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Brief Article

Synthesis and Evaluation of New Endomorphin-2 Analogues Containing (*Z*)-α,β-Didehydrophenylalanine (Δ^ZPhe) Residues

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New endomorphin-2 (EM-2) analogues incorporating (*Z*)-α,β-didehydrophenylalanine (Δ^ZPhe) in place of the native phenylalanine in EM-2 are reported. Tyr-Pro-Δ^ZPhe-Phe-NH_2 {[Δ^ZPhe^3]EM-2} (**1**), Tyr-Pro-Phe-Δ^ZPhe-NH_2 {[Δ^ZPhe^4]EM-2} (**2**), and Tyr-Pro-Δ^ZPhe-Δ^ZPhe-NH_2 {[Δ^ZPhe^{3,4}]EM-2}(**3**) have been synthesized, their opioid receptor binding affinities and tissue bioassay activities were determined, and their conformational properties were examined. Compound **2** shows high μ opioid receptor selectivity and μ agonist activity comparable to those of the native peptide. The conformation adopted in solution and in the crystal by *N*-Boc-Tyr-Pro-Δ^ZPhe-Phe-NH_2 (**8**) is reported.

Introduction

The alteration of the backbone sequence of native bioactive peptides through incorporation of unnatural amino acids or exogenous fragments of a different structure is a common strategy adopted in medicinal chemistry to study their structure−activity relationships and to obtain compounds with improved potency, selectivity, and potential therapeutic value. Recent and significant examples of this approach can be found in the field of endomorphins, two endogenous neuropeptides [endomorphin-1, Tyr-Pro-Trp-Phe-NH_2 (EM-1[a]); endomorphin-2, Tyr-Pro-Phe-Phe-NH_2 (EM-2)] whose main proposed biological function is pain control through activation, with high affinity and selectivity, of μ-opioid receptors.[1,2] A systematic evaluation of a variety of synthetic endomorphin analogues have provided insights into various structural and conformational features that are critical for the bioactivity of this family of neuropeptides.[3] The role exerted by the proline residue at position 2 of the tetrapeptide backbone and the spatial orientation adopted by the two Phe aromatic side chains at positions 3 and 4 appear to be particularly relevant.[4] Several papers dealing with the incorporation at position 2 of EM with higher and lower homologues of proline[5] and cyclic β-residues[6] document the attention dedicated to this first point. Also clearly documented is the influence that the mutual spatial orientation of the aromatic rings exerts on the interaction between EMs and their receptors.[7] The interest focused on this specific effect is based on the well recognized influence of the nonbonded aromatic/aromatic and backbone/aromatic interactions on the conformational stability of the peptide.

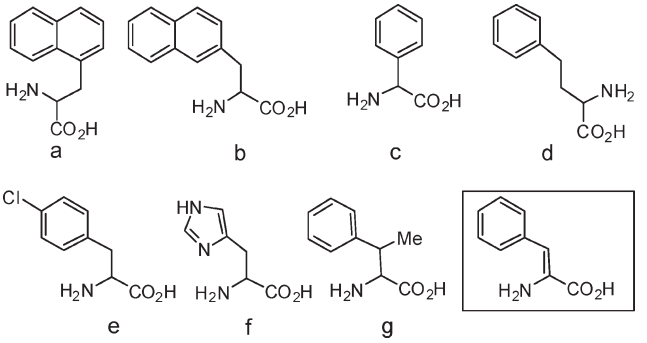

Figure 1. Structures **a**−**g** illustrate examples of aromatic amino acids so far used to replace native residues at position 3 and/or position 4 of endomorphins. The structure in the box refers to Δ^ZPhe, the achiral α,β-didehydroamino acid adopted in the present study: (**a**) 3-(1′-naphthyl)alanine (1-Nal);[8] (**b**) 3-(2′-naphthyl)-alanine (2′-Nal);[8] (**c**) phenylglycine (Phg);[9,7b] (**d**) homophenylalanine (Hfe);[9] (**e**): *p*-Cl-phenylalanine;[10] (**f**) histidine;[7a] (**g**) β-methylphenylalanine (β-MePhe).[11]

In accordance with the above-reported observations, a literature examination reveals that several papers have described structural modifications performed on the EM native sequences, which specifically focused on replacement of the Phe^3 and Phe^4 residues. In Figure 1 most of the so far incorporated Phe mimic residues are illustrated.

Of particular interest is the enhancement of activity and selectivity of EM analogues obtained through incorporation

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[a] Abbreviations: DCM, dichloromethane; DMAP, 4-(dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-(3-dimethylaminopropyl)carbodiimide; EM-1, Tyr-Pro-Trp-Phe-NH_2 (endomorphine-1); EM-2, Tyr-Pro-Phe-Phe-NH_2 (endomorphine-2); ^{3}H-DAMGO, [^{3}H]-[D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]-enkephalin; ^{3}H-DPDPE, [^{3}H]*c*[D-Pen^2,D-Pen^5]enkephalin; GPI/LMMP, guinea pig ileum/longitudinal muscle myenteric plexus (μ opioid receptors); hMOR, human μ opioid receptor; HOBt, 1-hydroxybenzotriazole; MVD, mouse vas deferens (δ opioid receptors); NMM, *N*-methylmorpholine; rDOR, rat δ opioid receptor; TEA, triethylamine; TFA, trifluoroacetic acid.

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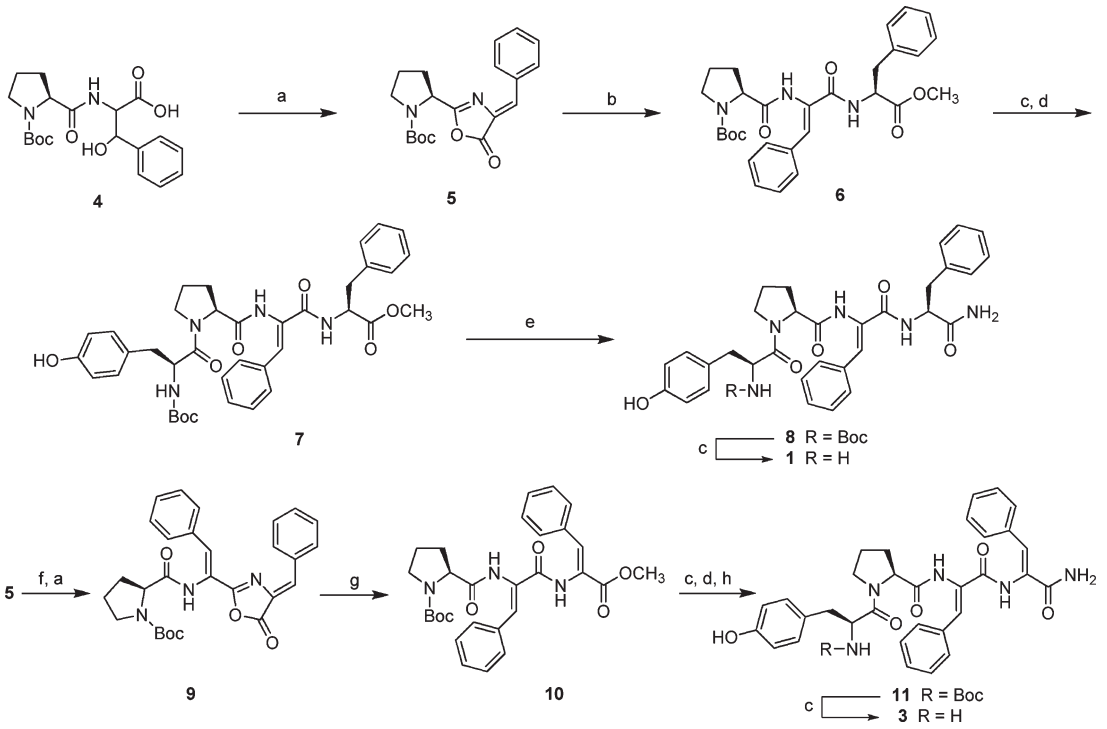
Scheme 1. Synthesis of the EM-2 Analogues Tyr-Pro-Δ^ZPhe-Phe-NH_2 {[Δ^ZPhe^3]EM-2} (**1**), Tyr-Pro-Δ^ZPhe-Δ^ZPhe-NH_2 {[Δ^ZPhe^{3,4}]EM-2}(**3**), and Boc-Tyr-Pro-Δ^ZPhe-Phe-NH_2 (**8**)[a]



[a] Reagents and conditions: (a) $(CH_3CO)_2O/CH_3COONa$, room temp, 24 h, 95%; (b) HCl·Phe-OMe, DIEA, DMAP, DCM, room temp, 12 h, 86%; (c) TFA/DCM (1:1), room temp, 1 h, quantitative; (d) Boc-Tyr-OH, EDC, HOBt, NMM, DCM, room temp, 12 h, 40%; (e) NH_3/MeOH, room temp, 48 h, 95%; (f) DL-3-β(OH)Phe-OH, 1 N NaOH, acetone, room temp, 12 h, 90%; (g) DMAP, MeOH, room temp, 12 h, 60%; (h) NH_3/MeOH, room temp, 48 h, 56%.

of β-methylated Phe residues (Figure 1) performed by Tomboly and co-workers.[11] The interesting rationale followed by these authors is based on the reduction of the conformational mobility of the Phe aromatic side chains by biasing, according to the original proposals of Hruby et al.,[12,13] the population of the χ_1 (torsion angle) rotamers. By following a related and still unexplored approach, we decided to synthesize and investigate the properties of a series of EM analogues incorporating α,β-unsaturated phenylalanine residues at positions 3 and 4. In analogues with this structure, the aromatic ring and each of its two adjacent backbone amide groups are bound to a sp^2 hybridized C^β and C^α atom, respectively. As a consequence, the achiral ΔPhe residue exerts conformational constraint on the backbone and restricts at the same time the β-aromatic substituent to the *Z* or *E* orientation. These steric and stereochemical features and the chemical properties connected with the electronic distribution within the involved peptide bonds render α,β-dehydroamino acids an appealing tool for the development of variants of naturally occurring bioactive peptides. The consequences of this structural alteration appear quite relevant in the case of the tetrapeptidic EMs molecule. Here in fact three closely located aromatic side chains are present and their location involves the "message" Tyr-Pro-Phe- N-terminal moiety and the Phe-NH_2 "address" C-terminal fragment.[14]

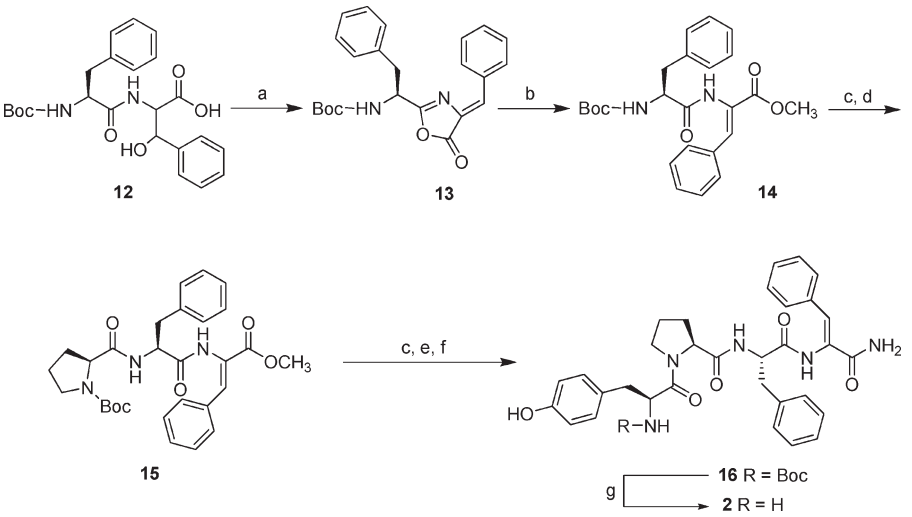
Chemistry

On the basis of the above considerations, the syntheses and biological activities of three EM-2 analogues Tyr-Pro-Δ^ZPhe-Phe-NH_2 {[Δ^ZPhe^3]EM-2} (**1**), Tyr-Pro-Phe-Δ^ZPhe-NH_2 {[Δ^ZPhe^4]EM-2} (**2**), and Tyr-Pro-Δ^ZPhe-Δ^ZPhe-NH_2 {[Δ^ZPhe^{3,4}]EM-2}(**3**) are here reported, together with the X-ray crystal structure of Boc-Tyr-Pro-Δ^ZPhe-Phe-NH_2 (**8**), the N-protected analogue of **1**. Incorporation of the Δ^ZPhe residue has been accomplished by performing an acetic anhydride mediated azlactonization dehydration reaction on dipeptide or tripeptide units containing C-terminal β-hydroxy-D,L-phenylalanine.[15] Scheme 1 outlines the synthesis of the [Δ^ZPhe^3] and [Δ^ZPhe^{3,4}] EM-2 analogues. The common intermediate **5** is used to obtain the final tetrapeptides **1** and **3**. In the case of the synthesis of the tetrapeptide **3** the incorporation of the two consecutive Δ^ZPhe residues was accomplished by ring-opening of the C-terminal unsaturated azlactone **5** with the sodium salt of β-hydroxy-D,L-phenylalanine followed by azlactonization and subsequent treatment with MeOH/DMAP. The analogue **2**, containing the unsaturated Phe residue at position 4, has been obtained by following an analogous synthetic strategy which is illustrated in Scheme 2.

Results and Discussion

Table 1 summarizes binding affinities and functional bioactivities for μ and δ opioid receptors of the here studied EM-2 analogues. Data reported indicate that **1** and **3** bind weakly to μ receptors (K_i^μ = 202 and 128, respectively) and are, although with substantial μ selectivity, weakly active in the GPI assay. Conversely, the analogue **2** shows potent μ binding affinity and high μ versus δ selectivity (K_i^δ /$K_i^\mu \approx$ 1200) with high potency in the GPI assay, comparable to the parent EM-2.[16] Thus, binding and bioassays data indicate that the incorporation of the Δ^ZPhe at position 3 or at positions 3 and 4 simultaneously is a detrimental structural modification. The corresponding alteration, performed at

Scheme 2. Synthesis of the EM-2 Analogue Tyr-Pro Phe-Δ^ZPhe-NH_2 {[Δ^ZPhe^4]EM-2} (**2**)[a]



[a] Reagents and conditions: (a) $(CH_3CO)_2O/CH_3COONa$, room temp, 24 h, 75%; (b) DMAP, MeOH, room temp, 12 h, 95%; (c) TFA/DCM (1:1), room temp, 1 h, quantitative; (d) Boc-Pro-OH, EDC, HOBt, NMM, DCM, room temp, 12 h, 44%; (e) Boc-Tyr-OH, EDC, HOBt, NMM, DCM, room temp, 12 h, 40%; (f) NH_3/MeOH, room temp, 48 h, 55%.

Table 1. Binding Affinity and in Vitro Activity for Compounds **1**−**3** and EM-2

	receptor affinity[a,b] (nM)			functional bioactivity, IC_{50} (nM)	
compd	K_i^δ	K_i^μ	selectivity, δ/μ	MVD[b]	GPI[b]
EM-2[c]		9.6 ± 0.98[c]		510 ± 35[c]	15 ± 2[c]
1 [Δ^zPhe^3]EM-2	nc[d]	200 ± 16		1900 ± 430	170 ± 13
2 [Δ^zPhe^4]EM-2	> 10000	8.4 ± 1.2	> 1200	390 ± 87	25 ± 2.8
3 [Δ^zPhe^{3,4}]EM-2	7300 ± 890	130 ± 36	57	1100 ± 120	330 ± 59

[a] Displacement of [^{3}H]DAMGO (μ-selective) and [^{3}H]DPDPE (δ-selective) using membrane preparations from transfected cells expressing rat μ-opioid receptor and human δ-opioid receptor, respectively. [b] ±SEM. [c] Data from ref 16. Binding affinity based on competition against [^{3}H]naloxone in rat brain membranes. Data were not presented on the affinity of EM2 at the δ-opioid receptor in that preparation, as EM was shown to be highly selective for μ-opioid receptors (see also ref 1). [d] nc = no competition, i.e., compound (up to 10^{-4} M) did not displace the specific binding of radioligand.

position 4 only, leads to ligand **2** which maintains high affinity and biological activity, together with the μ selectivity typical of the native ligand.

To gain further information on the preferred conformation of the new ligands, we examined the 2D ^{1}H NMR structures of the new ligands **1**−**3**, the conformation adopted in solution and in the crystal structure of **8**, the N^α-Boc derivative of **1** (see Scheme 1). Single crystals of **8** were successfully obtained by slow evaporation from a solution in MeOH. The X-ray structure of the molecule (Figure 2) presents a trans Tyr−Pro amide bond and adopts a H-bond stabilized β-turn structure with the Pro and Δ^ZPhe residues at the $i+1$ and $i+2$ corner positions, respectively, of the turn. As for the endomorphin μ-receptor agonists, only two other crystal structures have been reported, i.e., [D-Tic^2]EM-2[17] and [Chx^2]EM-2,[18] in addition to the C-terminal free acid Tyr-Pro-Phe-Phe-OH,[19] the latter completely devoid of μ-opioid receptor agonist activity. The X-ray crystal structure of **8** (Figure 2) gives then the first available information on the solid state conformation adopted by an N-protected EM analogue.

As shown in Table 2 and Figure 3, **1** and its N^α-Boc protected derivative **8** show strong sequential NOEs ProC^αH···Δ^Z-Phe^{3}NH. This effect is observed, for **1** and **8**, by two inter-residue NOEs which are not found in the case of **2** and **3**: the first between the NH groups of Δ^ZPhe^3 and Phe^4 and the second between the ProC^αH and Phe^{4}NH (i.e., between the "$i+2$ and $i+3$" and "$i+1$ and $i+3$" residues, respectively, of a β-turn). These data strongly suggest that the N^α-Boc derivative **8** maintains in DMSO-d_6 solution the folded conformation found in the crystal and that the same conformational preference is also shown by its N-terminal free analogue **1**. It is

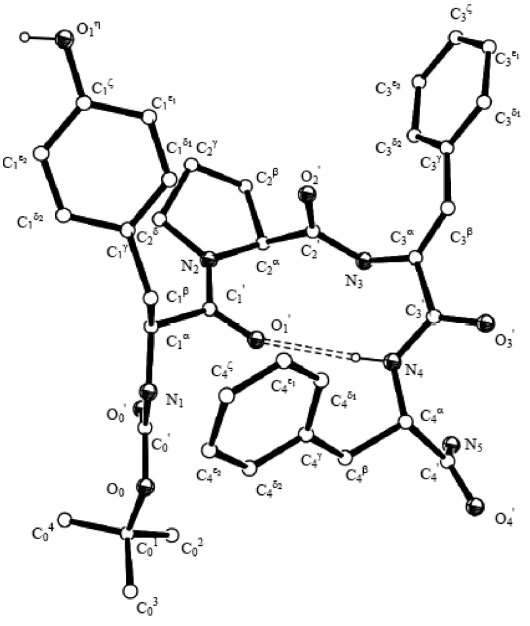
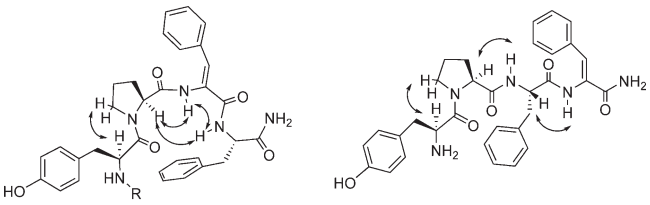


Figure 2. X-ray crystal structure of Boc-Tyr-Pro-Δ^ZPhe-Phe-NH_2 (**8**), with numbering of the atoms. The intramolecular H-bond is shown as a dashed double line.



1 R = H
8 R = Boc

2

Figure 3. Relevant interproton correlations as deduced by ROESY experiments of **1**, **2**, and **8**.

Table 2. Observed NOE Cross Peaks and Intensities of Analogues **1**−**3** and **8** in DMSO-d_6[a]

	1	2	3	8
Δ^ZPhe^3 NH···Phe^4 NH	m			m
Pro C$^\alpha$H···Phe^4 NH	m[b]			m
Pro C$^\alpha$H···Δ^ZPhe^3 NH	s[b]		s	s
Pro C$^\alpha$H···Phe^3 NH		s		
Phe^3 C$^\alpha$H···Δ^ZPhe^4 NH		m		
Tyr C$^\alpha$H···Pro C$^\delta$H$_2$	m	m	m	m

[a] NOE intensities are classified as weak (1.6−5.0 Å), medium (1.6−3.6 Å), and strong (1.6−2.9 Å). [b] The Pro C$^\alpha$H is partially overlapped with Phe C$^\alpha$H.

worth noting that the findings on **1** and **8** are those expected on the basis of literature on peptides containing α,β-dehydroamino acid where the strong tendency of the Δ^ZPhe residue to occupy the $i + 2$ position of β-turns is well documented.[20] No NOEs indicative of folded structures could be found in the spectra of the two peptides **2** and **3**. Furthermore, the presence of the sequential NOEs ProC$^\alpha$H···PheNH and PheNH···Δ^Z-PheNH in the spectrum of **2** is consistent with an extended structure.

Conclusions

As revealed by literature data, the tetrapeptide EMs are characterized by considerable conformational flexibility involving the backbone and the aromatic side chains. Although the optimal three-dimensional arrangement of the EM pharmacophoric elements favoring selectivity and potency as μ agonist is not yet resolved, the relevant role of a proper spatial orientation of the aromatic rings and in particular of the benzylic side chains at position 3 and 4 is well established. Here we have examined the properties of new EM analogues in which Phe mimics, with the aromatic ring locked in the *Z* spatial orientation, have been incorporated. A remarkable difference is observed in the receptor affinity and functional bioactivity between **2**, possessing the aromatic at position 3 free to adopt a proper spatial orientation, and the analogue **1** in which the Phe at position 3 is forced to the rigid *Z* orientation imposed by the olefinic geometry of the residue. These results emphasize the importance of the correct orientation of the aromatic rings and suggest at the same time that the preformed modification of Phe^3 in the Tyr-Pro-Phe- "message" moiety leads to ligand **1** in which the folded conformation of the backbone and the third aromatic ring spatial orientation are unfavorable features for proper interaction with the receptor. The analogue **2**, which maintains unchanged the native EM "message" sequence and an extended backbone conformation, exhibits a high activity comparable to that of EM-2. In this analogue the aromatic side chain at position 3 can adopt a proper orientation in the receptor pocket. This interpretation appears to be in agreement with the previously described EM analogues containing β-MePhe stereoisomers.[11] Here, the highest activity is shown by the ligand Tyr-Pro-Phe-(2*S*,3*S*)-β-MePhe-NH$_2$ which maintains the native Phe^3 residue and possesses at position 4 an aromatic side chain properly oriented by the stereochemistry of the β-MePhe residue.

Studies are now in progress to further investigate conformational and pharmacological consequences of EM analogues obtained by following this general approach.

Experimental Section

Chemistry. General Methods. Preparative layer chromatography (PLC) were performed on Merck 60 F_{254} silica gel plates. The purity of all tested compounds was determined by combustion analysis to be ≥95% pure. HRMS data are also obtained.

Synthesis. General Procedures for Preparation of Peptide Amides. The peptide methyl ester is allowed to stand in a pressure bottle at room temperature for 48 h in anhydrous methanol previously saturated with ammonia gas at 0 °C. The solution is then concentrated to dryness in vacuo at a temperature not exceeding 40 °C. Compound **8** was purified by crystallization from MeOH, and the amides **11** and **16** were purified by PLC.

General Procedure for Deprotection of Boc Derivatives 1, 2, and 3. The Boc group was removed by treatment with TFA in DCM (1:1) for 1 h at room temperature. Removal of solvent and precipitation of the residue with ether gave the TFA salt.

NMR Experiments. All 1D and 2D ^{1}H NMR experiments were performed at 400 MHz on a Bruker Avance 400 NMR spectrometer with a constant temperature at 298 K. The ROESY spectra were obtained using standard pulse programs, with a mixing times of 300 ms. The 2D NMR matrixes were created and analyzed using the TOPSPIN 3.0a computer program (Bruker Biospin, 2009). Each two-dimensional spectrum was acquired in a 1024 × 1024 data matrix complex points in F_1 and F_2. Zero filling in F_1 and sine windows in both dimensions were applied before Fourier transformation. Chemical shifts (δ) are quoted in parts per million (ppm) downfield from tetramethylsilane, and values of coupling constants are given in Hz.

TFA·H$_2$N-Tyr-Pro-Δ^ZPhe-Phe-NH$_2$ (1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.85−2.13 (4H, m, Pro C^{3}H$_2$ and Pro C^{4}H$_2$), 2.7−3.2 (4H, m, Phe^4 C$^\beta$H$_2$ and Tyr C$^\beta$H$_2$), 3.41 and 3.61 (2H, m, Pro C^{5}H$_2$), 4.21 (1H, m, Tyr C$^\alpha$H), 4.5 (2H, m, Phe^4 C$^\alpha$H and Pro C$^\alpha$H), 6.6−7.6 (17H, m, aromatics, Δ^ZPhe^3 C$^\beta$H and CONH$_2$), 7.77 (1H, d, J = 8, Phe^4 NH), 8.09 (3H, br, Tyr NH$_3^+$), 9.36 (1H, s, Tyr OH), 9.82 (1H, s, Δ^ZPhe^3 NH). HRMS for [M + H]$^+$: *m*/*z* calcd 570.2716; found 570.2710.

TFA·H$_2$N-Tyr-Pro-Phe-Δ^ZPhe-NH$_2$ (2). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.69−2.08 (4H, m, Pro C^{3}H$_2$ and Pro C^{4}H$_2$), 2.7−3.2 (4H, m, Phe^3 C$^\beta$H$_2$ and Tyr C$^\beta$H$_2$), 3.4−3.68 (2H, m, Pro C^{5}H$_2$), 4.17 (1H, m, Tyr C$^\alpha$H), 4.41 (1H, m, Pro C$^\alpha$H), 4.63 (1H, m, Phe^3 C$^\alpha$H), 6.6−7.55 (17H, m, aromatics, Δ^ZPhe^4 C$^\beta$H and CONH$_2$), 8.09 (3H, br, Tyr NH$_3^+$), 8.3 (1H, d, J = 7.8, Phe^3 NH), 9.38 (1H, s, Tyr OH), 9.7 (1H, s, Δ^zPhe^4 NH). HRMS for [M + H]$^+$: *m*/*z* calcd 570.2716; found 570.2721.

TFA·H$_2$N-Tyr-Pro-Δ^ZPhe-Δ^ZPhe-NH$_2$ (3). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.71−2.2 (4H, m, Pro C^{3}H$_2$ and Pro C^{4}H$_2$), 2.68−3.07 (2H, m, Tyr C$^\beta$H$_2$), 3.4−3.68 (2H, m, Pro C^{5}H$_2$), 4.17 (1H, m, Tyr C$^\alpha$H), 4.53 (1H, m, Pro C$^\alpha$H), 6.6−7.8 (18H, m, aromatics, Δ^ZPhe^3 C$^\beta$H, Δ^ZPhe^4 C$^\beta$H, and CONH$_2$), 8.09 (3H, br, Tyr NH$_3^+$), 9.35 (1H, s, Tyr OH), 9.42 (1H, s, Δ^ZPhe^4 NH), 10.13 (1H, s, Δ^ZPhe^3 NH). HRMS for [M + H]$^+$: *m*/*z* calcd 568.2560; found 568.2555.

Boc-Tyr-Pro-Δ^ZPhe-Phe-NH$_2$ (8). White solid: mp 228−232 °C, 95% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.3 [9H, s, C(CH$_3$)$_3$], 1.85−2.31 (4H, m, Pro C^{3}H$_2$ and Pro C^{4}H$_2$), 2.58−3.2 (4H, m, Phe^3 C$^\beta$H$_2$ and Tyr C$^\beta$H$_2$), 3.58−3.7 (2H, m, Pro C^{5}H$_2$), 4.17 (1H, m, Tyr C$^\alpha$H), 4.39 (1H, m, Pro C$^\alpha$H), 4.5 (1H, m, Phe^3 C$^\alpha$H), 6.6−7.55 (18H, m, aromatics, Tyr NH, Δ^ZPhe^4 C$^\beta$H and CONH$_2$),

7.90 (1H, d, J = 8.4, Phe^3 NH), 9.18 (1H, s, Tyr OH), 9.7 (1H, s, Δ^ZPhe^4 NH). HRMS for $[M + H]^+$: m/z calcd 670.3241; found 670.3248.

Boc-Tyr-Pro-Δ^ZPhe-Δ^ZPhe-NH$_2$ (11). Eluent mixture: chloroform/methanol, 95:5; 56% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.3 [(9H, s, C(CH$_3$)$_3$], 1.71−2.26 (4H, m, Pro C^{3}H$_2$ and Pro C^{4}H$_2$), 2.62−2.75 (2H, m, Tyr C$^\beta$H$_2$), 3.48−3.72 (2H, m, Pro C^{5}H$_2$), 4.25 (1H, m, Tyr C$^\alpha$H), 4.46 (1H, m, Pro C$^\alpha$H), 6.6−7.8 (19H, m, aromatics, Tyr NH, Δ^ZPhe^3 C$^\beta$H, Δ^ZPhe^4 C$^\beta$H, and CONH$_2$), 9.22 (1H, s, Tyr OH), 9.52 (1H, s, Δ^ZPhe^4 NH), 10.12 (1H, s, Δ^ZPhe^3 NH). HRMS for $[M + H]^+$: m/z calcd 668.3084; found 668.3091.

Boc-Tyr-Pro-Phe-Δ^ZPhe-NH$_2$ (16). Eluent mixture: chloroform/methanol, 95:5; 55% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.3 [(9H, s, C(CH$_3$)$_3$], 1.7−2 (4H, m, Pro C^{3}H$_2$ and Pro C^{4}H$_2$), 2.5−3.1 (4H, m, Phe^4 C$^\beta$H$_2$ and Tyr C$^\beta$H$_2$), 3.48 and 3.58 (2H, m, Pro C^{5}H$_2$), 4.21 (1H, m, Tyr C$^\alpha$H), 4.32 (1H, m, Pro C$^\alpha$H), 4.53 (1H, m, Phe^4 C$^\alpha$H), 6.6−7.51 (18H, m, aromatics, Tyr NH, Δ^ZPhe^3 C$^\beta$H and CONH$_2$), 8.31 (1H, d, J = 7.2, Phe^4 NH), 9.66 (1H, s, Δ^ZPhe^3 NH), 9.45 (1H, s, Tyr OH). HRMS for $[M + H]^+$: m/z calcd 670.3241; found 670.3238.

Binding and Functional Assays. All binding assays used crude membrane preparations from transfected HEK293 cells expressing the human δ-opioid receptor or HN9.10 cells expressing the rat μ-opioid receptor. Binding affinities of the compounds were determined by competitive binding analysis against the δ-selective agonist [^{3}H]DPDPE and the μ-selective agonist [^{3}H]DAMGO in the respective membrane preparations. Data from three independent experiments were fitted by nonlinear regression analysis using GraphPad Prism. K_i values were calculated from IC_{50} by the Cheng and Prusoff equation.[21] The in vitro tissue bioassays (MVD and GPI/LMMP) were performed as described previously.[22] IC_{50} values represent mean values from no less than four experiments. IC_{50} values, relative potency estimates, and their associated standard errors were determined by fitting the data to the Hill equation by a computerized nonlinear least-squares method.

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Supporting Information Available: Elemental analysis of final products, details on experimental procedures for the synthesis of intermediates, X-ray crystallographic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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