Development of a Model for the δ Opioid Receptor Pharmacophore. 2. Conformationally Restricted Phe³ Replacements in the Cyclic δ Receptor Selective Tetrapeptide Tyr-c[D-Cys-Phe-D-Pen]OH (JOM-13)†

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Received August 23, 1994®

The *in vitro* pharmacological properties and conformational features of analogs of the δ opioid receptor selective tetrapeptide Tyr-c[D-Cys-Phe-D-Pen]OH (JOM-13) in which the Phe³ residue was replaced by each of the four stereoisomers of β-methylphenylalanine (β-MePhe) were investigated. Both analogs in which the α carbon of the Phe³ replacement has L-stereochemistry display high affinity for δ receptors with the (2*S*,3*S*)-MePhe³ analog exhibiting approximately 8-fold higher affinity than the (2*S*,3*R*)-MePhe³ diastereomer. Surprisingly, one analog with D-stereochemistry in residue 3, the (2*R*,3*R*)-MePhe³ analog, also displays high affinity for the δ receptor and is extraordinarily selective for this receptor. All analogs were agonists in the mouse vas deferens (MVD) and guinea pig ileum (GPI) smooth muscle bioassays, displaying MVD and GPI potencies consistent with their δ and μ opioid receptor affinities, respectively. The use of β-MePhe as a replacement for Phe³ was based upon the desire to reduce the conformational flexibility of the Phe³ side chain by imposing a steric rotational constraint in the form of the β-methyl substituent and to thus deduce the residue 3 side chain orientation in the δ receptor-bound conformation from the correlation between δ receptor binding affinities and conformational preferences. Molecular mechanics computations revealed, however, that the conformational constraints imposed by the β-methyl group in the (2*S*,3*S*)-MePhe³ and (2*S*,3*R*)-MePhe³ analogs were too modest to allow unequivocal determination of δ receptor-bound residue 3 side chain conformation. However, analysis of the high-affinity (2*R*,3*R*)-MePhe³ analog revealed a strong preference for a single side chain conformer ($\chi^1 \sim 60°$). Low-energy conformers of this analog could only be effectively superimposed with low-energy conformers of the parent peptide in which the Phe³ side chain conformation was limited to $\chi^1 \sim -60°$. This observation eliminates the last remaining uncertainty regarding conformational features of the pharmacophore elements in the δ receptor-bound state, allowing the proposal of a complete model.

The cyclic tetrapeptide Tyr-c[D-Cys-Phe-D-Pen]OH, **1** (JOM-13), is a high-affinity opioid agonist with substantial selectivity for the δ opioid receptor.[1] The presence of conformational constraints, in the form of the 11-membered disulfide-containing ring which includes the *gem*-disubstituted penicillamine β carbon, makes this peptide a suitable tool for the elucidation of the bioactive conformation at the δ receptor, which for this and other opioid receptors must still be approached indirectly by extrapolation of conformational preferences of the ligand in the absence of receptor. Experimental (NMR, X-ray crystallography) and theoretical (molecular mechanics calculations) studies confirmed that the 11-membered cycle of **1** has limited flexibility.[2,3] Results from these studies indicated the existence of two closely related, isoenergetic conformations of the 11-membered cycle formed by the disulfide between residues 2 and 4. The two conformations are identical for the main chain atoms within the cycle, differing only in conformational features about the disulfide itself. These results clearly indicate that the 11-membered cycle of **1** forms a structurally well-defined scaffold which allows the appropriate orientations of the opioid pharmacophoric elements of the peptide, the Tyr¹ amino and phenolic functions and the Phe³ aromatic side chain, to be maintained. However, the conformations of these elements, themselves, remain very flexible with many distinct, low-energy arrangements of the pharmacophoric elements accessible. In order to better define the bioactive conformational features of the pharmacophoric elements of **1**, we have investigated analogs of this peptide in which the flexible Tyr¹ residue was replaced by structurally related, conformationally restricted amino acids.[4,5] Examination of accessible conformational space common to analogs with high or moderate δ receptor affinity led to a model of the bioactive conformation for **1** and its analogs in which the side chain torsion angle $\chi^1 \sim 180°$ and for which the main chain torsion angles ψ of residue 1 and φ of D-Cys² are both ~160°.[5] Left unresolved by this study is the likely orientation of the Phe³ side chain in the δ receptor-bound conformation since any of the three low-energy, staggered rotamers about the $C^\alpha-C^\beta$ bond ($\chi^1 = 60°$, *gauche*⁻; $\chi^1 = -60°$, *gauche*⁺; $\chi^1 = 180°$, *trans*) can be accommodated. In the present report we describe the synthesis, pharmacological evaluation, and conformational analysis of analogs

† Abbreviations and definitions recommended by IUPAC-IUB Commission of Biochemical Nomenclature have been used. Other abbreviations: β-MePhe, β-methylphenylalanine; MVD, mouse vas deferens; GPI, guinea pig ileum.

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® Abstract published in *Advance ACS Abstracts*, November 1, 1994.

0022-2623/94/1837-4384$04.50/0

Table 1. Binding Affinites (±SEM) and Selectivities of Residue 3-Modified Tetrapeptides

compd no.	peptide	K_i (nM) [^{3}H]DAMGO	K_i (nM) [^{3}H]DPDPE	$K_i(\mu)/K_i(\delta)$
1	Tyr-c[D-Cys-Phe-D-Pen]OH (JOM-13)	51.5 ± 4.4	0.74 ± 0.08	69
2	[(2*R*,3*S*)-MePhe^3]JOM-13	>10000	237 ± 11	>42
3	[(2*S*,3*R*)-MePhe^3]JOM-13	1000 ± 194	12.3 ± 0.92	81
4	[(2*R*,3*R*)-MePhe^3]JOM-13	>10000	4.80 ± 0.87	>2100
5	[(2*S*,3*S*)-MePhe^3]JOM-13	259 ± 20.3	1.52 ± 0.12	170
6	[D-Phe^3]JOM-13	2530 ± 650	70 ± 5.5	36

of **1** in which the Phe^3 residue is replaced, in turn, by each of the stereoisomers of β-methylphenylalanine (β-MePhe). Although only modest steric effects are observed for the two β-Me-L-Phe^3 analogs, rotational freedom about the C^α–C^β bond in the surprisingly potent and δ receptor selective (2*R*,3*R*)-MePhe^3 analog is greatly reduced, leading to an increased preference for a single rotamer with $\chi^1 \sim 60°$. Comparison of energetically favorable conformations of the (2*R*,3*R*)-MePhe^3 analog with those of the parent peptide, **1**, allows the final features of the δ receptor pharmacophore to be proposed.

Results and Discussion

Pharmacological Evaluation of β-MePhe^3 Analogs. Table 1 summarizes the binding affinities observed at μ (vs [^{3}H]DAMGO) and δ (vs [^{3}H]DPDPE) opioid receptors for the four β-MePhe-substituted analogs of **1** and compares these results to those for **1** and the previously reported[6] D-Phe^3 analog, **6**. Binding at κ receptors was also investigated; however none of the compounds showed significant affinity (K_i > 10 000 nM vs [^{3}H]U69,593). As seen from Table 1, both β-MePhe-substituted peptides, **3** and **5**, in which this residue has L-stereochemistry display high δ receptor binding affinity. The (2*S*,3*S*)-MePhe^3 analog, **5**, binds particularly well, exhibiting similar affinity as the parent peptide, **1**, while the (2*S*,3*R*)-MePhe^3 analog, **3**, experiences an order of magnitude loss in δ affinity. Striking differences in binding behavior are noted for the two analogs in which the β-MePhe residue has D-stereochemistry at the α carbon. Analog **2**, in which this stereochemistry is 2*R*,3*S*, binds rather weakly to the δ opioid receptor, an observation similar to that for the D-Phe^3 analog, **6**. By contrast the (2*R*,3*R*)-MePhe^3 analog, **4**, binds surprisingly well to the δ opioid receptor while evincing insignificant affinity for the μ receptor. Consequently, the δ receptor selectivity of **4** is exceedingly high. In our hands, the δ selectivity of **4** is an order of magnitude higher than that displayed by the deltorphins[7,8] and is comparable to that shown by the antagonist tetrapeptide TIPP and its analogs.[9,10]

The β-MePhe-substituted analogs **2**–**5** were further examined in bioassays to assess their pharmacological selectivities and to determine whether these analogs function as agonists or antagonists. The standard μ receptor sensitive guinea pig ileum (GPI)[11] and δ receptor sensitive mouse vas deferens (MVD)[12] assays were employed, and the observed results are summarized in Table 2 along with previously reported data for **1**. As can be seen from Table 2, all four β-MePhe-substituted analogs are agonists in the MVD assay and exhibit potencies consistent with their observed δ receptor binding affinities. In the GPI assay, only **3** and **5**, the two analogs with L-stereochemistry in residue 3, display measurable agonist potency. This, too, is consistent with the binding results since only these analogs displayed significant affinities for the μ opioid receptor (Table 1). As was found in the binding results, these two analogs differ in their bioactivities. Analog **5**, with 2*S*,3*S*-stereochemistry, is virtually indistinguishable from the L-Phe^3-containing parent peptide, **1**, while the potency of analog **3**, with 2*S*,3*R*-stereochemistry, is attenuated. Notable, too, among these compounds is the unexpectedly high MVD potency shown by **4**, which also follows the binding result.

Table 2. Bioassay Potencies (±SEM) and Selectivities of Residue 3-Modified Tetrapeptides

compd no.	IC_{50} (nM) GPI	IC_{50} (nM) MVD	IC_{50}(GPI)/IC_{50}(MVD)
1	463 ± 189	4.2 ± 1.0	110
2	>30000	1110 ± 164	>26
3	3730 ± 181	51.8 ± 18.8	72
4	>30000	9.72 ± 2.97	>3090
5	755 ± 78.3	4.13 ± 0.66	182

The excellent agreement between bioassay and corresponding receptor binding results is further evident in the extent to which bioassay selectivities mirror the binding selectivities. Consistent with the observations from the binding studies, the bioassay selectivity of **4** is quite striking. As was noted above, the δ receptor binding selectivity of this analog, in our hands, is comparable to that of the TIPP series of peptides developed by Schiller and co-workers.[9,10] However, as is clear from Table 2, **4** differs from these antagonist TIPP peptides by being a full agonist.

Conformational Analysis of Residue 3-Modified Analogs of JOM-13. Molecular mechanics calculations were done for five analogs of JOM-13: the four β-MePhe^3-substituted analogs, **2**–**5**, and the D-Phe^3 analog, **6**. As was earlier described for **1**,[3] conformational analyses of **2**–**6** were performed in two steps. First, low-energy conformers of the cyclic portion of the tetrapeptides were determined by conformational search/energy minimization studies of CH_3CO-c[D-Cys-Ala-D-Pen]OH[3] and CH_3CO-c[D-Cys-D-Ala-D-Pen]OH. These low-energy conformers were then combined with allowed conformations of the Tyr^1 residue and the β-MePhe^3 side chain and minimized again. For analogs **3** and **5**, with L-stereochemistry for residue 3, the previously reported analysis of CH_3CO-c[D-Cys-Ala-D-Pen]OH[3] results in three low-energy families of conformers, A, B, and C, all of which share very similar main chain torsion angles in the disulfide-containing cyclic fragment but differ in the geometry of the disulfide bridge (Table 3). As discussed previously,[3] the two most highly populated conformational families, A and B, are also observed in NMR and X-ray diffraction studies of **1**. The set of lowest energy conformers (with relative energies ΔE < 3.0 kcal/mol) for the [(2*S*,3*R*)MePhe^3]JOM-13 analog, **3**, is shown in Figure 1.

Unlike the conformationally well-defined cycle, all exocyclic elements of the (2*S*,3*R*)-MePhe^3 and (2*S*,3*S*)-

Table 3. Relative Energies, ΔE (kcal/mol), and Torsion Angles (deg) of Lowest Energy Conformations for Cyclic Structures A–C of Tyr-c[D-Cys-(2*S*,3*R*)-MePhe-D-Pen]OH, **3**, and A–F of Tyr-c[D-Cys-(2*R*,3*R*)-MePhe-D-Pen]OH, **4**

		3			**4**					
		A	B	C	A	B	C	D	E	F
ΔE		0.0	0.4	2.0	0.0	0.4	0.2	3.1	3.8	4.1
Tyr^1	ψ	148	147	148	151	149	150	−51	2	−31
	χ^1	−173	−179	−175	−176	180	−177	60	69	61
	χ^2	65	72	74	62	72	72	104	104	95
D-Cys^2	φ	168	166	168	174	174	175	69	77	161
	ψ	40	49	47	37	46	45	−179	−164	125
	χ^1	−60	176	177	−63	175	178	172	−47	−59
	χ^2	−148	147	71	−145	139	68	159	−49	46
	χ^3	93	−104	87	93	−106	89	−105	−92	−133
MePhe^3	φ	−80	−79	−76	−68	−68	−67	93	82	71
	ψ	−46	−32	−54	−53	−43	−61	−17	−78	21
	χ^1	176	178	173	45	43	46	−65	52	−64
	χ^2	64	64	65	63	61	66	79	61	83
D-Pen^4	φ	142	141	134	141	139	134	135	150	73
	χ^1	−69	−69	48	−68	−68	52	−63	−51	−57
	χ^2	53	96	−143	54	104	−145	63	150	79

Figure 1. Superposition (stereoview) of low-energy (ΔE < 3 kcal/mol) conformers from family A (solid line) and B and C (dashed lines) of **3** (Tyr-c[D-Cys-(2*S*,3*R*)-MePhe-D-Pen]OH). C$^\alpha$ atoms of the Tyr^1, D-Cys^2, and Phe^3 residues were used for the superposition.

MePhe^3 analogs (i.e., the Tyr^1 residue and β-Me-L-Phe^3 side chain) are flexible (Figure 1). The first peptide group (between Tyr^1 and D-Cys^2) assumes different conformations because of the existence of two local energy minima for both the ψ angle of the Tyr^1 and the φ angle of the D-Cys^2 residues. This leads to a variety of orientations of the entire Tyr^1 residue relative to the rest of the molecule. In addition, all staggered rotamers of the Tyr^1 and β-Me-L-Phe^3 side chains are represented in the sets of low-energy conformations for both β-Me-L-Phe^3 analogs, **3** (Figure 1) and **5**. This is identical to the situation described previously for the parent peptide, **1**.[3]

In order to eliminate the conformational uncertainty of the exocyclic Tyr region of **1**, we examined analogs of this peptide in which the flexible Tyr^1 residue was replaced by structurally related, conformationally restricted amino acids.[4,5] Analysis of accessible conformational space common to analogs with moderate or high δ receptor affinity led to the conclusion that δ receptor binding of **1** and its analogs requires $\chi^1 \sim 180°$ for residue **1** and both ψ of residue **1** and φ of D-Cys^2 to be ~160°.[5] The present study was undertaken to relieve the remaining conformational uncertainty: the orientation of the phenyl side chain of residue 3. The use of β-MePhe to restrict orientation about χ^1 is well known[13,14] and based upon the expectation that the second β substituent, due to its steric overlap with the two adjacent main chain peptide groups connected to the α carbon atom of the β-methylated residue, will favor orientations in which the sole β proton is oriented between the α nitrogen and carbonyl groups. However, the extent to which such orientations are favored is highly dependent on the main chain φ and ψ torsion angles of the β-methylated residue. For analogs **3** and **5**, the side chain conformers of the β-Me-L-Phe^3 residue (for which $\varphi \sim -70°$ and $\psi \sim -30°$ in structures A–C, Table 3) have relative energies which are similar to those in the parent peptide, **1** (Table 4), indicating that, for these peptides, the β-methyl substitution has only a minor effect as a conformational constraint. Nonetheless, some tentative conclusions can be proposed. As seen in Table 4, the side chain preferences for the (2*S*,3*R*)-MePhe^3 analog, **3**, are virtually indistinguishable form those of the parent peptide, **1**. The significantly lower δ binding affinity of **3** (~17-fold) compared with **1** suggests that the β-methyl substituent may give rise to an adverse steric interaction with the receptor. By contrast, the side chain preferences for the (2*S*,3*S*)-MePhe^3 analog, **5**, differ from those of **1** and **3** in that the $\chi^1 = -60°$ rotamer is favored by 1.5–2.2 kcal/mol. Since this analog displays δ binding affinity similar to **1**, a reasonable interpretation is that the common bioactive conformation of **1**, **3**, **5**, and related analogs features $\chi^1 \sim -60°$ for residue 3. In this case higher δ binding affinity for **5** vs **1** would be expected since the "correct" side chain conformer is favored in the former. That this result is not observed may again reflect an

Table 4. Relative Energies (kcal/mol) of Side Chain Rotamers for Residue 3 in Peptides Tyr-c[D-Cys-X-D-Pen]OH[a]

compd no.	residue 3	side chain (χ^1) rotamer			$K_i(\delta)$ (nM)
		−60°	180°	60°	
1	L-Phe	0.1	0.0	0.9	0.74
3	(2*S*,3*R*)-MePhe	0.2	0.0	2.0	12
5	(2*S*,3*S*)-MePhe	0.0	2.2	1.5	1.5
		60°	180°	−60°	
6	D-Phe	0.0	2.0	5.0	70
2	(2*R*,3*S*)-MePhe	1.2	0.0	4.7	240
4	(2*R*,3*R*)-MePhe	0.0	6.1	6.3	5

[a] The energies were calculated for the lowest energy structure A of the D-Cys-X-D-Pen cycle and the lowest energy conformation of the first residue (χ^1 and ψ of Tyr^1 ~ 180° and 160°, respectively; φ for D-Cys^2 ~ 160°).

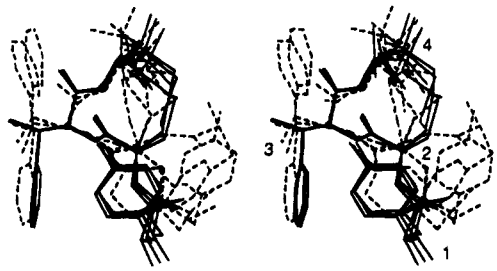

Figure 2. Superposition of representative conformers of **4** (Tyr-c[D-Cys-(2*R*,3*R*)-MePhe-D-Pen]OH). Each structure, A–F, of the tripeptide cycle [D-Cys-(2*R*,3*R*)-MePhe-D-Pen] is represented by its lowest energy conformer (from Table 3). Three lowest energy conformers (A–C structures, solid line; ΔE < 1.0 kcal/mol) are identical to ones of the parent peptide, **1**. Alternative cycle structures (D–F, dashed line) have relative energy >3 kcal/mol.

adverse steric interaction between the β-methyl substituent and the receptor.

While the results obtained for the β-Me-L-Phe^3 analogs, **3** and **5**, are consistent with a bioactive conformation which includes χ^1 ~ −60° for residue 3, they do not provide unequivocal evidence that this is so. Such evidence, however, follows from the results obtained for the β-Me-D-Phe^3-containing analogs, **2** and **4**. For analogs **2**, **4**, and **6**, the replacement of D- for L-stereochemistry in residue 3 does not alter the rigid main chain structure of the tripeptide cycle; the three lowest energy conformational families observed for **2**, **4**, and **6** (conformers A–C) are identical to those for **1** and its β-Me-L-Phe^3 analogs, **3** and **5**. Three additional conformational families, D, E, and F, of somewhat higher energies (3–4 kcal/mol relative to conformer A) are also observed and shown for analog **4** in Figure 2 and Table 3. It should be noted that these additional conformational families are not unique to analogs with D-stereochemistry in residue 3; they were also observed in the conformational analyses of **1**, **3**, and **5**, however at higher energy (>4 kcal/mol higher than A). For the analogs with D-stereochemistry in residue 3, the local main chain conformation of this residue in structures A–C (φ ~ −70°, ψ ~ −30° as in a right-handed α-helix) becomes less energetically preferred, decreasing the energy difference between them and alternative structures, D–F, of the tripeptide cycle. Given their considerably (>4 kcal/mol) higher energies, it is unlikely that conformer families D–F are of significance for the bioactivity of the analogs with L-stereochemistry for residue 3. Energetic considerations also suggest that the bioactive conformations of analogs with D-stereochemistry for residue 3 do not lie within conformer families D–F. Further, because of alterations in the backbone in conformers D–F which affect the separation between the critical Tyr^1 and residue 3 aromatic side chains, no energetically reasonable conformation within these families allows for good superposition with candidate conformers for **1** (i.e., those from families A, B, or C with χ^1 of Tyr^1 ~ 180° and ψ of residue **1** and ϕ of residue 2 ~ 160°). Accordingly, it seems reasonable to conclude that the bioactive conformers for analogs **2**, **4**, and **6**, like those for **1** and related analogs with L-stereochemistry for residue 3, are also represented in conformational families A–C. As shown in Table 4, in the lowest energy conformer in each conformational family A, B, or C for the high-affinity δ receptor analog **4**, χ^1 for residue 3 lies near 60°. In fact, this is observed for all low-energy members of these conformational families and arises from very unfavorable steric interactions between the backbone carbonyl group of the previous residue and the phenyl (for χ^1 ~ −60°) or β-methyl substituents (for χ^1 ~ 180°) of the (2*R*,3*R*)-MePhe^3 residue when the φ and ψ angles of this residue are ~−70° and ~−50°, respectively (Table 3). These unfavorable interactions result in the χ^1 ~ 60° rotamer being favored by >6 kcal/mol in the cyclic structures A (Table 4), B, and C.

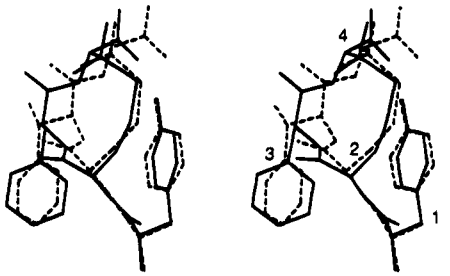

Figure 3. Superposition of candidate δ receptor-bound conformers of the parent peptide, **1** (solid line), and its (2*R*,3*R*)-MePhe^3 analog, **4** (dashed line). Relative energies of the conformers are 0.1 and 0.0 kcal/mol, respectively.

The stabilization of the χ^1 ~ 60° rotamer of residue 3 in analog **4** is of great significance for the completion of the model for the binding conformation of **1** and its analogs. Figure 3 shows the superposition of low-energy conformers of family A for tetrapeptides **4** and **1**, which incorporate the previously deduced[5] torsion angles for the Tyr^1 side chain (χ^1 ~ 180°) and backbone (Tyr^1 ψ ~ 160°, D-Cys^2 φ ~ 160°). In this superposition, the residue 3 side chain of **4** is fixed at the highly favored χ^1 ~ 60° rotamer which allows superposition with **1** (and related analogs with L-stereochemistry of residue 3) *only* if χ^1 ~ −60° (which is similar geometrically to χ^1 ~ 60° for D-residues) for these L-amino acid side chains. Attempts to effect superpositioning of **4** with analogs with L-stereochemistry for residue 3 and χ^1 for this residue ~60° or -180° fail to provide good overlap of the important residue 3 aromatic rings. While Figure 3 only depicts the superposition of conformers from family A, it should be recalled that families B and C share identical backbone conformations with family A; hence the conformational inferences discussed above apply equally well.

As discussed earlier, the higher δ binding affinity of **5** compared with **3** is suggestive of the importance of the χ^1 ~ −60° side chain orientation for residue 3 (when it has L-stereochemistry), since this rotamer is favored in the former but not the latter. The results described above for analog **4** confirm this. The binding and computational results for analogs **2** and **6** (with D-

stereochemistry for residue 3) are also consistent with this interpretation. These analogs, which display 49- and 15-fold lower δ affinity, respectively, than **4**, share the same main chain conformational features of **4**, including the A–F structures (Table 3) of the tripeptide cycle and similar flexibility of the first exocyclic peptide group. As a result, compounds **2** and **6**, which display reduced affinity, can be superimposed with the parent peptide, **1**, in exactly the same manner as can the high-affinity peptide **4** (Figure 3). This superposition, however, results in slightly different orientations of the Phe aromatic rings in peptides with D-stereochemistry of residue 3 compared with the parent peptide, **1** (Figure 3). As a result, for all 14 atoms used for the superposition (carbons of Tyr^1 and Phe^3 aromatic rings and N$^\alpha$ and O$^\eta$ atoms of Tyr^1), the coordinate root mean square deviation (rmsd) between peptides **1** and **4** is 0.6 Å. Rotation of the Phe^3 side chain around the C$^\beta$–C$^\gamma$ bond (χ^2 torsion angle) by 30° (with an energy increase of 1.4 kcal/mol) reduces the rmsd value to 0.36 Å. This different orientation of the residue 3 aromatic group may explain the reduced δ affinity ($K_i \sim 70$ nM) of the D-Phe^3 analog, **6**. The binding affinity of analog **2** is somewhat lower than that of **6** due to an additional stabilization of an "improper" side chain rotamer with χ^1 of residues 3 ~ 180° (Table 4). Conversely, the strong stabilization of a proper orientation of the phenyl side chain ($\chi^1 \sim 60°$) in peptide **4** restores high-affinity δ receptor binding. Thus, as can be seen from Table 4, the degree to which the $\chi^1 \sim 60°$ rotamer is preferred correlates well with δ receptor binding affinity for these three analogs with D-stereochemistry for residue 3.

Conclusions

The conformational constraint imposed upon the side chain torsion angle χ^1 of residue 3 in the (2*R*,3*R*)-MePhe^3 analog, **4**, allows the final uncertainty in the development of a model of the binding conformation at δ opioid receptors to be relieved. The expectation that structurally related analogs with similar high binding affinities will share the same geometric arrangement of pharmacophore elements requires that the χ^1 torsion angle of the Phe^3 residue in the lead compound **1** (and its analogs with L-stereochemistry for residue 3) be ~−60°. Together with conformational elements deduced for the structurally well-defined 11-membered cycle[3] and the exocyclic Tyr^1 residue in the bound form,[5] this observation leads to the binding conformation model of **1** presented in Table 5 and shown in Figures 3 and 4. All three important pharmacophoric elements of **1**, i.e., the Tyr^1 NH_3^+ group and the aromatic rings of the Tyr^1 and Phe^3 residues, are situated on the same side of the molecule and form an almost continuous surface (front side in Figure 4). This part of the molecule is presumably embedded into the binding cleft between the seven transmembrane α-helices of the δ opioid receptor, which belongs to the superfamily of G-protein coupled receptors. The opposite surface of the molecule (back side in Figure 4) consists of hydrophilic main chain carbonyl groups which probably point toward the water solution from the binding site. The charged N- and C-terminal NH_3^+ and COO^- groups of **1** and its analogs are oriented in opposite directions (Figure 4). The COO^- group probably points from the binding cleft toward the extracellular space, while the NH_3^+ group is buried deep in the binding pocket, most likely interacting with the aspartic acid residue (Asp128) of the third transmembrane α-helix of δ opioid receptors. This aspartic acid was shown experimentally to be the counterion of the positively charged nitrogen in cationic amine receptors[15] and is conserved in δ, μ, and κ opioid receptors.[16]

Table 5. Comparison of Candidate δ Receptor-Bound Conformations and Relative Energies (for families A–C) of the Parent Peptide, **1**, Identified in the Present Study and the Model of Nikiforovich et al.[17]

		A	B	C	Nikiforovich et al.[17,a]
Δ*E* (kcal/mol)		0.1	0.0	1.8	5.8[b]
Tyr^1	ψ	138	141	137	149
	χ^1	−167	−175	−172	180
	χ^2	80	81	84	90
D-Cys^2	φ	165	160	164	78
	ψ	41	48	45	49
	χ^1	−58	177	180	167
	χ^2	−148	148	72	*c*
	χ^3	94	−103	86	77
Phe^3	φ	−85	−85	−84	−74
	ψ	−40	−23	−44	−41
	χ^1	−59	−59	−57	180
	χ^2	93	95	101	88
D-Pen^4	φ	141	138	130	125
	χ^1	−71	−71	46	47
	χ^2	52	94	−143	*c*

[a] Lowest energy δ receptor-bound conformer from Nikiforovich et al.[17] Other conformers differ only in the χ^1 torsion angle of the Tyr^1 residue. [b] This energy was calculated after minimization with the CHARMm force field to compare it with energies of other conformers in the table. Torsion angles, taken from the original publication,[17] were not significantly altered during CHARMm minimization. [c] These torsion angles were not represented in ref 17.

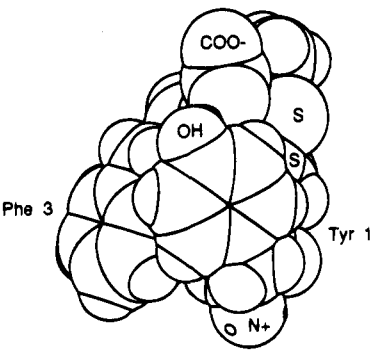

Figure 4. Space-filling model of a candidate δ receptor-bound conformation of the parent peptide, 1.

Table 5 presents torsion angles for the proposed bound conformer of **1** for all three low-energy conformational families, A–C. The proposed conformers differ only in the region of the disulfide; geometrical relationships among the pharmacophore elements are identical in all three conformers. Interestingly, the proposed bound conformers of **1** in families A and B and the corresponding conformers of **4** (Table 3) are the lowest energy conformers observed for each analog in these families. Our model can be compared to one previously proposed by Nikiforovich et al. based on superpositions of JOM-13 with other more flexible δ selective opioid peptides.[17] As can be seen from Table 5, both models agree on conformational features of the main chain within the tripeptide cycle, the model of Nikiforovich et al. representing an example of the higher energy C conformation of the disulfide bridge. In the model proposed by Nikiforovich et al., the side chain conformation for the Tyr^1 residue was not specified; several conformers, which differ only in χ^1 for Tyr, were proposed as candidate bound conformations. The conformation represented in Table 5, for which this χ^1 =

180°, in agreement with our findings, was, by a small margin, the lowest energy conformer reported in the earlier work.[17] The models differ in the proposed φ angle of D-Cys^2 and the side chain conformations of the Phe^3 residue (Table 5); while Nikiforovich et al. propose a χ^1 value of ~180°, the results reported here indicate that $\chi^1 \sim -60°$. As a result of these different φ and χ^1 angles, the centers of the aromatic rings of the critical Tyr and Phe residues are spaced 10.9 Å apart in the previously developed model[17] in comparison with 5.7 Å in the present (Figure 4) conformation and thus represent two distinctly different models for the pharmacophore.

Materials and Methods

General Synthetic Approach. Commercially available (Sigma, Aldrich) β-MePhe, a mixture of all four stereoisomers, was used for the synthesis of peptides **2**–**5**. In order to prepare, isolate, and stereochemically identify these four peptides, the following approach was employed. First, the mixture of four isomers of β-MePhe was separated into two pairs of enantiomers by recrystallization and preparative reverse phase high-performance liquid chromatography (RP-HPLC), and each pair of enantiomers was used for the synthesis of the corresponding pair of diastereomeric peptides. Each pair of peptides was readily separated by HPLC to yield the four desired peptides; however at this stage the stereochemistry of the β-MePhe residue in each peptide was unknown. In order to assign the stereochemistry of all four peptides, samples of the two pairs of β-MePhe enantiomers, separated previously, were trifluoroacetylated and treated with carboxypeptidase A, which selectively hydrolyzes the L-amino acid. The hydrolyzed L- and unhydrolyzed D-amino acids were separated by extraction, and the free D-amino acid was generated by acid hydrolysis. In this manner all four stereochemically pure isomers of β-MePhe were obtained and stereochemically assigned by comparison with previously reported physicochemical data.[18] In order to extend the stereochemical assignments to the β-MePhe-containing peptides, a sample of one resolved amino acid from each pair of enantiomers was used to resynthesize the corresponding peptides. Comparison of HPLC elution profiles then allowed unequivocal stereochemical assignment of all four peptides prepared from the unresolved amino acid enantiomer pairs.

Separation of β-MePhe into Enantiomeric Pairs. Repeated recrystallization of the hydrochloride form of the β-MePhe mixture, as reported by Hruby et al.,[14] resulted in the substantial purification of the (2*R*,3*R*)-MePhe and (2*S*,3*S*)-MePhe enantiomeric pair (stereochemical assignments followed from analytical tests on the final isolated, stereochemically pure amino acids, described below). In a typical experiment, 10.0 g of a mixture of all four isomers of β-MePhe·HCl was dissolved with heating in 6 mL of water. Upon cooling, the resulting precipitate was found to be enriched in the 2*R*,3*R*/2*S*,3*S* enantiomeric pair (81% 2*R*,3*R*/2*S*,3*S* based on HPLC intensities, see below). Repeating the recrystallization on this enriched precipitate yielded 2.1 g of the 98.5% pure (2*R*,3*R*)-MePhe and (2*S*,3*S*)-MePhe racemate, which was used for the synthesis of peptides **4** and **5**.

While recrystallization was effective in yielding the sufficiently pure 2*R*,3*R*/2*S*,3*S* racemate, only ~75% pure 2*R*,3*S*/2*S*,3*R* racemate could be obtained from the filtrate after repeated recrystallizations. After several other approaches proved unsuccessful, the partially purified 2*R*,3*S*/2*S*,3*R* racemate was converted to the N$^\alpha$-Boc derivative and purified by semipreparative HPLC under isocratic conditions [0.1% (w/v) trifluoroacetic acid (TFA) in water/0.1% (w/v) TFA in acetonitrile (74:26); flow rate, 10 mL/min]. The resulting diastereomerically pure N$^\alpha$-Boc-(2*R*,3*S*)/(2*S*,3*R*)-MePhe racemate was used for the synthesis of peptides **2** and **3**.

(2*S*,3*R*)-Methylphenylalanine (7). Enzymatic resolution, *via* carboxypeptidase A, was based upon the method described by Samanen et al.[13] In a typical experiment, 2.6 g (14.5 mmol) of partially purified β-MePhe (81% 2*R*,3*S*/2*S*,3*R* racemate, 19% 2*R*,3*R*/2*S*,3*S* racemate) was dissolved in 40 mL of TFA and cooled to 0 °C in an ice bath. Trifluoroacetic anhydride (12.2 g, 58.1 mmol) was added in three portions over ~4 min, and the reaction was stirred for 75 min, after which the solution was evaporated to dryness. The resulting residue was dissolved in a 1:1 mixture of ethyl acetate and water and extracted twice more with ethyl acetate. The ethyl acetate fractions were pooled, dried with anhydrous $MgSO_4$, and evaporated to dryness to yield 2.95 g (10.7 mmol, 74% yield) of (trifluoroacetyl)-β-methylphenylalanine (76% (2*R*,3*S*/2*S*,3*R* racemate, 24% 2*R*,3*R*/2*S*,3*S* racemate) which was then subjected to semipreparative HPLC, as described above, to provide diastereomerically pure (trifluoroacetyl)-(2*R*,3*S*)/(2*S*,3*R*)-MePhe.

A 2.5 g (9.08 mmol) sample of (trifluoroacetyl)-(2*R*,3*S*)/(2*S*,3*R*)-MePhe was dissolved in 125 mL of 0.1 M ammonium acetate and the pH adjusted to 8.0 with NH_4OH. Carboxypeptidase A (2.5 mg) was added and the solution stirred at room temperature for 6 days with periodic readjustment of the pH to 8.0 with NH_4OH and with daily monitoring of the progress of the reaction by HPLC. The digestion mixture was then filtered, and the filtrate was adjusted to pH 3.0 with HCl and extracted with 4 × 25 mL of ethyl acetate. The ethyl acetate extracts were combined, extracted with H_2O, dried with $MgSO_4$, and evaporated under vacuum. The ethyl acetate extract yielded 1.04 g (3.78 mmol) of recovered (trifluoroacetyl)-(2*R*,3*S*)-MePhe. The aqueous phase, which, in addition to (2*S*,3*R*)-MePhe, also contained buffer salts and some enzyme, was rotary evaporated, redissolved in the minimum amount of water, adjusted to pH 7.0 with 1 N NaOH, and left to precipitate overnight at 4 °C. The yield was 0.45 g (2.5 mmol, 55% yield) of (2*S*,3*R*)-MePhe. $[\alpha]_D = -7.6°$ (c 0.79, H_2O) (lit.[18] $[\alpha]_D = -5.3$ (c 0.75, H_2O)). Stereochemical purity was further assessed with Marfey's reagent, N$^\alpha$-(2,4-dinitro-5-fluorophenyl)-L-alaninamide,[19] using the RP-HPLC protocol described by Szókan et al.[20] A 1–2 mg sample of amino acid was dissolved in 100 μL of H_2O, 200 μL of 1% (w/v) Marfey's reagent in acetone, and 40 μL of 1 M $NaHCO_3$ and heated at 40 °C for 1 h. After cooling, the contents were neutralized with 20 μL of 2 N HCl, diluted with methanol, and analyzed by RP-HPLC using the solvent system 0.02 M sodium acetate (solvent A) and methanol (solvent B) employing a gradient of 47–55% B in 40 min. The derivatized sample of (2*S*,3*R*)-MePhe eluted at 15.5 min and was determined to be 93.4% isomerically pure by integration of the peaks of the chromatogram.

(2*R*,3*S*)-Methylphenylalanine (8). A 0.98 g (3.56 mmol) sample of (trifluoroacetyl)-(2*R*,3*S*)-MePhe recovered from the carboxypeptidase resolution above was dissolved in 10 mL of 3 N HCl and slowly heated to reflux. After 1 h of gentle reflux, the solution was allowed to cool and rotary evaporated to dryness. The resulting residue was redissolved in water and rotary evaporated two additional times to remove residual HCl. The product was redissolved in 3 mL of water, adjusted to pH 7.0 with 4 N NaOH, and stored overnight at 4 °C. The resulting precipitate was collected and the filtrate evaporated to dryness, washed with cold water to remove salts, and combined with the precipitate to yield 0.3 g (1.66 mmol, 47% yield) of the title product. $[\alpha]_D = 7.5°$ (*c* 1.1, H_2O) (lit.[18] $[\alpha]_D = 5.1°$ (c 1.1, H_2O)). Isomeric purity (Marfey's reagent): 99.4%.

(2*S*,3*S*)-Methylphenylalanine (9). The title compound was prepared by enzymatic resolution as described above for (2*S*,3*R*)-MePhe. The (2*R*,3*R*)/(2*S*,3*S*)-MePhe racemic mixture (5.1 g, 28.5 mmol) was converted to the (trifluoroacetyl)-(2*R*,3*R*)/(2*S*,3*S*)-MePhe racemic mixture (6.5 g, 23.7 mmol, 83% yield) which was employed as a substrate for carboxypeptidase A (6 mg) to yield, after workup, 1.4 g (7.8 mmol, 55% overall yield) of the title compound. $[\alpha]_D = -29.1°$ (c 1.03, H_2O) (lit.[18] $[\alpha]_D = -26.7°$ (*c* 1.0, H_2O)). Isomeric purity (Marfey's reagent): 98.8%.

(2*R*,3*R*)-Methylphenylalanine (10). A 2.4 g (8.72 mmol) sample of (trifluoroacetyl)-(2*R*,3*R*)-MePhe recovered from the carboxypeptidase resolution above was subjected to acid hydrolysis as described for (trifluoroacetyl)-(2*R*,3*R*)-MePhe to yield 0.43 g (2.96 mmol, 34% yield) of the title compound. $[\alpha]_D = 26.8°$ (c 1.00, H_2O) (lit.[18] $[\alpha]_D = 21°$ (*c* 1.0, H_2O)). Isomeric purity (Marfey's reagent): 99.8%.

General Methods for Peptide Synthesis and Analysis. Peptides were synthesized by standard solid phase procedures

as previously described for the lead tetrapeptide, **1**,[1] using chloromethylated poly(styrene) (Merrifield) resin cross-linked with 1% divinylbenzene. TFA was employed for deprotection, and dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) were used as coupling agents. α-Amino functions were protected with the *tert*-butyloxycarbonyl (Boc) group, and *p*-methylbenzyl protection was employed for the labile side chain sulfhydryl groups of Cys and Pen. Deprotection and cleavage from the resin were accomplished by treatment with anhydrous hydrogen fluoride in the presence of 5% cresol and 5% *p*-thiocresol,[21] with stirring for 45 min at 0 °C. HF was subsequently removed by vacuum. Following extraction with 9:1 DMF:80% HOAc and dilution with 0.1% TFA in water, the resulting linear, free sulfhydryl-containing peptides were purified by RP-HPLC on a Vydac 218TP C-18 column (2.5 cm × 22 cm) using the solvent system 0.1% (w/v) TFA in water/0.1% (w/v) TFA in acetonitrile. Peptide diastereomers which had been synthesized from racemic β-MePhe were separated at this step and then oxidized separately. For the pair of sulfhydryl-containing peptide precursors of **2** and **3**, separation was effected by RP-HPLC, as above, using a gradient of 5–40% organic component in 70 min. Under these conditions, the precursor of **2** eluted at ~37 min (23% organic component), while that of **3** eluted at ~52 min (31% organic component). For the corresponding pair of precursors of **4** and **5**, a gradient of 5–55% organic component in 75 min was employed for RP-HPLC separation, with elution of the sulfhydryl precursor of **4** at ~33 min (27% organic component) and that of **5** at ~39 min (31% organic component).

Following lyophilization, peptides to be oxidized were dissolved in 1 or 2 mL of dimethyl sulfoxide (DMSO) or a 9:1 mixture of DMF and 80% acetic acid and diluted to a peptide concentration of 1 mg/mL with water. This solution was stirred under nitrogen at 0 °C, and the pH was adjusted to 8.5 with NH_4OH. Potassium ferricyanide (4:1 mole ratio vs peptide) was dissolved in cold water and added all at once to the slightly basic peptide solution. After 15 s the reaction mixture was acidified to pH 4.0 with glacial acetic acid and the peptide solution was stirred with BioRad AG3-X4 resin in the chloride form for 2 min (resin:$K_3Fe(CN)_6$ ≃ 100:1, w/w) and filtered. Oxidized peptides were purified by semipreparative RP-HPLC on a Vydac 218TP C-18 column (2.5 cm × 22 cm) using the solvent system 0.1% (w/v) TFA in water/0.1% (w/v) TFA in acetonitrile, by a gradient of 10–50% organic component in 40 min at a flow rate of 10 mL/min. Both disulfide and disulfhydryl peptides prepared from single isomers of β-MePhe were cochromatographed with samples of disulfide and disulfhydryl peptides that had been prepared from racemic β-MePhe mixtures in order to conclusively determine which isomer of β-MePhe was in each of the four peptides.

Final peptide purity was determined by analytical RP-HPLC on a Vydac 218TP C-18 column (4.6 mm × 250 mm) by a gradient of 0–70% organic component over 70 min, with a flow rate of 1 mL/min. All analytical RP-HPLC gradients were run using the solvent system 0.1% (w/v) TFA in water/0.1% (w/v) TFA in acetonitrile. Peaks were monitored at 220, 230, 254, and 280 nm and analyzed with Waters Maxima 820 software. Peaks which also appeared in chromatograms in which no peptide was injected were considered to be artifacts and ignored. Peptide purity was then evaluated by integration of peaks and found to range from 95% to >99% for the peptides reported here. All newly reported peptides were also subjected to thin layer chromatography (TLC) on precoated silica gel plates in three solvent systems (solvent ratios are v:v): (A) *n*-butanol:acetic acid:water (4:1:5, organic phase only), (B) *n*-butanol:water (containing 3.5% acetic acid and 1.5% pyridine) (1:1, organic phase only), and (C) *n*-amyl alcohol:pyridine:water (7:7:6). In all cases, a single spot was detected using three methods of visualization (ninhydrin, ultraviolet absorption, iodine vapor) for each solvent system.

Final product confirmation was obtained by fast atom bombardment mass spectrometry (FAB-MS). In all cases, the anticipated molecular weights were confirmed by FAB-MS. The molecular weights determined from FAB-MS along with retention times from HPLC and R_f values from TLC analyses are summarized in Table 6.

Table 6. Physicochemical Data for Cyclized Peptides

compd no.	HPLC elution time (min)[a]	TLC R_f[b] A	B	C	MW
2	34.0	0.58	0.40	0.56	575
3	30.4	0.59	0.50	0.60	575
4	34.9	0.52	0.37	0.57	575
5	29.8	0.71	0.66	0.64	575

[a] HPLC elution time using a linear gradient of 0–70% organic component in 70 min at a flow rate of 1 mL/min. Solvent system was 0.1% (w/v) TFA in water/0.1% (w/v) TFA in acetonitrile. The solvent front eluted at 3.0 min. [b] R_f values for thin layer chromatograms in solvent systems: (A) *n*-butanol:acetic acid:water (4:1:5, organic component only), (B) *n*-butanol:water (containing 3.5% acetic acid and 1.5% pyridine) (1:1, organic component only), and (C) *n*-amyl alcohol:pyridine:water (7:7:6).

Receptor Binding Assays. Receptor binding assays on guinea pig brain membrane homogenates were performed at 25 °C using a previously described protocol.[22] Binding affinities of test ligands for μ, δ, and κ opioid receptors were determined by competition with the radiolabeled receptor selective ligands [^{3}H]DAMGO, [^{3}H]DPDPE, and [^{3}H]U69,593, respectively. For μ and δ receptor binding, IC_{50} values were obtained by linear regression from plots relating inhibition of specific binding to the log of 11 different ligand concentrations, using the computer program LIGAND[23] (Biosoft Software). K_i values were similarly calculated using values for K_D of each ligand, determined by analysis of saturation binding experiments. Values of K_D were determined for each membrane preparation used and were in the following ranges: K_D = 1.18–1.72 nM for [^{3}H]DPDPE; K_D = 1.06–2.68 nM for [^{3}H]-DAMGO. For each analog, K_i values reported in Table 1 represent the mean of two to four independent determinations, each performed in triplicate. For binding to κ receptors, expected to be weak for all analogs, the protocol was altered to include only five ligand concentrations (in duplicate).

GPI and MVD Bioassays. Electrically induced smooth muscle contractions of mouse vas deferens and strips of guinea pig ileum longitudinal muscle–myenteric plexus were used as bioassays.[24] Tissues from male ICR mice weighing 25–40 g or male Hartley guinea pigs weighing 250–500 g were tied to gold chain with suture silk, suspended in 20 mL baths containing 37 °C oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (magnesium free for MVD), and allowed to equilibrate for 15 min. The tissues were then stretched to optimal length, previously determined to be *1g* tension (0.5 g for MVD), and allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum wire electrodes at 0.1 Hz, 0.4 ms pulses (2.0 ms pulses for MVD), and supramaximal voltage. Drugs were added to the baths in 14–60 μL volumes. The agonists remained in contact with the tissue for 3 min before the addition of the next cumulative doses, until maximum inhibition was reached. Percent inhibition was calculated by using the average contraction height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure of the agonist. IC_{50} values represent the mean of two to four tissues. IC_{50} estimates and their associated standard errors were determined by fitting the mean data to the Hill equation using a computerized nonlinear least squares method (PCNOCIN and NONLIN84, Statistical Consultants Inc.).

Computational Methods. The search for low-energy conformations of **2–5** was done in two stages, as previously described for **1**.[3] First, the low-energy conformers of the common cyclic fragments CH_3CO-c[D-Cys-Ala-D-Pen]OH and CH_3CO-c[D-Cys-D-Ala-D-Pen]OH were calculated. Second, these conformers of the cyclic fragment were combined with conformers of the Tyr^1 residue and the Phe^3 side chain and minimized again.

In the first stage of the computations, all possible combinations of backbone torsion angles φ and ψ within the cycles CH_3-CO-c[D-Cys-Ala-D-Pen]OH or CH_3CO-c[D-Cys-D-Ala-D-Pen]OH (with a step of 30° within sterically allowed regions of the Ramachandran plot) and rotamers of D-Cys^2 and D-Pen^4 side

chains ($\chi^1 = \pm 60°$ and 180°) were generated and minimized initially with the ECEPP/2 force field[25] using the program CONFORNMR.[26] "Soft" parabolic disulfide bond closing functions $U(r - r)^2$ were used with ECEPP/2 ($U = 30$ kcal/mol Å^2 for S–S bond and C^β–S–S valence angles) since it was observed that the use of the usual closing functions ($U = 100$ kcal/mol Å^2) within the small conformationally strained cycle of these tetrapeptides led to an apparent increase of relative energy for some conformers which was inconsistent with results obtained with the CHARMm force field. Low-energy conformers identified in this fashion ($\Delta E < 10$ kcal/mol) in which at least one torsion angle differed by >30° were selected and minimized additionally with the QUANTA 3.3/CHARMm force field.[27]

In the second stage of computations, the low-energy conformers of the fragments CH_3CO-c[D-Cys-Ala-D-Pen]OH and CH_3CO-c[D-Cys-D-Ala-D-Pen]OH ($\Delta E < 4$ kcal/mol with CHARMm) were combined with conformers of the β-methylated L- or D-Phe^3 side chain and the Tyr^1 residue (including combinations of sterically allowed values for ψ of Tyr^1 and φ of D-Cys^2 torsion angles with a 50° step) and minimized again with the CHARMm force field. For all calculations, a compromise value of dielectric constant, $\epsilon = 10$, was used and the adopted basis Newton–Raphson method of minimization was employed. This intermediate value of ϵ has previously been found to be appropriate for the conformational analysis of peptides[28] and the computations of electrostatic energy in proteins.[29] The QUANTA 3.3 Molecular Similarity system was used for all superpositions.

Acknowledgment. This work was supported by the National Institute on Drug Abuse through Grant DA03910 and Grant DA00118 (Research Scientist Development Award) to H.I.M. Financial support for D.L.H. was provided by The American Foundation for Pharmaceutical Education, The National Institutes of Health (Training Grant GM07767), and The Horace H. Rackham School of Graduate Studies at The University of Michigan.

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