of synthetic analogues on the other, are necessary tools to form a working hypothesis of the structural and conformational elements necessary for peptide biological activity and/or selectivity at a particular receptor. One of the most successful SAR approaches for peptide analogues has been the creation of constrained cyclic analogues.[15,16] The successful creation of constrained analogues and the development of more receptor-selective analogues often has involved introduction of S–S bridges between sulfur-containing amino acids incorporated into the peptide chain. In opioid peptides such modifications have provided analogues with high selectivity for μ-,[17-19] δ-,[20-23] and κ-opioid[24] receptor types.

Reducing the number of possible conformers of linear peptides through cyclization was used in one of the first reported SAR studies of deltorphin.[10,11] The two cyclic analogues were less potent in the one δ receptor assay (MVD) used and showed dramatic decreases in receptor selectivity but provided important information about δ receptor requirements. We have used energy-minimization approaches, as well as independent intuitive approaches based on model building, to examine the possible similarities between opioid peptides and benzomorphan alkaloids.[8,20,25-30] These approaches led to structurally different models, but they have common short distances between the side-chain residues in the 2- and 5-positions. The results from these studies have stimulated us to synthesize constrained analogues of the deltorphins and dermenkephalins in which the side chains of amino acids in positions 2 and 5 were connected through a disulfide bridge. The first analogue of this series, [D-Cys^2,Cys^5]-DTI, 4, was highly potent for δ receptor types but was nonselective as a result of significant increases of affinity for μ receptor types.[25] In our proposed model of "δ active"

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Table 1. Binding Affinities of Deltorphins and Dermenkephalin Analogues

compound	IC_{50} ± SEM (nM) δ[a]	IC_{50} ± SEM (nM) μ[b]	selectivity ratios: μ/δ
1, Tyr-D-Ala-Phe-Asp-Val-Val-GlyNH$_2$ (DT I)	0.60 ± 0.30[8]	2140 ± 690[8]	3570
2, Tyr-D-Ala-Phe-Glu-Val-Val-GlyNH$_2$ (DT II)	0.73 ± 0.33[8]	1680 ± 430[8]	2300
3, Tyr-D-Met-Phe-His-Leu-Met-AspNH$_2$ (DERM)	0.47 ± 0.10[8]	1900 ± 160[8]	4000
4, Tyr-D-Cys-Phe-Asp-Cys-Val-GlyNH$_2$	0.87 ± 0.04[25]	5.15 ± 0.55[25]	5.9
5, Tyr-D-Cys-Phe-Asp-Pen-Val-GlyNH$_2$	2.2 ± 0.46	3760 ± 600	1700
6, Tyr-D-Pen-Phe-Asp-Pen-Val-GlyNH$_2$	3.7 ± 0.9	26000 ± 250	7000
7, Tyr-D-Pen-Phe-Asp-D-Pen-Val-GlyNH$_2$	210 ± 52	1200 ± 170	5.6
8, Tyr-D-Pen-Phe-Glu-Pen-Val-GlyNH$_2$	5.7 ± 0.7	4900 ± 900	860
9, Tyr-D-Pen-Phe-His-Pen-Nle-AspNH$_2$	3.4 ± 0.53	10200 ± 1400	3000
10, Tyr-D-Pen-Phe-Ser-Pen-Val-GlyNH$_2$	10 ± 1.8	3300 ± 580	330
11, Tyr-D-Pen-Phe-Asp-Pen-Nle-GlyNH$_2$	4.83 ± 1.3	54600 ± 3500	11400
12, Tyr-D-Pen-Phe-His-Pen-Nle-Asp-OH	5.11± 1.2	1900 ± 300	360

[a] Versus [^{3}H][*p*-Cl-Phe^4]DPDPE. [b] Versus [^{3}H]CTOP.

Table 2. Bioassays of the Deltorphins and Dermenkephalin Analogues

compound	IC_{50} (nM) MVD	IC_{50} (nM) GPI	selectivity ratios: GPI/MVD
1, Tyr-D-Ala-Phe-Asp-Val-Val-GlyNH$_2$ (DT I)	0.36 ± 0.04[8]	2890 ± 250[8]	8000.
2, Tyr-D-Ala-Phe-Glu-Val-Val-GlyNH$_2$ (DT II)	0.67 ± 0.17[8]	3180 ± 240[8]	4700.
3, Tyr-D-Met-Phe-His-Leu-Met-AspNH$_2$ (DERM)	0.28 ± 0.02[8]	3400 ± 610[8]	12000.
4, Tyr-D-Cys-Phe-Asp-Cys-Val-GlyNH$_2$	0.23 ± 0.04[25]	2.98 ± 0.37[25]	13.
5, Tyr-D-Cys-Phe-Asp-Pen-Val-GlyNH$_2$	0.25 ± 0.05	1100 ± 580	4400.
6, Tyr-D-Pen-Phe-Asp-Pen-Val-GlyNH$_2$	6.30 ± 1.40	68000 ± 8900	11000.
7, Tyr-D-Pen-Phe-Asp-D-Pen-Val-GlyNH$_2$	920 ± 130	5500 ± 1600	6.0
8, Tyr-D-Pen-Phe-Glu-Pen-Val-GlyNH$_2$	2.24 ± 0.39	7100 ± 1900	3200.
9, Tyr-D-Pen-Phe-His-Pen-Nle-AspNH$_2$	12.70 ± 1.16 (E_{max} = 75%)	22000 ± 4100	1700.
10, Tyr-D-Pen-Phe-Ser-Pen-Val-GlyNH$_2$	91.8 ± 20	13500 ± 2800	150.
11, Tyr-D-Pen-Phe-Asp-Pen-Nle-GlyNH$_2$	8.80 ± 1.30	137000 ± 6900	16000.
12, Tyr-D-Pen-Phe-His-Pen-Nle-Asp-OH	43.5% at 6000	4500 ± 950	<1.5

conformation we stressed the importance of the lipophilic surface at the C-terminus of deltorphin for δ selectivity. The importance of the lipophilicity of amino acid residues in positions 5 and 6 also has been explicitly and implicitly suggested by a number of other authors,[7,9-13] and thus we have hypothesized that increasing the lipophilicity of the amino acid residue in position 5 to simulate valine or leucine might increase receptor selectivity of the cyclic deltorphin analogues. To further check this hypothesis, to evaluate the effect cyclization on δ-opioid receptor potency and selectivity, and to analyze how increases in the lipophilicity of an amino acid in position 5 might influence the opioid receptor affinity and selectivity of deltorphins and dermenkephalin, we have synthesized and tested cyclic deltorphin analogues with a lipophilic penicillamine residue in position 5, and various substitutions in positions 2, 6, and 7 in the two deltorphins and in dermenkephalin.

Results and Discussion

The peptides synthesized in this work were prepared by the solid-phase method of peptide synthesis following procedures similar to those previously used for other deltorphin peptides.[8,27] Our unpublished results and results reported by others[6] suggested that the Met^6 residue of dermenkephalin could be replaced with Nle. Therefore, in some analogues we have introduced a norleucine-6 as a substitute for methionine-6. The receptor potencies and selectivities of the deltorphins and their analogues were evaluated in vitro by both receptor binding to rat brain homogenates and by bioassays that measured the ability of the peptides to inhibit electrically evoked contractions of the myenteric plexus longitudinal muscle preparation of the guinea pig ileum (GPI) (μ receptor preferring) and of the mouse vas deferens (MVD) (δ receptor preferring).[31]

The substitution of Cys^5, in the previously reported [D-Cys^2,Cys^5]DT I^{25}, with L-Pen^5 (compound **5**) resulted in an analogue with somewhat reduced binding potency compared to **4** but a dramatic 240-fold increase in selectivity for the δ receptor (Table 1). The binding affinity for δ receptors was similar to the analogue containing Cys^5 ^{25} (less than 3-fold difference; compare **4** and **5**, Table 1). Thus the increased selectivity is primarily due to a loss of affinity at the μ opioid receptor. The substitution of D-Cys^2 with D-Pen^2 (compare **5** and **6**) resulted in somewhat reduced δ binding affinity as was generally the case for the other cyclic analogues and a larger decrease in potency in the MVD bioassay (compounds **5** and **6**, Table 2). Again, the decrease in affinity for μ receptors was much more dramatic. As a result, the deltorphin I analogues **6** and **11** have among the highest δ receptor

selectivities known in binding assays. Similar selectivities were observed in the MVD vs GPI bioassays for linear Atc[3]-substituted deltorphin I analogues by Schiller et al.[11] Indeed compounds 6 and 11 interact very weakly with the μ receptor. The respective D-Pen[2],Pen[5] cyclic deltorphin II analogue 8 and the dermenkephalin analogue 9 possess reasonably high δ receptor selectivities in both binding and bioassays (Tables 1 and 2). This provides additional support for a common "δ active" topographical model for the two deltorphins and dermenkephalin as previously suggested. The chirality of the amino acid in position 5 was previously shown to be important for δ receptor potency in linear dermenkephalins[12] and clearly is very important for δ receptor selectivity in the cyclic compounds. The substitution of L-Pen[5] with D-Pen[5] to give compound 7 resulted in a 600-fold *decrease* in δ binding selectivity and 1800-fold in the bioassays. This is a consequence both of a decrease in affinity (activity) for the δ receptor (Table 1 and 2) and an increased affinity (activity) for the μ receptor in both assays. Interestingly, the analogue of DERM with a free C-terminal carboxyl group (compound 12) possessed almost equal affinity for δ receptors as the analogue with a C-terminal amide (compound 9) (Table 1), but a 5-fold increase in activity in μ binding activity and in the μ receptor bioassay was noticed (Table 2). This preservation of δ binding affinity along with an increased affinity for μ receptors is contrary to the previously reported significant decrease in both δ and μ affinities of respective linear dermenkephalin analogues with free carboxyl groups at the C-terminus.[6] It is interesting to note, however, that 12 has very weak activity in the MVD bioassay. It is very likely that the disulfide bridge has stabilized the bioactive conformation of the peptide chain. In linear analogues, the C-terminal carboxyl group may mask the number of active conformations by ionic interactions with basic groups of amino acids in positions 1 and 4 of dermenkephalin.

Summary

A series of disulfide-containing analogues of dermenkephalin (DERM), deltorphin I (DT I), and deltorphin II (DT II) has been designed and synthesized in an effort to examine a previously proposed "bioactive conformation" for these compounds and to stabilize the proposed bioactive conformation of these linear peptides by construction of a δ receptor compatible cyclic system. On the basis of preliminary modeling studies, these cyclic analogues can form conformations compatible with those we previously proposed for the deltorphins and dermenkephalins.[25,29,30] Both D-Cys[2] and D-Pen[2] residues were used in the 2-position to replace D-Met[2] in dermenkephalin and D-Ala[2] in DT I and DT II. In position 5, the Leu[5] of dermenkephalin and the Val[5] of deltorphin I and deltorphin II were replaced by D-Pen[5] or L-Pen[5] and the disulfide bridge was closed. Generally, L-Pen[5]-substituted analogues were both much more potent in binding to δ receptors and in biological activity in the MVD (δ) assay than D-Pen[5] analogues, and they also were much more selective. However their potencies were reduced relative to the native ligands. These results suggest that both stereochemical and topographical relationships are critical to δ receptor binding of these compounds and especially in distinguishing those properties important to δ- vs μ-opioid receptor recognition. More extensive biophysical work will be needed to determine precisely what these differences might be. The new, more stable cyclic dermenkephalin and deltorphin analogues reported should provide powerful tools for further development of δ receptor ligands of increased specificity, stability, and potency.

Experimental Section

Molecular Models. The models used in this study for examining the possible "bioactive conformation" are those previously reported.[25,29,30] The conformation for the disulfide-containing analogues were created by extending side chain of D-alanine[2] and valine[5] with the respective sulfide-containing side chain. Good overlaps with previously suggested models for the "δ active conformation" could be obtained.

Peptide Synthesis. t-Boc-Tyr(O-BzlCl$_2$)-D-Pen(S-pMeBzl)-Phe-Asp(β-O-Chx)-Pen(S-pMeBzl)-Val-Gly-Resin. *p*-Methylbenzhydrylamine (pMBHA) resin (0.7 mequiv/g of resin) (Bachem, Torrance, CA) was used as the solid-phase support. The synthesis was accomplished on a 9500 Peptide Synthesizer (MilliGen/Biosearch, Novato, CA).

The above resin (1.0 g) was placed into a solid-phase synthesis reaction vessel and Boc-Gly, Boc-Val, Boc-Pen(S-pMeBzl), Boc-Asp(β-O-Chx), Boc-Phe, Boc-D-Pen(S-pMeBzl), and Boc-Tyr-(2,6-diCl-Bzl) were then incorporated into the peptide resin sequentially according to the protocol for N^{α}-Boc amino acids similar to those previously used in our laboratory for other peptides[8,25-27,32] to yield N^{α}-Boc-Tyr(O-BzlCl$_2$)-D-Pen(S-pMeBzl)-Phe-Asp(β-O-Chx)-Pen(S-pMeBzl)-Val-Gly-Resin.

Tyr-D-Pen-Phe-Asp-Pen-Val-GlyNH$_2$·TFA (6). The protected peptide-resin 1 (2.0 g) was reacted with 15 mL of anhydrous HF, 2 mL of cresol/thiocresol (1:1, v/v) at 0 °C for 1 h in the HF-reaction apparatus (Peninsula Lab. Inc., Belmont, CA). The mixture was evaporated in vacuo, and the dried mixture of free peptide and resin was washed three times with 30-mL portions of ether which was then discarded. The resin was then extracted three times with 50-mL portions of 30% acetic acid. The combined extracts were then lyophilized to a powder. This powder, containing the free sulfhydryl form of the peptide, was dissolved in 20 mL of methanol. The methanol solution was slowly added (using a syringe pump with the speed of 1 mL/h) into 400 mL of distilled water (the pH was adjusted to 8.5 with 3 N ammonium hydroxide) containing 0.7 mmol of $K_3Fe(CN)_6$. After 26 h, the pH was decreased to 5 with a few drops of glacial acetic acid, and the ferrocyanide and excess ferricyanide were removed by stirring the solution with 30 mL (settled volume) of anion-exchange resin Amberlite IRA-45 (Cl^- form). After the mixture was stirred for 30 min, the resin was filtered off and washed with aqueous acetic acid. The completion of the oxidation was evaluated by HPLC (Hewlett-Packard 1090 monitored at 230, 254, and 280 nm) of the filtrate using a Hewlett-Packard Model 1090 HPLC system with simultaneous evaluation at 230, 254, and 280 nm. The aqueous acetic acid solution of the crude product was lyophilized.

The solid powder of the crude product was dissolved in ca. 10 mL of methanol and was purified by preparative reverse-phase high-pressure liquid chromatography (HPXL, Rainin, MA) in a 0.05% aqueous TFA, acetonitrile gradient system. After the proper fractions were collected, the organic solvent was removed by rotary evaporation and the aqueous solution lyophilized. Yield: 191 mg (28%) of the homogeneous title compound. FAB-MS: calcd [M + H] 860, found 860. Amino acid analysis: Asp 0.95 (1.0), Gly 1.00 (1.0), Phe 1.05 (1.0), Tyr 0.92 (1.0), Val 0.83 (1.0). TLCs: R_f(A) = 0.68 (1-butanol/acetic acid/water, 4:1:1); R_f(B) = 0.80 (1-butanol/acetic acid/pyridine/water, 13:3:12:10); R_f(C) = 0.85 (2-propanol/ammonia/water, 4:1:1); R_f(D) = 0.61 (1-butanol/acetic acid/ethyl acetate/water, 5:1:3:1). HPLC capacity factor (k') = 8.33 (Vydac 218TP104C18 reverse phase column, 25 × 0.46 cm, with 0.1% TFA/acetonitrile gradient from 85/15 to 55/45, v/v in 30 min at a flow rate of 1.5 mL/min.)

Tyr-D-Cys-Phe-Asp-Pen-Val-GlyNH$_2$·TFA (5). The compound was prepared in the same manner as peptide 6. FAB-MS: calcd [M + H] 832, found 832. Amino acid analysis: Asp 1.00 (1.0), Cys 0.81 (1.0), Gly 1.00 (1.0), Phe 1.03 (1.0), Tyr 0.80 (1.0), Val 0.88 (1.0). TLC's: R_f(A) = 0.61; R_f(B) = 0.79; R_f(C) = 0.75; R_f(D) = 0.51. HPLC k' = 6.71.

Tyr-D-Pen-Phe-Asp-D-Pen-Val-GlyNH$_2$·TFA (7). The compound was prepared in the manner as peptide 6. FAB-MS: calcd [M + H] 860, found 860. Amino acid analysis: Asp 1.00 (1.0), Gly 1.05 (1.0), Phe 1.06 (1.0), Tyr 0.94 (1.0), Val 0.94 (1.0). TLC's: R_f(A) = 0.68; R_f(B) = 0.80; R_f(C) = 0.85; R_f(D) = 0.61. HPLC k' = 8.33.

Tyr-D-Pen-Phe-Glu-Pen-Val-GlyNH$_2$·TFA (8). The compound was prepared by the same methods as peptide 6. FAB-MS: calcd [M + H] 874, found 874. Amino acid analysis: 0.98 (1.0), Gly 1.00 (1.0), Phe 1.05 (1.0), Tyr 0.82 (1.0), Val 0.77 (1.0). TLC's: R_f(A) = 0.68; R_f(B) = 0.80; R_f(C) = 0.78; R_f(D) = 0.61. HPLC k' = 7.68.

Tyr-D-Pen-Phe-His-Pen-Nle-AspNH$_2$·TFA (9). The compound was prepared by the same methods as peptide 6. FAB-MS: calcd [M + H] 954, found 954. Amino acid analysis: Asp 1.05 (1.0), His 0.93 (1.0), Phe 1.00 (1.0), Tyr 0.85 (1.0). TLC's: R_f(A) = 0.44; R_f(B) = 0.76; R_f(C) = 0.76; R_f(D) = 0.19. HPLC k' = 8.31.

Tyr-D-Pen-Phe-Ser-Pen-Val-GlyNH$_2$·TFA (10). The compound was prepared by the same methods as peptide 6. FAB-MS: calcd [M + H] 832, found 832. Amino acid analysis: Gly 1.00 (1.0), Phe 1.02 (1.0), Ser 0.91 (1.0), Tyr 0.79 (1.0), Val 0.71 (1.0). TLC's: R_f(A) = 0.66; R_f(B) = 0.82; R_f(C) = 0.86; R_f(D) = 0.57. HPLC k' = 7.71.

Tyr-D-Pen-Phe-Asp-Pen-Nle-GlyNH$_2$·TFA (11). The compound was prepared by the same methods as peptide 6. FAB-MS: calcd [M + H] 874, found 874. Amino acid analysis: Asp 1.04 (1.0), Gly 1.00 (1.0), Phe 1.09 (1.0), Tyr 0.89 (1.0). TLC's: R_f(A) = 0.62; R_f(B) = 0.79; R_f(C) = 0.84; R_f(D) = 0.70. HPLC k' = 8.94.

Tyr-D-Pen-Phe-His-Pen-Nle-Asp·TFA (12). The compound was prepared by the same methods as peptide 6. FAB-MS: calcd [M + H] 955, found 955. Amino acid analysis: Asp 1.09 (1.0), His 0.98 (1.0), Phe 1.00 (1.0), Tyr 0.79 (1.0). TLC's: R_f(A) = 0.44; R_f(B) = 0.80; R_f(C) = 0.70; R_f(D) = 0.22. HPLC k' = 7.88.

Radioreceptor Binding Assay. Adult male Sprague–Dawley rats (200–300 g) were sacrificed, their brains were immediately removed and placed on ice, and the membranes were prepared as previously reported.[33] Whole brain was homogenized in 20 volumes of 50 mM Tris-HCl stock buffer (pH = 7.4) with a glass–Teflon homogenizer. The homogenate was centrifuged (48000g for 15 min), resuspended, and preincubated (25 °C for 30 min) to remove endogenous opioids. The homogenate was centrifuged and resuspended again (0.5% final concentration). Binding affinities of all of the analogues were measured against [^{3}H][p-ClPhe^4]DPDPE (42.7 Ci/mmol)[33] and [^{3}H]CTOP (64.1 Ci/mmol)[19,34] (New England Nuclear, Boston, MA) by a rapid filtration technique. A 100-μL aliquot of the rat brain homogenate was incubated at 25 °C for 180 min with either 0.75 nM [^{3}H][p-ClPhe^4]DPDPE or 0.5 nM [^{3}H]CTOP in a total volume of 1 mL of 50 mM Tris-HCl pH (pH = 7.4) containing bovine serum albumin (1 mg/mL), bacitracin (50 μg/mL), bestatin (30 μM), captopril (10 μM), and 0.1 mM toluenesulfonyl fluoride and were done in duplicate. Naltrexone hydrochloride (10 μM) was used to define nonspecific tissue binding. The binding reaction was terminated by rapid filtration through presoaked (0.5% polyethylenimine solution) GF/B Whatman glass fiber strips with a Brandel Cell Harvester followed immediately by three rapid washes with 4-mL aliquots of ice-cold saline solution. The filters were removed and soaked in 10 mL of scintillation fluid at 4 °C for at least 6 h before bound radioactivity was measured. All experiments were done in duplicate, and at least three experiments were done for each ligand. Thus n = 6 or more for each new compound assayed. The data was analyzed by a nonlinear least-squares regression analysis computer program. The program was generously written by Susan Yamamura.

GPI and MVD in Vitro Bioassays. The bioassays of all of the analogues were based on electrically induced smooth muscle contractions of mouse vas deferens and strips of guinea pig ileum longitudinal muscle myenteric plexus.[31] Tissues came from male ICR mice weighing 25–30 g and from male Hartley guinea pigs weighing 150–400 g. The tissues were first tied to gold chains 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 and allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum plate electrodes at 0.1 Hz, 0.4-ms pulses (2.0-ms pulses for MVD) at supramaximal voltage. Drugs were added to the baths in 14–60 μL volumes to produce cumulative dose–response curves. The peptidase inhibitor cocktail contained 3.1 mg/mL bestatin, 5 mg/mL bacitracin, and 2.2 mg/mL captopril. Twenty microliters of each inhibitor was added to the bath 3 min prior to the initial aliquot of the test drug. Percent inhibition was calculated by using an average contraction height for 1 min preceding the addition of the peptide divided by contraction height 3 min after the exposure to the peptide. All experiments were done in duplicate and repeated two or three times. IC_{50} values are the mean of not less than four tissues. IC_{50} estimates and their associated standard errors were determined by fitting the mean data to the Hill equation using a computerized least-squares method.[35] The standard errors of the IC_{50} values for μ activity appear large in some cases, as the weak μ agonist character of these analogues did not permit completion of dose–response curves in the GPI.

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References

(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission on Nomenclature (*J. Biol. Chem.* 1972, *247*, 977–983). All optically active amino acids are of L variety unless otherwise noted. Other abbreviations used are Nle, norleucine; Pen, penicillamine; TFA, trifluoroacetic acid; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; DICI, diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole; NAI, *N*-acetylimidazole; pMeBzl, *p*-methylbenzyl; Chx, cyclohexyl; HPLC, high-performance liquid chromatography.

(2) A preliminary report of some of this work was presented at the 22nd European Peptide Symposium, Interlaken, September 1992, and a brief account has recently been published in the proceedings of the Symposium. Misicka, A.; Lipkowski, A. W.; Horvath, R.; Davis, P.; Kramer, T. H.; Yamamura, H. I.; Porreca, F.; Hruby, V. J. Topographical requirements for δ opioid ligands: The synthesis and biological properties of cyclic analogs of deltorphin and dermenkephalin. *Peptides 1992*; Schneider, C. H., Eberle, A. N., Eds.; ESCOM Sci. Publ.: Leiden, 1993; pp 651–652.

(3) Kreil, G.; Barra, D.; Simmaco, M.; Erspamer, V.; Falconieri-Erspamer, G.; Negri, L.; Severini, C.; Corsi, R.; Melchiorri, P. Deltorphin, a novel amphibian skin peptide with high selectivity and affinity for δ opioid receptors. *Eur. J. Pharmacol.* 1989, *162*, 123–128.

(4) Erspamer, V.; Melchiorri, P.; Falconieri-Erspamer, G.; Negri, L.; Corsi, R.; Severini, C.; Barra, D.; Simmaco, M.; Kreil, G. Deltorphins: A family of naturally occurring peptides with high affinity and selectivity for δ opioid binding sites. *Proc. Natl. Acad. Sci. U.S.A.* 1989, *86*, 5188–5192.

(5) Jiang, Q.; Mosberg, H. I.; Porreca, F. Antinociceptive effects of [*D*-Ala^2]Deltorphin II, a highly selective δ agonist in vivo. *Life Sci.* 1990, *47*, PL43–47.

(6) Lazarus, L. H.; Salvadori, S.; Santagada, V.; Tomatis, R.; Wilson, W. E. Function of negative charge in the "address domain" of deltorphins. *J. Med. Chem.* 1990, *34*, 1350–1355.

(7) Charpentier, S.; Sagan, S.; Delfour, A.; Nicolas, P. Dermenkephalin and deltorphin I reveal similarities within ligand-binding domains of μ- and δ-opioid receptors and an additional address subsite on the δ-receptor. *Biochem. Biophys. Res. Commun.* 1991, *179*, 1161–1168 and references therein.

(8) Misicka, A.; Lipkowski, A. W.; Horvath, R.; Davis, P.; Kramer, T. H.; Yamamura, H. I.; Hruby, V. J. Topographical requirements for delta opioid ligands: Common structural features of dermenkephalin and deltorphin. *Life Sci.* 1992, *51*, 1025–1032 and references therein.

(9) Sasaki, Y.; Ambo, A.; Suzuki, K. [D-Ala²]deltorphin II analogs with high affinity and selectivity for delta-opioid receptor. *Biochem. Biophys. Res. Commun.* **1991**, *180*, 822–827.
(10) Schiller, P. W.; Nguyen, T. M. D.; Weltrowska, G.; Lemieux, C.; Chung, N. In *New Leads in Opioid Research—Proc. of the INRC 1990*; van Ree, J. M., Mulder, A. H., Wiegant, V. M., van Wimersma Greidanus, T. B., Eds.; Excerpta Medica: Amsterdam, 1990; pp 288–290.
(11) Schiller, P. W.; Weltrowska, G.; Nguyen, T. M. D.; Wilkes, B. C.; Chung, N. N.; Lemieux, C. Conformationally restricted deltorphin analogues. *J. Med. Chem.* **1992**, *35*, 3956–3961.
(12) Lazarus, L. H.; Salvadori, S.; Balboni, G.; Tomatis, R.; Wilson, W. E. Stereospecificity of amino acid side chain in deltorphin defines binding to opioid receptors. *J. Med. Chem.* **1992**, *35*, 1222–1227 and references therein. See also: Lazarus, L. H.; Salvadori, S.; Grieco, P.; Wilson, W. E.; Tomatis, R. Unique sequence in deltorphin C confers structural requirement for δ opioid receptor selectivity. *Eur. J. Med. Chem.* **1992**, *27*, 791–797.
(13) Melchiorri, P.; Negri, L.; Falconieri-Erspanier, G.; Severine, C.; Corsi, R.; Soaje, M.; Erspamer, V.; Barra, D. Structure-activity relationships of the δ-opioid selective agonists, deltorphins. *Eur. J. Pharmacol.* **1991**, *195*, 201–207.
(14) Balboni, G.; Marastoni, M.; Picone, D.; Salvadori, S.; Taucredi, T.; Temussi, P. A.; Tomatis, R. New features of the δ opioid receptor: Conformational properties of Deltorphin I analogues. *Biochem. Biophys. Res. Commun.* **1990**, *169*, 617–622.
(15) Hruby, V. J.; Al-Obeidi, F.; Kazmierski, W. Emerging approaches in the molecular design of receptor selective peptide ligands: Conformational, topographical and dynamic considerations. *Biochem. J.* **1990**, *268*, 249–262 and references cited therein.
(16) Hruby, V. J. Conformational restrictions of biologically active peptides via amino acid side chain groups. *Life Sci.* **1982**, *31*, 189–199.
(17) Kazmierski, W.; Hruby, V. J. A new approach to receptor ligand design: Synthesis and conformation on a new class of potent and highly selective μ opioid antagonists utilizing tetrahydroisoquinoline carboxylic acid. *Tetrahedron* **1988**, *44*, 697–710.
(18) Schiller, P. W.; Nguyen, T. M.-D.; Maziak, L. A.; Wilkes, B. C.; Lemieux, C. Structure-activity relationships of a cyclic opioid peptide analogues containing a phenylalanine residue in the 3-position. *J. Med. Chem.* **1987**, *30*, 2094–2099.
(19) Hawkins, K. N.; Knapp, R. J.; Lui, G. K.; Gulya, K.; Kazmierski, W.; Wan, Y. P.; Pelton, J. T.; Hruby, V. J.; Yamamura, H. I. [³H]-[H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2], a potent and highly selective peptide for mu opioid receptors in rat brain. *J. Pharmacol. Exp. Ther.* **1989**, *248*, 73–81.
(20) Toth, G.; Russell, K. C.; Landis, G.; Kramer, T. H.; Fang, L.; Knapp, R.; Davis, P.; Burks, T. F.; Yamamura, H. I.; Hruby, V. J. Ring substituted and other conformationally constrained tyrosine analogues of [*D*-Pen²,*D*-Pen⁵]enkephalin with delta opioid receptor selectivity. *J. Med. Chem.* **1992**, *35*, 2384–2391.
(21) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. Bis-penicillamine enkephalins possess highly improved specificity toward delta opioid receptors. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5871–5874.
(22) Toth, G.; Kramer, T. H.; Knapp, R.; Lui, G.; Davis, P.; Burks, T. F.; Yamamura, H. I.; Hruby, V. J. [D-Pen²,D-Pen⁵]enkephalin analogues with increased affinity and selectivity for delta opioid receptors. *J. Med. Chem.* **1990**, *33*, 249–253.
(23) Hruby, V. J.; Toth, G.; Gehrig, C. A.; Kao, L.-F.; Knapp, R.; Lui, G. K.; Yamamura, H. A.; Kramer, T. H.; Davis, P.; Burks, T. F. Topographically designed analogues of [D-Pen²,D-Pen⁵]enkephalin. *J. Med. Chem.* **1991**, *34*, 1823–1830.
(24) Kawasaki, A. M.; Knapp, R. J.; Kramer, T. H.; Wire, W. S.; Vasquez, O. S.; Yamamura, H. I.; Burks, T. F.; Hruby, V. J. Design and synthesis of highly potent and selective cyclic dynorphin A analogues. *J. Med. Chem.* **1990**, *33*, 1874–1879.
(25) Misicka, A.; Nikiforovich, G.; Lipkowski, A. W.; Horvath, R.; Davis, P.; Kramer, T. H.; Yamamura, H. I.; Hruby, V. J. Topographical requirements for delta opioid ligands: The synthesis and biological properties of a cyclic analogue of deltorphin I. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 547–552.
(26) Misicka, A.; Lipkowski, A. W.; Nikiforovich, G. V.; Kazmierski, W.; Knapp, R. J.; Yamamura, H. I.; Hruby, V. J. Computer modeling of opioid selective ligands: Possible new topographical relationships to bioactivity are examined with new analogues designed to the δ opioid receptor. In *Peptides: Chemistry and Biology*; Smith, J., Rivier, J., Eds.; ESCOM Publ.: Leiden, 1992; pp 140–141.
(27) Misicka, A.; Lipkowski, A. W.; Fang, L.; Knapp, R. J.; Davis, P.; Kramer, T.; Burks, T. F.; Yamamura, H. I.; Carr, D. B.; Hruby, V. J. Topographical requirements for delta opioid ligands: Presence of a carboxyl group in position 4 is not critical for deltorphin high delta receptor affinity and analgesic activity. *Biochem. Biophys. Res. Commun.* **1991**, *180*, 1290–1297.
(28) Portoghese, P. S.; Sultana, M.; Nagase, H.; Takemori, A. E. Application of the message-address concept in the design of highly potent and selective non-peptide delta opioid receptor antagonists. *J. Med. Chem.* **1988**, *31*, 281–282.
(29) Nikiforovich, G. V.; Hruby, V. J. Examination of the conformational meaning of "δ-address" in the dermenkephalin sequence. *Biochem. Biophys. Res. Commun.* **1990**, *173*, 521–527.
(30) Nikiforovich, G. V.; Hruby, V. J.; Prakash, O.; Gehrig, C. A. Topographical requirements for δ selective opioid peptides. *Biopolymers* **1991**, *34*, 941–955. (b) Nikiforovich, G. V.; Prakash, O.; Gehrig, C.; Hruby, V. J. Conformations of the Dermenkephalin backbone in DMSO solution by a novel approach to solution conformations of short linear peptides. *J. Am. Chem. Soc.* **1993**, *115*, 3399–3406 and references therein.
(31) Kosterlitz, H. W.; Lord, F. A. H.; Paterson, S. J.; Waterfield, A. A. Effects of changes in the structure of enkephalins and of narcotic analgesic drugs on their interactions with mu- and delta-receptors. *Br. J. Pharmacol.* **1980**, *68*, 333–342.
(32) For more details, see for example: Toth, G.; Russell, K. C.; Landis, G.; Kramer, T. H.; Fang, L.; Knapp, R.; Davis, P.; Burks, T. F.; Yamamura, H. I.; Hruby, V. J. Ring substituted and other conformationally constrained tyrosine analogs of [D-Pen²,D-Pen⁵]-enkephalin with δ opioid receptor selectivity. *J. Med. Chem.* **1992**, *35*, 2384–2391.
(33) Vaughn, L. K.; Knapp, R. J.; Toth, G.; Wan, J.-P.; Hruby, V. J.; Yamamura, H. I. A high affinity, highly selective ligand for the delta opioid receptor: [³H]-[D-Pen²,pCl-Phe⁴,D-Pen⁵]Enkephalin. *Life Sci.* **1989**, *45*, 1001–1008.
(34) Shook, J. E.; Pelton, J. T.; Wire, W. S.; Hirning, L. D.; Hruby, V. J.; Burks, T. F. Pharmacological evaluation of a cyclic somatostatin analog with antagonist activity at mu-opioid receptors in vivo. *J. Pharmacol. Exp. Ther.* **1987**, *240*, 772–777.
(35) Statistical Consultants Inc. *Am. Stat.* **1986**, *40*, 52.