Articles

Phe³-Substituted Analogues of Deltorphin C. Spatial Conformation and Topography of the Aromatic Ring in Peptide Recognition by δ Opioid Receptors

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In order to study the contribution of the electronic, hydrophobic, and conformational properties of the amino acid residue at position 3 in deltorphin C on binding to δ and μ opioid receptors, a series of 5- and 6-membered ring and bicyclic amino acid replacements at position 3 were prepared by solution synthesis methods. In general, the substitutions were deleterious for high δ affinity (K_i^δ) and δ selectivity (K_i^μ/K_i^δ). However, several notable exceptions were recognized: peptides containing the constrained, bicyclic structures Aic³ and (*R* or *S*) Atc³ enhanced δ affinity, but only the latter increased δ selectivity 4-fold (=2475) relative to deltorphin C(=661); at the other extreme, δ affinity of N$^\alpha$MePh³ fell 900-fold. Bioassays of [N$^\alpha$MePhe³]-, [(*R* or *S*)C$^\alpha$MePhe³]-, [Tic³]-, [Aic³]-, and [(*R* or *S*) Atc³]deltorphin C using guinea pig ileum (GPI) and mouse vas deferens (MVD) for μ and δ bioactivity, respectively, revealed a significant correlation (r = 0.916) between MVD bioactivity and δ binding in brain membranes. [(*R* or *S*)Atc³]deltorphin C also exhibited the highest biological selectivity (GPI/MVD) (=3,522), which was 3-fold greater than that observed for deltorphin C. Molecular modelling of [N$^\alpha$MePhe³]- and [(*S*)Atc³]deltorphin C established that these amino acid replacements for Phe³ produce alterations in the backbone (ϕ,ψ) and side-chain ($\chi1,\chi2$) dihedrals which critically affect the flexibility of the peptide and possibly limit accessible conformations for its alignment within the δ opioid receptor. The data provide evidence that the δ receptor is sensitive to changes in the composition, conformation, and orientation of the side chain of residue 3 of a linear opioid heptapeptide.

Introduction[1]

The deltorphin family of δ opioid agonists consists of three heptapeptides isolated from amphibian skin;[2,3] they were first recognized in the sequence of cDNA clones of the dermorphin gene[4] and during a screening for additional deltorphin-like peptides.[5] The remarkable structural feature of these peptides consists of two major characteristics: (a) the presence of a naturally occurring D-amino acid enantiomer in the second position from the N-terminus and (b) an N-terminal sequence common to the μ specific dermorphin family of opioid peptides,[6] namely H-Tyr¹-D-Xaa²-Phe³, where D-Xaa can be either D-Met as in deltorphin A or D-Ala as in deltorphins B and C and dermorphin.[6] Of the opioid peptides known in the literature, the deltorphins exhibit the highest affinity and selectivity for the δ receptor sites in binding assays with brain membranes[2,3,7,8] and isolated GPI and MVD preparations.[3,8,9] The central administration of deltorphins in rodents resulted in pronounced effects on myoclonic seizures, and epileptic discharge[10] revealed antinociceptive efficacy in some strains of mice,[11] which developed tolerance, long-term sensitization,[12] and induced several behavioral modifications,[13] while in humans, they inhibited hypoglycemia-stimulated ACTH secretion.[14]

Modifications in the side chains of individual amino acids in deltorphins demonstrated that considerable latitude was possible at some positions in these heptapeptides without substantial or deleterious effects;[15] changes in the electronic and hydrophobic configuration significantly effected receptor binding properties.[14,16-18] Alteration in the hydrophobicity of the fifth residue[15] or the combinatorial effect at positions 5 and 6 disclosed a pivotal hydrophobic core in deltorphins.[15,16,18] Inversion of the spatial orientation of the side chains around the α-carbon axis in residues 1 through 5 led to substantial losses in receptor recognition.[19,20] The crucial role of a D-enantiomer at position 2 was evident following the change in the stereocenter to the L-conformer which resulted in losses in δ affinity by several orders of magnitude.[6-8,20] Substitutions in the N-terminal region were tolerated in several linear deltorphin analogues including modifications on the Tyr¹ phenolic ring,[21,22] replacement of D-Ala² by other D-amino acids[20] or the bicyclic residue Tic,[19] and the substitution of Aic for Phe³;[19,23] on the other hand, an extension on Tyr¹ in deltorphin C was not well-tolerated.[24] Similarly, the systematic replacement of Phe³ in cyclic, conformationally constrained opioid tetrapeptide analogues indicated that several bicyclic substituted analogues

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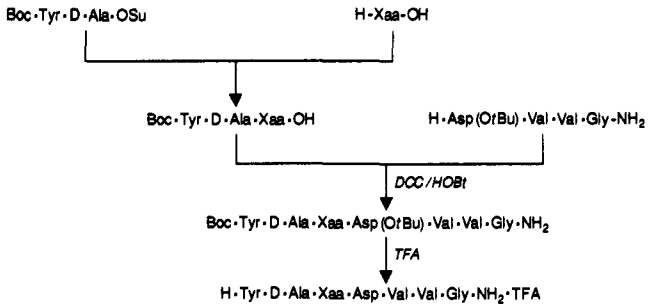
Scheme I. Synthesis of Deltorphin C Analogues **2**, **3**, and **7–12**[a]



[a] Xaa³ = Phg (**2**), Hfe (**3**), Tyr (**7**), TyrOMe (**8**), His (**9**), Trp (**10**), Nap(1) (**11**), and Nap(2) (**12**).

maintained or enhanced opioid receptor binding properties.[22,23,25,26]

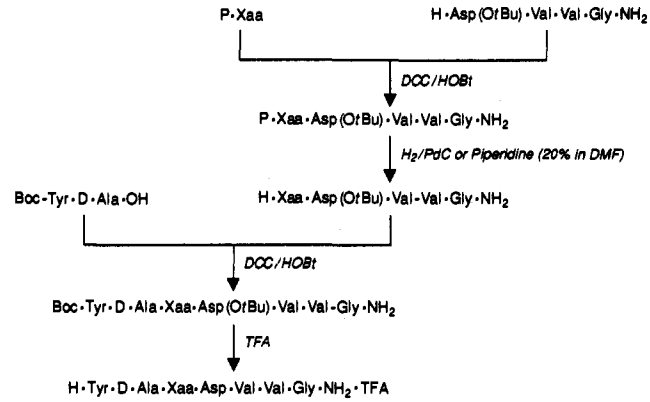
Extensive solution conformation analyses of the deltorphins through ^{1}H-NMR spectroscopy indicated the existence of a type II' β-turn in the N-terminal sequence[27–30] and possible helical portions within the C-terminal region.[28] Initial modeling endeavors on these peptides suggested the possible involvement of the C-terminal region in receptor recognition[31] and the inclusion of the aromatic benzyl ring of Phe³ in that process.[23,27] In this report, we sought to explore further possible changes in receptor binding activity in brain membranes and the effect of selected analogues on the bioactivity of GPI and MVD upon replacement of phenylalanine at this critical region of deltorphin C. This goal was approached by the systematic alteration in the spatial orientation of the side-chain ring of residue 3 through alterations in electronic configuration, hydrophobicity, and the molecular volume of bicyclic, constrained residues that would either compromise or accelerate compatibility with the receptor. Molecular models were developed for solvated deltorphin C, [N$^\alpha$MePhe³]deltorphin C (**5**), and [(*S*)Atc³]deltorphin C (**15**) based on the nuclear Overhauser enhancement spectroscopy data of Amodeo et al.[28] Simulated annealing analyses generated low-energy conformers for each peptide, in order to compare and study the interactive forces that might possibly contribute to receptor recognition of the ligands exhibiting the lowest and highest δ affinities.

General Methods for Peptide Synthesis

Amino acids and their derivatives were purchased from Bachem Feinchemikalien, Novabiochem, and Aldrich. The following peptides and constrained amino acids were synthesized as described in detail elsewhere: deltorphin C (compound 1),[32] Boc-Tyr-D-Ala-OSu,[33] the azlactone derivative related to the dipeptide Z-D-Ala-Phe(β-OH)-OH,[34] Tic,[35,36] and Aic.[37] Commercially available 7,8-benzo-1,3-diazaspiro[4.5]decane-2,4-dione was converted to (*R* or *S*) Atc according to the preparation of Aic.[37] The Z derivatives of Aic, Tic, and (R or S) Atc and the Fmoc derivative of N$^\alpha$MePhe were prepared by reacting with dibenzyl dicarbonate[38] and 9-fluorenylmethyl chloroformate,[39] respectively. Z-Tic-OH and Z-(*R* or *S*)Atc-OH were crystallized as DCHA salts; the quantitative analyses are given below.

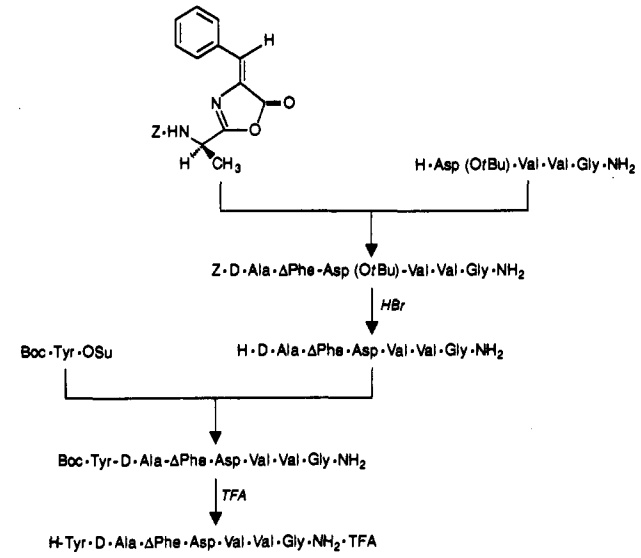
Peptides **2**, **3**, and **7-12** were prepared by condensation of protected N-terminal tripeptides, Boc-Tyr-D-Ala-Xaa-OH, with the corresponding C-terminal tetrapeptides, H-Asp(O*t*Bu)-Val-Val-Gly-NH$_2$ (Scheme I): modified N-terminal tripeptides were prepared by condensation of

Scheme II. Synthesis of Deltorphin C Analogues **5**, **6**, **6a**, **8–15**, and **15a**[a]



[a] Xaa³ = N$^\alpha$MePhe (**5**), (*R* or *S*)C$^\alpha$MePhe (**6**, **6a**), Tic (**13**), Aic (**14**), and (*R* or *S*)Atc (**15**, **15a**). P = Z or Fmoc.

Scheme III. Synthesis of a Deltorphin Analogue Containing ΔPhe³ (**4**)



N-protected (Boc) Tyr-D-Ala as the succinimidyl ester with various modified phenylalanines, such as Phg, Hfe, Nap-(1), Nap(2), His, Trp, Tyr, and Tyr(OMe). Constrained Phe³ analogues, peptides **5**, **6**, **6a**, **13-15**, and **15a**, were synthesized by condensation of N-terminal dipeptide Boc-Tyr-D-Ala-OH with the corresponding C-terminal pentapeptides H-Xaa-Asp(O*t*Bu)-Val-Val-Gly-NH$_2$ (Scheme II): modified pentapeptides were synthesized by condensation of N-protected (Z, Fmoc) phenylalanine analogues, Tic, Aic, (*R* or *S*)Atc, (*R* or *S*)C$^\alpha$MePhe, and N$^\alpha$MePhe, with the C-terminal synthesized as stated above. Racemic mixtures of C$^\alpha$MePhe and Atc were used in the synthesis of their respective peptides; the diastereoisomeric peptides were isolated and arbitrarily assigned as peptides **6** and **6a** or **15** and **15a**, respectively, on the basis of their distinct HPLC retention times. The ΔPhe analogue (peptide **4**) was obtained by double-coupling steps through treatment of the azlactone derivative of dipeptide Z-D-Ala-Phe(β-OH)-OH[34] as shown in Scheme III withH-Asp(O*t*Bu)-Val-Val-Gly-NH$_2$: the N-protected dehydrohexapeptide Z-D-Ala-ΔPhe-Asp(O*t*Bu)-Val-Val-Gly-NH$_2$ intermediate was deprotected followed by a second coupling with Boc-Tyr-OSu to yield the protected heptapeptide amide.

Table I. Binding Affinities of Phe[3]-Substituted Deltorphin C Analogues[a]

no.	peptide	K_i^δ (nM)	K_i^μ (nM)	K_i^μ/K_i^δ
1	H-Tyr-D-Ala-**Phe**-Asp-Val-Val-Gly-NH_2	0.31 ± 0.05 (9)	205 ± 20.5 (5)	661
2	H-Tyr-D-Ala-**Phg**-Asp-Val-Val-Gly-NH_2	2.85 ± 0.11 (3)	3750 ± 125 (4)	1314
3	H-Tyr-D-Ala-**Hfe**-Asp-Val-Val-Gly-NH_2	4.84 ± 0.99 (4)	326 ± 87.3 (3)	67
4	H-Tyr-D-Ala-**ΔPhe**-Asp-Val-Val-Gly-NH_2	4.23 ± 1.13 (4)	114 ± 20.7 (4)	27
5	H-Tyr-D-Ala-**N$^\alpha$MePhe**-Asp-Val-Val-Gly-NH_2	284 ± 35.4 (3)	19000 ± 3,520 (4)	67
6	H-Tyr-D-Ala-(***R* or *S*)C$^\alpha$MPhe**-Asp-Val-Val-Gly-NH_2	69.1 ± 8.04 (4)	1360 ± 178 (3)	20
6a	H-Tyr-D-Ala-(***R* or *S*)C$^\alpha$MePhe**-Asp-Val-Val-Gly-NH_2	48.6 ± 0.65 (3)	3400 ± 545 (3)	70
7	H-Tyr-D-Ala-**Tyr**-Asp-Val-Val-Gly-NH_2	34.0 ± 1.7 (3)	1180 ± 162 (4)	35
8	H-Tyr-D-Ala-**Tyr(OMe)**-Asp-Val-Val-Gly-NH_2	45.4 ± 9.7 (4)	526 ± 37.7 (3)	12
9	H-Tyr-D-Ala-**His**-Asp-Val-Val-Gly-NH_2	45.5 ± 1.9 (4)	3720 ± 936 (6)	82
10	H-Tyr-D-Ala-**Trp**-Asp-Val-Val-Gly-NH_2	2.34 ± 0.25 (3)	323 ± 28.6 (5)	138
11	H-Tyr-D-Ala-**Nap(1)**-Asp-Val-Val-Gly-NH_2	0.61 ± 0.10 (4)	279 ± 36.2 (3)	457
12	H-Tyr-D-Ala-**Nap(2)**-Asp-Val-Val-Gly-NH_2	10.7 ± 1.8 (4)	770 ± 121 (4)	72
13	H-Tyr-D-Ala-**Tic**-Asp-Val-Val-Gly-NH_2	90.2 ± 23.3 (4)	7810 ± 1190 (4)	87
14	H-Tyr-D-Ala-**Aic**-Asp-Val-Val-Gly-NH_2	0.20 ± 0.03 (3)	56.2 ± 9.2 (3)	281
15	H-Tyr-D-Ala-(***R* or *S*)Atc**-Asp-Val-Val-Gly-NH_2	0.07 ± 0.01 (4)	178 ± 37.7 (4)	2475
15a	H-Tyr-D-Ala-(***R* or *S*)Atc**-Asp-Val-Val-Gly-NH_2	0.12 ± 0.03 (4)	82.3 ± 8.9 (3)	686

[a] Radioreceptor assays for δ ([^{3}H]DPDPE) and μ ([^{3}H]DAGO) sites on rat brain synaptosomes are detailed in the Experimental Section. The affinity constants (K_i) were derived from the IC_{50} values according to Cheng and Prusoff.[52]

Results and Discussion

The parent peptide deltorphin C (1) displayed high affinity and selectivity for δ receptors (Table I).[3,18,24,40] We compared low-energy models of deltorphin C with that of [N$^\alpha$MePhe^3]deltorphin C (5) and the *S*-conformer of [Atc^3]deltorphin C, presumed to represent the proper diastereomer in the peptide although the stereocenter for Atc was not experimentally determined.

Receptor Binding Characteristics. 1. Linear Peptides. Substitution of Phe^3 in deltorphin C by amino acids **2–9** (Figure 1) reduced δ affinity 9-900-fold (Table I); μ affinities were similarly reduced 1.6-93-fold in the corresponding peptides, although a nearly 2-fold enhancement was observed with compound **4**. Shortening the side chain of Phe^3 through the loss of CH_2 (phenylglycine **2**) resulted in double δ selectivity to 1314 primarily due to an 18-fold suppression in μ binding since δ affinity fell 9-fold. Interestingly, the inclusion of Phg^3 in a cyclic μ selective tetrapeptide related to deltorphin C (H-Tyr-D-Orn-Phg-Asp-NH_2) also reduced μ affinity.[4,42] Even though Phg exhibits reduced orientational freedom compared to Phe,[41] peptide **2** is still capable to exhibit δ selectivity.

Lengthening the side chain and extending the benzyl ring further from the backbone by the replacement of Phe^3 with homophenylalanine (compound **3**) (Figure 1) resulted in a greater loss in δ than μ binding, such that δ selectivity decreased by 1 order of magnitude (Table I). The added length and positioning of the Hfe aromatic ring might physically impede access to or binding in a hydrophobic pocket of the receptor. Opposite conclusions were obtained with a conformationally restricted tetrapeptide containing Hfe,[41,42] which further points to conformational differences between cyclic and linear peptides in their recognition by the δ receptor. Formation of a rigid ethylene bond (peptide **4**), which restricts movement of the aromatic ring around the χ_1 angle, decreased δ affinity 14-fold and doubled μ affinity (Table I).

Thus, data for peptides **1–4** indicate that the benzyl ring of residue 3 occupies or aligns within a hydrophobic receptor region, and rotational freedom, electronic milieu, and distance from the peptide backbone are important elements in the binding phenomenon. The modest decrease in δ affinity could result from a steric interaction between Phe^3 and the methyl group of D-Ala^2 and might alter the configuration of the N-terminal β-turn; the close proximity of these residues was indicated from nuclear Overhauser enhancement spectroscopy measurements.[28,30,43]

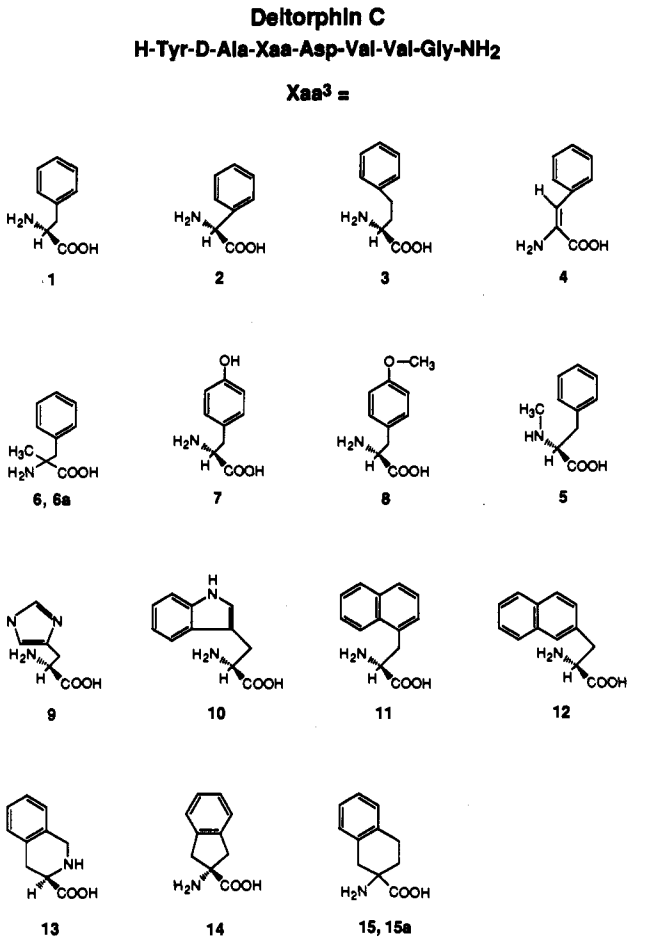


Figure 1. Structures of amino acid substituents in position 3 (Xaa^3) in deltorphin C analogues. Arabic numerals refer to peptides in which the complete sequence of these compounds are found in Table I.

Replacement of Phe^3 by amino acid residues **5–9** reduced δ affinities in the range of 100-900-fold. Methylation of the α-carbon of Phe^3 (analogues **6** and **6a**) reduced δ more than μ affinities and, as a consequence, diminished δ selectivities (Table I). C$^\alpha$-Methylation changes the conformational flexibility of the peptide backbone through a limitation in the torsion angles.[23] Similarly, the change in the chirality around the α carbon of Phe^3 from the L- to D-conformer in deltorphin C,[19] as well as in deltorphin

Table II. Analytical Data of Deltorphin C Analogues

no.	analogue	TLC R_f I	TLC R_f II	HPLC[a] K'	FAB-MS MH^+	amino acid analysis[b] Y	A	D	V	G	H
2	[Phg³]	0.48	0.34	1.26	755	0.87	0.95	0.97	1.84	1.0	
3	[Hfe³]	0.53	0.43	2.51	783	0.85	0.96	0.90	1.79	1.0	
4	[ΔPhe³]	0.51	0.48	2.41	767	0.88	0.98	0.94	1.81	1.0	
5	[N$^\alpha$MePhe³]	0.59	0.25	5.12	783	0.90	1.02	0.91	1.82	1.0	
6	[(*R* or *S*)C$^\alpha$MePhe³]	0.46	0.30	3.03	783	0.85	0.97	0.90	1.79	1.0	
6a	[(*R* or *S*)C$^\alpha$MePhe³]	0.65	0.48	6.47	783	0.81	0.94	0.89	1.80	1.0	
7	[Tyr³]	0.56	0.44	10.99*	785	1.89	0.94	0.93	1.84	1.0	
8	[Tyr(OMe)³]	0.54	0.43	2.69	799	1.71	0.94	0.87	1.90	1.0	
9	[His³]	0.35	0.40	1.60*	759	0.89	0.93	0.89	1.89	1.0	0.9
10	[Trp³]	0.54	0.35	3.04	808	0.95	0.99	0.91	1.90	1.0	
11	[Nap(1)³]	0.71	0.60	14.37	819	0.89	0.94	0.89	1.89	1.0	
12	[Nap(2)³]	0.73	0.61	13.71	819	0.91	0.97	1.02	1.79	1.0	
13	[Tic³]	0.55	0.27	2.74	781	0.92	0.89	1.03	1.80	1.0	
14	[Aic³]	0.53	0.23	5.06	781	0.89	1.02	1.01	1.91	1.0	
15	[(*R* or *S*)Atc³]	0.55	0.28	6.44	795	0.91	0.99	1.01	1.90	1.0	
15a	[(*R* or *S*)Atc³]	0.57	0.30	7.23	795	0.89	1.01	1.02	1.89	1.0	

[a] HPLC used a C_{18} reversed-phase column (180 × 4.6 mm) under isocratic elution conditions at a ratio of 80:20 for mobile phases A:B, or at a ratio of *100:0, as detailed in the Experimental Section. [b] The values for phenylalanine (F) were eliminated because modified phenylalanines are not detected after amino acid hydrolysis. The complete sequence of the peptides can be found in Table I.

A,[20] drastically reduced δ and μ binding affinities with concomitant losses in δ selectivity.[19,20]

The most deleterious amino acid replacement on receptor binding occurred through methylation at the α-nitrogen of Phe³ (compound 5): that peptide had a 900-fold loss in δ affinity and 90-fold in μ affinity relative to compound 1. A less detrimental effect on affinity was noted in a cyclic μ selective tetrapeptide containing N$^\alpha$-MePhe³.[23] The loss in δ affinity by the methyl group can be attributed to several interrelated factors: e.g., an inability of the methyl amide to form H-bonds, cause steric impedance,[23] reduce flexibility through modification of inter- and intramolecular hydrophobic forces, and stabilize the peptide in a conformation unacceptable to the receptor. Examination of the K' values (Table II) revealed that an additional methyl group in compounds 5, 6, and 6a increased the retention time on the reversed-phase matrix, presumably due to enhanced hydrophobicity.

Replacement of a phenolic side chain in position 3 (7) diminished δ and μ affinities. Although decreased binding with Tyr³ was also apparent in the cyclic opioid tetrapeptide H-Tyr-D-Cys-Tyr-D-Pen-OH, its loss in δ selectivity was considerably less[25] than for compound 7. The hydrophilic hydroxyl moiety of Tyr at position 3 changes the electronic properties of the aromatic ring;[25] methylation of that functionality (peptide 8) further modified the lipophilic characteristics of the peptide and enhanced μ affinity by a factor of 2 without affecting δ affinity. A 5-membered hydrophilic ring substitution, His³ (analogue 9), resulted in δ and μ receptor binding properties identical to that of peptide 6a; the partial positive charge on the indole ring of His³ may, in part, explain the reduction in binding.

2. Bicyclic Residues. Bicyclic amino acid substituents (compounds 10-15a) were incorporated into position 3 of deltorphin C in order to further determine the influence of increased constraint at position 3. Taken as a whole, these substitutions were less restrictive on δ affinity than those observed with the 5- and 6-membered ring substitutions (1–9), indicating that the receptor site apparently accommodates and tolerates a bulky bicyclic ring substituent and a greater constraint. Substitution by Trp³ (10) decreased δ affinity 7.5-fold and reduced μ binding 50% to give a compound with modest δ selectivity. Substitution of Trp³ in a cyclic tetrapeptide manifested similar results.[26] The indole ring of Trp³ was anticipated to affect the electronic milieu involved in binding; however, the spatial configuration of Trp may also play a decisive role as visualized by a comparison of the differences in the δ affinities between Nap(1)³ (11) and Nap(2)³ (12): the orientation of Trp³ approximates that of Nap(1)³ rather than Nap(2)³, which appears similar to Tyr(OMe)³ (8) (Figure 1). In the conformationally constrained tetrapeptides (JOM-13), however, Nap(2)³ gave higher δ selectivity than Nap(1)³.[26] Apparently, conformational restriction in a peptide through cyclization between residues 2 and 4[25,26,41] constrains the spatial orientation of the side chain in position 3 relative to that in the flexible deltorphin C.[44,45]

The replacement of Phe³ by Tic³ results in spatially freezing the aromatic ring to its α-nitrogen atom through a methylene group, yielding a gauche(+) orientation of the side chain.[46] Tic represents one possible closed-ring structure of N$^\alpha$MePhe³ (5) and therefore it was not entirely surprising that it (8) displayed deleterious effects on opioid binding (Table I). These data confirm other observations on several other Tic-containing peptides, such as [Tic³]-deltorphin C,[19] H-Tyr-D-Orn-Tic-Glu-NH_2, a μ selective tetrapeptide analogue derived from deltorphin B,[23] and [Tic³]dermorphin.[46] The increased δ affinity observed in [Aic³]deltorphin C (14) indicates that the restriction in side chain conformation of Aic³ affects the spatial orientation of the peptide relative to the receptor. Moreover, the enhanced μ affinity in the Aic³ analogue (14) (Table I) could be explained in terms of a conformational constraint imposed by the side chain.[23] Furthermore, the modest loss in δ receptor selectivity on brain membranes (Table I) parallels that reported for the bioactivity on peripheral tissue with this analogue.[47]

Of the two chiral isomers of Atc³ (analogues 15 and 15a), peptide 15 exhibited a 4-fold higher δ selectivity (=2475) than deltorphin C(=661); compound 15a was equivalent to deltorphin C. Enhancement in δ selectivity in analogue 15 was primarily due to an increased δ affinity. Atc is known to be limited to two side-chain conformations on the basis of limited accessibility to a range of torsion angles,[23] which, in the case of deltorphin C, enhanced δ binding characteristics (Table I) and bioactivity.[47] The δ receptor selectivity of peptide 15 is the highest value

Table III. Bioassay of Selected Deltorphin C Analogues on Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD)[a]

no.	analogue	IC_{50} (nM) MVD	GPI	GPI/MVD
1	deltorphin C	0.34 ± 0.12	420 ± 95	1235
5	[N$^{\alpha}$MePhe^3]	>10 μM	>10 μM	
6	[(*R* or *S*)C$^{\alpha}$MePhe^3]	554 ± 59	>10 μM	>18
6a	[(*R* or *S*)C$^{\alpha}$MePhe^3]	498 ± 35	>10 μM	>20
13	[Tic^3]	156 ± 15	>10 μM	>65
14	[Aic^3]	0.41 ± 0.03	369 ± 40	900
15	[(*R* or *S*)Atc^3]	0.46 ± 0.02	1620 ± 170	3522
15a	[(*R* or *S*)Atc^3]	0.38 ± 0.03	230 ± 20	606

[a] The IC_{50} values (nM) represent the mean ± SE of four independent experiments as described in the Experimental Section. Bioselectivity is the ratio GPI/MVD. The complete sequence of the peptides is listed in Table I. The K_e for the antagonists naloxone or ICI 174,864 (*N,N*-dial Tyr-Aib-Aib-Phe-Leu-OH) fell within the range of 1–2 nM.

reported to date for a deltorphin analogue, being only slightly greater than that of [*p*-BrPhe^3]deltorphin C (=2003).[24]

Biological Activity. Bioassays were conducted only with analogues (compounds **5, 6, 6a, 13, 14, 15, 15a**) which demonstrated a marked deviation in δ receptor binding from that of deltorphin C (Table I). As seen in Table III, the analogues with low affinity for brain δ and μ receptors (compounds **5, 6, 6a, 13**) (Table I) interacted very weakly with mouse vas deferens (MVD) and (GPI), guinea pig illeum producing low bioselectivity ratios (GPI/MVD). On the other hand, analogues with high δ receptor affinities exhibited bioactivity on MVD comparable to deltorphin C (Table III); the bioselectivity of analogue **15** was approximately 3-fold higher than that of deltorphin C.

The bioactivity of peptides **15** and **15a** on pharmacological peripheral tissue preparations indicated that elevated δ selectivity occurred as a result of suppressed recognition at μ receptor sites.[47] In order to determine whether MVD bioactivity correlated with δ receptor binding, the data were analyzed as K_i^{δ} versus log IC_{50} MVD; *r* (correlation coefficient) equaled a significant 0.916 ($P < 0.005$). Thus, these compounds appear to interact in parallel to both peripheral and central δ receptors. Further, the peptides clearly behaved as δ agonists in the MVD assay since the K_e for the antagonists naloxone or *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH were within the range of 1-2 nM.

Molecular Modeling. The backbone torsional angles φ and ψ, and the side chain torsional angles χ1 and χ2 of the low-energy conformations of deltorphin C (1), N$^{\alpha}$-MePhe^3 (**5**), and (*S*)Atc^3 are listed in Table IV; other parameters are summarized in Table V.

Disparities that appeared between the low-energy structures of deltorphin C and the N$^{\alpha}$MePhe^3-containing analogue revealed that the side-chain torsional angle χ1 for Tyr^1 decreased from χ1 = 179.2° in deltorphin C to χ1 = −85.3° for N$^{\alpha}$MePhe^3. The constraint imposed on the side-chain-rotational ability of Tyr^1 by the incorporation of a methyl group on the amide nitrogen of Phe^3 was not anticipated. This suggests that steric interaction occurs between the phenolic ring of Tyr^1 and the methyl hydrogens which constrains the aromatic ring and the methyl carbon to the same plane (Figure 2); the torsional angles (χ1 and χ2) of the aromatic side-chain of Phe^3 were unaffected by the methyl group. The change in the torsional angle of the phenolic ring of Tyr^1, however, appeared to affect the backbone torsional angle φ for

Table IV. Backbone and Side-Chain Dihedrals for Low-Energy Conformers of Deltorphin C, [N$^{\alpha}$MePhe^3]-, [(*S*)Atc^3]deltorphin C

residue	degrees φ	ψ	χ1	χ2
	Deltorphin C			
Tyr^1		−58.676	179.2	63.4
D-Ala^2	74.588	−92.711	146.5	
Phe^3	−70.095	158.454	138.3	−77.7
Asp^4	−52.795	146.660	51.0	−89.8
Val^5	−92.536	−57.473	66.8	
Val^6	−162.065	40.946	165.7	
Gly^7	−101.95			
	[N$^{\alpha}$MePhe^3]deltorphin C			
Tyr^1		−49.952	−85.3	75.3
D-Ala^2	118.939	−104.779	135.3	
N$^{\alpha}$MePhe^3	−67.322	154.217	174.8	−76.3
Asp^4	−56.361	131.490	74.5	−103.7
Val^5	−55.141	−35.808	87.6	
Val^6	−61.009	−28.072	95.3	
Gly^7	−49.606			
	[(*S*)Atc^3]deltorphin C			
Tyr^1		46.633	−75.9	−62.0
D-Ala^2	−73.379	−2.471	123.3	
(*S*)Atc^3	68.909	60.894	−56.8	
Asp^4	25.538	−52.759	−172.6	77.6
Val^5	73.899	−77.184	158.2	
Val^6	73.744	−72.239	164.1	
Gly^7	−56.994			

[a] Solvated low-energy conformers were generated from molecular dynamics simulations using HyperChem as described in the Experimental Section.

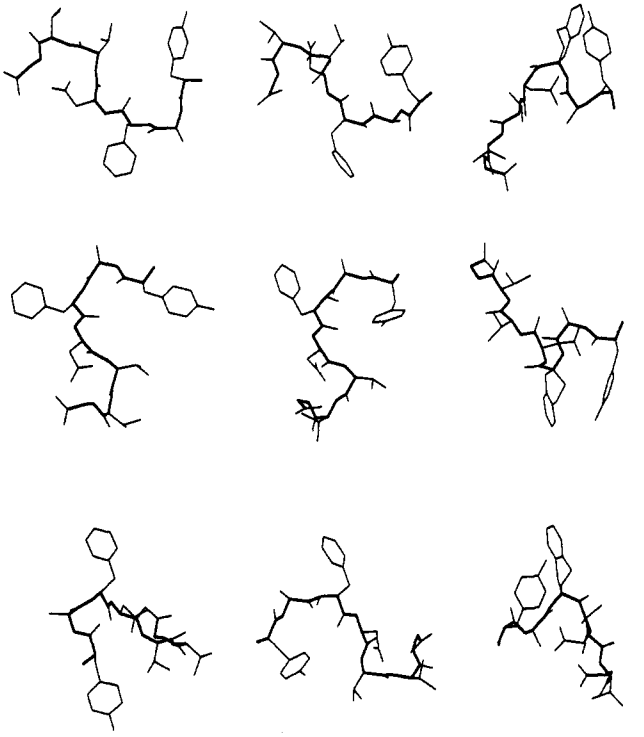


Figure 2. Three-dimensional representations of deltorphin C in first column, [N$^{\alpha}$MePhe^3]deltorphin C in the second column, and [(*S*)Atc^3]deltorphin C in the third column (right to left) oriented with the *z* axis perpendicular to the plane of the paper. Three views of each peptide are represented with alignment of the following residues: Tyr^1 for the top row, D-Ala^2 for the middle row, and Phe^3, N$^{\alpha}$MePhe^3, or (*S*)Atc^3 for the bottom row.

D-Ala^2, Val^5, Val^6, and Gly^7 by about a factor of 2, while φ for Phe^3 and N$^{\alpha}$MePhe^3 are limited to values around −70°. These changes in spatial orientation are indicated in the molecular models (Figure 2). (In corroboration of

Table V. Low-Energy Parameters (kcal/mol) of Deltorphin C, [N$^{\alpha}$MePhe³]-, and [(*S*)Atc³]deltorphin C[a]

peptide	atoms	energy (total)	gradient (kcal/mol Å)	dihedral	van der Waals	electrostatic	bond	angle	H-bond
DEL C	106	-237.329	0.097	9.237	10.914	-236.560	1.55	5.855	-1.325
N$^{\alpha}$MePhe³	110	-182.601	0.099	22.494	13.689	-234.728	1.423	15.989	-1.469
(*S*)Atc³	110	-196.158	0.098	16.827	17.090	-241.893	1.403	12.985	-2.571

[a] The potential energy parameters of solvated low-energy conformers in the AMBER force field calculated using HyperChem as described in the Experimental Section. The complete sequence of the peptides is shown in Table I.

this observation, a change in the chirality of Tyr¹ in a deltorphin analogue produced a marked decrease in δ affinity and selectivity.[20] The restriction placed on the side chain of Tyr¹ that induced modifications in the backbone conformation confirm that the loss of receptor binding of N$^{\alpha}$MePhe³ (5) (Table I) is consistent with the importance of the proper spatial orientation of the peptide in solution and the conformation of the crucial aromatic rings.[31] The interesting increase in δ receptor affinity observed by the substitution of 4-aminotetrahydro-2-benzazepin-3-one for the dipeptide Phe³-Gly⁴ in demorphin further illustrates the importance of the orientation of aromatic side chain of Phe³ and its relative orientation to Tyr¹.[46]

In comparison to deltorphin C, (*S*)Atc³ exhibited higher energy/mol for several variables (Table IV) and conspicuous changes in the torsional angles for the backbone and side chains for several amino acid residues. Of particular note is the reverse in backbone orientation observed in the φ values and changes in χ1 values, which translated for the most part into a low-energy conformation distinctly different from that of either deltorphin C or N$^{\alpha}$MePhe³ (Figure 2). The spatial orientation of Tyr¹ in relation to (*S*)Atc³, due to the constraints placed on the molecule by the Atc residue, places the aromatic rings in relatively close proximity in a molecule that approximates the solution conformation of several unrelated cyclic and structurally restricted opioids.[23,31,48]

Several low-energy structures exist for a molecule and indicates that other conformations with similar energies could feasibly describe the spatial orientation of these three molecules during receptor interaction. Furthermore, higher energy conformations cannot be ignored since an adopted configuration during receptor interaction may not be of the lowest energy. In fact, a higher energy conformer of deltorphin C may account for the unexpected structural differences with the Atc³-substituted analogue; in particular, the folding of the side chain of Asp⁴ toward that of Phe³, forming a pseudobicyclic ring compound through weak intra- and intermolecular hydrogen bonding interactions, may describe the receptor interactive structure better than the lowest energy conformer. This conformation would be similar to the bicyclic ring structure of Atc³, which exhibits significant δ receptor interaction. In contrast, N$^{\alpha}$MePhe³, which does not favor δ receptor interaction, cannot feasibly accommodate this type of pseudocyclization due to a steric interaction between the amide methyl group hydrogens, the peptide backbone, and the Asp⁴ residue.

Experimental Section

Peptide Purification. Open-column chromatography (2 × 70 cm, 0.7-1 g of material) was run on silica gel 60 (70-230 mesh) using a gradient from 10% to 60% MeOH in CH_2Cl_2. Preparative reverse-phase HPLC was conducted with a Waters Delta Prep 3000 system using a Delta Pak C_{18} 3000 A (300 × 30 mm, 15 μm, spherical) column. Peptides were eluted with a gradient of 10% to 80% B at a flow rate of 30 mL/min; mobile phases consist of solvent A (10% acetonitrile in 0.1% TFA, v/v) and solvent B (60% acetonitrile in 0.1% TFA, v/v).

Analytical HPLC was performed on a Bruker liquid chromatography LC 21-C instrument using a Spherisorb 5 ODS2 C_{18} column (250 × 4.6 mm, 5-μm particle size) and equipped with a Bruker LC 313 UV variable-wavelength detector. Recording and quantification were accomplished with a chromatographic data processor coupled to an Epson computer system (QX-10).

Analytical Determinations. Melting points were determined on a Kofler apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer 241 polarimeter with a 10-cm water-jacketed cell using either DMF or MeOH as the solvents at 1% peptide concentrations. The capacity factors (*K'*) of the peptides (Table II) were determined using a Vydac C_{18} reverse-phase column (180 × 4.6 mm) operating under isocratic conditions with ratios of 80:20 or 100:0 (v/v) for mobile phases A and B given above and at flow rates of 1 mL/min. All analogues revealed less than 1% impurities when monitored at 220 nm.

Amino acid analyses were carried out using PITC (Pico-Tag) methodology (Waters-Millipore, Waltham, MA) as the amino acid derivatization reagent. Lyophilized samples of peptides (50-1000 pmol) were place in heat-treated borosilicate tubes (50 × 4 mm), sealed, and hydrolyzed using 200 μL of 6 N HCl containing 1% phenol in the Pico-Tag workstation for 1 h at 150 °C. A Pico-Tag column (15 × 3.9 mm) was employed to separate the PITC-amino acid derivatives. The peptide containing Trp (compound 10) was determined at 291 nm. Hydrogenation of the dehydro residue in the ΔPhe³ analogue (4) was followed by acid hydrolysis to yield Phe; acid hydrolysis without hydrogenation yields phenylpyruvic acid and ammonia instead of Phe.[49]

TLC used precoated plates of silica gel F254 (Merck, Darmstadt, Germany) in the following solvent systems: (I) 1-butanol/acetic acid/H_2O (3:1:1, v/v/v), (II) EtOAc/pyridine/acetic acid/H_2O (12:4:1.2:2.2, v/v/v/v), (III) CH_2Cl_2/MeOH/toluene (85:10:5 v/v/v), and (IV) $CHCl_3$/MeOH/benzene/H_2O (8:8:8:1, v/v/v/v). Ninhydrin (1%), fluorescamine, and chlorine spray reagents were employed to detect the peptides.

Quantitative Analyses. N$^{\alpha}$-Z-Tic-OH·DCHA: mp 125-127 °C; TLC R_f 0.94 (I), 0.88 (II), 0.33 (III), 0.44 (IV). Anal. Calcd for $C_{18}H_{17}NO_4$: C, 69.44; H, 5.51; N, 4.5. Found: C, 69.38; H, 5.66; N, 4.45. **N$^{\alpha}$-Z-Tic-OH·DCHA**: mp 148-150 °C; TLC R_f 0.95 (I), 0.88 (II), 0.31 (III), 0.58 (IV), 0.58 (IV). Anal. Calcd for $C_{30}H_{40}N_2O_4$: C, 73.14; H, 8.18; N, 5.69. Found: C, 72.97; H, 8.17; N, 5.71. **Na$^{\alpha}$-Z-(*R* or *S*)Atc-OH·DCHA**: mp 162-165 °C; TLC R_f 0.91 (I), 0.89 (II), 0.27 (III), 0.64 (IV). Anal. calcd for $C_{31}H_{32}N_2O_4$: C, 73.49; H, 8.36; N, 5.53. Found: C, 73.61; H, 8.21; N, 5.71. **N$^{\alpha}$-Fmoc-(*R* or *S*)C$^{\alpha}$MePhe-OH**: mp 182-185 °C; TLC R_f 0.9 (I), 0.95 (II), 0.32 (III), 0.64 (IV). Anal. Calcd for $C_{25}H_{23}NO_4$: C, 74.8; H, 5.77; N, 3.49. Found: C, 75.01; H, 5.91; N, 3.47.

Deprotection Procedures. Method A. Boc and O*t*Bu protecting groups were removed by treating the peptide with aqueous 90% TFA (1:10, w/v) containing anisole (1 mL) for 30-40 min. The solvent was removed *in vacuo* at 0 °C and the residue triturated with diethyl ether or petroleum ether; the resulting solid was collected and dried.

Method B. Hydrogenation was carried out in acetic acid/MeOH (1:3, v/v) at atmospheric pressure and room temperature in the presence of 10% palladium on charcoal (catalyst to peptide ratio, 1:9, w/w). The reaction mixture was filtered through a Cellite bed and evaporated to dryness. The residue was treated as described above.

Method C. The Fmoc protecting group was removed by treating the peptide with 20% piperidine in DMF for 30 min. The solvent was evaporated and the residue triturated with ether, collected, and dried.

Table VI. Analytical Properties of Boc-Tyr-D-Ala-Xaa-OH

Xaa[3]	TLC R_f[a] I	TLC R_f[a] II	mp (°C)	$[\alpha]^{20}_D$[b] (deg)
Phg	0.74	0.70	132–135	+9.1
Hfe	0.80	0.90	130–132	+3.2
Tyr	0.85	0.75	114–116	+10.1
Tyr(OMe)	0.89	0.76	107–109	+9.7
His	0.94	0.83	79–81	+11.2
Trp	0.87	0.75	110–112	+16.8
Nap(1)	0.88	0.78	118–120	-3.1
Nap(2)	0.93	0.78	104–106	-1.8

[a] The solvent systems for TLC are listed in the experimental Section. [b] Optical rotations were recorded in MeOH.

Table VII. Analytical Properties of P-Xaa-Asp(OtBu)-Val-Val-Gly-NH_2

Xaa[3]	TLC R_f[a] I	TLC R_f[a] II	TLC R_f[a] III	mp (°C)	$[\alpha]^{20}_D$[b] (deg)
N$^{\alpha}$MePhe	0.88	0.96	0.38	98–102	-24.9
(*R* or *S*)C$^{\alpha}$MePhe	0.79	0.92	0.49	130–133	-5.3
Tic	0.78	0.84	0.37	138–142	-31.6
Aic	0.79	0.98	0.61	197–201	-6.8
(*R* or *S*)Atc	0.82	0.75	0.38	182–185	+3.2

[a] TLC solvent systems I, II, and III are given in the Experimental Section. [b] Optical rotations were taken in MeOH.

Specific Syntheses. Scheme I. Procedures for the Synthsis of the Protected Peptides Boc-Tyr-D-Ala-Xaa-OH. To a solution of H-Xaa-OH (2 mmol) in DMF (10 mL) at 0 °C were added NMM (2.2 mmol or 4.4 mmol in the case of His) and Boc-Tyr-D-Ala-OSu (1.8 mmol). This reaction mixture was stirred for 2 h at 0 °C and 24 h at room temperature. The solution was diluted with EtOAc (100 mL) and washed with 10% citric acid (2 × 20 mL) and brine. The organic layer was dried over sodium sulfate and concentrated *in vacuo* and the residue crystallized from diethyl ether. Table VI lists the analytical properties of the tripeptides.

Scheme II. Procedures for the Synthesis of Protected Pentapeptides P-Xaa-Asp(OtBu)-Val-Val-Gly-NH_2. To a solution of P-Xaa-OH (2 mmol) in DMF (10 mL) were added in the following order at 0 °C: the amino component (2 mmol), NMM (2 mmol if the amino component is in the protonated form), HOBt (2.2 mmol), and DCC (2.2 mmol). The reaction mixture was stirred for 2 h at 0 °C and 24 h at room temperature. *N,N'*-Dicyclohexylurea was filtered off and the solution diluted with EtOAc (100 mL) and successively washed with brine, 0.5 N $KHSO_4$, brine, 5% $NaHCO_3$, and brine. The organic phase was crystallized from diethyl ether or purified by column chromatography (*supra vide*). The analytical properties of the pentapeptides are given in Table VII.

Schemes I and II. Procedures for the Synthesis of Protected Heptapeptides Boc-Tyr-D-Ala-Phe-Asp(OtBu)-Val-Val-Gly-NH_2. To a solution of the carboxy component (1 mmol) in DMF (10 mL), were sequentially added the following at 0 °C: the amino component (1 mmol), NMM (1 mmol if the amino component is in the protonated form), HOBt (1.1 mmol), and DCC (1.1 mmol). The heptapeptides were worked up as detailed in the previous paragraph and their analytical properties are reported in Table VIII.

Synthesis of [ΔPhe[3]]deltorphin C (peptide IV). Step 1. Z-D-Ala-ΔPhe-Asp(Ot-Bu)-Val-Val-Gly-NH_2. To a solution of Z-D-Ala-ΔPhe-azlactone (1.75 g, 5 mmol)[34] in THF (50 mL) was added dropwise a solution of H-Asp (OtBu)-Val-Val-Gly-NH_2 (2.67 g, 6 mmol) in DMF (8 mL) at 0 °C. After 4 h at room temperature, the reaction mixture was evaporated to remove the solvent, diluted with EtOAc (150 mL), washed with basic and acidic solutions, and dried. Partial evaporation furnished a crystalline product that was recrystallized from EtOAc/diethyl ether. Analysis of the synthetic product follows: 71% yield (2.82 g); mp 141–143 °C; $[\alpha]^{20}_D$-18.5° (MeOH); TLC R_f 0.88 (I), 0.85 (II), 0.36 (III). The Z protecting group was removed by treating the dehydrohexapeptide with 32% HBr in acetic acid (1:10, w/v) for 30-40 min. The solvent was evaporated in vacuo at 0 °C and the residue triturated with diethyl ether. The resulting solid was crystallized from ethanol–diethyl ether, collected, and dried.

Table VIII. Analytical Properties of Boc-Tyr-D-Ala-Xaa-Asp(OtBu)-Val-Val-Gly-NH_2

Xaa[3]	TLC R_f[a] I	TLC R_f[a] II	TLC R_f[a] III	mp (°C)	$[\alpha]^{20}_D$[b] (deg)
Tyr	0.91	0.94	0.38	189–191	-3.9*
Tyr(OMe)	0.94	0.89	0.41	182–184	-3.5*
His	0.85	0.91	0.35	188–190	-8.7*
Trp	0.87	0.90	0.30	192–194	-2.4*
Nap(1)	0.96	0.89	0.40	207–209	-2.4*
Nap(2)	0.95	0.90	0.39	205–207	-2.8*
Phg	0.91	0.87	0.32	215–220	-10.7**
Hfe	0.90	0.95	0.25	202–205	-13.7**
Tic	0.78	0.84	0.37	138–142	-31.6*
(*R* or *S*)C$^{\alpha}$MePhe	0.86	0.89	0.40	139–141	+4.0**
Aic	0.80	0.89	0.39	145–147	+6.4**
(*R* or *S*)Atc	0.85	0.79	0.22	115–120	-11.2**
N$^{\alpha}$MePhe	0.88	0.89	0.42	135–138	-31.4*

[a] The solvent systems for TLC are listed in the Experimental Section. [b] Optical rotations were taken in either *MeOH or **DMF.

Step 2. Boc-Tyr-D-Ala-ΔPhe-Asp-Val-Val-Gly-NH_2. The deprotected peptide H-D-Ala-ΔPhe-Asp-Val-Val-Gly-NH_2·HBr (2.96 g, 4 mmol) and TEA (0.56 mL, 4 mmol) were dissolved in DMF (20 mL) at 0 °C. To this solution was added Boc-Tyr-OSu (1.5 g, 4 mmol) and the mixture was stirred for 1 h at 0 °C and then overnight at room temperature. The reaction mixture was diluted (200 mL EtOAc) and washed with acidic solution, brine, and evaporated; the residue was crystallized from EtOAc–diethyl ether. Analysis of the product gave the following: 84% yield (3.11 g); mp 200–202 °C; $[\alpha]^{20}_D$-13.8° (DMF); TLC R_f 0.92 (I), 0.85 (II), 0.36 (III). Hydrogenation and acid hydrolysis of the peptide gave the following amino acid composition: Tyr 0.89, Ala 0.97, Phe 0.95, Asp 1.01, Val 1.79, Gly 1.0.

Radioreceptor Assays. Synaptosomal preparations (P_2) were prepared from the brains (minus cerebella) of Sprague–Dawley CD male rats according to the method of Chang and Cuatrecasas[50] as detailed elsewhere.[7,15,18,32,51] The synaptosomes were preincubated to remove endogenous opioids,[51] washed three times by centrifugation, and stored at -80 °C in 50 mM HEPES, pH 7.5, containing 20% glycerol, 50 μg/mL soybean trypsin inhibitor, and 1 mM dithiothreitol. Under these conditions, synaptosomes could be thawed to room temperature and refrozen without deleterious effects on binding.[51]

Receptor binding was measured by the following rapid filtration technique: the membrane bound-[^{3}H]ligand complex was entrapped on Whatman glass-fiber filters (GF/C) under vacuum and washed with 3 × 2 mL of ice-cold buffered BSA within 5 s. Binding at steady-state conditions was conducted at room temperature (22 °C) for 2 h in duplicate polypropylene tubes to minimize absorption to vessel surfaces. The assays contained 1.6 mg protein and 0.62 nM [^{3}H]DPDPE (34.3 Ci/mmol, New England Nuclear–Du Pont, Boston, MA) to label δ sites or 0.68 nM [^{3}H]DAGO (60 Ci/mmol, Amersham, Arlington, IL) for μ sites.[18] The difference in the radioligand bound to that displayed by either 1 μM DPDPE or DAGO, respectively, is defined as specific binding. Filters were dried at 75–80 °C; bound radioactivity was measured by liquid scintillation spectrometry using CytoScint (ICN, IRvine, CA) after a 1–2-h equilibration. The K_i values were calculated from the IC_{50} values according to the method of Cheng and Prusoff.[42]

Bioassays. Segments of GPI (2–3 cm each) were mounted in 20-mL organ baths and bathed in Kreb's solution containing 70 μM hexamethonium bromide and 0.125 μM mepyramine maleate and aerated with 95% O_2/5% CO_2 at 36 °C as described earlier.[24] The suspended tissue was transmurally stimulated with square-wave electric pulses of 0.5 ms at 0.1 Hz; the strength of the stimulus was 1.5-fold that which produced a maximal twitch (~30 V). Contractions were isotonically recorded at a magnification ratio of 1:15. The IC_{50} values are defined as the concentration of peptide (nM) required to inhibit the amplification of the electrically induced twitch by 50%. A single MVD was dissected and suspended in 4 mL of modified Kreb's solution aerated with 95% O_2/5% CO_2 at 33 °C.[24] The twitch was induced by field

stimulation (0.1 Hz for 1 ms at 40 V) and recorded with an isometric transducer.

Dose–response curves were obtained by the addition of 10–100-μL quantities of the peptides in Kreb's solution. The tissues were thoroughly washed 3 or 4 times over a 10-min interval between each dose. log–dose curves were prepared for each tissue using dermorphin or morphine standards and the IC_{50} values normalized according to published methods.[24] The K_e for the antagonists naloxone or ICI 174,864 (*N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH) were in the range of 1–2 nM.

Molecular Modeling. Molecular dynamics calculations were performed using the HyperChem (release 2) computer modeling program (AutoDesk) on a Dell 486D personal computer operating at 50 MHz; the application of the personal computer to molecular modeling was validated by Garrett et al.[53] The atomic coordinates and parameters of the amino acid residues N$^{\alpha}$MePhe and (*S*)Atc were assigned according the Brookhaven Protein Data Bank format and incorporated into HyperChem. Starting structures of deltorphin C, and the [N$^{\alpha}$MePhe³]- and [(*S*)Atc³]-containing analogues were generated using dihedrals derived from the nuclear Overhauser enhancement spectroscopy data described by Amodeo et al.[28] Analyses were completed using periodic boundary conditions and the all-atom AMBER force field with distance dependent dielectric (ϵ) for initial structures and constant dielectric ($\epsilon = 1$) for solvated structures. Geometry optimizations incorporated the Polak Ribiere algorithm stopping with root mean square gradient of 10^{-2} kcal/mol Å or less. Solvated low-energy structures were generated by optimization of initial starting structures in equilibrated TIP3P water molecules. Simulated annealing analysis generated more than 6000 conformers per peptide and utilized a mechanism similar to that reported by Tancredi et al.[30] Six temperatures were used in the annealing process, 1000, 500, and 250 K, each with 2-ps run times, and 100, 50, and 10 K, each with 1-ps run times. Snapshots and data were collected at every time step, i.e., at 0.2 fs for the first three temperatures and at 0.5 fs for the latter three temperatures. The lowest energy conformers were chosen from each run, optimized, and used in the next annealing cycle. Final structures were selected based on the lowest energy conformations that were generated during the complete analysis.[45]

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(1) The abbreviations used throughout this paper are in accordance to the recommendations of the IUPAC–IUB Commission for Amino Acids and Peptides and other abbreviations are as follows: DEL C, deltorphin C; Aic, 2-aminoindane-2-carboxylic acid; Atc, 2-aminotetralin-2-carboxylic acid; Boc, *tert*-butoxycarbonyl; C$^{\alpha}$MePhe, C$^{\alpha}$–methylphenylalanine; DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; Δ, dehydro(α,β-unsaturated); ΔPhe, dehydrophenylalanine in the *Z* isomer; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, (fluoren-9-ylmethoxy)carbonyl; GPI, guinea pig ileum; Hfe, homophenylalanine; HPLC, high-performance liquid chromatography; MVD, mouse vas deferens; N$^{\alpha}$MePhe, N$^{\alpha}$-methylphenylalanine; Nap(1), 3-(1′-naphthyl)alanine; Nap(2), 3-(2′-naphthyl)alanine; NMM, *N*-methylmorpholine; OSu, *N*-succinimidyl ester; O*t*Bu, *tert*-butyl ester; Phg, phenylglycine; PITC, phenyl isothiocyanate; TEA, triethylamine; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TLC, thin-layer chromatography; and Z, benzyloxycarbonyl.

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