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Conformational Sampling of Bioactive Conformers: A Low-temperature NMR Study of ^{15}N-Leu–Enkephalin

PIETRO AMODEO[1], FRED NAIDER[2], DELIA PICONE[3], TEODORICO TANCREDI[1] and PIERO A. TEMUSSI[3,*]

[1] Istituto Chimica Molecole di Interesse Biologico (associated to the Italian National Institute for the Chemistry of Biological Systems), CNR, Arco Felice, Italy
[2] New York University, College of Staten Island, New York, USA
[3] Dipartimento di Chimica, Università di Napoli Federico II, Napoli, Italy

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Abstract: Conformational studies of enkephalins are hampered by their high flexibility which leads to mixtures of quasi-isoenergetic conformers in solution and makes NOEs very difficult to detect in NMR spectra. In order to improve the quality of the NMR data, Leu–enkephalin was synthesized with ^{15}N-labelled uniformly on all amide nitrogens and examined in a viscous solvent medium at low temperature. HMQC NOESY spectra of the labelled Leu–enkephalin in a $DMSO_{d6}/H_2O$) mixture at 275 K do show numerous NOEs, but these are not consistent with a single conformer and are only sufficient to describe the conformational state as a mixture of several conformers. Here a different approach to the structure–activity relationships of enkephalins is presented: it is possible to analyse the NMR data in terms of limiting canonical structures (i.e. β- and γ-turns) and finally to select only those consistent with the requirements of δ selective agonists and antagonists. This strategy results in the prediction of a family of conformers that may be useful in the design of new δ selective opioid peptides. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Opioids; enkephalin; selectivity; conformation; NMR

INTRODUCTION

Opioid peptides are small linear molecules which, with the exception of dynorphin (17 residues) and β-endorphin (31 residues), typically have from four (morphiceptin) to seven (deltorphins) residues. The availability of a very large number of synthetic analogues of the opioid peptides has favoured the collection of information on the relationship between primary structure and biological activity (SAR) [1]. However, in spite of the high flexibility of this class of molecules, data concerning their conformation–activity relationship (CAR) have also been obtained.

Most of the information on CAR has been gathered using constrained peptides. Specifically, partially rigid cyclic peptides [2–5] and the family of dermorphin/deltorphins have enhanced conformational preferences due to an intrinsic constitutional constraint, mainly the presence of a D-residue in the second position [6,7]. In contrast, the CAR of enkephalins, the prototype opioid peptides, is poorly understood [8]. The reason is twofold: the enhanced flexibility (with respect to other opioid peptides) afforded by the two adjacent Gly residues leads to a large number of quasi-isoenergetic conformers and to a very low specificity towards recep-

Abbreviations: BW373U86, (+)-4-[(αR)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl-3-oxybenzyl]-*N*,*N*-diethylbenzamide; CAR, conformation-activity relationship; EM, energy minimization; HMQC NOESY, hetero multiple quantum coherence nuclear Overhauser effect spectroscopy; MeNTI, methylnaltrindole; NOESY, nuclear Overhauser effect spectroscopy; SAR, structure–activity relationship; SIOM, 7-spiroindanyloxymorphone; SQC-NOESY, single quantum coherence nuclear Overhauser effect spectroscopy..

* Correspondence to: Dipartimento di Chimica, Università di Napoli Federico II, via Mezzocannone 4, I-80134 Napoli, Italy.

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tor subtypes. Theoretical models of the conformational preferences of enkephalins [9,10] and conformations found in solution (see [8] and paper quoted therein) cover a fairly wide range of conformational families: from fully extended conformers to several types of folded conformers. As pointed out by Paine and Scheraga [10], 'the differences in the conformations reflect the fact that enkephalin is an extremely flexible molecule. It, therefore, can pack in crystals in different ways, depending on the nature and proportion of solvent molecules that co-crystallise'. The choice, among these structures, of a 'bioactive conformation' is indeed an 'elusive goal' [12], unless one can find a reliable guiding principle.

In the conformational study of opioids, one should try to take advantage of the features of both peptides and alkaloids. While opioid peptides can be more active and in some cases are more selective than their alkaloid counterparts, the latter are generally quite rigid. One can argue that bioactive peptide conformations ought to be consistent with the shape of more rigid compounds and thus one can use the shape of alkaloids to select potentially bioactive conformers. In turn, the conformations of peptides that are consistent with the shape of the rigid moulds may reveal novel features of the interaction with the receptors. This is true even in the case of enkephalins that are non-selective agonists, since their μ and δ activities are nonetheless very high. Therefore, we may try to use μ and δ selective moulds independently.

The strategy we have generally followed (e.g. see [13]) implies (i) determination of the most likely peptide conformers in solution; (ii) comparison with rigid alkaloids to check whether the shape of the message domain is consistent with that of the rigid compounds; and (iii) use of the global shape of the peptide to improve the mapping. This procedure is not a vicious circle, since in the peptides there are features that we cannot possibly find in structural studies of the alkaloids. To name one trivial difference, peptides are in general much larger in shape than the corresponding alkaloids. On the other hand, the 'certification' of potential bioactive conformations of the peptide by comparison with rigid alkaloid counterparts tells us that we can use the shape of the peptide conformer with a higher degree of reliability for indirect receptor mapping.

When dealing with the CAR of enkephalins, besides the problems coming from conformational flexibility, there are those connected to the choice of a rigid agonist. For many years the only available reference rigid agonist was morphine. This alkaloid has a fairly high μ selectivity whereas enkephalins have δ selectivity, albeit low. In addition, morphine has only one aromatic ring while all opioid peptides have message domains containing two aromatic rings corresponding to the T and P subsites proposed by Portoghese [14,15]: those of Tyr^1 (common to all opioids) and of Phe^4 (in enkephalins, dynorphins, endorphin, etc.) or Phe^3 (in dermorphin and deltorphins). Portoghese and co-workers have recently described several non-peptidic δ- and κ-selective opioids containing two aromatic rings [16–20]. Owing to their rigidity these compounds can well reproduce the minimum requirements of volume, shape and spatial distribution of electronic features of an idealized opioid.

We can then adopt a reverse strategy (with respect to common practice): instead of using spectroscopic data to define the mixture of conformers and *then* trying to identify 'the' or 'a' bioactive conformation among the many conformers that populate the solutions of enkephalin, we can use spectroscopic data simply to check whether there are conformers consistent with the shape of rigid alkaloid opioids among the conformational distribution of the peptide and then check whether these conformers are consistent with NMR data and energy requirements. Here we show that this is indeed possible: we looked and found in solution the presence of conformers consistent with the shape of these new, δ selective rigid compounds. Although this procedure cannot possibly inform us on all low-energy conformers existing in solution (nor on the dynamics of the interaction) it can yield the minimum conformational requirements of our peptide when complexed with the receptor. As the quality of the spectroscopic data is important for a successful application of our strategy we tried several approaches to improve the NMR data. These included the use of Leu–enkephalin uniformly ^{15}N-labelled on all amide nitrogens, to deal with residual solvent resonance, and measurement in cryoprotective solvents at 275 K. Indeed, our results show that the HMQC NOESY [21] spectra of labelled Leu–enkephalin in a $DMSO_{d6}/H_2O$ mixture at low temperature are better than those collected in previous experiments on this opioid peptide [9,11,12].

MATERIALS AND METHODS

Materials

The ^{15}N-labelled amino acids (glycine, leucine and phenylalanine) were purchased from Cambridge Isotope laboratories (Woburn, MA, USA) as their N^{α}-Boc derivatives and were 99% isotopically pure. The tyrosine residue was incorporated as the N^{α}-Boc (2-bromobenzyloxycarbonyl) derivative and was not isotopically enriched. The peptide was assembled on a Merrifield resin (chloromethylpolystyrene) using the diisopropylcarbodiimide-1-hydroxybenzotriazole activation method.

The C-terminal amino acid (Boc-[^{15}N]-Leu-OH) was esterified to the resin by nucleophilic attack of the carboxylate group on the chloromethylated polystyrene using $KF \cdot H_2O$) as a catalyst in dimethylformamide at 50–60°C for 5 h. Unreacted benzylchloride functionalities were capped by reaction with excess sodium acetate under similar conditions. The loaded resin was found to have 0.63 meq/g of leucine using quantitative ninhydrin analysis. The protecting groups were removed using trifluoroacetic acid in dichloromethane and the final peptide was cleaved from the resin using HF. All coupling reactions were monitored using the Kaiser test [22]. The crude peptide was 82% homogeneous, as judged by HPLC, and was purified to homogeneity using a C_{18}-reversed-phase column and an acetonitrile/water/trifluoroacetic acid gradient. The final peptide was $>99.5\%$ pure on a C_{18}-column using both an acetonitrile/water/trifluoroacetic acid gradient and a methanol/water/trifluoroacetic acid gradient, and co-migrated with an authentic sample of Leu–enkephalin purchased from Sigma Chemical Company (St Louis, MO, USA). The isotopically labelled enkephalin had the expected monoisotopic molecular weight ($[M + H^+]$ calculated = 560.27; found 560.3) and amino acid ratios Tyr (0.97); Gly (1.99); Phe (0.98); Leu (1.07).

NMR

NMR samples were prepared by dissolving appropriate amounts of ^{15}N-labelled enkephalin in water and diluting the aqueous solution with $DMSO_{d6}$ to a final concentration of 1 mM.

^1H-NMR spectra were run at 500 MHz on a Bruker AMX-500 spectrometer. One-dimensional (1D) NMR spectra were recorded in the Fourier mode, with quadrature detection, and the water signal was suppressed by a low-power selective irradiation in the homogated mode. SQC-NOESY [23] and HMQC-NOESY [21] experiments were run in the phase-sensitive mode using quadrature detection in ω_1 by time-proportional phase incrementation of the initial pulse [24]. Data block sizes were 2048 addresses in t_2 and 512 equidistant t_1 values. NOESY experiments were run at mixing times in the range 100–800 ms. The resonance of the CH_2 protons of Gly, obviously present only in the normal NOESY, together with other peaks present in both experiments (HMQC-NOESY and NOESY) was utilized to scale intensities in the HMQC-NOESY and for distance calibration. NOEs of potential diagnostic value were measured at 100 ms and translated into inter-atomic distances by the method by Esposito and Pastore [25], using the distance between the CH_2 protons of Gly (0.178 nm) for calibration. Before Fourier transformation, the time domain data matrices were multiplied by Lorentz–Gauss functions in both dimensions.

EM

Energy calculations were based on the all-atoms parametrization of the AMBER force field, as implemented in the SYBYL package [26,27].

Conformational searches were performed by systematic variation of the dihedral angles (see Results) using the search module of SYBYL package (version 6.3). The acceptance criteria were based on inter-atomic distances between pairs of non-bonded atoms, plus, in restrained search only, on constraints derived from NOE data. In particular, a conformation was rejected whenever either it presented one or more inter-atomic distances below a threshold represented by the scaled van der Waals distances, with scaling factors of 0.85, 0.75 and 0.70, respectively, for distal (1–5 or more), vicinal (1–4) and H-bond interactions, or when restrained distance values outside their allowed ranges were detected. As the 6.3 version of SYBYL does not allow either EM or the use of the AMBER force field during the search, a command procedure, written in the SYBYL SPL language, has been developed in order to remove the main sterical strain by performing 20 unrestrained EM steps on each conformation obtained in the search. In this way the reliability of a selection based on energetic thresholds is considerably increased.

A total of eight distance restraints were used in both conformational search and restrained EM calculations. The five interprotonic distance ranges

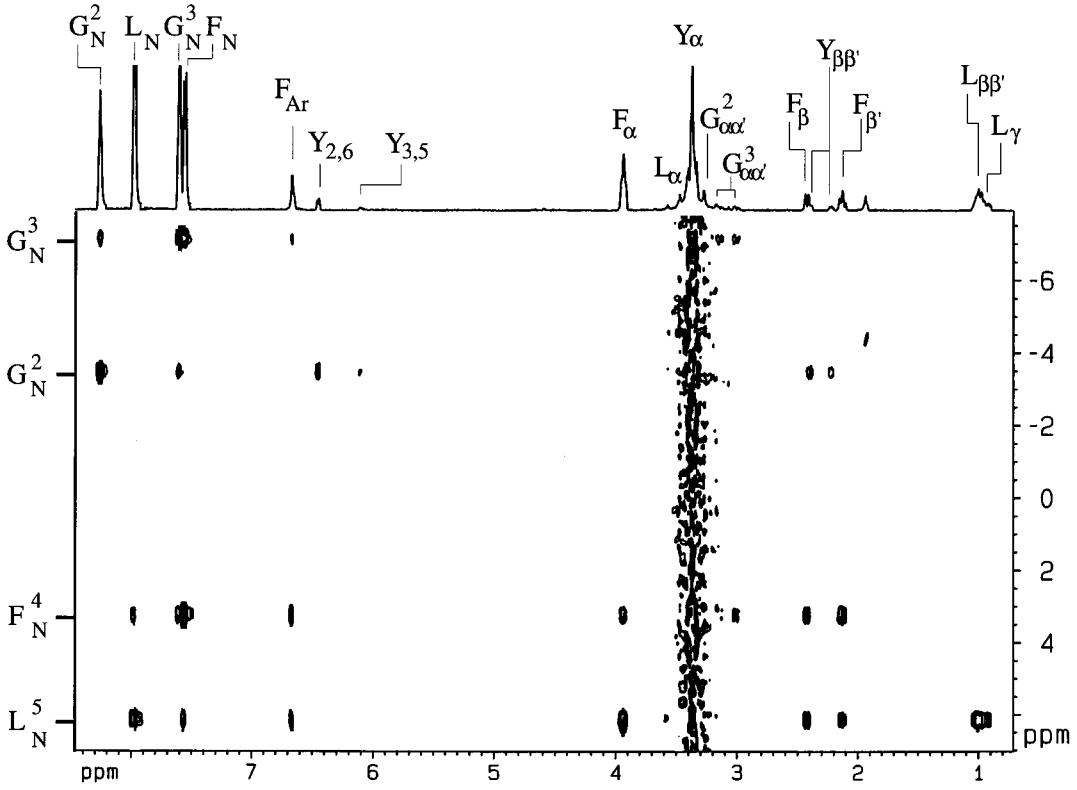

Figure 1 HMQC-NOESY spectrum of ^{15}N-Leu–enkephalin in a 90/10 (v/v) $DMSO_{d6}/H_2O$ cryomixture at 275 K.

listed in the 'Expl' column of Table 3 (see below), plus two 0.40 nm lower limits, corresponding to absent NOEs (N2–N5 and N3–N5, respectively) were used as experimentally derived restraints. In addition, the relative orientation of the two aromatic side-chains was constrained by imposing an allowed range from 0.15 to 0.45 nm to the corresponding C^β–C^β distance, as derived from rigid molecular moulds. A quadratic penalty function was applied in restrained EM calculations for distance values outside their allowed ranges, with a single value (2000 kJ/mol/nm^2) for the force constant of the eight restraints.

The final unrestrained EMs in each series of calculations have been performed according to the following scheme: the all-atoms parameterizations of AMBER force field were used in a series of EM calculations. The computational procedure can be divided into two steps: (i) an EM calculation is performed, using a quasi-Newton method, stopping when the gradient norm is 10^{-3} or less; and (ii) a final refinement is obtained by a full Newton–Raphson minimization, with a convergence criterium on the gradient norm of 10^{-6} or less.

RESULTS AND DISCUSSION

The flexibility of enkephalins leads to inextricable mixtures of quasi-isoenergetic conformers and makes NOEs very difficult to detect in neat solvents such as water or DMSO. A proper choice of the solvent medium can improve this situation. We have shown [28,29] that the NMR problems linked to flexibility can be partially overcome by running NMR spectra at low temperature in a cryoprotective solvent mixture. The use of a highly viscous solvent medium can affect the equilibrium among isoenergetic conformers, selecting the more compact conformers [30]. A widely employed alternative to influence the conformational distribution of enkephalin and other opioid peptides in solution is the use of micellar systems [31–33]. The rationale is that these peptide hormones might interact with their receptors through a lipid-mediated entry into the binding site of these proteins [34–36]. However, this appears rather unlikely for opioids as it has been recently shown that interactions with extracellular loops of the seven helix bundle receptor are crucial in the recognition process of dynorphin [37], and all characterized opioid recep-

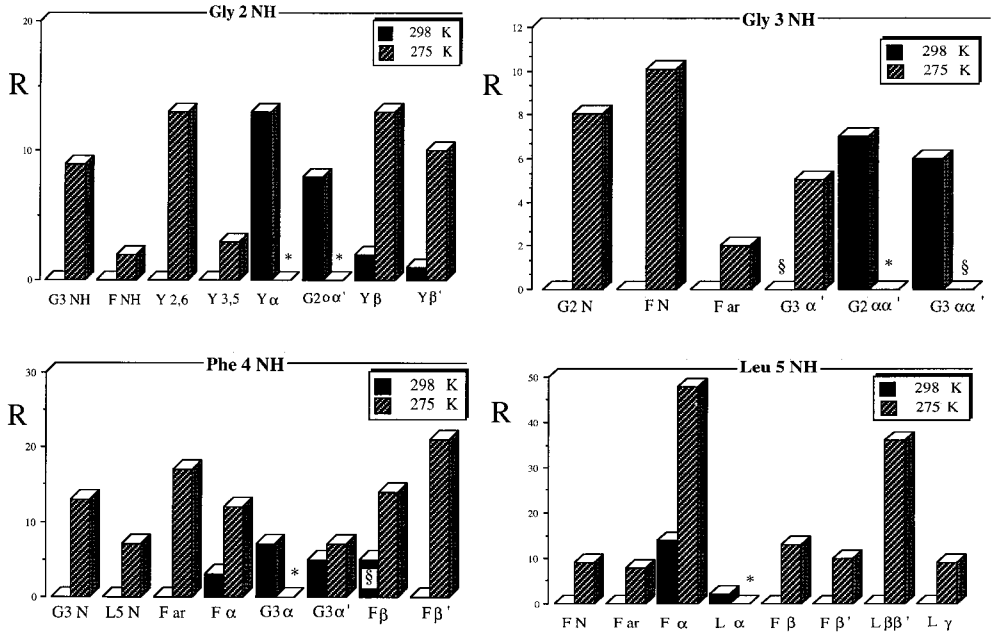

Figure 2 Comparison of the NOEs of ^{15}N-Leu–enkephalin measured in $DMSO_{d6}$ at 298 K and in the $DMSO_{d6}/H_2O$ cryomixture at 275 K. Columns marked with * indicate peaks obscured by presaturation of the HDO resonance, those marked with § indicate peaks affected by superpositions. The parameter in the ordinate (R) is the ratio of the volume of the cross peak to the volume of the corresponding diagonal peak.

tors share the same structural motif. Accordingly, we chose to rely on the best characterized among the cryoprotective mixtures, i.e. DMSO/water [28–30].

In the present study, to improve the quality of the NMR data, we have synthesized a Leu–enkephalin uniformly ^{15}N-labelled on all amide nitrogens. Figure 1 shows the HMQC-NOESY spectrum of ^{15}N-Leu–enkephalin in a 90/10 (v/v) $DMS)_{d6}/H_2O$ cryomixture at 275 K. The NOEs observed are more numerous than those measured in DMSO and in the corresponding NOESY spectrum of unlabelled enkephalin in the same mixture [11,28,29]. Figure 2 shows the comparison of the NOEs of ^{15}N-Leu–enkephalin measured in DMSO at 298 K and in the $DMSO_{d6}/H_2O$ cryomixture at 275 K. The increment induced by the higher viscosity is not uniform for all cross peaks, in agreement with our interpretation of viscosity as a possible natural mechanism of conformation preselection [30].

The simultaneous observation of all possible NH–NH cross peaks is difficult to reconcile with a single structure. Rather than trying to interpret all NOEs in terms of an exhaustive mixture of conformers, we attempted to identify the largest number of solution structures whose shape is consistent with the main topological characteristics of rigid agonists. We have limited our search to δ selective agonists since they, like the opioid peptides, are characterized by the presence of two aromatic subsites, whereas nearly all known μ-selective rigid agonists contain a single aromatic ring.

Among the several naltrindole derivatives (agonists and antagonists) with good selectivity prepared by Portoghese and co-workers [16–20,38], we chose 7-spiroindanyloxymorphone (SIOM), the first selective non-peptide δ_1 opioid agonist [19]. The molecular model, built on the basis of the crystal structure of a related compound [39], shows that SIOM is not totally rigid since the spiro moiety can be arranged in slightly different conformations. However, a conformational search yielded the same conformer proposed by Portoghese *et al.* as the absolute minimum [19]. In addition to SIOM, we employed BW373U86, a novel δ opioid agonist [40] with a piperazinyl-diphenylmethane skeleton characterized by a μ/δ selectively comparable to that of DPDPE [41]. An EM computation on BW373U86 yielded the structure shown in Figure 3 as the absolute minimum, which is presented together with the model of SIOM. It is interesting to note that the two molecu-

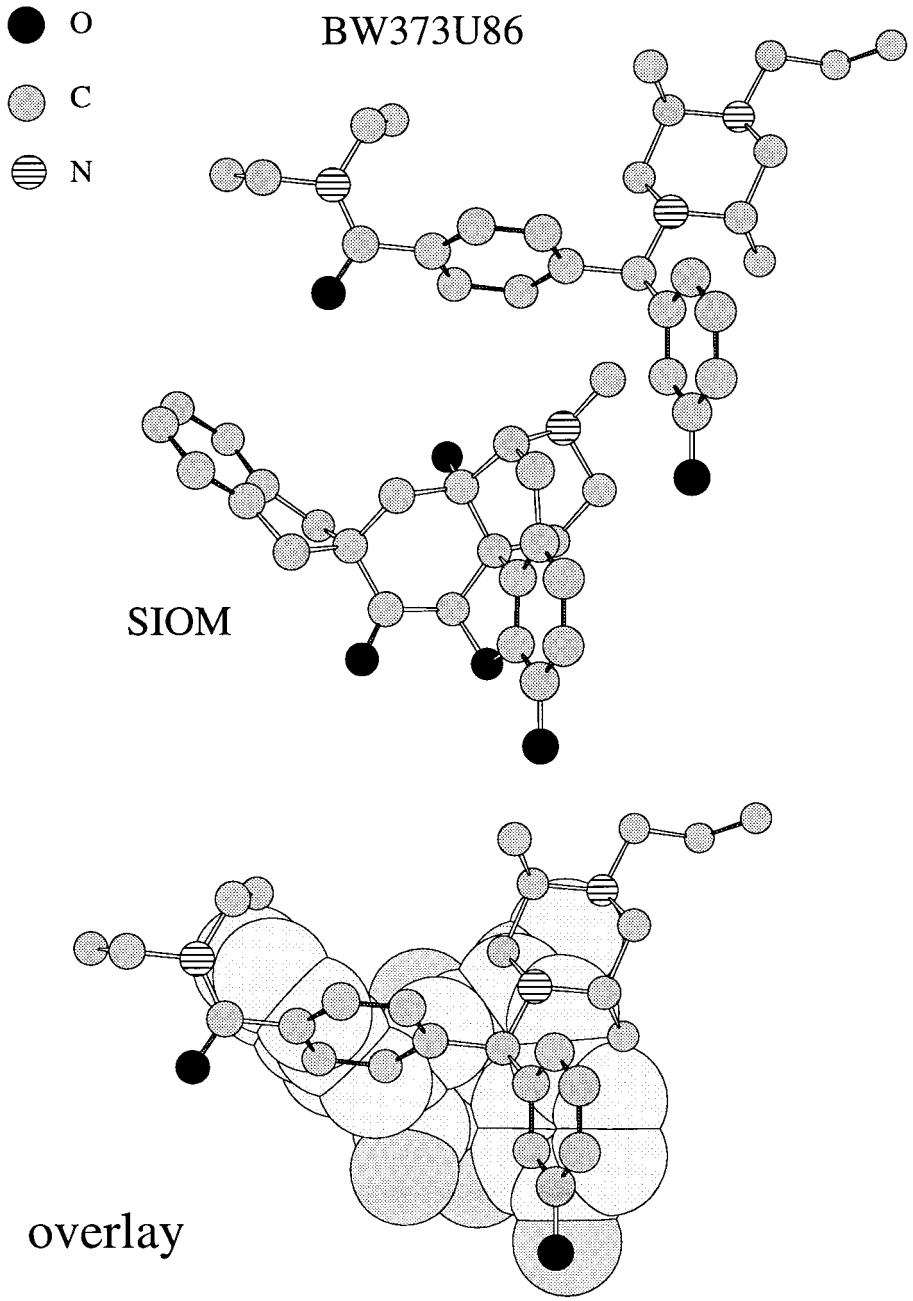

Figure 3 Molecular models of BW373U86 and SIOM, and overlay of the two models (SIOM is represented as a space-filling model in the overlay).

lar models, in spite of the rather different chemical constitution, have very similar topologies as evidenced by the overlay. In particular, in the superposition the single basic nitrogen of SIOM falls exactly midway between the two basic nitrogens of the piperazine ring of BW373U86 and the rings have the same orientations. It may also be in order to note that the proposed 'reverse strategy' requires some assumption in the 3D comparison of peptide conformations with rigid moulds. In our case the underlying assumption is that the relative position of the T and P subsites [14,15] is critical for selectivity (and activity). The relevance of this topological feature has been stressed by Portoghese *et al.* [38] for δ selective agonists, but is also crucial for δ selective antagonists [42,43].

Table 1 Relevant Backbone Torsion Angles of the Main Structures Extracted from the Automatic Search

	I	II	III	IV	V	VI	VII	VIII
$\psi 1$	−66.1	−66.0	−66.1	−66.1	165.4	23.9	33.4	−66.1
$\phi 2$	100.0	90.0	59.6	75.0	−75.0	−59.9	−59.9	−119.5
$\psi 2$	−30.0	−30.0	60.5	70.5	−45.1	−30.1	−30.0	60.0
$\phi 3$	−150.3	179.7	109.7	105.3	−135.2	−120.2	179.7	130.2
$\psi 3$	−70.3	60.3	−30.2	60.2	−75.2	30.2	60.2	60.2
$\phi 4$	60.2	−135.3	60.2	−136.3	60.2	−120.2	−135.3	−134.8
$\psi 5$	60.2	60.2	60.2	60.2	60.2	59.7	60.24	60.2
$\phi 6$	−115.4	−115.4	−115.4	−115.4	−115.4	−115.4	−112.4	−115.4

As mentioned above, the NMR data seem difficult to reconcile with a single structure, nonetheless, before undertaking the 'reverse strategy', we checked whether the NMR data can be explained by a single conformer. To find this conformer we combined the NOE-derived distance restrains with constrains derived from the shape of the rigid compounds. Thus, we established a searching-mask that would lead to single structures consistent both with all NMR constraints and the shape of rigid opiates. In this systematic conformational search (CS), a 30° grid was used for ϕ and ψ torsion angles, and a 120° grid for side-chains' χ torsion angles. NOE-derived distance restrains were included by using a square-well potential, and a hard-sphere approach was used for non-bonded interactions. Restraints from rigid compounds were included as distance restraints involving atoms in the side chains of the two aromatic residues. CS was followed by a relaxation of steric strains of the selected structures by partial EM. The 784 structures so obtained were incorporated into 'conformational families' by including in a 'family' all the structures sharing similar values of the ϕ, ψ angles of the residues 2–4. This analysis reduced the number of structures to 47. For each conformer different arrangements of ψ_1, ϕ_4, ψ_4 and of all side-chains are possible, even if not explicitly addressed in the search. EM of the most stable structure in each family, accounting for this problem, shows that the families collapse into only eight distinct conformers.

The backbone torsion angles that characterize the eight conformers are reported in Table 1. All conformers are obviously consistent with the shapes of BW373U86 and SIOM since the topological constraints corresponding to their shapes have been incorporated in the search. As an example, Figure 4 shows the overlays of two of the eight final conformers found in this search, i.e. IV and VI, with BW373U86 and SIOM respectively. It should be noted that some of the pairs of torsion angles of Table 1 depart largely from those typical of regular structures, i.e. they fall into forbidden regions of the Ramachandran map. This may be possible for the two glycines that can adopt values of ϕ and ψ typical of D-residues, but it is very unlikely for the other residues. However, this result may be an artefact due to the many constraints imposed by the simultaneous presence of NMR distance data and rigid template forcing. In fact, once these constraints are relieved, the peptide backbones undergo conformational transitions that lead to lower-energy conformers characterized by internal coordinates inconsistent with at least one of the NMR constraints. The inevitable conclusion is that we must try to account for the NMR data with mixtures of conformers.

The selection of the conformers of these mixtures was achieved by means of an empirical search that starts from all possible canonical structures (β turns and γ turns for *trans* peptide bonds), followed by a comparison with the structures of rigid opioid agonists, unrestrained minimization, and a final check for consistency with NMR data. Such a procedure does not require that each conformer be consistent with all NMR data simultaneously. Thus, it represents a rudimentary form of an ensemble calculation; a more rigorous treatment is prevented by the scarcity of NMR data. It is implied that the solution contains several conformers of very similar energy, a likely assumption for a molecule as flexible as enkephalin.

Starting structures were selected by choosing all possible combinations of pairs of torsion angles (ϕ and ψ) corresponding to canonical turns and cen-

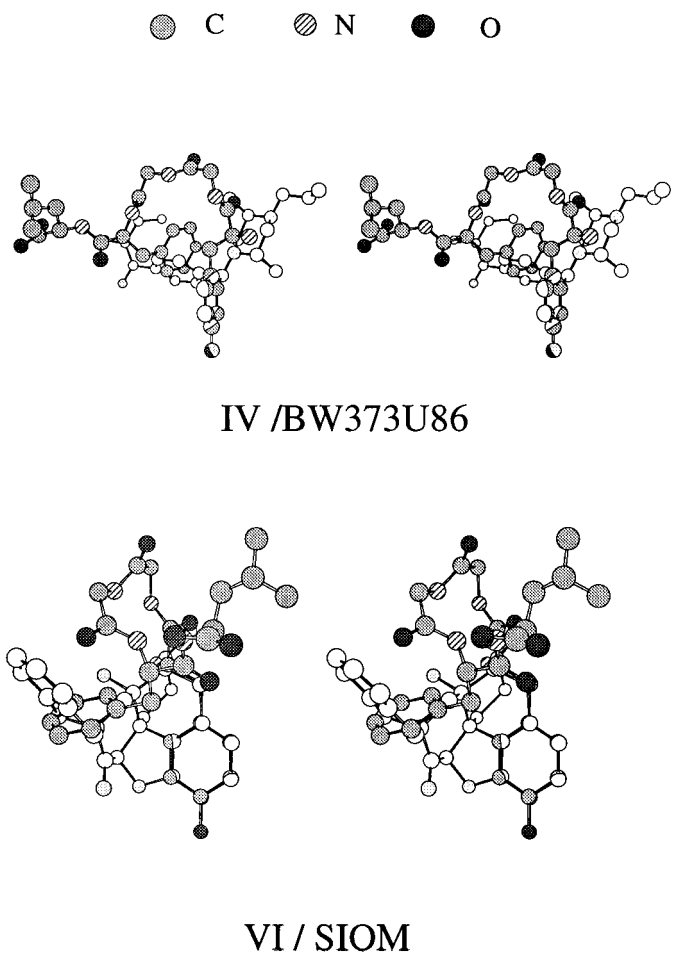

Figure 4 Overlay of conformers IV and VI, found in the automatic search (see Table 1), with BW373U86 and SIOM, respectively. The rigid moulds are represented with lighter grey balls.

tring them on Gly[2], Gly[3], and Phe[4]. That is, we chose ϕ, ψ pairs typical of β or γ turns, -60, -90, 80, 77 and $-77°$ plus a value typical of extended structures ($-120°$) for ϕ angles and, correspondingly, -30, 0, -65, 65 and 120° for ψ angles. We have taken into account the fact that the torsion angles of Gly are not restricted to those typical of (*S*) residues by including also 'primed' β-turn angles typical of (*R*) residues. The resulting conformations were first energy-minimized by restraining these angles to the chosen 'canonical' values. They were then compared with the shapes of SIOM and BW373U86 in order to check the consistency of the orientation of the aromatic rings with respect to the corresponding rings of the rigid compounds. The conformers consistent with the shapes of SIOM and BW373U86 were then subjected to a further unconstrained minimization cycle to test whether they could retain the consistency with the shapes of SIOM and BW373U86. The backbone torsion angles characterizing the lowest energy conformers within a range of *ca.* 10 kcal/mol that result from this procedure are reported in Table 2. Conformer A is characterized by a succession of two inverse γ turns centred on residues 3 and 4; conformer B is characterized by an inverse γ turn centred on residue 4, preceded at residue 3 by a pair of ϕ, ψ angles typical of the $i+2$ residue of a type I β-turn; both conformers C and D are characterized by an inverse γ turn centred on residue 4, preceded at residue 3 by a pair of ϕ, ψ angles typical of the $i+2$ residue of a type I′ β turn and by a fully extended conformation at residue 2; conformer E, which is the lowest energy structure, is characterized by a γ turn followed by an inverse γ turn centred on residues 3 and 4 respectively; conformer F is characterized by a distorted inverse

Table 2 Relevant Backbone Torsion Angles of the Main Structures Extracted From the Discrete Search Procedure Along With Their Relative Energies. The Torsion Angles of the Starting Conformers Are Reported in Brackets

	A (I, I)[a]	B (I, II)	C (II, I)	D (II, II)	E (I′, II)	F ($\gamma\gamma^i$, II)
E (kJ/mol)	36	40	41	40	0.0	24
$\psi 1$	179 (180)	179 (180)	172 (180)	173 (180)	−177 (180)	164 (180)
$\phi 2$	162 (−60)	173 (−60)	158 (−60)	159 (−60)	95 (60)	76 (77)
$\psi 2$	−50 (−30)	−54 (−30)	162 (120)	166 (120)	130 (30)	−91 (−65)
$\phi 3$	−74 (−90)	−84 (−90)	77 (80)	76 (80)	72 (90)	−73 (−77)
$\psi 3$	77 (0)	3 (0)	−8 (0)	−4 (0)	−79 (0)	122 (65)
$\phi 4$	−90 (−60)	−88 (−60)	−87 (−60)	−85 (−60)	−74 (−60)	−35 (−60)
$\psi 5$	48 (−30)	67 (120)	52 (−30)	61 (120)	93 (120)	−60 (120)
$\phi 6$	−80 (−90)	68 (9)	−138 (−90)	60 (80)	73 (80)	−78 (80)

[a] I, II and I′ indicate different types of β turns. γ^i indicates the 'inverse' type of γ turn.

Table 3 Comparison between Relevant Backbone NOEs and the Corresponding Distances of the Structures from the Discrete Search Procedure

	Expl	A	B	C	D	E	F
E (kJ/mol)		36	40	41	40	0.0	24
N2–N3	2.74–3.35	2.44	2.41	4.30	4.33	3.99	3.94
N3–N4	2.60–3.17	2.71	2.66	2.76	2.70	3.94	4.53
N4–N5	2.76–3.37	3.23	3.62	3.37	3.61	4.43	4.46
N5–α4	2.50–3.06	2.70	2.46	2.65	2.49	2.13	2.45
N2–N4	3.50–4.29	2.37	2.26	3.47	3.65	4.17	5.22

γ turn at residue 3 preceded at residue 2 by a ϕ, ψ pair typical of a γ turn. It can be seen that the final conformations are rather different from the starting ones, based on successions of 'canonical' angles. However, it must be stressed that the goal was not to *impose* canonical turns, but rather to use them as reasonable starting points.

Table 3 shows a comparison between relevant backbone NOEs and the corresponding distances of the structures extracted from the search procedure. Two of these structures (A and B) alone account fairly well for all NOEs, but they are of relatively high energy. Obviously, the experimental NOEs represent average values for all rapidly interconverting conformers in the distribution. It is clear that suitable averages among all low-energy structures can account for the observed NOEs even better than single conformers, but we have not attempted to quantify ensembles of different conformers since the experimental data are too scanty to justify detailed comparisons. Rather, we have concentrated our attention on the pair of structures that account directly for the NOEs (A and B) and on the pair of lower energy structures, E and F.

Figure 5 shows the best overlays of these structures of Leu–enkephalin with the shapes of either SIOM or BW373U86. As previously shown (Figure 3), in spite of the different chemical constitution the global shapes of these two rigid molecules are very similar; thus, they can be used interchangeably for overlaying the peptide conformers. The overlays were performed in the simplest possible way in order not to influence the comparison. We superimposed the 1,4-carbons of the aromatic ring and the adjacent α and β carbons of Tyr with the corresponding atoms of the tyramine moiety of SIOM, and the 1,4-carbons of the Tyr ring and the adjacent α carbon with the two 1,4-carbons of the phenolic ring and first basic nitrogen of the piperazine ring of BW373U86.

Owing to the particular emphasis that has been placed on the relative spatial position of the two aromatic rings corresponding to the side chains of Tyr and Phe [38], it is interesting to analyse the overlays also from this point of view. It can be

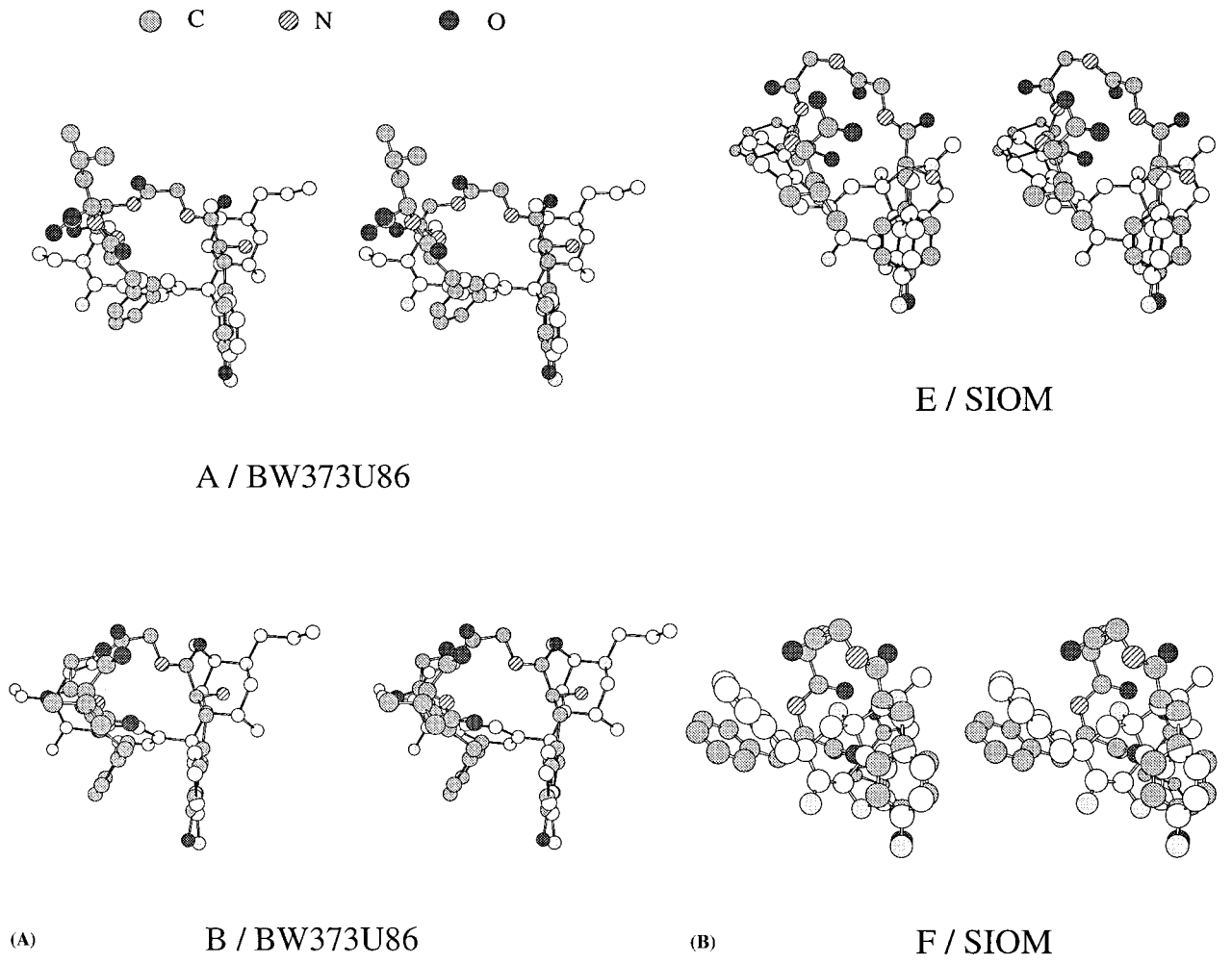

Figure 5 Best fit of four significant structures of Leu–enkephalin (A, B, E and F of Table 2) with the shapes of BW373U86 (A and B) and SIOM (E and F). The rigid moulds are represented with lighter grey balls.

seen from Figure 5 that even if conformers A and B are apparently rather different (i.e. conformer B is a much more compact structure), the second aromatic ring of BE373U86 falls in the same region occupied by the side chain of Phe[4]. The main difference between the two overlays is the fact that in A the ring of Phe is nearly parallel to the aromatic ring of BW373U86, whereas in B it is almost perpendicular. Conformers E and F are similar and yielded a good overlay with SIOM. It can be appreciated that also in these cases the second aromatic ring of SIOM falls in the same region occupied by the side chain of Phe[4].

It is interesting to note that the conformers of Table 2 are all consistent with the shape of rigid moulds but differ to some extent from models previously proposed [8–11]. In particular, conformer B does resemble the double bend conformation [8] in the central backbone torsion angles (ψ_2, ϕ_3, ψ_3, ϕ_4), but all other conformers have less similarities with either single bend or double bend structures that have been proposed previously. Although it is difficult to assess whether any of the conformers of Table 2 is *the* bioactive conformation of Leu–enkephalin, it is very interesting that our search has unveiled a new region of accessible conformational space for this molecule, consistent with rigid mould requirements. It goes without say that, owing to the method adopted in the search, the conformers of Table 2 do not include μ and κ selective conformers and it is likely that some of the several bioactive conformations previously proposed [8–11] act as μ and κ selective agonists.

CONCLUSIONS

The search for the so-called bioactive conformation of a small peptide hormone in solution is such a difficult task that it has been defined as an elusive goal [44]. This is dramatically emphasized by the experimental behaviour of enkephalins that are more flexible that other opioid peptides. In fact, it is very difficult even to extract the exact composition of the mixture of conformers present in solution. Nonetheless, in the present work we have shown that it is possible to derive meaningful information from NMR data collected in a viscous environment, provided the data are filtered by an appropriate conformational sieve, represented, in this case, by the topology of rigid moulds. We can conclude that for very flexible peptides the directed search described in this paper, i.e. a form of 'template forcing', seems to be more efficient than traditional methods in finding tentative bioactive conformations in solution. We can now use the shapes of the conformers of Table 2 to design new δ selective peptides.

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