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Development of a Model for the δ-Opioid Receptor Pharmacophore. 4. Residue 3 Dehydrophenylalanine Analogues of Tyr-c[D-Cys-Phe-D-Pen]OH (JOM-13) Confirm Required *gauche* Orientation of Aromatic Side Chain

We have previously proposed a model for the δ-opioid receptor binding conformation of the high affinity tetrapeptide Tyr-c[D-Cys-Phe-D-Pen]OH (JOM-13) based on experimental and theoretical conformational analysis of this peptide and a correlation of conformational preferences of further conformationally restricted analogues of this tetrapeptide with their receptor binding affinities. A key element of this model is the requirement that the Phe³ side chain exist in the $\chi^1 = -60°$ conformation. Conformational calculations on the residue 3 dehydrophenylalanine analogues of JOM-13 suggest that while the dehydro(Z)phenylalanine analogue can be superimposed easily with the proposed binding conformer of JOM-13, the dehydro-(E)phenylalanine analogue cannot. These results lead to the prediction that the dehydro(Z)-phenylalanine analogue should display similar δ-receptor binding affinity as JOM-13 while the dehydro(E)phenylalanine analogue is expected to bind less avidly. Synthesis and subsequent opioid receptor binding analysis of the dehydrophenylalanine analogues of JOM-13 confirm these predictions, lending support to the δ-pharmacophore model. © *1996 John Wiley & Sons, Inc.*

INTRODUCTION

The incorporation of conformational constraints into analogues of flexible, receptor-active peptides is a well-established approach for the identification of the peptide pharmacophore, since the resulting conformationally more well-defined analogue is less subject to the dynamic averaging that compromises attempts to elucidate the solution and bioactive conformations of the flexible, native peptide ligand and thus serves as a better probe of the bioactive conformation at the specific receptor. The cyclic tetrapeptide, Tyr-c[D-Cys-Phe-D-Pen]OH, (JOM-13) (Pen, penicillamine is β,β-dimethylcysteine), a high affinity, δ-opioid receptor selective agonist, is an example of such a conformationally restricted analogue.[1] We have recently investigated the conformational features of JOM-13 using a combination of experimental (x-ray crystallography, ^{1}H-nmr spectroscopy) and theoretical (molecular mechanics computations) techniques.[2] This peptide has a single energetically preferred backbone confor-

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mation for the cyclic tripeptide portion of the molecule and two major conformers of the disulfide bridge. Although the cyclic part of JOM-13 is conformationally well defined, the key elements of the δ-receptor pharmacophore (exocyclic Tyr^1 residue and Phe^3 side chain) are still very flexible in solution. Consequently, additional constraints were incorporated into the Tyr^1 and Phe^3 side chains of the parent tetrapeptide and the binding affinities of the resulting analogues were correlated with their conformational preferences, with the underlying assumption that such structurally related analogues must share a common bioactive conformation, found within the intersection of conformational space available to those analogues that exhibit good binding affinity. This analysis led us to propose a detailed model for the binding conformation of JOM-13 and its analogues.[3,4] The proposed δ-bound conformation is compact; the Tyr and Phe side chains are close together (distance between the centers of the aromatic rings is 5.7 Å) and have *trans* ($\chi^1 \sim 180°$) and *gauche* ($\chi^1 \sim -60°$) conformers, respectively, while the main-chain fragment between Tyr^1 and the tripeptide cycle is in an extended conformation (ψ of Tyr^1 and φ of D-Cys^2 are $\sim 160°$). Comparison of this model with low energy conformers of other conformationally constrained opioid peptides and alkaloids displaying high δ-receptor affinities yielded excellent agreement and was consistent with the view that, among the analogues compared, peptide and nonpeptide ligands share a similar binding mode, but that agonists and antagonists differ in the orientation and position of the Phe^3 aromatic ring (or its equivalent).[5]

A salient feature of our proposed δ-pharmacophore model is the requirement that the Phe^3 side chain of JOM-13 have a *gauche* ($\chi^1 \sim -60°$) orientation. In order to further examine this aspect of the model, we evaluated analogues of JOM-13 in which the orientational freedom of the phenyl side chain of residue 3 is attenuated by replacement of phenylalanine by dehydrophenylalanine (ΔPhe). Conformational search and molecular mechanics studies, described below, of the diastereomers Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH and Tyr-c[D-Cys-Δ^E-Phe-D-Pen]OH (Figure 1) indicate that the former analogue can be readily superimposed with the proposed δ pharmacophore of JOM-13. By contrast, attempts to superimpose the $\Delta^E Phe^3$ analogue with JOM-13 fail to simultaneously align the corresponding Tyr residues and the Phe and Δ^EPhe side chains. If the proposed δ-pharmacophore model is correct, δ-opioid receptor binding affinity comparable to that of JOM-13 would be predicted for Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH, while lower affinity would be predicted for Tyr-c[D-Cys-Δ^EPhe-D-Pen]OH. The syntheses and receptor binding evaluation of the two ΔPhe^3 analogues were undertaken to test these predictions.

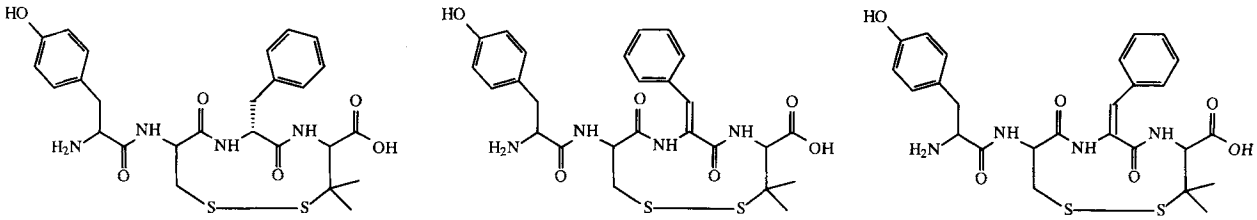
RESULTS AND DISCUSSION

Conformations of N-Acetyl-Dehydrophenylalanine Methylamides

Introduction of the double bond between the C^α and C^β atoms decreases the flexibility of the ΔPhe backbone and side chain due to the stabilization of a planar configuration of the conjugated C^α–C^β and peptide carbonyl double bonds. This planar arrangement requires that the φ, ψ, and χ^1 torsion angles be close to 0° or 180°. However, the conformations of the ΔPhe residue cannot be precisely planar (with both φ and ψ = 0° or 180°) due to steric hindrances between the two adjacent peptide groups and between the peptide groups and the $C^\beta H$ group and aromatic ring of the ΔPhe side chain. The strongest overlap arises between the ΔPhe aromatic ring and the carbonyl or NH of the adjacent peptide group (the preceding peptide group in the case of Δ^ZPhe or the following peptide group in the case of Δ^EPhe). As a result, planar conformations with $\varphi \sim 180°$ or 0° become energetically unfavorable in N-acetyldehydro(*Z*)phenylalanine methylamide, while conformers with $\psi \sim 180°$ or 0° are unfavorable in N-acetyldehydro(*E*)phenylalanine methylamide. Calculated low energy conformers of the *Z*- and *E*-isomers of N-acetyldehydrophenylalanine methylamide are presented in Table I. N-acetyldehydro(*Z*)phenylalanine methylamide has 4 main-chain conformations ($\varphi \sim \pm 85°$ and $\psi \sim 180°$ or 0°) and two isoenergetic side-chain rotamers with $\chi^2 = \pm 45°$ (Table I). N-acetyl-dehydro(*E*)phenylalanine methylamide has 8 local minima in the φ,ψ map ($\varphi \sim \pm 120°$ and $\psi \sim \pm 150°$ or $\pm 30°$) and with $\chi^2 = -130°$ or $+130°$ depending on the combination of the φ and ψ angles (Table I). Several of the calculated conformers of the N-acetyl-dehydrophenylalanine methylamides have been observed in crystal structures of dehydrophenylalanine-containing peptides and derivatives (Table I).

Conformations of the Dehydrophenylalanine-Containing Tetrapeptides

Like the parent peptide, JOM-13,[1] both dehydrophenylalanine-containing analogues consist of a



Tyr-c[*D*-Cys-Phe-*D*-Pen]OH (JOM-13)

Tyr-c[*D*-Cys-Δ^{Z}Phe-*D*-Pen]OH

Tyr-c[*D*-Cys-Δ^{E}Phe-*D*-Pen]OH

FIGURE 1 Structures of JOM-13, Tyr-c[D-Cys-Δ^{Z}Phe-D-Pen]OH, and Tyr-c[D-Cys-Δ^{E}Phe-D-Pen]OH.

relatively rigid disulfide-bridged cycle and a flexible exocyclic Tyr[1] residue. Molecular mechanics calculations reveal three low energy structures—A, B, and C—for the tripeptide cycle of the dehydrophenylalanine-containing analogues that have almost identical torsion angles for the main chain within the cycle (i.e., ψ of D-Cys[2], φ and ψ of ΔPhe[3], and φ of D-Pen[4]) and that differ from each other only in the conformation of the disulfide bridge (χ angles of Cys and Pen residues, Tables II and III; Figures 2 and 3). Two higher energy structures, E and D, differ from A and B, respectively, only in the orientation of the peptide group between the D-Cys[2] and ΔPhe[3] residues (concerted change of Cys[2] ψ and ΔPhe[3] φ angles; Tables II and III; Figures 2 and 3). Almost identical A–D conformers were calculated theoretically for JOM-13[2] and its analogues with the D-substituted Phe[3] residue.[4] Two of these conformers (A and B) were also observed in x-ray crystallography and ^{1}H-nmr spectroscopy studies of JOM-13.[2] Thus, introduction of the C^{α}-C^{β} double bond in both ΔPhe[3]-containing

Table I Torsion Angles (Degrees) and Relative Energies ΔE (kcal/mol) for Conformers of N-Acetyl Dehydrophenylalanine Methylamides (AcΔPheNHMe)[a]

	Calculated with CHARMm					
	φ	ψ	χ^1	χ^2	ΔE	X-Ray Ref.[b]
AcΔ^{Z}PheNHMe	−104	179	3	48	0	[9]
	−69	172	−3	−53	0.2	[10]
	104	−179	−3	−48	0	[10]
	69	−172	3	53	0.2	
	94	−4	−3	−48	2.5	[15]
	69	6	3	53	2.5	[12]
	−97	4	3	48	2.5	
	−69	−6	−3	−53	2.5	[17]
AcΔ^{E}PheNHMe	−124	145	173	129	0	[23]
	−128	−147	−173	−130	0.2	
	124	−145	−173	−129	0	
	127	145	173	130	0.2	
	−117	36	−174	−131	2.3	
	−121	−30	175	130	2.7	
	109	−35	176	131	2.3	
	120	33	−175	−130	2.7	

[a] The conformers listed represent local minima identified from φ, ψ plots and refined by energy minimization with CHARMm. All torsion angles are defined according to IUPAC nomenclature.

[b] Reference denotes crystallographic structure of dehydrophenylalanine-containing peptide or derivative with similar dehydrophenylalanine conformation.

Table II Relative Energies, ΔE (kcal/mol) and Torsion Angles (Degrees) of Conformations Representing Different Structures (A–E) of the Disulfide-Bridged Cycle in Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH[a]

		A	B	C	D	E
ΔE		0.9	0	1.6	3.1	4.0
Tyr^1	ψ	150	148	152	148	−39
	χ^1	−176	179	−174	−179	−176
	χ^2	60	69	75	68	65
D-Cys^2	φ	171	170	175	165	155
	ψ	39	48	43	−168	−175
	χ^1	−59	178	176	170	−64
	χ^2	−146	149	74	158	−164
	χ^3	93	−106	87	−108	89
$\Delta^Z Phe^3$	φ	−90	−88	−88	89	88
	ψ	−24	−8	−14	−18	−5
	χ^1	2	2	1	−2	−2
	χ^2	44	45	43	−46	−46
D-Pen^4	φ	131	124	107	132	85
	ψ	52	58	33	75	3
	χ^1	−69	−69	44	−62	−69
	χ^2	52	93	−133	66	31

[a] Conformers from the A, B, and C families in the table are possible δ-bound ones,[b] while those in the D and E columns are the lowest energy conformers from these families. All torsion angles are defined according to IUPAC nomenclature.

[b] The coordinate rmsd, relative to the pharmacophore model of JOM-13 (4), for 14 functionally important atoms (all nonhydrogen atoms of Tyr^1 and $\Delta^Z Phe^3$ aromatic rings and the O^η and N^α atoms of Tyr^1) are 0.32, 0.36, and 0.16 Å for the indicated conformations from families A, B, and C, respectively. The lowest energy conformers from families A and C have relative energies 0.7 and 1.1 kcal/mol, respectively.

Table III Relative Energies ΔE (kcal/mol) and Torsion Angles (Degrees) of Lowest Energy Conformations Representing Different Structures (A–E) of the Disulfide-Bridged Cycle in Tetrapeptide Tyr-c[D-Cys-Δ^EPhe-D-Pen]OH[a]

		A	B	C	D	E
ΔE		0	0.1	0.9	2.2	3.2
Tyr^1	ψ	151	158	156	146	150
	χ^1	−177	−171	−175	−178	−171
	χ^2	60	65	70	72	66
D-Cys^2	φ	177	−180	177	162	167
	ψ	38	46	42	−169	−179
	χ^1	−60	−177	177	170	−66
	χ^2	−147	159	72	158	−160
	χ^3	93	−105	87	−109	93
$\Delta^E Phe^4$	φ	−78	−99	−76	93	99
	ψ	−38	29	−36	−29	−43
	χ^1	174	−175	174	174	175
	χ^2	132	52	131	131	−48
D-Pen^4	φ	136	93	121	140	137
	ψ	45	55	31	71	34
	χ^1	−67	−67	46	−62	−72
	χ^2	54	76	−138	67	39

[a] All torsion angles are defined according to IUPAC nomenclature.

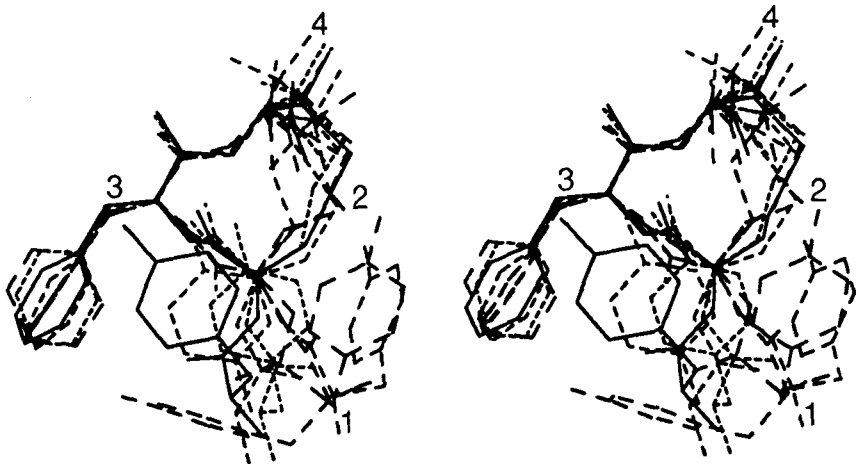


FIGURE 2 Superposition (stereoview) of 6 low-energy conformers of Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH. C^α atoms of D-Cys^2, Δ^ZPhe^3, and D-Pen^4 were used for superposition.

peptides does not significantly change conformations of the rigid tripeptide ring c[D-Cys-Xaa-D-Pen]. The only minor changes are a small stabilization (by ~ 0.7 kcal/mol, Table II) of conformer B in the Δ^ZPhe^3 analogue (conformers A and B have almost identical calculated energies in JOM-13[2]) and a decrease of the energy gap between the lower energy conformers A, B, C, and the higher energy D and E structures of the dehydrophenylalanine-containing analogues compared with the parent peptide.

As discussed below, the "folded" local conformation found for the Δ^ZPhe residue in the lower energy structures A–C of Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH (with φ and ψ angles for the Δ^ZPhe residue corresponding to an α- or 3_{10}-helix) is typical of many Δ^ZPhe-containing peptides that have been studied. Calculations for N-acetyl-dehydro(*Z*)phenylalanine methylamide indicate, however, that for the residue alone, "extended" conformations (with $\psi \sim 180°$) are slightly more preferred energetically than "folded" ones (with $\psi \sim 0°$; Table I). Both types of conformations are present in Δ^ZPhe-containing di- and tetrapeptides in $CDCl_3$ solution as indicated by the presence of nuclear Overhauser effects from both NH and $C^\alpha H$ protons of the Δ^ZPhe residue to the NH proton of the following residue.[6,7] Crystal structures of Δ^ZPhe-containing dipeptides also belong to both "extended"[8-10] or "folded"[11-15] types. In longer peptides the "folded" conformations become preferred due to the formation of intramolecular hydrogen bonds. The "type II" β-turn (i.e., a single turn

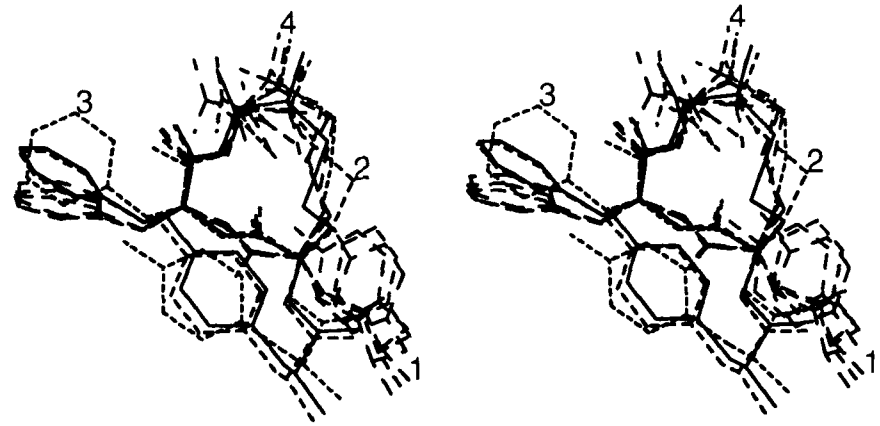


FIGURE 3 Superposition (stereoview) of 6 low-energy conformers of Tyr-c[D-Cys-Δ^EPhe-D-Pen]OH. C^α atoms of D-Cys^2, Δ^EPhe^3, and D-Pen^4 were used for superposition.

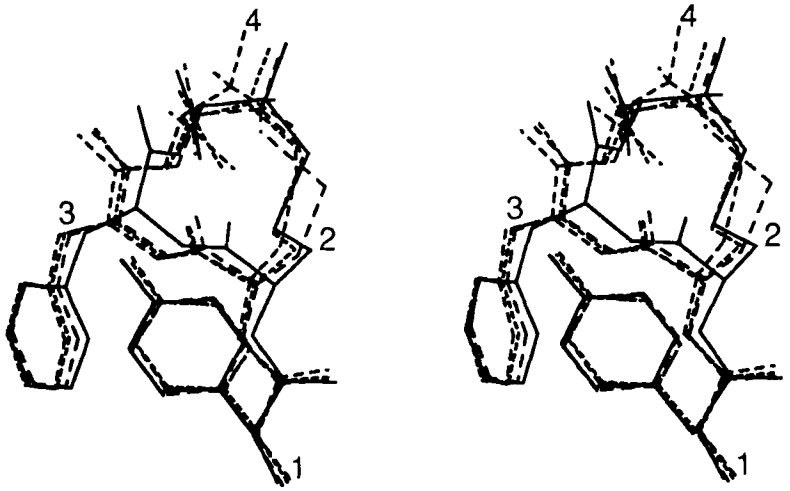


FIGURE 4 Superposition (stereoview) of proposed δ-bound conformation of JOM-13 (solid line) and low energy conformers A, B, and C of Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH from Table II. C atoms of phenyl rings of Tyr^1 and $\Delta^Z Phe^3$ (or of Phe^3) and O^η and N^+ of Tyr^1 were used for superposition.

of a 3_{10}-helix) is formed in Δ^ZPhe-containing tri- and tetrapeptides,[16,17] while longer right-handed ($\varphi = -58°$ to $-67°$, $\psi = -16°$ to $-28°$[18-20]) or left-handed ($\varphi = 53°$ to $58°$, $\psi = 23$ to $26°$[21]) 3_{10}-helices are stabilized in crystals of peptides with length ≥ 5 residues.

As noted above, the energetically preferred conformations A, B, and C, presented in Table II for both ΔPhe^3-containing analogues, are very similar to ones identified previously for JOM-13 and its D-Phe^3 analogues.[4] Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH and Tyr-c[D-Cys-Δ^EPhe-D-Pen]OH, with distinct conformationally constrained orientations of the residue 3 aromatic ring, allow the verification of the previously developed δ-bound model for JOM-13,[3,4] and in particular, the previously proposed δ-bound conformation of the Phe^3 side chain ($\chi^1 \sim -60°$ when the Phe residue has L stereochemistry and $\chi^1 = +60°$ when the residue has D stereochemistry).[4] The pharmacophore elements (Tyr^1 residue, Phe^3 aromatic side chain, and C-terminal COO^- group) of the lowest energy conformer, B, of Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH (in which the aromatic side chain of residue 3 is fixed in the *Z* configuration) and the two alternative low energy conformations with different orientations of the S-S bridge (A and C in Table II with relative energies 0.9 and 1.6 kcal/mol, respectively) can be easily superimposed with the previously proposed δ-bound conformation of JOM-13 [rms deviation (rmsd) < 0.4 Å] for 14 functionally important atoms (all nonhydrogen atoms of the Tyr^1 and $\Delta^Z Phe^3$ aromatic rings and the O^η and N^α atoms of Tyr^1; Figure 4). The excellent superposition is possible despite different χ^1 angles of residue 3 in the $\Delta^Z Phe^3$ analogue (χ^1 close to 0°, Table II) and the parent peptide ($\chi^1 \sim -60°$) because of concomitant changes in ψ angle of $\Delta^Z Phe^3$ (also close to 0°) compared with Phe of JOM-13 ($\psi \sim -60°$).

In contrast to the superposition of Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH with the proposed δ-pharmacophore model for JOM-13, the low energy conformations of Tyr-c[D-Cys-Δ^EPhe-D-Pen]OH (with $\Delta E < 4$ kcal/mol) in which the residue 3 aromatic side chain is in the *E* configuration, corresponding to $\chi^1 \sim 180°$, cannot be well superimposed with the model. This is due, in part, to the increased distance between the Tyr^1 residue and Phe^3 aromatic ring observed for most low energy conformers of Tyr-c[D-Cys-Δ^EPhe-D-Pen]OH; however some low energy conformers do exist in which this interring distance is close to that predicted from the binding model of JOM-13 (5.7 Å). Nonetheless, even in these conformers overlap with the binding model for JOM-13 is relatively poor (Figure 5) with the best observed rmsd = 1.43 Å for the same atom set. When the important carboxy terminal —COOH group is also considered, good superpositioning of Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH with JOM-13 is maintained, while this group occupies distinctly different regions of space in Tyr-c[D-Cys-Δ^EPhe-D-Pen]OH. These modeling results lead to the prediction that, if the proposed binding conformation of JOM-13 is indeed correct, then Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH should display

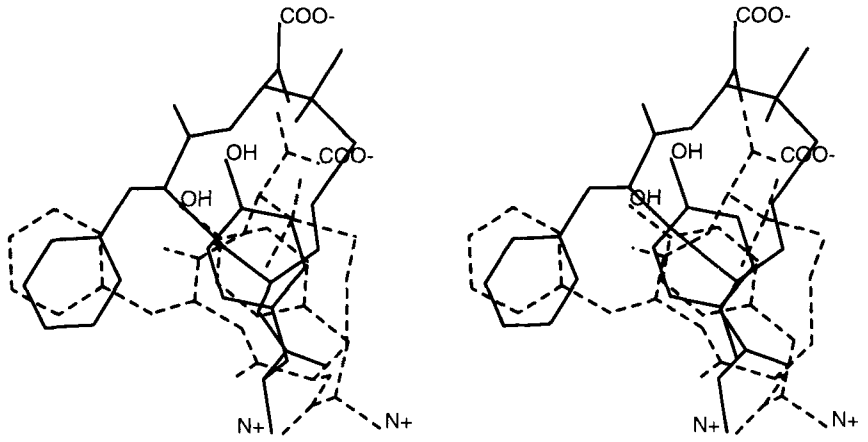


FIGURE 5 Superposition (stereoview) of proposed δ-bound conformation of JOM-13 (solid line) and best fit (rmsd = 1.43 Å) low-energy (ΔE = 1.2 kcal/mol) conformer of Tyr-c([D-Cys-Δ^EPhe-D-Pen]OH (dashed line). C atoms of phenyl rings of Tyr^1 and Δ^EPhe^3 (or of Phe^3) and O$^\eta$ and N$^+$ of Tyr^1 were used for superposition.

comparable δ-receptor binding affinity as JOM-13, but that Tyr-c[D-Cys-Δ^EPhe-D-Pen]OH should bind less well. The opioid receptor binding affinities observed for the dehydrophenylalanine-containing analogues are consistent with these predictions. As shown in Table IV, the δ-binding affinity of Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH is indeed similar to that displayed by JOM-13, while the δ affinity of Tyr-c[D-Cys-Δ^EPhe-D-Pen]OH is considerably lower. We have previously proposed distinct binding conformations for δ-opioid agonists and antagonists, differing chiefly in the orientation of the Phe side chain (or its equivalent; 5). The excellent superposition of Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH with JOM-13, which is a δ-receptor agonist (1), suggests that Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH should also function as an agonist. This prediction is borne out by observations in the mouse vas deferens bioassay in which Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH exhibits full agonist activity and comparable potency as the parent peptide (IC_{50} = 9.8 and 4.2 nM for Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH and JOM-13, respectively; F. Porreca and P. Davis, personal communication).

The results reported here strongly support the δ-receptor binding model developed from the study of JOM-13 and its analogues, especially the proposed requirement that the Phe^3 side chain assumes a conformation with $\chi^1 \sim -60°$. It is also interesting to note that the μ-receptor binding affinity of Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH is decreased ca. 15-fold relative to JOM-13, while the μ affinity of Tyr-c[D-Cys-Δ^EPhe-D-Pen]OH is similar to that of JOM-13, suggesting that δ and μ receptors prefer different orientations of the Phe^3 aromatic side chain. The stabilization of the aromatic side-chain orientation appropriate for the δ receptor in Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH results in a 5-fold improvement in δ selectivity relative to JOM-13.

METHODS

Computational Methods

Molecular mechanics calculations for Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH and Tyr-c[D-Cys-Δ^EPhe-D-Pen]OH were done in three stages using the CHARMm force field.[22] First, all conformers of N-acetyldehydro(Z)phenylalanine methylamide and N-acetyl-dehydro(E)phenylalanine methylamide were identified. Ramachandran maps, E(φ ψ), were computed using a grid of 20°. Second, conformations of the disulfide-containing tripeptide cycles, c[D-Cys^2-Δ^ZPhe^3-D-Pen^4]OH and c[D-Cys^2-Δ^EPhe^3-D-Pen^4]OH, were calculated using standard search procedures: all combinations of the φ and ψ main-chain torsion angles of D-Cys^2, ΔPhe^3, and D-Pen^4 residues (with 60° increments in the allowed regions of the Ramachandran plot) and side-chain rotamers (χ^1 = −60°, 60°, and 180° for D-Cys^2 and D-Pen^2, and χ^2 = ±90° for Δ^ZPhe^3) were taken for energy minimization. Third, each low energy conformer of the tripeptide cycles (with ΔE < 5 kcal/mol) was combined with all possible conformations of the Tyr^1 residue (conformational search in the space of torsion angles ψ and χ^1 of Tyr^1 and φ of D-Cys^2) and minimized again, and conformers with relative energies < 4 kcal/mol were selected for subsequent analysis and superpositions.

The Ramachandran plots and the conformations of

the cycles were generated using the conformational search module of QUANTA 4.0. The energy minimization was performed using the adopted basis Newton–Raphson method and a compromise value of dielectric constant (ε = 10).[2-4] The clusters of low energy cycle conformers (ΔE < 5 kcal/mol) in which at least one torsion angle differed by >30° were selected using the cluster analysis feature of QUANTA 4.0. The molecular similarity system of QUANTA 4.0 was used for all superpositions.

Syntheses

Boc-D-Cys(S-p-Methylbenzyl)-β-Phenyl-D,L-Serine-OH. A solution of Boc-D-Cys(S-*p*-methylbenzyl)-OH (Boc: *t*-butyloxycarbonyl; 1.62 g, 5 mmol) in 25 mL of dry tetrahydrofuran was cooled in an ice bath and N-methylmorpholine (0.61 g, 6 mmol) was added followed by addition of isobutylchloroformate (0.68 g, 5 mmol). After stirring at 0°C for 30 min, a solution of β-phenlyserine hydrate (1.1 g, 6 mmol) containing 0.58 g of NaOH in 10 mL of water was added. The mixture was allowed to stir at 0°C for 2 h and at room temperature for an additional 4 h. The reaction mixture was concentrated in vacuo to give an oil that was partitioned between ethyl acetate and water. The aqueous layer was acidified with solid citric acid and extracted with ethyl acetate. The organic layer was washed with saturated NaCl and water, dried over anhydrous Na_2SO_4, and concentrated in vacuo to give an oil that was used without further purification in the next step. Crude yield = 2.1 g (94%).

Boc-D-Cys(S-p-Methylbenzyl)-Dehydro(Z)-phenyylalanine-Azalactone. Boc-D-Cys(S-*p*-methylbenzyl)-β-Phenyl-D,L-serine-OH (0.85 g, 1.87 mmol) was dissolved in 10 mL of dry acetic anhydride and sodium acetate (0.21 g, 2.8 mmol) was added. After stirring for 1 h, the reaction mixture was concentrated in vacuo to give a dark brown oil, which was purified by column chromatography (silica gel 60, particle size 70–230, EM Science) using ethyl acetate : hexane (2:3) to give the title product as a light yellow oil. Yield 0.6 g (75%), R_f = 0.9 (ethyl acetate : hexane, 2:3). ^{1}H-nmr ($CDCl_3$) 8.07–8.12 (m, 2H, aromatic), 7.42–7.51 (m, 3H, aromatic), 7.08–7.22 (m, 5H, *p*-methylbenzyl and C=CHPh), 5.3 (d, 1H, NH), 4.9 (m, 1H, α-CH), 3.71 (s, 2H, CH_2 of *p*-methylbenzyl), 2.90 (m, 2H, β-CH_2), 2.32 (s, 3H, CH_3 of *p*-methylbenzyl), 1.5 (s, 9H, Boc).

Boc-D-Cys(S-p-Methylbenzyl)-Dehydro(Z)phenylalanine-D-Pen (S-p-Methylbenzyl)-Benzyl Ester. Boc-D-Pen (S-*p*-methylbenzyl)-benzyl ester (0.35 g, 1.0 mmol) was dissolved in a cold saturated solution of HCl gas in ethyl acetate and allowed to stand for 1 h. The reaction mixture was concentrated in vacuo to remove any free hydrochloric acid. The resultant hydrochloride salt of D-Pen (S-*p*-methylbenzyl)-benzyl ester was taken up in cold ethyl acetate and washed 3 times with a cold solution of 10% aqueous K_2CO_3. The organic layer was dried over anhydrous Na_2SO_4 and filtered. To this solution was added Boc-D-Cys (S-*p*-methylbenzyl)-dehydro(*Z*)phenylalanine-azalactone (0.43 g, 0.95 mmol) and the reaction mixture was allowed to stir at 55°C overnight. The solvent was removed under vacuum to give the title product as an oil, which was purified by silica gel column chromatography using ethyl acetate : hexane (1:1) to give 0.71 g of the title compound. Yield = 90%, R_f = 0.65 (ethyl acetate : hexane; 1:1).

Boc-Tyr(2,6-Dichlorobenzyl)-D-Cys(S-p-Methylbenzyl)-Dehydro(Z)phenylalanine-D-Pen (S-p-Methylbenzyl)-Benzyl Ester. Boc-D-Cys (S-*p*-methylbenzyl)-dehydro(*Z*)phenylalanine-D-Pen (S-*p*-methylbenzyl)-benzyl ester (0.38 g, 0.48 mmol) was taken up in a cold solution of ethyl acetate saturated with HCl gas. The reaction mixture was allowed to stand at 0°C for 1 h and concentrated in vacuo to give an oil. The resultant oil was taken up in 15 mL of CH_2Cl_2 and diisopropylethylamine (0.11 mL, 0.57 mmol), followed by addition of Boc-Tyr (2,6-dichlorobenzyl)-OH (0.22 g, 0.57 mmol), dicyclohexylcarbodiimide (0.13 g, 0.57 mmol) and 1-hydroxybenzotriazole (0.09 g, 0.57 mmol). The resultant mixture was stirred at room temperature for 2 h, concentrated in vacuo and chromatographed on silica gel (1:1, ethyl acetate : hexane) to give 0.45 g of the title compound. Yield = 84%, R_f = 0.5 (ethyl acetate : hexane; 1:1).

Tyr-D-Cys-Dehydro(Z)phenylalanine-D-Pen-OH. The fully protected tetrapeptide from the previous step (0.15 g, 0.13 mmol) was treated with anhydrous liquid HF (8 mL) containing *p*-cresol (0.5 g), and *p*-thiocresol (0.5 g) for 45 min at 0°C. The solution was evaporated to leave an oil that was partitioned between diethyl ether and water containing 0.1% (trifluoroacetic acid: TFA; w/v). The aqueous layer was washed with ether (3 × 100 mL) and purified by reverse phase high performance liquid chromatography (RP-HPLC) on a Vydac 218TP C-18 column (2.5 × 22 cm) using the solvent system 0.1% (w/v) TFA in water/0.1% (w/v) TFA in acetonitrile, using a gradient of 10–45% organic component in 35 min. The fractions containing the title peptide were pooled and lyophilized to give about 80 mg of the pure sulfhydryl-containing peptide.

Tyr-c[D-Cys-Dehydro(Z)phenylalanine-D-Pen]OH. The free sulfhydryl containing peptide was dissolved in 1 mL of a 9:1 mixture of N,N-dimethylformamide and 80% acetic acid, and diluted to a peptide concentration of 1 mg/mL with water. This solution was stirred under nitrogen at room temperature, and the pH was adjusted to 8.5 with NH_4OH. Potassium ferricyanide (4:1 mole ratio vs peptide) was dissolved in water and added all at once to the slightly basic peptide solution. After 1 min the reaction mixture was acidified to pH 4.0 with glacial acetic acid and the peptide

Table IV Opioid Receptor Binding Affinities of JOM-13 and its ΔPhe³ Analogues

Analogue	$K_i(\mu)$ (nM)	$K_i(\delta)$ (nM)	$K_i(\mu)/K_i(\delta)$
Tyr-c[D-Cys-Phe-D-Pen]OH	51.5 ± 4.4	0.74 ± 0.08	69.2
Tyr-c[D-Cys-Δ^ZPhe-D-Pen]OH	783 ± 104	2.36 ± 0.16	332
Tyr-c[D-Cys-Δ^EPhe-*D*-Pen]OH	83.4 ± 10.1	44.9 ± 4.22	1.86

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. The oxidized peptide was purified by semipreparative RP-HPLC as described above, and pure fractions were pooled and lyophilized to provide the title peptide in 70% yield. Molecular weight [fast atom bombardment/mass spectroscopy (FAB/MS)]: 559 (expected, 559); RP-HPLC elution time: 32 min (Vydac 218TP C-18 column, 4.6 × 250 mm; gradient elution from 0–70% organic component in 70 min at a flow rate of 1 mL/min. Solvent system: 0.1% (w/v) TFA in water/0.1% (w/v) TFA in acetonitrile. Peaks were simultaneously monitored at 220, 230, 254, and 280 nm. Peptide purity, determined by integration of chromatogram, was > 99%.

Tyr-c[D-Cys-Dehydro(E)phenylalanine-D-Pen]-OH. Tyr-c[D-Cys-Dehydro(*Z*)phenylalanine-D-Pen]OH was converted to Tyr-c[D-Cys-dehydro(*E*)phenylalanine-D-Pen]OH via photoisomerization.[23] Ten milligrams of the dehydro(*Z*)phenylalanine containing peptide, described above, was dissolved in a 1:1 (v:v) mixture of methanol and dimethylformamide (10 mL). The resultant solution was purged with dry nitrogen gas for 15 min and photoisomerized using an ACE 450W uv immersion lamp for 24 h. The resultant dehydro(*E*)phenylalanine containing analogue was separated from the starting material by RP-HPLC on a Vydac 218TP C-18 column (1.0 × 22 cm) as described above, and pure fractions were pooled and lyophilized to give about 1 mg of the pure peptide. Yield of photoisomerization = 10%. Molecular weight (FAB/MS): 559 (expected, 559); RP-HPLC elution time: 29 min (same conditions as for Tyr-c[D-Cys-dehydro(*Z*)phenylalanine-D-Pen]OH, above). The success of the photoisomerization was confirmed by ^{1}H-nmr (in D_2O containing 2.0% CD_3COOD) in which the characteristic dehydrophenylalanine vinyl proton resonance, observed at 7.58 ppm in Tyr-c[D-Cys-dehydro(*Z*)phenylalanine-D-Pen]OH is shifted upfield (to 6.67 ppm) in Tyr-c[D-Cys-dehydro(*E*)phenylalanine-D-Pen]OH, consistent with observations for the corresponding N-phthaloyl dehydro(*Z*)phenylalanine t-butylamide [chemical shift (TFA solution), 7.74 ppm] and N-phthaloyl dehydro-(*E*)phenylalanine t-butylamide [chemical shift (TFA solution), 6.88 ppm].[24]

Receptor Binding Assays. Receptor binding assays on guinea pig brain membrane homogenates were performed at 25°C using a previously described protocol.[25] Binding affinities of test ligands for μ-, δ-, and κ-opioid receptors were determined by competition with radiolabeled receptor selective ligands [^{3}H]DAMGO, [^{3}H]DPDPE, [^{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[26] (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 analogue, K_i values reported in Table IV represent the mean of 2–4 independent determinations, each performed in triplicate. For binding to κ receptors, expected to be weak for all analogues, the protocol was altered to include only 5 ligand concentrations (in duplicate). As expected, neither JOM-13 nor its ΔPhe³ analogues displayed significant κ-receptor affinities (<50% displacement of [^{3}H]U69,593 at 10 μM concentration of test ligand).

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