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A proposal for the molecular basis of μ and δ opiate receptor differentiation based on modeling of two types of cyclic enkephalins and a narcotic alkaloid

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SUMMARY

The molecular basis underlying the divergent receptor selectivity of two cyclic opioid peptides Tyr-c[N^{δ}-D-Orn^2-Gly-Phe-Leu-] (c-ORN) and [D-Pen^2, L-Cys^5]-enkephalinamide (c-PEN) was investigated using a molecular modeling approach. Ring closure and conformational searching procedures were used to determine low-energy cyclic backbone conformers. Following reinsertion of amino acid side chains, the narcotic alkaloid 7α-[(1R)-1-methyl-1-hydroxy-3-phenylpropyl]-6,14-endoethenotetrahydro oripavine (PEO) was used as a flexible template for bimolecular superpositions with each of the determined peptide ring conformers using the coplanarity and cocentricity of the phenolic rings as the minimum constraint. A vector space of PEO, accounting for all possible orientations for the C_{21}-aromatic ring of PEO served as a geometrical locus for the aromatic ring of the Phe^4 residue in the opioid peptides. Although a vast number of polypeptide conformations satisfied the criteria of the opiate pharmacophore, they could be grouped into three classes differing in magnitude and sign of the torsional angle values of the tyrosyl side chain. Only class III conformers for both c-ORN and c-PEN, having tyramine dihedral angles $\chi_1 = -150° \pm 30°$ and $\chi_2 = -155° \pm 20°$, had significant structural and conformational properties that were mutually compatible while respecting the PEO vector space. Comparison of these properties in the context of the divergent receptor selectivity of the studied opioid peptides suggests that the increased distortion of the peptide backbone in the closure region of c-PEN together with the pendant β,β-dimethyl group, combine to generate a steric volume which is absent in c-ORN and that may be incompatible with a restrictive topography of the μ receptor. The nature and stereochemistry of substituents adjacent to the closure region of the peptides could also modulate receptor selection by interacting with a charged (δ) or neutral (μ) subsite.

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INTRODUCTION

The central and peripheral nervous systems contain a heterogeneous population of opiate receptors whose interaction with narcotic opiates or opioid peptides manifests the characteristic analgesic response [1–4]. Among the major opioid receptor families, the μ and δ subtypes have been studied extensively. Pharmacological and biochemical evidence suggest that they are anatomically different and could be distinguished on the basis of brain region distribution [5,6], differential affinity for alkaloid and opioid peptide ligands [5,7], divergent behavior toward allosteric modulators such as Na^+ and GTP [8,9] and variable reactivity toward irreversible site-directed alkylating agents [10].

Other important advances have been made in the pharmacologic characterization of opiate receptors through the rational design of synthetic opiate alkaloids [11] and highly potent and receptor-selective opioid peptide analogs [12,13]. Some of these, such as the conformationally constrained analogs of [Leu^5]-enkephalin prototyped by **1** (Fig. 1) have helped to define the limits of structural and conformational tolerance of the receptors with which they interact [14–17]. In binding studies morphine and related narcotic alkaloids typically have higher affinity for μ than δ receptors and the converse is true for Met and Leu enkephalin [18]; however, in spite of their peptidic nature, the cyclic compounds prototyped by **1** have been shown to display moderate to high selectivity for morphine receptors [19].

In another study Mosberg et al. [20,21] have described a related series of enkephalin analogs cyclized through a disulfide bridge between a D-penicillamine residue in position two (Pen^2) and a cysteine or another penicillamine residue at the C-terminal position (**2**, Fig. 1). Notwithstanding the structural analogies between cyclic peptides of type **1** and **2**, the former interacts selectively with the μ receptors while the latter with δ sites in functional isolated tissues preparations. As an explanation, Mosberg [22] has proposed a model whereby the β,β-gem dimethyl group of c-PEN confers steric properties that are deleterious to binding to μ receptors. Alternatively, the possibility that different bioactive conformations may be required by μ and δ receptors has been investigated by Loew et al. [23,24] using computational methods.

In the light of opposing conclusions drawn from the above investigations, we report the results of our own studies dealing with the conformational properties of the disulfide **2a** and the cyclic amide **1c** in order to derive a probable molecular basis for the divergent receptor selectivity. Our approach utilized the 'template forcing' method to investigate key geometrical congruencies between low-energy backbone conformers of the cyclic enkephalin analog **1c** (Fig. 1) and the model synthetic alkaloid 7α-[(1R)-1-methyl-1-hydroxy-3-phenylpropyl]-6,14-endoethenotetrahydro oripavine (PEO) (**3**, Fig. 1) that could serve to explain the mutual affinity for a common receptor [25]. That PEO and its congener etorphine do not show any significant receptor discrimination [26,27] could be exploited since other than the 7α-aralkyl substituent, the remainder of the opiate pharmacophore is locked in an unambiguous atomic arrangement. Accordingly these alkaloids

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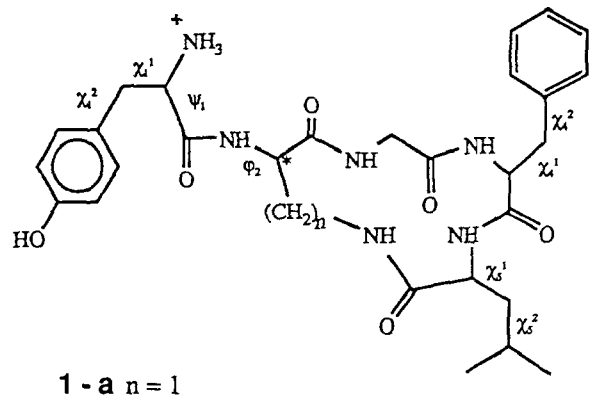
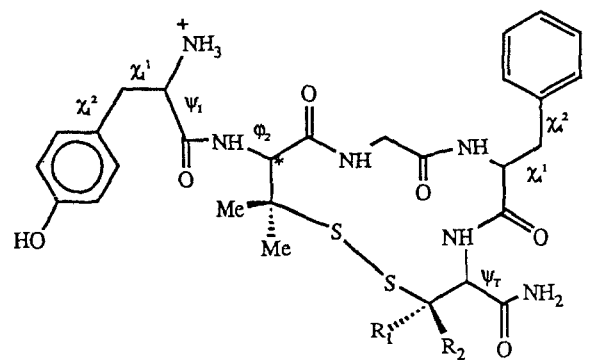
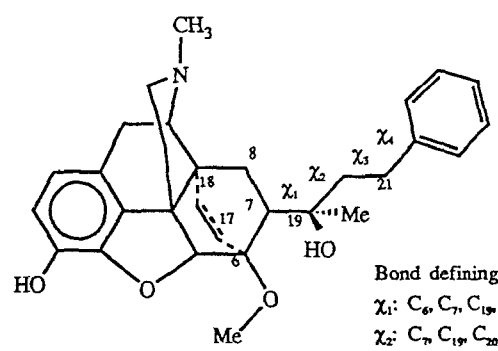
Abbreviations. Symbols and abbreviations are in accordance with recommendation of the IUPAC-IUB Joint Commission on Biochemical Nomenclature, Biochemistry, 9 (1970) 3471. c-ORN, Tyr-c[N^6-D-Orn^2-Gly-Phe-Leu-]; c-PEN, [D-Pen^2, L-Cys^5]-enkephalinamide; Pen, penicillamine = β,β-dimethyl cysteine; PEO, 7α-[(1R)-1-methyl-1-hydroxy-3-phenylpropyl]-6,14-endoethenotetrahydro oripavine; THT, 7α-[(1R)-1-methyl-1-hydroxy-3-methylbutyl]-6,14-endoethenotetrahydro thebaine; CSO, common space occupation: NMR, nuclear magnetic resonance spectroscopy.



1 - a n = 1
1 - b n = 2
1 - c n = 3 H-Tyr-c[N^{δ}-D-Orn^{2}-Gly-Phe-Leu-]
1 - d n = 4



2 - a $R_1 = R_2 = H$ ([D-Pen^{2}, L-Cys^{5}] enkephalinamide)
2 - b $R_1 = R_2 = CH_3$ ([D-Pen^{2}, L-Pen^{5}] enkephalinamide)



3 (PEO)

Bond defining torsional angles:
χ_1: C_6, C_7, C_{19}, C_{20}
χ_2: C_7, C_{19}, C_{20}, C_{21}
χ_3: C_{19}, C_{20}, C_{21}, C_{22}
χ_4: C_{20}, C_{21}, C_{22}, C_{23}

g. 1. Molecular structure of cyclic enkephalin analogs and PEO with some conformational variables under study.

have recognitive elements that define both the μ and δ receptor binding sites and, as such may be regarded as good semi-rigid models that bridge the otherwise extreme behavior of the two related series of cyclic enkephalin **1** and **2**. Therefore we also investigated all relevant conformers of the cyclic disulfide **2a** in relation to PEO. Comparison of the resulting low-energy superpositions served to explain the possible molecular basis for the divergent receptor selectivity of the two peptides.

MATERIALS AND METHODS

Molecular model and force field

Energy calculations and conformational searches were performed using the MAXIMIN2 minimization and the SEARCH function of the SYBYL 5.1 software package on a microVAX 2000 computer [28].

Conformational energies were computed by the molecular mechanics approach. Standard parameters supplied by the software were used in the energy function [29] but special parameters had to be added to take into account the torsional barrier of the disulfide bond which has a minimum at + or −90° [30]. The electrostatic component was computed assuming a distance-variable dielectric constant [31] and assigning partial atomic charges calculated by the Pullman method.

Conformational searches were executed using the SEARCH program by the grid search procedure, to explore the conformational space and calculate the conformational energy of given structures while varying specified torsional angles. A general cutoff of 0.85 times the van der Waals radius served to reject conformations with bad atomic contact (bump check). The SEARCH program also allowed us to invoke specific constraints such as distance and orientation of vectors.

The function MULTIFIT was used to achieve the multimolecular flexible fits; this option allows the pharmacophore of many molecules to superimpose while minimizing the total strain energy of all the molecules. Standard strength constants were used to achieve the fit between the pharmacophoric elements.

Molecular models for the peptide precursors were assembled from a standard fragment library supplied by the SYBYL system and all the amide bonds were assigned the *trans*planar arrangement. The fused ring structure of PEO was constructed based on the crystal structure of its congener 7α-[(1R)-1-methyl-1-hydroxy-3-methylbutyl]-6,14-endoethenotetrahydro thebaine (THT) [32] as described previously [25].

Ring searching and ring closure for the cyclopeptides

Since our primary concern resided in the low-energy conformation of the possible cyclic backbone structures of the respective cyclic amide **1c** (c-ORN) and disulfide **2a** (c-PEN), the α-carbon side chains were temporarily omitted and replaced by hydrogen atoms while the tyrosine residue was altogether removed for both molecules. A ring search and ring closure strategy based on distance constraints was used in order to investigate the largest conformational space. Accordingly, the SEARCH program was applied to the models represented in Fig. 2. The bond distance constraints were such that for c-ORN $d_1 = 1.54 \pm 0.15$ Å; $d_2 = d_3 = 2.51 \pm 0.30$ Å; $d_4 = 1.90 \pm 0.40$ Å; and for c-PEN $d_1 = 1.80 \pm 0.20$ Å; $d_2 = 2.85 \pm 0.45$ Å; $d_3 = 2.64 \pm 0.40$ Å; $d_4 = 1.90 \pm 0.40$ Å. In both cases the distance d_1 represents the bond formed by ring closure, and in order to avoid nonbonded interactions in the closure region, three atoms were dummied [33] as shown

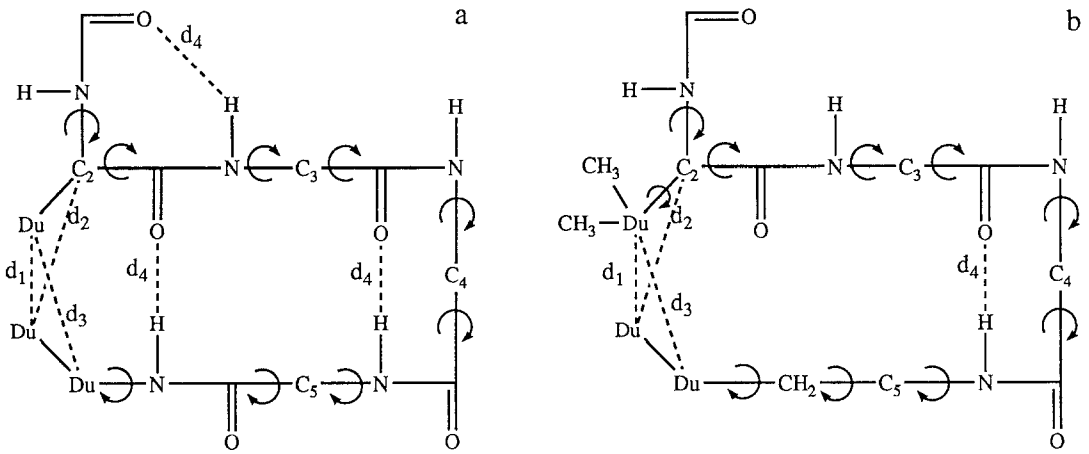


Fig. 2. Distance constraints and rotatable bonds as used in the ring closure and conformational search procedure: (a) model for ORN (b) model for PEN.

in Fig. 2. The purpose of the distance parameters d_2 and d_3 was to invoke valid valence values for the newly formed angles. The distance d_4 was imposed to simulate intramolecular hydrogen bonds as previously reported for c-ORN [34]. A screening of all possible cyclic conformers generated for c-PEN demonstrated that the potential candidates for future superpositions with the c-ORN molecule were also those that featured the intramolecular hydrogen bond Gly-C=O Leu-NH. This assumption was also reinforced by experimental evidences obtained for similar peptides [35] and supports our d_4 constraint.

In the subsequent SEARCH procedure to affect ring closure, degrees of freedom were assigned to the rotatable bonds for the abbreviated models shown in Fig. 2 and the corresponding torsional angles were allowed to vary from 0° to 360° by 30° increments. Following the systematic search all dummy atoms were replaced by appropriate groups and the conformers resulting from the ring closure were energy minimized using the MAXIMIN2 program in order to release local deformations. No special component was used to preserve the putative intramolecular hydrogen bonds. For subsequent studies the side chains and the exocyclic tyrosyl residue were reinserted into the respective ring conformers shown in Table 1.

Vector map for PEO

The high potency of PEO and its C_7 α-alkyl congeners has been attributed to a well-defined site on the opiate receptor which can accommodate a lipophilic alkyl or aralkyl group whose geometry is determined by the stereochemistry at C_7 and C_{19} (Fig. 1) [36]. In PEO the flexibility of the 7α-[(1R)-1-methyl-1-hydroxy-3-phenylpropyl] side chain precludes an accurate designation of the bioactive spatial geometry of the C_{21} phenyl ring. Consequently a systematic conformational search was conducted varying the torsional angles χ_1, χ_2, χ_3 and χ_4 (Fig. 1) from 0° to 360°by 30° increments as previously described [25]. Unlike the previous study no hydrogen bond distance constraint was invoked between the C_6-OCH_3 and C_{19}-OH groups. This variation permitted the evaluation of all possible vector loci corresponding to the C_{21}-aromatic ring. For each sterically

TABLE 1
CONFORMERS RESULTING FROM THE RING CLOSURE AND CONFORMATIONAL SEARCH APPLIED TO THE MODEL OF ORN AND PEN (SHOWN IN FIG. 2)

Ring conformer	Stereochemical code of the ring		Energy[a] (kcal/mol)	Deviation from the middle plane (rms values)
ORN1	C* A* C* A	a +a −s a	0.1	0.691
ORN2	C* F* C G	a +a −s a	1.4	0.420
ORN3	C* F C* D	−s −a +s +a	3.1	0.292
ORN4	C* A C A*	−s −a +s a	1.1	0.695
ORN5	C C C* D	a +s −s −s	2.1	0.685
ORN6	C A C A*	a +s −s −a	9.8	0.690
ORN7	C F C* D	a −s +a −a	0.0	0.522
ORN8	C A C A*	a −s +a +a	2.5	0.749
PEN1	E* A C G	a +s +s a +	6.7	0.710
PEN2	A* G C E	−s −s −s a −	10.5	0.611
PEN3	D* A C D*	−s −s a +a +	4.4	0.637
PEN4	C* C C* C*	−s −a −a +s +	4.7	0.452
PEN5	A* C C* C*	−a +s −s a −	8.1	0.600
PEN6	C* F C* A	a +s +s a +	6.9	0.405
PEN7	F* C* C A	a a −s +s −	6.2	0.790
PEN8	E* C* C G	a +s +s a +	0.0	0.847
PEN9	F* C* C E	−s −a a a +	10.3	0.517
PEN10	F* A* C* A*	a +s −s a +	11.1	0.643
PEN11	F* A* C* A*	−s −s −s a −	4.5	0.675

[a]Energy is relative to the global minimum set to zero. Absolute energies were −19.3 and −26.7 kcal/mol for ORN7 and PEN8, respectively.

allowed conformer the position of the vector normal to the centroid of the C_{21}-aromatic ring was recorded affording a vector map reflecting the preferential conformational space of the C_7 aralkyl chain.

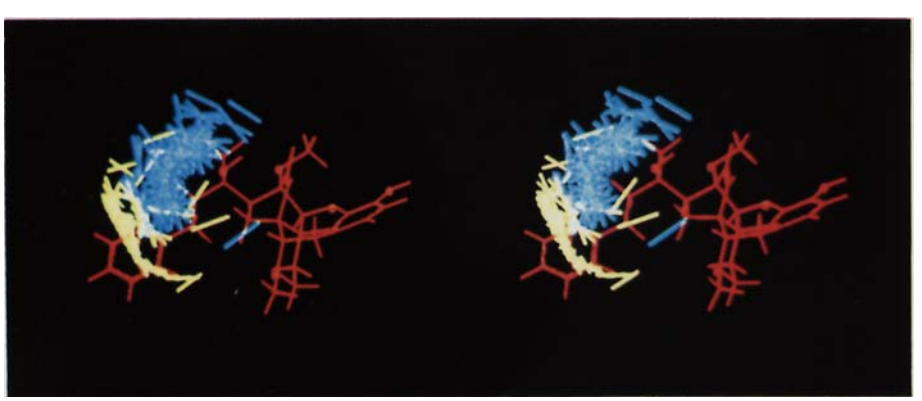


Fig. 3. Vector map corresponding to the conformational space of the C_{21}-aromatic ring of PEO (rotatable bonds are χ_1, χ_2, χ_3 and χ_4 as indicated in Fig. 1).

Superposition of pharmacophore

The coplanarity and cocentricity of the phenolic nuclei of c-ORN, c-PEN and PEO were strictly imposed while a distance deviation of up to 1.5 Å was permitted for the corresponding basic nitrogens. Furthermore, the vector map previously generated for PEO served as an additional orientation constraint for the proper alignment of the aromatic ring of the Phe^4 residue in the respective peptides. Using the computed backbone conformers (*vide supra;* Table 1) of the peptides, a systematic conformational search was performed for each PEN-PEO and ORN-PEO pair. Degrees of freedom were assigned to the rotatable bonds of the peptide side chains as indicated in Fig. 1. Subsequently each bimolecular conformer emerging from the conformational search was resubmitted to a bimolecular energy minimization using the function MULTIFIT. This operation permitted the mutual convergence towards the optimum bimolecular fit. The fitted structures were then allowed to relax freely of any imposed constraints to the nearest local minimum with a convergence criterion of 0.001 kcal/mol. The energy differences for conformers before and after multifitting can be used as an estimate for the conformational contributions to the binding energy.

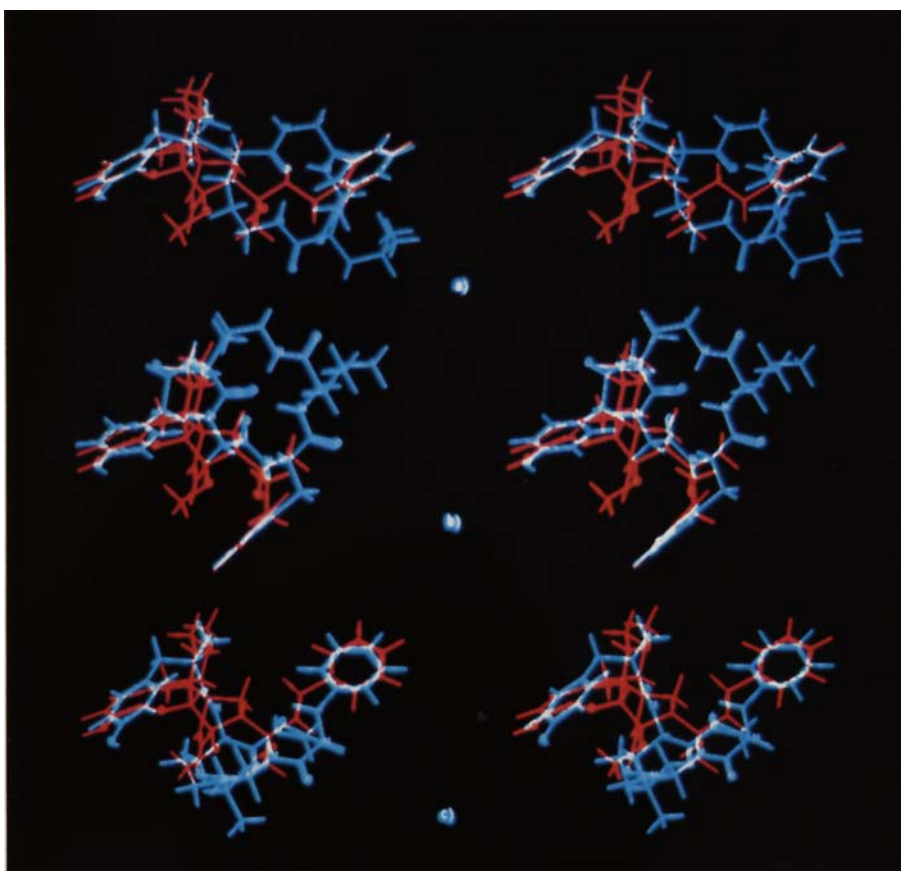


Fig. 4. Representative fit between PEO (red lines) and some ORN conformations (blue lines). (a) fit No. 6, (b) fit No. 3, (c) fit No. 5 (see Table 2)

Examination of the common and divergent conformational and steric properties exemplified by the bimolecular fitted pairs allowed the identification of those parameters that could account for the different pattern of receptor selectivity.

RESULTS AND DISCUSSION

The potent narcotic alkaloid 7α-[(1R)-1-methyl-1-hydroxy-3-phenylpropyl]-6,14-endoetheno-tetrahydro oripavine (PEO) is a good semi-rigid template to model more flexible cyclic opioid peptides [37]. Despite its typical phenanthrene-like nucleus, this alkaloid displays little receptor selectivity since it is equally effective at both μ and δ receptors. By inference, the flexibility of the 7α-aralkyl substituent could account for the observed indiscriminant behavior by interacting with similar complementary binding sites on two different receptors but in different orientations. Figure 3 shows a stereoscopic vector map corresponding to the geometrical space accessible to the C_{21} aromatic ring of PEO. Examination of the vector map indicates that the search generates a plethora of stable conformers scattered in almost two symmetrical vector regions; one concentrated in the pro-S and the other in the pro-R enantiotopic edge of the piperidine ring [38]. The pro-R region (blue) corresponds to that set of conformations with $\chi_1 = 300° \pm 30°$ whereas the pro-S

TABLE 2
CONFORMATIONAL AND ENERGY PARAMETERS CHARACTERIZATION OF ORN FITTED ON TO PEO

Fit no.	Cyclic peptide	Class	Strain energy[a]		Characteristic distances[b]				Hydrogen bonds[c]				F angle[d]	CSO[e]
			ORN	PEO	Tyr-Phe	N-Tyr	N-Phe	N-N	No. 1	No. 2	No. 3	No. 4		
1	ORN1	II	12.5	4.7	9.22	5.17	11.09	1.11	–	–	1 48	1.50	33	**
2	ORN1	III	6 7	4.6	10.90	5 13	11.68	1.10	–	–	1.55	1.58	42	***
3	ORN2	II	13.0	5.8	9.20	5 14	11.70	1.32	–	–	1 49	1.77	57	**
4	ORN3	I	13 9	1.3	10 35	3.91	7.11	0.75	2.01	–	1.44	1.60	101	
5	ORN3	III	6.2	0 7	10.31	5.15	8.18	1.17	1.91	–	1 41	1.57	31	*****
6	ORN4	I	17.4	1.2	10.99	4.48	7.72	0.51	1.90	–	1.48	1.80	62	
7	ORN4	III	4.1	0.4	7.73	5.18	4.33	0 70	1.91	–	1.40	1.99	39	*****
8	ORN5	II	13.2	2.3	9.81	5.14	11.68	1.53	–	–	2.50	1.62	70	**
9	ORN6	III	17.4	2.1	10.98	5.19	8 74	1.01	2.01	1.49	1.59	2.31	110	*
10	ORN7	III	14.6	3 0	9.94	5.16	10 15	0.72	–	1.58	1.89	1.61	109	**
11	ORN8	III	12 1	2.4	10 13	5.19	9.84	0.90	1.81	1.54	1 57	2.28	95	*

[a] Conformational energy in kcal/mol relative to the global minimum set to zero.

[b] Tyr-Phe, distance between centroid of aromatic nucleus of Tyr and Phe (Å).
N-Tyr, distance between nitrogen of Tyr and centroid of aromatic nucleus of Tyr (Å).
N-Phe, distance between nitrogen of Tyr and centroid of aromatic nucleus of Phe (Å).
N-N, distance between nitrogen of Tyr in ORN and tertiary nitrogen in PEO (Å).

[c] H-bond No. 1 C_{19}-OH → O-C6; H-bond No. 2 Gly-NH → O=C-Tyr; H-bond No. 3 (D)Orn $^{\delta}$NH → O=C (D)Orn; H-bond No. 4 Leu-NH → O=C-Gly

[d] Angular deviation between the vector joining the C_{21}-carbon and the centroid of phenyl ring and the vector joining the C^{β} of phenylalanine and the centroid of aromatic nuclei.

[e] Common space occupation, obtained by visual estimation of the global overlap between nonfitted atoms as observed after the bimolecular flexible fitting.

locus (yellow) coincides with $\chi_1 = 180° \pm 30°$. Interestingly if the putative hydrogen bond [39] between the C_6-OCH_3 and C_{19}-OH group is imposed, the resulting conformational sampling of the C_{21}-aromatic ring affords a locus concentrated primarily in the pro-S cluster. However, both illustrated vector spaces were considered in subsequent flexible fit procedures since the putative intramolecular hydrogen bond has been shown not to be an absolute requirement for high biological activity [40]. According to one hypothesis [41], the C_{21}-aromatic ring in PEO may have a functional equivalent in opioid peptides of the enkephalin type; serving both as the 'message' and 'address' component of the opiate pharmacophore [42]. Although this analogy has not been proven unequivocally, we have shown that using a single ring conformation [34] of the cyclic opioid peptide Tyr-c[N^{δ}-D-Orn^2, Leu^5] enkephalin (c-ORN), the Phe^4 residue satisfies this correspondence. Further, the alkaloid and peptide share other topological similarities that could serve to explain their affinity for a mutually exclusive site [25].

In the present study we have investigated other possible polypeptide backbone conformers using a thorough conformational ring search of c-ORN and have included the cyclic disulfide (D-Pen^2,L-Cys^5]-enkephalinamide: a δ opioid receptor selective ligand [20]. The choice of c-PEN (**2a**) was based on the observation that it is an established prototype upon which other more selective δ-ligands have been modeled [43] and it displays a similar level albeit opposite selectivity to the

TABLE 3
CONFORMATIONAL AND ENERGY PARAMETERS CHARACTERIZATION OF PEN FITTED ON TO PEO

Fit no.	Cyclic peptide	Class	Strain energy[a]		Characteristic distances[b]				Hydrogen bonds[c]		F angle[d]	CSO[e]
			PEN	PEO	Tyr-Phe	N-Tyr	N-Phe	N-N	No. 1	No. 2		
12	PEN1	III	9.6	1.0	10.12	5.18	8.71	1.67	1.86	1.51	56	***
13	PEN2	III	11.3	0.9	10.43	5.17	8.60	1.47	1.87	1.60	30	****
14	PEN3	III	13.8	0.6	10.84	4.88	6.52	0.83	1.97	1.71	16	*****
15	PEN3	III	5.2	0.0	7.22	5.15	3.68	0.79	1.90	1.78	29	*****
16	PEN4	I	12.8	1.7	11.62	4.40	8.44	0.38	1.98	1.61	62	
17	PEN5	III	13.6	8.3	11.12	5.01	11.20	1.20	–	1.64	53	**
18	PEN7	III	12.4	0.7	10.92	5.18	8.05	0.96	1.90	–	37	****
19	PEN8	III	8.1	0.6	10.27	5.16	6.78	0.91	1.93	1.74	49	***
20	PEN9	I	14.8	5.8	9.85	4.38	8.29	0.41	1.99	1 54	164	
21	PEN9	II	19.5	9.6	9.90	5.14	9.90	1.27	–	1.66	66	**
22	PEN10	II	18.4	3.2	10.83	5.13	12.44	1.39	–	1.54	57	**
23	PEN11	II	14.0	5.8	10.60	5.16	11.47	1.64	–	1.56	114	**

[a] Conformational energy in kcal/mol relative to the global minimum set to zero.

[b] Tyr-Phe, distance between centroid of aromatic nucleus of Tyr and Phe (Å).
N-Tyr, distance between nitrogen of Tyr and centroid of aromatic nucleus of Tyr (Å).
N-Phe, distance between nitrogen of Tyr and centroid of aromatic nucleus of Phe (Å).
N-N, distance between nitrogen of Tyr in PEN and tertiary nitrogen in PEO (Å).

[c] H-bond No. 1 C_{19}-OH → O-C6; H-bond No. 2 Cys-NH → O=C-Gly.

[d] Angular deviation between the vector joining the C_{21}-carbon and the centroid of phenyl ring and the vector joining the C^{β} of phenylalanine and the centroid of aromatic nuclei.

[e] Common space occupation, obtained by visual estimation of the global overlap between nonfitted atoms as observed after the bimolecular flexible fitting.

prototypic cyclic amide **1c** on isolated tissue preparations [16]. In addition the C-terminal residue is amidated and has the same relative stereochemistry as in c-ORN. The results of the ring closure by cyclization and conformational search of the peptide backbone component of c-ORN and c-PEN are shown in Table 1. The conformational search generated eight low-energy ring backbones for the cyclic amide and eleven correspondingly low-energy conformations for the cyclic disulfide. The resulting conformers are represented in Table 1, along with corresponding relative energies, using the stereochemical code adopted by Zimmerman et al. [44]. The small letter codes correspond to the values for χ_1, χ_2, χ_3 and χ_4 of the side chain torsional angles of the D-Orn^2 residue of the cyclic amide analog and the χ_1 and χ_2 values of the torsional angle of the respective side chains of the D-Pen^2 and L-Cys^5 residue in the cyclic disulfide analog. The letter codes are defined as s = synperiplanar and a = antiperiplanar where s = $0° \pm 30°$, +s = $60° \pm 30°$, −s = $-60° \pm 30°$, a = $180° \pm 30°$, +a = $10° \pm 30°$, −a = $-120° \pm 30°$. For the disulfide torsional angle the + sign stands for $+90° \pm 10°$ and the − sign for $-90° \pm 30°$.

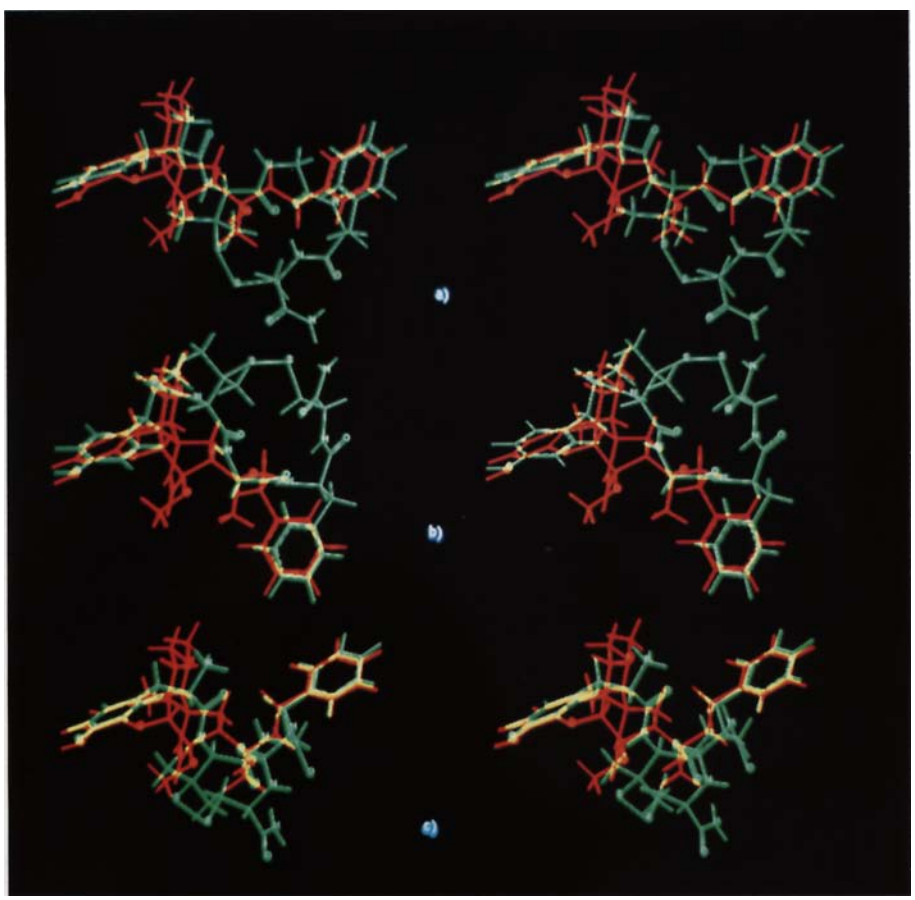


Fig 5 Representative fit between PEO (red lines) and some PEN conformations (green lines). (a) fit No. 16, (b) fit No. 21, (c) fit No 14 (see Table 3).

The last column in Table 1 lists the rms deviation [45] from the plane constructed by fitting the best plane through the atoms comprising the cyclic backbone (i.e. N, C^{α}, C′ and atoms of the closure region). The values provide an estimate of planarity of the peptide backbone. It is interesting to note that the most planar structures are found in the c-ORN series (ORN3 and ORN2 with rms values of 0.292 and 0.420, respectively) while the most distorted is found for the global minimum in the c-PEN series (ORN8, rms = 0.847). The latter is stabilized by an extra H-bond between Pen-C=O and Phe-NH.

The final step in this study involved the mutual superposition of the essential pharmacophoric elements of the cyclic peptides with those of PEO. Using each ring conformer in Table 1 and the coplanarity and cocentricity of the phenolic ring on one hand and a PEO vector map on the other as minimum constraints, we obtained a large number of relaxed peptide conformers which could be grouped into three classes. These could be differentiated by the values of the torsional angles χ_1 and χ_2 of the tyramine moiety. Class I had $\chi_1 = -90° \pm 15°$ and $\chi_2 = -150° \pm 15°$; class II had $\chi_1 = 155° \pm 15°$ and $\chi_2 = 115° \pm 15°$; class III had $\chi_1 = -150° \pm 30°$ and $\chi_2 = -155° \pm 20°$.

The results in Tables 2 and 3 demonstrate that both [D-Pen^2, L-Cys^5]-enkephalinamide and Tyr-c[N^{δ}-D-Orn^2, Leu^5]-enkephalin could be modeled upon the alkaloid with only minor deviation from their lowest-energy conformation despite obvious differences in their backbone structure, however, the ORN and PEN conformations having the greatest common occupational space with PEO are concentrated in class III. This result is also illustrated in Figs. 4 and 5.

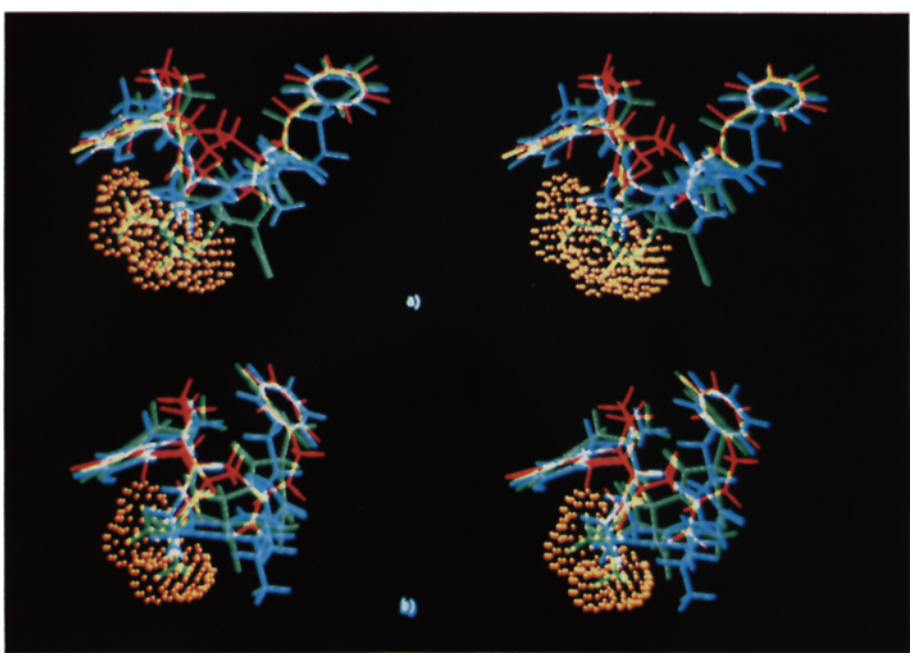


Fig. 6. Triple fit of PEO (red), ORN (blue) and PEN (green) depicting the exclusion volume (yellow) of the disulfide bond and adjacent β,β-dimethyl group of PEN. (a) tyrosine→phenylalanine in the extended conformation (fits No. 5 and 14) and (b) tyrosine→phenylalanine in the folded conformation. (fits No. 7 and 15).

Characteristics of the bimolecular superpositions

Tables 2 and 3 list key geometrical and energetic characteristics of some bimolecular superpositions corresponding to ORN-PEO and PEN-PEO, respectively. Interestingly, there was no major reorientation of the polypeptide backbone during the bimolecular fitting, consequently the various designated intramolecular hydrogen bonds remained intact. The C_6-OCH_3, C_{19}-OH hydrogen bond of PEO was also conserved in many superpositions; however, the greater global overlap with PEO coincided with the accessory aromatic ring in the pro-S vector space and having a conserved C_6-OCH_3 < C_{19}-OH (see fits No. 5,7 and 14,15). The data also show that the Phe^4 aromatic ring of both peptides can assume either an extended (exo) or folded (endo) orientation with distances inter-ring of 7.0 – 11Å as already observed in related analogs [46]; the energy being slightly lower in the folded conformation. This last phenomenon is presumably due to the better intramolecular van der Waals contacts in a folded structure while it is compensated in the extended conformation by intermolecular contacts in the receptor microenvironment. The basic nitrogens of the fitted peptide-alkaloid pairs do not coincide resulting in generally more extended tyramine conformations in the cyclic peptides compared to the alkaloid.

The last column in Tables 2 and 3 denotes the common space occupation factor (CSO) which was estimated by visual inspection and was assigned a value proportional to the degree of global overlap within the fitted pairs of molecules. Figures 4 and 5 illustrate this point and depict representative bimolecular superpositions within the calculated classes of conformers. For both series of peptides those conformers comprising class III, exemplified by Figs. 4c (ORN) and 5c (PEN) have the greatest common space occupation. In the conformation shown in Figs. 4c and 5c for fit 5 and 14, respectively, both aromatic rings of the peptides are located on the β face of the polypeptide backbone in an exo configuration separated by approximately 10 Å. The phenolic ring and Phe^4 side chain create a highly lipophilic surface in agreement with the arrangement proposed by Hruby et al. [47] for the solution conformation of the related peptide [D-Pen^2, D-Pen^5]-enkephalin. The putative hydrogen bond between the Gly^3-NH and the Tyr-C=O is not conserved in this orientation for ORN3 but appears in PEN3 (Figs. 4c and 5c). The exocyclic tyrosyl residue is redirected over the cyclic backbone. For both cyclic peptides the polypeptide backbone sweeps underneath the α face of the alkaloid such that the closure region of the respective peptides is proximal to the 6,14-endoetheno bridge. However, because the PEN backbone is more distorted (0.637 PEN3 vs 0.292 ORN3), the disulfide moiety descends further below the plane of PEO. In contrast to the conformers resulting from class III superpositions, those emerging from class I (Figs. 4a and 5a; $\chi_1 = -90° \pm 15°$, $\chi_2 = -150° \pm 15°$) and class II (Figs. 4b and 5b; $\chi_1 = 155° \pm 15°$, $\chi_2 = 115° \pm 15°$) have their respective cyclic backbone redirected in a region bearing little correspondence with PEO. Based on the reasonable assumption that, as in other neurotransmitter receptor superfamilies [48], the opioid receptor binding site is finite with conserved structural and stereochemical requirements in the active site of the μ and δ receptor subtypes, the departure of the ring backbone as shown by Figs. 4a,b (ORN) and 5a,b (PEN) would not be expected to be conductive to favorable receptor interaction.

It has been shown previously that substitution of the Phe^4 residue, in either cyclic or acyclic enkephalin analogs, with electron-withdrawing or electron-donating groups causes parallel qualitative and quantitative potency shifts at both receptors (in vitro) [49]. This result illustrates that the Phe^4 aromatic ring is subject to the same electronic effects at both receptors suggesting that the critical binding site associated with the side chain of the Phe^4 residue in PEN and ORN subserves

a similar role and may be a functional residue that inextricably links μ and δ receptor subtypes. This argument is supported by the present model and is illustrated by a triple 'extended' fit in Fig. 6a involving ORN3, PEN3 and PEO. Indeed the triple superposition shows that the aromatic ring of the Phe^4 residue in the peptides and the C_{21}-aromatic ring of the alkaloid are coplanar while the fitting angle, defined as the angular deviation of the bond bearing the C^{β}-aromatic ring of the Phe^4 residue on one hand and the C_{21}-aromatic ring in the alkaloid, is sufficiently small rendering the ring substituent positions congruent, and therefore subject to an analogous effect. However, small perturbations in the polypeptide backbones can confer substantially large fitting angles (Tables 2 and 3) such that the ring positions are not equivalent; however, the validity of the correspondence discussed above should be extended to examine the effects of biological activity of similar ring substitutions on the C_{21}-aromatic ring of PEO.

Comparison with other receptor models

Early studies based on active analog design have generated several models regarding the determinant features of the μ and δ opioid receptors pharmacophore; among these, the greater compactness of the μ binding site and the nature of the side chain of the fourth residue in opioid peptides have formed the basis of distinction [12]. One current model of the μ receptor has been proposed by Keys et al. [24] using theoretical energy calculations to study morphiceptin and other μ-selective morphiceptin analogs. The conclusion from that study and a subsequent one was that the interaction with the μ and δ receptor necessitates different polypeptide conformations. The results from the present study which suggest no dramatic conformational difference between PEN and ORN using PEO as a model template, are at variance with the conclusions derived by Loew et al. In the latter study the μ pharmacophore is defined by the conformational properties of morphiceptin (Tyr-Pro-Phe-Pro-NH_2) whose primary structure having two proline residues and a Phe residue in position three, is a significant departure from c-PEN and c-ORN which have an accessory aromatic ring in position four and whose positional relation of side chains is secured by intramolecular cyclization. Furthermore the present investigation allowed full freedom to the torsional angles χ_1 and χ_2 of the tyramine residue in the cyclic peptides while in the Loew model these values were confined to 267° and 193°, respectively, corresponding to those found in fused morphine and other alkaloids. Indeed we found that the rigid tyramine constraint described above redirects the polypeptide backbones away from the PEO contour analogous to Class I superposition with the added effect that the Phe^4 aromatic ring fell outside of the vector map for the C_{21} ring of PEO. A tyramine conformation different from rigid alkaloids is also justified on other grounds; for example: (1) the synthetic hybrids of enkephalinamide and metazocine, sharing in common the tyramine moiety, have been shown to be inactive [50], and (2) the nitrogen locus, which is variable in analgesics [51–53], could also be different in these opioid peptides and, by extension, would not manifest parallel structure–activity relationships as rigid alkaloids [54]. Indeed the model proposed by Portoghese et al. [53] reconciles some of these apparent anomalies.

It is now generally accepted that the conformational heterogeneity of structurally diverse analogs of opioid peptides preclude a reliable assignment of the steric and geometric requirements of the various receptor subclasses owing to the propensity of such linear peptides to adapt to the topography of the active site [17]. On the other hand rigidification of the enkephalin backbone by cyclization through an amide or disulfide bridge can be expected to reduce the number of intrinsic interconverting conformations. Cyclic peptides of the types **1** and **2** as well as variants having the

phenylalanine residue in position three have been the subject of considerable investigation by NMR and theoretical energy calculations [47, 55–58]. Notwithstanding that rigidification of the enkephalin backbone by cyclization via disulfide or amide bridge would be expected to reduce the number of interconverting conformations, the degree of receptor selectivity is highly dependent on the mode of cyclization. Accordingly, although NMR data suggests that the overall conformations corresponding to [D-Pen^2, L-Cys^5]-enkephalinamide and [D-Cys^2, L-Cys^5]-enkephalinamide are similar, the latter is equally effective at both δ and μ receptor sites [59]. Within the cyclic amide opioid peptides prototyped by Tyr-c[N^{ω}-D-Xxx-Gly-Phe-Leu] where Xxx is A_2pr, A_2Bu or Orn, similar conformations exist for all three [56]. The receptor model derived from the present study provides a rationale for the above anomalies. Figure 6 shows two triple superpositions involving (a) PEO, ORN3 (fit No. 5), PEN3 (fit No. 14) and (b) PEO, ORN4 (fit No. 7) and PEN3 (fit No. 15) adopting a class III tyramine conformation and with characteristic torsional angles listed in

TABLE 4
TORSIONAL ANGLES[a] OF TWO SELECTED CONFORMATIONS OF ORN AND PEN CORRESPONDING TO THE PROPOSED ACTIVE CONFORMATIONS

Residue	Torsional angle	ORN fit No. 5[b] (Xxx = Orn, Yyy = Leu)	ORN fit No. 7[c] (Xxx = Orn, Yyy = Leu)	PEN fit No. 14[b] (Xxx = Pen, Yyy = Cys)	PEN fit No. 15[c] (Xxx = Pen, Yyy = Cys)
Tyr^1	ψ	96	−98	162	162
	χ^1	−162	−178	−127	180
	χ^2	−136	−159	−146	−131
(D)Xxx^2	ϕ	159	−14	76	108
	ψ	−56	−64	−76	−84
	χ^1	−48	−51	−39	−39
	χ^2	−113	−123	−73	−73
	χ^3	94	74	88[d]	89[d]
	χ^4	98	150	–	–
Gly^3	ϕ	−66	−45	−63	−62
	ψ	162	−31	−64	−50
Phe^4	ϕ	74	−86	−66	−77
	ψ	−64	60	78	74
	χ^1	−44	−44	−73	32
	χ^2	110	145	92	116
Yyy^5	ϕ	−161	52	167	167
	ψ	62	46	−56	−61
	χ^1	−173	−176	137	134
	χ^2	60	61	−172	−169

[a] Angles are expressed in degrees.
[b] Corresponding torsional angles for PEO are $\chi_1 = -177°$, $\chi_2 = 58°$, $\chi_3 = 177°$, $\chi_4 = 81°$.
[c] Corresponding torsional angles for PEO are $\chi_1 = -177°$, $\chi_2 = 58°$, $\chi_3 = -91°$, $\chi_4 = 123°$.
[d] C-S-S-C dihedral angle in the c-PEN series.

Table 4. The stereoscopic view clearly demonstrates that the large distortion of the backbone in the area of the disulfide bond of PEN forces this region and the adjacent gem dimethyl group outside of the common occupational volume of the three molecules and suggests that this structural element, barring any major reorientation of the polypeptide backbone at the receptor microenvironment, may be incompatible with the μ site. Therefore the results of the approach used in this study are more in agreement with a steric effect which is deleterious to receptor binding as suggested by Mosberg [22] based on NMR studies. Expectedly the observed unfavorable steric bulk is exacerbated by substitution of an additional Pen residue in position five, consequently the resulting analog [D-Pen^2, L-Pen^5]-enkephalinamide would be expected to lose further affinity for the μ receptor as has been previously reported [20]. Interestingly the exclusion volume associated with the β-gem dimethyl groups in the present model resides below the plane of the phenolic ring which effectively deshields one methyl group in agreement with the anisotropic effect previously observed [22]. The above observations may also serve to explain the finding by Portoghese et al. [42] that alkaloids derived by fusion of an indole or benzofuran to the 6,7-position of naltrexone or oxymorphone confers antagonist and agonist δ receptor selectivity, respectively. According to the present model the additional aromatic ring of the above alkaloids coincides with the exclusion volume shown in Fig. 6 and may account for the observed δ selectivity. Thus it is conceivable that the greater distortion of the polypeptide backbone and the bulky steric volume of the pendant β,β-dimethyl groups of Pen^2 residue in [D-Pen^2, L-Cys^5]-enkephalinamide or the bis-gem dimethyl group of the [D-Pen^2, L-Pen^5]-enkephalinamide congener are not compatible with a restrictive topography of the μ site.

Finally, it should be noted that in the depicted triple superposition shown in Fig. 6, the pendant Leu^5 side chain of c-ORN and the carboxamide function of c-PEN also maintain a similar orientation relative to their respective polypeptide backbone but, while the former is predisposed to confer additional lipophilic character to the β surface, the latter is not. The coincidence of these side chains stems from the fact that the absolute configurations of L-cys and L-Leu are R and S, respectively, but while c-ORN is cyclized through the terminal carboxy groups, c-PEN is cyclized through a disulfide bond involving side chains. Therefore it is tempting to speculate that receptor interaction may be related to the hydrophobicity or hydrophilicity of this side chain. Indeed the carboxylate derivative of c-PEN and congener display further reduced potency at the μ receptor [21], but related data for c-ORN is unavailable.

CONCLUSION

The molecular basis of opioid receptor selectivity relies on the structural, conformational and stereoelectronic requirements of the receptor active site in a membrane microenvironment. Clearly no singular approach can assimilate the above criteria, however, we have arrived at a tentative model for the μ and δ receptor pharmacophore which does not necessitate drastically different conformations for the related Tyr-c[N^{δ}-D-Orn^2, Leu^5]-enkephalin and [D-Pen^2,L-Cys^5]-enkephalinamide. For each opioid peptide two conformers emerge as good candidates representative of the biologically active conformation: for the c-ORN, fits No. 5 and No. 7 (Table 2), and for c-PEN, fits No. 14 and No. 15 (Table 3). The torsional angles of these conformers are provided in Table 4. In the present model, the interplay of steric and conformational effects in the vicinity of the closure region of D-Pen generates an exclusion volume which may be incompatible with the μ

binding site; this result is in agreement with Mosberg's proposal who ascribed the receptor selectivity of PEN to a deleterious effect of the gem dimethyl group. The exclusion volume resides in juxtaposition to a site that could interact with a charged substituent on the δ ligand but with a neutral lipophilic residue on the μ counterpart. This model does not exclude the possibility that the respective binding sites may be buried within the cell surface whereby the membrane serves to screen the effective net charge [60]; however, it is noteworthy that other studies have alluded to similar models based on other lines of evidence [61]. In the absence of sequence and structural data for individual receptors, the model derived above provides the basis for a heuristic approach for the design of receptor-selective opioid peptides.

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