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δ-Selective Opioid Peptides Containing a Single Aromatic Residue in the Message Domain: An NMR Conformational Analysis

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Abstract: The sequence of deltorphin I, a δ-selective opioid agonist, has been systematically modified by inserting conformationally constrained $C^{\alpha,\alpha}$ disubstituted apolar residues in the third position. As expected, substitution of Phe with Ac_6c, Ac_5c and Ac_3c yields analogues with decreasing but sizeable affinity. Surprisingly, substitution with Aib yields an analogue with almost the same binding affinity of the parent compound but with a greatly increased selectivity. This is the first case of a potent and very selective opioid peptide containing a single aromatic residue in the message domain, that is, only Tyr^1. Here we report a detailed conformational analysis of [Aib^3]deltorphin I and [Ac_6c^3]deltorphin I in DMSO at room temperature and in a DMSO/water cryomixture at low temperature, based on NMR spectroscopy and energy calculations. The peptides are highly structured in both solvents, as indicated by the exceptional finding of a nearly zero temperature coefficient of Val^5 NH resonance. NMR data cannot be explained on the basis of a single structure but it was possible to interpret all NMR data on the basis of a few structural families. The conformational averaging was analysed by means of an original computer program that yields qualitative and quantitative composition of the mixture. Comparison of the preferred solution conformations with two rigid δ-selective agonists shows that the shapes of [Aib^3]deltorphin I and [Ac_6c^3]deltorphin I are consistent with those of rigid agonists and that the message domain of opioid peptides can be defined only in conformational terms.

Keywords: opioid peptides; selectivity; antagonism; conformation; NMR

INTRODUCTION

Most structural studies on the topology of opioid receptors are indirect studies, based either on conformational analysis of (flexible) peptide hormones or on constitutional studies of (rigid) alkaloids. The indirect mapping of the main opioid receptors (μ, δ and κ) has greatly benefited from systematic (synthetic) studies on alkaloid analogues. In particular, Portoghese and coworkers have recently described several non-peptidic (and very rigid) δ- and κ-selective opioids [1–4]. 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. Of particular importance is the emphasis on the relative spatial position of the two aromatic rings corresponding to the side chains of Tyr and Phe: a recent work by the same authors actually associates μ/δ selectivity of agonist (or antagonist) naltrindole derivatives with the relative orientation of the two aromatic rings [5].

Abbreviations: $DMSO_{d6}$, perdeuterated dimethylsulphoxide; DQF-COSY, double-quantum filtered correlation spectroscopy; GPI, guinea pig ileum; MVD, mouse vas deferens; MeNTI, methylnaltrindole; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame nuclear Overhauser effect spectroscopy; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIP, Tyr-Tic-Phe-NH_2 TIPP, Tyr-Tic-Phe-Phe-NH_2; TOCSY, total correlation spectroscopy.

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Alkaloids, however, do not constitute completely satisfactory molecular moulds, i.e. they cannot substitute natural hormones (opioid peptides) in all respects. First of all they are generally smaller (in volume) than peptides and thus may have a smaller interaction area. Secondly, their structure cannot be easily subdivided in two clearly distinct domains [6]. Schwyzer suggested [6] that the sequence of most peptide hormones can be subdivided in two parts: a message domain, which is necessary for recognition, and an address domain, which imparts selectivity with respect to receptor subtypes. In the case of opioids the message domain was originally identified with the initial tetrapeptide fragment, Tyr-Gly-Gly-Phe, common to the sequences of enkephalin, dynorphin and endorphin [7,8]. Later on, the discovery of potent μ-selective and δ-selective natural peptides, containing a D residue, i.e. dermorphin [9,10] and deltorphins [11,12] respectively, has shown that the message domain can be shorter, albeit containing the same two aromatic residues: Tyr-D-Xaa-Phe-. Accordingly, in natural opioid peptides the address domain can vary from one residue (Leu or Met in the case of enkephalin) to four residues for dermorphin and deltorphins and up to 26 residues in the case of endorphin. In the explanation of the rationale for the synthesis of non-peptidic δ- and κ-selective opioids [1–4], Portoghese and coworkers identified the address domain of peptides with Phe^3 or Phe^4, to make it correspond to the second aromatic ring of naltrindoles. In spite of the success in designing an impressive number of new opioids, this interpretation of the message/address concept, as applied to rigid alkaloids, is not consistent with all peptide data. The systematic variation of the message domain of DTI presented in the present work can help to elucidate the meaning of the message/address concept.

In the indirect mapping of receptor sites, one should try to take advantage of the features of both classes of compounds (peptides and alkaloids). The best strategy can be summarized as follows: (a) determination of the most likely conformers of peptides, possibly on the basis of experimental data; (b) comparison with rigid alkaloids to check whether the shape of the message domain is consistent with that of the rigid compounds; and (c) use of the global shape of the peptide to improve the mapping.

The critical point of this strategy resides in the difficulty of structural determinations for peptides; these compounds are so flexible that they normally exist (in solution) as mixtures of several quasi-isoenergetic conformers. The means of reducing their flexibility are essentially two: the introduction (in their sequence) of conformational constraints, e.g. by cyclization or by insertion of non-proteic residues etc., and the use of environmental constraints. In the present work, we have pursued both methods, by introducing non-proteic restricted amino acid residues in the sequence of deltorphin I and by studying the conformation of the resulting peptides in highly viscous media.

Conformational studies on deltorphin I [13,14], Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2, together with previous indications furnished by dermorphin *N*-tetrapeptide analogues [15], e.g. one containing cyclohexylalanine (Cha) in lieu of Phe^3 and endowed of a moderate μ activity, suggest that the outer shape of the whole molecule is more important than the specific constitution of the message domain. In particular, a continuous hydrophobic surface in the C-terminal domain seems to contribute significantly to the marked δ selectivity of deltorphins [14]. Thus, it may be possible to find potent δ selective peptides lacking Phe^3. In other words, it seems interesting to explore the possibility of reducing the number of aromatic residues in the message domain to one, i.e. to the single Tyr^1.

We have investigated this through the design of a series of deltorphins systematically substituted in the third position with bulky ($C^{\alpha,\alpha}$-dialkylated) non-aromatic residues. This substitution not only limits the accessible conformational space but offers the additional advantage of testing the ability of bulky aliphatic residues to play the role of Phe, as already demonstrated for other classes of bioactive peptides [16]. As expected on the basis of side-chain volumes, substitution of Phe with Ac_6c, Ac_5c and Ac_3c yields analogues with decreasing but sizeable affinity [17]. The linear decrease of binding affinity that parallels the decrease of the size of the Ac_xc ring is consistent with a one-to-one replacement of the Phe side chain in the bioactive conformation. Surprisingly, substitution with Aib^3 yields an analogue with almost the same binding affinity of the parent compound but with a greatly increased selectivity. Such a behaviour cannot possibly be explained on the basis of constitutional differences since the side chains of Aib have the same encumbrance of those of Ac_3c, and calls for a detailed comparison of the conformations [Aib^3] deltorphin and [Ac_xc^3] deltorphins.

Here we present a study of the conformational state of [Aib^3]DT I and [Ac_6c^3] DT I based on a combination of ^{1}H-NMR in DMSO at room temperature, DMSO/water cryomixture at low temperature and molecular mechanics (MM) and molecular dynamics (MD) calculations.

MATERIALS AND METHODS

Materials

[Aib^3]DT I was synthesized according to published methods using solid-phase synthesis techniques [18,19] with a Milligen 9050 synthesizer. Amino acids were purchased from Novabiochem AG (Germany). Peptide synthesis was accomplished on a Rink resin [4-(2′,4′-dimethoxy-phenyl)-Fmoc-aminomethyl-phenoxy resin, 0.47 mmol/g; 0.1 g] obtained from Bachem (Torrance, CA, USA). The resin was mixed with glass beads (1:15 w/w) obtained from Sigma. The peptide is assembled using Fmoc-protected amino acids (four-fold excess) and 1,3-diisopropylcarbodiimide (DIPCDI, four-fold excess) and 1-hydroxybenzotriazole (HOBt, four-fold excess) as coupling agents, 1 h for each coupling. Double coupling is required in the sequences Val-Val,Asp-Val and D-Ala-Aib. The peptides were cleaved from the resin by treatment with TFA/H_2O/Et_3SiH (88:5:7; v/v) at room temperature for 1 h.

[Ac$_x$c^3]DT I analogues (Ac$_x$c is a $C^{\alpha,\alpha}$-dialkylated glycine whose side chain is made by a cycle of x carbons; x=6,5,3) were synthesized by solution methods. H-Ac$_x$c-OMe were condensed by soluble carbodiimide with Boc-D-Ala-OH (Boc is a *tert*-butyloxycarbonyl) to give protected dipeptides, Boc-D-Ala-Ac$_x$c-OMe. Protections at C- and N-termini were removed by treatment with 1 M NaOH and trifluoroacetic acid and dipeptides condensed with Boc-Tyr-OSu (OSu is a *N*-hydroxysuccinimido ester) to give N^{α}-protected Boc-Tyr-D-Ala-Ac$_x$c-OH that we used in the final condensation with H-Asp(OBut)-Val-Val-Gly-NH_2 [20] via soluble carbodiimide. Protected heptapeptides Boc-Tyr-D-Ala-Ac$_x$c-Asp(OBut)-Val-Val-Gly-NH_2 were deprotected with trifluoroacetic acid at room temperature.

Crude peptides obtained by solid-phase and solution methods were purified by reverse-phase chromatography using a Waters Delta Prep 3000 (30 × 3 cm, 15 μm particle size column). [Aib^3]DT I is eluted with a gradient of 0% to 60% B over 25 min at a flow rate of 30 ml/min using mobile phase A (10%, v/v acetonitrile in 0.1% TFA) and B (60%, v/v acetonitrile in 0.1% TFA). [Ac$_x$c]DT I analogues were eluted with a gradient of 0–100% B in 25 min at a flow rate of 30 ml/min. Analytical HPLC analyses were performed on a Bruker liquid chromatography LC-21 instrument fitted with a Waters Pico-Tag C_{18} column (150 × 3.9 mm, 5 μm particle size) or a Vydac C_{18} column (150 × 4.6 mm, 5 μm particle size) and equipped with a Bruker LC 313 UV variable wavelength detector. Recording and quantification were accomplished with a chromatographic data processor coupled to an Epson computer system (QX10). Analytical determinations and the capacity factor (*K*&prime) of the peptides were determined using HPLC conditions in the above solvent systems in a linear gradient from 0% to 100% B in 25 min at a flow rate of 1 ml/min. All analogues showed less than 1% impurities when monitored at 220 nm. TLC used precoated plates of silica gel F254 (Merck, Darmstadt, Germany) in the following solvent systems: (a) 1-butanol/HOAc/H_2O (3:1:1); (b) EtOAc/pyridine/HOAc/H_2O (6:2:0.6:1.1). Ninhydrin (1%, Merck), fluorescamine (Hoffman-La Roche) and chlorine reagent were used as detection sprays. Molecular weights of compounds were determined by a triple stage quadrupole mass spectrometer (TSQ-700 Finnigan MAT, San Jose, CA, USA) equipped with a pneumatic electron-spray (ion-spray) interface. Data were acquired onto a DEC station 5000/125 computer (Digital equipment Corporation, Maynard, MA, USA).

Optical rotations were determined using a Perkin-Elmer 241 polarimeter with a 10 cm cell; peptide analogues were dissolved in methanol at a concentration of 1% and determined at 20°C.

Amino acid analyses were performed by means of Millipore Pico-Tag methodology; lyophilized samples of peptides were hydrolysed using 6N HCl containing 1% phenol for 1 h at 150°C and amino acids reacted with PITC. The PITC amino acid derivatives were separated on a Pico-Tag column (15 × 3.9 mm) and monitored at 254 nm.

The analytical properties of Phe^3-substituted deltorphin 1 analogues are summarized in Table 1. The synthesis and analytical properties of deltorphin I have already been reported in [19].

Bioassays

A 2–3 cm segment of guinea pig ileum (GPI) was mounted in a 20 ml organ bath using the method of Kosterlitz and Watt [21]. The tissue was bathed in Krebs solution containing 70 μM hexamethonium bromide and 0.25 μM mepyramine maleate and aerated with 95% O_2/5% CO_2 at 36°C. The ileum was stimulated transmurally with square-wave electrical pulses of 0.5 ms duration at a frequency of 0.1 Hz. Unless otherwise stated, the strength of the stimulus was 1.5 times that which produced a maximal twitch (30 V). The contractions were recorded isotonically at a magnification ratio of 1:15. A single vas deferens (MVD) from a mature mouse (30–

Table 1 Analytical Properties of Phe^3-substituted Deltorphin I Analogues

Peptide	TLC R_F values		HPLC (K')	ES/MS (M+)	$[\alpha]$D	Amino acid analysis				
	A[a]	B[a]				Y	a	D	V	G
[Ac$_6$c^3]DT I	0.5	0.14	3.43[b]	707	−21.5	0.91	1.02	0.94	1.87	1.0
[Ac$_5$c^3]DT I	0.34	0.53	10.6	747	−25.2	0.94	0.97	0.92	1.7	1.0
[Ac$_3$c^{3}DT I	0.31	0.48	9.2	733	−21	0.90	1.0	0.89	1.8	1.0
[Aib^3]DT I	0.21	0.46	6.69	705	−23.5	0.89	0.97	0.90	1.8	1.0

[a]See text for composition of the mobile phases.
[b]On Waters Pico-Tag C_{18} column. All other data were obtained on a Vydac C_{18} column.
[c]Aib and Ac$_x$c residues were not quantitatively determined as PITC derivatives.

35 g) was dissected and suspended in 4 ml modified Krebs solution [22] aerated with 95% O_2/5% CO_2 at 33°C. The twitch was recorded by means of an isometric transducer. Each analogue in 10–100 μl Krebs solution was tested for its ability to inhibit electrically stimulated contractions on GPI and MVD. The concentration of the peptide (nM) required to inhibit the amplification of the electrically induced twitch by 50% is given as an IC_{50} value.

^{1}H-NMR

Samples were prepared by dissolving appropriate amounts of the peptide in $DMSO_{d6}$ and diluting to a final concentration of 3 mM for both the DMSO and the DMSO/water (80:20; v:v) cryomixture solution.

NMR spectra were run at 500 MHz on a Bruker AMX-500 instrument. 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. DQF-COSY [23], TOCSY [24], NOESY [25] and ROESY [26] experiments were run in the phase-sensitive mode using quadrature detection in ω_1 by time-proportional phase incrementation of the initial pulse [27]. Data block sizes were 2048 addresses in t_2 and 512 equidistant t_1 values. Before Fourier transformation, the time domain data matrices were multiplied by Lorentz-Gauss functions in the F_2 dimension and by a shifted sine-bell in the F_1 dimension. Mixing times of 70 ms and 120 ms were employed for TOCSY and ROESY experiments respectively. NOESY experiments were run at mixing times in the range of 75–300 ms. NOEs of potential diagnostic value were measured at 100 ms and translated into interatomic distances by the method of Esposito and Pastore [28], using the distances between the CH_2 protons of Asp (0.180 nm), between the terminal NH_2 protons (0.178 nm) and between *ortho* and *meta* protons of Tyr (0.247 nm) for calibration.

Molecular Mechanics and Molecular Dynamics

Energy and MD calculations were based on the all-atom parametrization of the AMBER force field [29,30] (as implemented in the SYBYL package). The tentative fit of experimental constraints to a single low-energy conformer was performed through a combination of restrained simulated annealing (SA) calculations, combined with local conformational searches (CS) and final restrained and unrestrained energy minimizations (EM). Both SA and EM calculations have been performed with a distance-dependent dielectric constant ($\varepsilon = r$) and no distance cut-off for non-bonded interactions. Distance restraints from NOESY spectra have been applied using a harmonic potential outside the fixed distance ranges, with a force constant value of 100 kcal mol^{-1}Å^{-2}, while a null restraining potential is applied inside that range. The harmonic term is switched to a linear one when the absolute value of a calculated violation exceeds 0.5 Å at either range limit. During SA runs, in addition to the molecular temperature, the value of the restraining potential force constant has also been varied, using a time-dependent weight function.

Conformational Averaging

The calculation of NOEs in terms of a mixture of conformers poses difficult problems that are not encountered, as a rule, in structural determinations of more rigid biological macromolecules. The two most important ones are the following. It is not possible to use simple 'distance constraints' since the requirements of some of the interproton distances

are self-contradictory. Secondly, the evaluation of errors connected with experimental NOEs is necessary to have meaningful averages.

We have adopted the following procedure. First, the type of dependence of errors (σ_η) on measured NOEs from the actual values (η) of NOEs must be determined experimentally, although it is very difficult to evaluate the actual functional dependence, since there are, in principle, many sources of error. It is clear that the real situation has to be intermediate between two extreme cases: σ_η proportional to η (constant relative errors); and σ_η constant (i.e. constant absolute errors). Therefore, we have hypothesized a functional dependence of the type $\sigma_\eta = K\eta^n$. As a first rough approximation to σ_η values we simply used the differences between cross-peak volumes measured, for the same NOE, in opposite quadrants of the NOESY (taking actual values both from the two peptides of this work and from previous spectra of other linear peptides) and tabulated them vs. $\langle\eta\rangle$, the NOE averaged from the values in the two quadrants. The non-linear fit of equation $\sigma\eta = K\eta^n$ yields a value of n close to 0.4. In the case of coupling constants (Js) we have similarly hypothesized a functional dependence on the type $\sigma_J = KJ^n$, where n is chosen, arbitrarily, to have an intermediate situation ($n = 0.6$) between constant absolute errors ($n = 0$) and constant relative errors ($n = 1$). This is so because it is necessary to attribute larger errors to larger Js to comply with the nature of Karplus type equations that show the largest discrepancies among different versions for higher Js, even if this choice contradicts the experimental behaviour of nearly constant errors in the normally accessible experimental range (3–10 Hz).

The weights (w_k) to be used in the calculation of quadratic deviations are thus, as usual:

$$w_k = 1/(\sigma_\eta)k^2$$

Second, the penalty function Q, to be associated with any particular combination of conformers (with given weights) is composed of two terms. The first term, Q_1, is the sum of the squares of deviations between calculated and experimental NOEs, weighted by factors w_k, as defined above.

Q_1 was normalized dimensionally dividing by $\sum_i w_i\eta_i^2$. The following assumptions are necessary: averaged NOEs are simply the sum of individual contributions from each conformer, i.e. the exchange rate among conformers was assumed to be intermediate between very fast and very slow exchanges so that the relaxation is not directly influenced by exchange, yet single sharp lines can be observed; the correlation times are the same for all conformers.

A second term, Q_2, is the sum of the square of deviations between calculated and experimental coupling constants, again weighted by appropriate weight factors and normalized as above. J values corresponding to individual conformers were calculated by means of a Karplus type equation [31].

These two terms are then linearly combined in order to have a global penalty function:

$$Q = \beta Q_1 + Q_2$$

where β is a fixed constant so chosen as to make Q_1 and Q_2 contributions comparable.

The relative statistical weights attributed to NOEs and Js were chosen according to the following criteria. First of all we normalized them with respect to the amount of experimental data available, in such a way that the average value of a single NOE be comparable to that of a single J. Thus, one would have similar values of Q_1 and Q_2 only when the number of Js is comparable to that of NOEs. Secondly, we chose a value of β that takes into account the fact that NOEs carry more weight. In most actual calculations β typically had a value around 3.

Third, the choice of conformers and of the corresponding weight α_i that minimizes the global deviation function is determined by means of an algebraic method, introducing a Lagrange multiplier in such a way that the sum of weights of conformers is 1.

The extremum conditions are:

$$\delta(\ +\ \lambda\textstyle\sum_i \alpha_i)/\delta\alpha_j = 0$$

($j = 1 \ldots N$; N = number of conformers employed in the average.)

$$\textstyle\sum_i \alpha_i = 1$$

Once the partial derivatives have been solves, these conditions represent a system of $N+1$ algebraic equations in $N+1$ variables ($\alpha_1, \alpha_2, \ldots, \alpha_N, \lambda$). The system is solved by a direct elimination method. In general, the solution may not have physical meaning since one or more weights may occur as a negative number. Unless there is an unlikely linear dependence among the constraints, there is but one solution ($\alpha_1, \ldots, \alpha_N, \lambda$) for a given set of conformers, that is, only one extremum of Q consistent with the condition $\sum_i \alpha_i = 1$. Accordingly, a solution with

negative weights can only be interpreted as an indication that the particular combination of conformers that gives rise to this solution has no minimum within the domain ($0 < \alpha_i < 1$; $-\infty < \lambda < \infty$) and thus that one or more conformers must be rejected. Such a rejection is then performed in all possible ways, i.e. by grouping the N_{tot} initial conformers 1 by 1, 2 by 2, etc., up to an arbitrary composition comprising N_{max} conformers. The weights that minimize the deviation function are recalculated for each subset of conformers and the sets (and the corresponding weights) corresponding to the lowest minima of Q are stored.

In the actual case of this work, owing to the comparatively small number of conformers examined, we have chosen an $N_{max} = N_{tot}$, i.e. all possible combinations of the base conformers were examined.

The averaging procedure described in this section is reminiscent of other methods of so-called 'ensemble calculation' recently designed for the treatment of NMR data of flexible peptides [32–35]. All of them have merits but we judged them not perfectly suited to our problem. The method described by Nikiforovich *et al.* [32] examines the so-called feasible space in the search of mixtures that have violations smaller than a given threshold for all parameter; however, it uses no quality or matching function. The method of Blackledge *et al.* [33], which is similar to the previously published MEDUSA [34], is powerful but also very specific in the choice of the starting conformers and best suited to cases with a limited number of conformers. Besides, it does not use any statistical weighting of NOEs, a feature we consider necessary in the case of poor uniformity of the NOE data, i.e. of simultaneous fitting of long and short contacts. While our work was in progress a method similar to ours was presented [35]; nonetheless we preferred to employ our method since we had a home-made program for it and also because it is probably simpler to use.

RESULTS AND DISCUSSION

Table 2 summarizes the *in vitro* agonist activity (μ and δ) of the four DTI analogues synthesized for this study together with the previously reported binding properties [17]. It can be seen that the role of Phe^3 can be played by other apolar residues. Substitution of Phe with Ac_6c leads to a sharp decrease of the *in vitro* δ activity (the MVD IC_{50} is lowered by nearly three orders of magnitude) but, surprisingly, to a binding constant only one order of magnitude smaller than that of the parent natural peptide and to an even greater selectivity: the μ/δ ratio is 661 and 1667 for deltorphin I and [Ac_6c^3]DT I respectively. Substitution of Phe^3 with the smaller rings (Ac_5c and Ac_3c) yields analogues with decreasing yet sizeable binding constants and fairly high selectivity. Surprisingly, substitution with Aib^3, a residue whose side chains have minimal encumbrance, yields an analogue with nearly the same binding constant of the parent compound, reasonably high *in vitro* activity and a greatly increased selectivity (the μ/δ ratio jumps to 5600). In this instance, we cannot possibly imagine that the side chains of Aib can play the role of Phe^3 since the difference in steric encumbrance between the two side chains is enormous. Besides, the steric encumbrance of the Aib side chains is certainly smaller than any of the Ac_xc side chains (with the exception of Ac_3c) in the related deltorphin analogues. The apparent conclusion seems to be that in [Aib^3]DT I the message domain cannot by Tyr-D-Ala-Aib-, i.e. the analogue of the natural message domain (Tyr-D-Ala-Phe-), although a message domain constituted of a single residue (Tyr) seems rather odd. The only possible explanation of this outstanding result is that the backbone conformation of the message domain has a shape consistent with the encumbrance of Phe^3 and/or places hydrophobic groups in the same spatial position normally occupied by the aromatic ring of Phe, or by the second aromatic ring of naltrindoles. Such a possibility is obviously favoured by the extra rigidity afforded by the Aib residue [36] but calls for a detailed conformational analysis since it is not obvious, *a priori*, where the apolar side chains can be positioned.

In order to ascertain the conformational preferences of these deltorphin analogues, we undertook an NMR structural study of [Ac_6c^3]DT I and [Aib^3]DT I, the two extremes of Table 2 from the point of view of the encumbrance of the third residue side chain. The solution study was performed both in neat $DMSO_{d6}$ at room temperature and in a $DMSO_{6d}$/water cryomixture at low temperature. The NOESY spectra in $DMSO_{d6}$ are unusually rich of backbone and side-chain cross-peaks for linear peptides of this size but the combination of high-viscosity cryoprotective media and low temperature led to a further increase of diagnostically useful NOEs. As mentioned in the Introduction, the conformational freedom of linear peptide hormones can be limited not only by the insertion of appropriate, conformationally constrained, residues but also by using 'environmental constraints'. We have previously shown, for several

Table 2 Binding Affinities[a] and *in vitro* Potencies of Phe[3]-substituted Deltorphin I Analogues

Peptide	K_δ(nM)	K_μ(nM)	μ/δ[a]	MVD IC_{50}(nM)	GPI IC_{50}(nM)
DT I	0.31	205	661	0.46 ± 0.24	420 ± 95
[Ac_6c^3]DT I	3.3	5,500	1,667	540 ± 130	> 10 μM
[Ac_5c^3]DT I	10	6,800	680	1,640 ± 260	> 10 μM
[Ac_3c^3]DT I	17	10,200	600	458 ± 40	> 10 μM
[Aib^3]DT I	0.80	4,500	5,600	57 ± 5.5	> 10 μM

[a]Values taken from [16].

peptides, notably deltorphin I, that the use of biocompatible media with viscosities of the order of 7 to 10 *cp*, not only leads to a quantitative increase of all NOEs observed at lower viscosity, as expected by the theory of microviscosity, but also to the selective growth of (conformationally sensitive) effects involving backbone protons with respect to intraresidue ones [37,38]

Sequential assignment of all protons was straightforward, also because the sequences of the two peptides have only two residues of the same type (Val). It was achieved by means of the usual systematic application of DQF-COSY, TOCSY and NOESY experiments. Table 3 summarizes the chemical shifts of all protons together with $J_{NH\text{-}C\alpha H}$ coupling constants and the temperature coefficients of the amide protons.

The average values of chemical shifts and coupling constants point to a mixture of conformers, as expected for a linear peptide this size. On the other hand, temperature coefficients, in particular that of Val^5 NH and to a minor extent that of Asp^4 NH, indicate that at least the central part of the sequence is highly structured. It should be noted that the nearly zero temperature coefficient of Val^5 NH was observed both in neat DMSO and in the DMSO/water cryoprotective mixture over an unusually large temperature range (− 20 to + 40°C), a circumstance that rules out the possibility of an accidental zero, e.g. resulting from the compensation between opposing trends in the temperature dependence. This finding, in fact, represents a powerful indication that solvent inaccessibility of Val^5 NH is connected to a strong conformational preference. The contradictory behaviour shown by coupling constants and temperature coefficients is confirmed by the NOESY spectra of both peptides. Figure 1 shows a comparison of the low field portion of the NOESY spectra of [Aib^3]DT I and [Ac_6c^3]DT I in the DMSO/water cryomixture at 278 K. It can be seen that both spectra are unusually rich in intra- and interresidue effects, as would be the case for a peptide in a single, ordered conformation, but some of the NOEs, notably key NN and αN backbone effects, are mutually exclusive. In particular, the simultaneous observation of short d_{NN}, between the amide protons of Asp^4 and Val^5, and $d_{\alpha N}$, between the $C^\alpha H$ proton of Asp^4 and the amide proton of Val^5, can hardly be reconciled with a single stable structure.

Nonetheless we tested the hypothesis of a single ordered structure by means of MD calculations that included all backbone NOEs (both observed and not observed) as distance constraints. Starting structures were either completely random or based on sequences of regular β turns consistent with most of the observed backbone NOEs. The procedure used in these calculations, consisting essentially of restrained simulated annealing (SA) calculations, combined with local conformational searches (CS) and final restrained and unrestrained energy minimizations (EM), is illustrated in the Methods section.

The results of the MD calculations show that there are several different structures consistent with experimental NOEs. However, unconstrained minimization does not discriminate between them and, worst of all, reveals that a few of the constraints used in the MD procedure are not entirely conserved. Consistent use of all available NOE-derived constraints amounts, in our case, to the introduction of severe structural biases: the minimum obtained from the constrained minimization is characterized by internal strains (indicated by unusual values of internal coordinates) that are relieved in the unrestrained minimization at the expense of NOE violations. To illustrate the difficulties encountered in fitting experimental data with single structures, Table 4 shows a comparison between relevant NOE-derived distances and the corresponding calculated distances for a single conformer of [Aib^3]DT I derived from SA. The same table shows also the comparison

Table 3 Chemical Shifts, $J_{NH-C\alpha H}$ Coupling Constants and NH Temperature Coefficients ($\Delta\delta/\Delta T$) of [Aib^3]- and [Ac$_6$c^3]deltorphin I in DMSO/Water (80:20, v:v) Cryomixture at 278 K

Residue[a]	[Ac$_6$c^3]		[Aib^3]	
	δ(p.p.m.) (p.p.b. K^{-1})	J(Hz)	δ(p.p.m.) (p.p.b. K^{-1})	J(Hz)
Y^{1}α	3.87		3.86	
Y^1 β/β′	2.88		2.87	
Y^1 δ/δ′	6.99		6.99	
Y^1 ε/ε′	6.68		6.68	
a^2 NH	8.56 (− 7.0)	6.2	8.52 (− 7.0)	6.6
a^{2}α	4.24		4.20	
a^2 β	1.08		1.06	
Ac$_6$c^3 NH/Aib^3 NH	8.10 (− 7.0)		8.39 (− 7.0)	
Ac$_6$c^3 ring/Aib^3 β/β′	1.86, 1.6–1.3, 1.13		1.27	
D^{4}NH	7.80 (− 4.9)	7.6	7.86 (− 4.9)	7.9
D^{4}α	4.45		4.42	
D^{4}β	2.68		2.68	
D^{4}β′	2.52		2.52	
V^{5}NH	7.52 (− 0.5)	8.2	7.53 (− 0.5)	8.4
V^{5}α	3.94		3.93	
V^{5}β	1.93		1.94	
V^{5}γ	0.76		0.77	
V^{5}γ	0.74		0.75	
V^{6}NH	8.02(− 8.3)	7.1	8.01(− 8.3)	7.9
V^{6}α	3.94		3.93	
V^{6}β	1.92		1.94	
V^{6}γ	0.82		0.82	
V^{6}γ	0.80		0.81	
G^{7}NH	8.24 (8.2)	6.1, 5.6	8.23 (− 8.2)	6.0, 5.6
G^{7}α	3.66		3.66	
G^{7}α′	3.55		3.56	
NH1f	7.34		7.34	
NHhf	7.15		7.15	

[a]In this and following tables the one-letter code for amino acid residues was used. D-Ala is abbreviated as a.

of calculated ϕ angles and the corresponding ranges derived from experimental $J_{NH-C\alpha H}$ coupling constants.

In order to explain all observed NOEs we hypothesized that there are two or more conformational families in fast equilibrium. We refer to *families* of conformations rather than to single, rigid, conformers sine for a given low-energy backbone conformation it may be possible, in general, to find a number of related conformers of similar energy that differ by small deviations of the internal coordinates of either the backbone, the side chains or both.

As previously shown for deltorphin I itself [14], this is the natural solution to explain contradictory NOEs such as the quoted d_{NN} between the amide protons of Asp^4 and Val^5 and $d_{\alpha N}$ between the C$^\alpha$H proton of Asp^4 and the amide proton of Val^5, but, in our case, it is also necessary to explain at the same time the zero temperature coefficient of Val^5 NH since this proton is involved in both distances and, moreover, it can never be exposed to the solvent. In an aqueous medium temperature coefficients of exposed amide protons have usually very high values (*ca.* 10 p.p.b./K); even the presence of populations as small as 10% of extended conformers (with an exposed Val^5 NH) would lead to a coefficient larger than 1 p.p.b./K. These two experimental observations, in addition to the fairly large number of NOEs, make the description of the (presumably complex) conformational mixture an actual possibility.

Starting from these requirements, and taking into account the preferences induced by the presence of a D residue in second position, that is, the likelihood of 'primed' turns (characterized by torsion angles of opposite sign with respect to canonical turns) involving the first four residues, we generated several

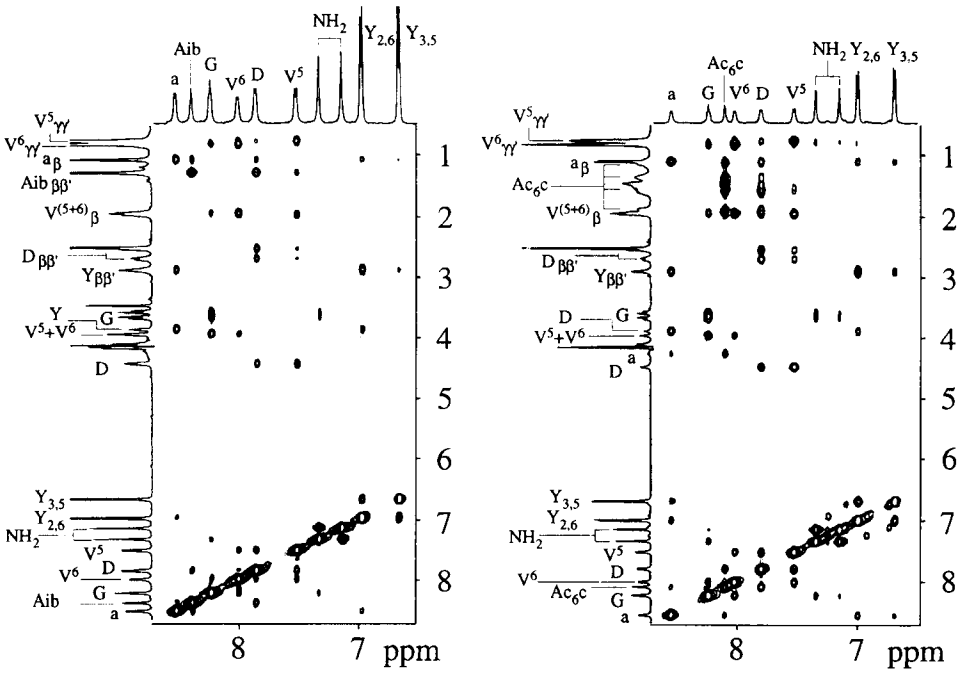

Figure 1 Comparison of the low field portion of the 500 MHz ^{1}H NOESY spectra of [Aib^3]DT I and [Ac$_6$c^3]DT I in the DMSO $_{d6}$/water (80:20; v:v) cryomixture at 278 K. Mixing time 100 ms.

families of low-energy conformers that, in turn, can be used as a basis set to find a suitable mixture consistent with all NMR constraints.

Owing to the complexity of the problem we factorized the search by first building families whose combination can satisfy only the requirements of the N-terminal sequence (i.e. the first four residues) and subsequently using this set for a full search on the four C-terminal residues (with an obvious overlap on the fourth residue). In the generation of the 'N-terminal' families the conformation of the C-terminal sequence was provisionally kept fixed at 3_{10} helix. We started from conformations that satisfy the short $d_{\alpha N}$ between the C$^\alpha$ H proton of Asp^4 and the amide proton of Val^5. These conformers were generated in two different ways: either by systematic combinations of backbone torsion angles consistent with canonical turns or by simulated annealings employing all NOE constraints but the one corresponding to the short d_{NN} between the amide protons of Asp^4 and Val^5.

Energy minimization led to two families with well-defined N terminal structures: one characterized by a I′ β-turn over the first four residues, followed by an inverse γ-turn centred on Asp^4 (dubbed I′/αN), and another characterized by a II′ β-turn over the first four residues, followed by an inverse γ-turn centred on Asp^4 (dubbed II′/αN). These conformers can be easily converted into two other families characterized by a short d_{NN} between the amide protons of Asp^4 and Val^5 by simply changing the ψ4 torsion angle from positive (*ca.* 65°) to negative values (*ca.* − 30°). Conversion of 'αN conformers' into the corresponding 'NN conformers' was achieved by constrained minimization in which the value of ψ4, after external incrementation, was kept fixed. After final unconstrained energy minimization two new stable conformers that satisfy the Asp^4/Val^5 d_{NN} distance were obtained. These conformers, dubbed I′/NN and II′/NN, are characterized by a fairly regular I′ turn (over the first four residues) followed by a type III turn (from Asp^4 to Gly^7) and by a type II′ turn linked to a type III turn (from Aib^3 to Val^6), respectively.

As already mentioned, these four conformers constituted the basis for a full conformational search on the C-terminal sequence and, finally, for the generation of unconstrained global conformations. Seventeen final conformers differing by less than 3 kcal/mol in internal energy were generated by these procedures. Some of them have N-terminal conformations still consistent with the starting conformers, whereas others diverged completely. The corresponding conformers of [Ac$_6$c^3]DT I, although generated independently, are very similar to those of [Aib^3]DT I since the space occupied by the methyl groups of Aib^3 is compatible, in all cases with

Table 4 Comparison[a] Between Relevant NOE-derived Distances and the Corresponding Calculated Distances for a Single Conformer of [Aib^3]DT I Derived from Sa

	Experimental	Restrained structure	Unrestrained structure
		r(Å)	
$Y^1C^{\alpha}H$–a^2NH	2.30	2.405	2.445
a^2NH–Aib^3NH	3.16	3.504	**4.621**
Aib^3NH–D^4NH	2.81	2.700	2.933
$Aib^3C^{\beta}H_3/C^{\beta'}H_{3\text{–}D^4NH}$	3.36	3.022	2.927
$Aib^3C^{\beta}H_3/C^{\beta'}H_3$–$V^5NH$	4.56	4.813	4.475
D^4NH–$C^{\alpha}H$	2.61	2.374	2.244
D^4NH–V^5NH	2.75	2.624	3.118
$D^4C^{\alpha}H$–V^5NH	2.55	2.648	**3.567**
V^5NH–V^6NH	2.97	2.941	3.630
V^6NH–$C^{\alpha H}$	3.05	2.872	2.920
		Dihedral (degrees)	
ϕ_2	[58 to 81 [139 to 162] [−113 to −76] [−64 to −27]	85.8	69.9
ϕ_4	[36 to 104] [−155 to −128] [−92 to −65]	58.6	81.6
ϕ_5	[39 to 101] [−152 to −123] [−97 to −68]	−133.5	−79.0
ϕ_6	[36 to 104] [−155 to −128] [−92 to −65]	**−102.9**	−73.8

[a] Severe violations are shown in bold face.

the greater encumbrance of Ac_6c^3. The conformation of the cyclohexyl moiety is a chair characterized, in all cases, by the axial position of the amide nitrogen; only in the case of conformer no. 14 the isomer with the nitrogen in the equatorial position is isoenergetic. Figure 2 shows a comparison of the relevant internal coordinates of the seventeen best conformers of [Aib^3]DT I; it is interesting to note that, in spite of large apparent differences in global shape (*vide infra*), the spread of backbone torsion angles is only significant at the two extremes of the sequence, whereas the values characterizing the central part of the sequence are rather uniform.

These conformers can be used for a global minimization with respect to all NMR parameters, i.e. observed NOEs and coupling constants, in order to find the best set(s) that can reproduce all experimental parameters. This approach is reminiscent of other methods of so-called 'ensemble calculation' to treat NMR data of flexible peptides that have been recently described but is based on an original computer program that has some advantages over published methods.

Before starting the minimization procedure, however, they were all tested for consistency with absent backbone NOEs (henceforth called 'antiNOEs', a kind of widely used 'negative' constraint) and solvent accessibility of Val^5 NH, a crucial feature of our NMR data. Most conformers of [Aib^3]DT I have a few 'violations' of absent NOEs but not severe enough that they can not be compensated for by proper mixing with other conformers. The only two conformers whose 'antiNOEs' cannot be averaged with others to make the average NOEs weak enough to be undetectable are nos 16 and 17: even populations as small as 10%, with the remaining 90% conformers characterized by very long distances (> 5 Å) would lead to average distances of the order of 3.2 Å. Accordingly, conformers nos 16 and 17 were henceforth discarded. The data for [Ac_6c^3]DT I are almost

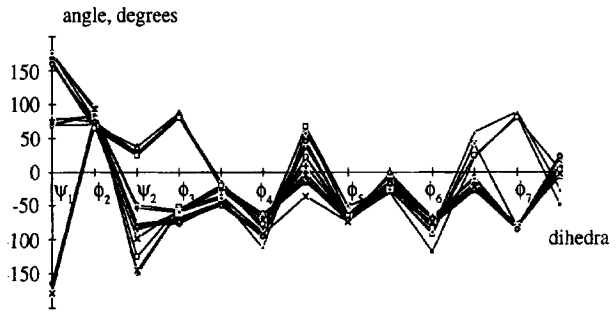

Figure 2 Variation of the relevant internal coordinates of the seventeen best conformers of [Aib^3]DT I as a function of position along the sequence.

identical. All final fifteen conformers are characterized by compact structures and it is reassuring to find that Val^5 NH appears, on visual inspection of space-filling models, inside a central cavity. This observation was substantiated by a more objective criterion, i.e. the calculation of exposed area by program GEPOL [39].

The fifteen families of [Aib^3]DT I and the corresponding ones of [Ac$_6$c^3]DT I were used as a database to generate a set of NOEs averaged over a small number of conformers in equilibrium by means of a computer program based on Lagrange multipliers that optimizes the fit with experimental NOEs and $J_{NH-C\alpha H}$ coupling constants as a function of relative populations. The best agreement with experimental data was obtained for the set of conformers **1**, **2**, **5**, **8** and **9** (henceforth called ensemble no. I) with populations of 0.18, 0.12, 0.14, 0.31 and 0.25 respectively. Similar agreements however, were also obtained for sets **1**, **5**, **8**, **9** (ensemble no. II, with relative populations 0.20, 0.21, 0.33, 0.26); **2**, **3**, **5**, **8**, **9**, **15** (ensemble no. III, with relative populations 0.14, 0.09, 0.14, 0.32, 0.20, 0.10); **1**, **2**, **8**, **9**, **15** (ensemble no. IV, with relative populations 0.19, 0.18, 0.32, 0.28, 0.02) and **1**, **2**, **8**, **9** (ensemble no. V, with relative populations 0.20, 0.18, 0.32, 0.29).

Comparisons of relevant experimental backbone distances with the corresponding distances of ensembles nos I and III, along with allowed ϕ ranges derived from $J_{NH-C\alpha H}$ coupling constants, are reported in Table 5. The comparison between backbone distances, derived from experimental NOEs, and the corresponding distances averaged over different structures of the five best ensembles (nos I, II, III, IV, V) is also shown schematically in Figure 3. The overall agreement is satisfactory but does not allow a clear distinction among the five solutions. This finding confirms that there is not a prevailing single conformer in solution or even a prevailing ensemble; thus, all the structures represented in the five ensembles (**1**, **2**, **3**, **5**, **8**, **9**, **15**) can be considered as representative of the global conformational mixture in solution. A corollary is that all of them can also be regarded as putative bioactive conformations and were consequently used for comparison with conformationally rigid δ selective opiates to find their consistency with the hypothetical bioactive conformation.

There are several naltrindole derivatives prepared by Portoghese and coworkers [1–5, 40] with a fairly good δ selectivity, both agonists and antagonists. The best candidate in our case is probably 7-spiroindanyloxymorphone (SIOM), the first selective non-peptide δ_1 opioid agonist [40]. The same authors have also shown that the δ selectivity of several related naltrexone-derived antagonists is critically dependent on the precise relative orientation of the two aromatic rings [5]. The molecular model, built on the basis of the crystal structure of a related compound [41], shows that SIOM is not totally rigid since the spiro moiety can be arranged in slightly different conformations. An exhaustive search based on internal energy yielded, an absolute minimum, the structure shown in Figure 4, i.e. the same conformer proposed by Portoghese *et al.* [40]. In order to test the general applicability of non-peptidic scaffolds as benchmarks for the bioactive conformations of peptide opioids we searched for other non-peptidic opiates with good δ selectivity and affinity. A good candidate is BW373U86, a novel δ opioid agonist [42] with a piperazinyl diphenylmethane skeleton characterized by a μ/δ selectivity comparable to that of DPDPE [43]. Also in this case, an exhaustive search based on internal energy yielded, as an absolute minimum, the structure shown in Figure 4 along with its overlay with SIOM. It can be appreciated that, although the constitution of the two compounds is widely different, superposition of the phenolic rings leads to a very good superposition of the other two aromatic rings. In addition, other hydrophobic groups, i.e. the two ethyl groups of BW373U86, occupy the region of space of the second aromatic ring of SIOM.

Superpositions of either (non-peptidic) compound with the models of our peptides were generated by overlaying only a portion of the tyramine moiety of the peptides, N, C$^\alpha$, C$^\beta$ and the C-1 and C-4 carbons of the aromatic ring, with the corresponding atoms of SIOM (or with the equivalent atoms of BW373U86 as observed in the overlay of the two). In fact, it must be noted that the *ortho* and *meta* carbon atoms of the aromatic ring of Tyr (of any opioid peptide) cannot

Table 5 Comparison Between Relevant NOE-derived Distances and the Corresponding Calculated Distances for Two of the Ensembles of Conformers **1,2,5,8,9** of [Aib^3]DT I (Ensembles nos I and III). The Comparison Between Allowed Dihedral Ranges and Calculated Ranges are also Reported

	Experimental	Ensemble no. I	Ensemble no. III
	r/Å		
$Y^1C^{\alpha}H$–a^2NH	2.30	2.4265	2.4398
a^2NH–Aib^3NH	3.16	2.9611	3.0240
Aib^3NH–D^4NH	2.81	2.6851	2.6946
$Aib^3C^{\beta}H_3/C^{\beta'}H_3$–$D^4NH$	3.36	3.4097	3.4226
$Aib^3C^{\beta}H_3/C^{\beta'}H_3$–$V^5NH$	4.56	4.4956	4.1937
D^4NH–$C^{\alpha H}$	2.61	2.9481	2.9452
D^4NH–V^5NH	2.75	2.7071	2.7282
$D^4C^{\alpha}H$–V^5NH	2.55	2.8348	2.9082
V^5NH–V^6NH	2.97	2.6213	2.6093
V^6NH–$C^{\alpha H}$	3.05	2.9558	2.9625
	Dihedral (degrees)		
$\phi_{;2}$	[58 to 81] [139 to 162] [−113 to −76] [−64 to 27]	[66 to 94] [126 to 154] [−103 to −37]	[66 to 92] [128 to 154] [−104 to −36]
ϕ_4	[36 to 104] [−155 to 128] [−92 to −65]	[32 to 108] [−158 to −133] [−87 to −62]	[32 to 108] [−158 to −134] [−86 to −62]
ϕ_5	[39 to 101] [−152 to −123] [−97 to −68]	[20 to 49] [91 to 120] [−168 to −145] [−75 to −52]	[20 to 49] [91 to 120] [−168 to −145] [−75 to −52]
ϕ_6	[36 to 104] [−155 to −128] [−92 to −65]	[31 to 109] [−159 to −134] [−86 to −61]	[33 to 107] [−157 to −132] [−88 to −63]

possibly overlap with the corresponding atoms of any alkaloid related to morphine since the orientation of the ring depending from the χ_2 torsion angle is forced by cyclization (in all alkaloids) to values close to 0°, which are energetically inaccessible in linear molecules. We found that only conformers **2**, **5** and **9** among those characterizing the equilibrium mixture are consistent with the shape of this rigid non-peptidic agonist. Figure 5 shows the overlays of these conformers with the model of SIOM. The model of conformer **9** is overlaid also with the model of BW373U86. It is interesting to note that the backbone conformation of the N-terminal part of **2** and **9** is consistent with the II′ β-turn previously proposed for DT I itself [14]. The structures of the corresponding conformers of [Ac_6c^3]DT I are very similar; their overlays with the model of SIOM are shown in Figure 6. The overlay of conformer **1** of [Ac_6c^3]DT I, i.e. one of the final conformers that do not fit the shape of SIOM, is also shown for comparison. It is interesting to note that in all corresponding structures, the side chain of Ac_6c has no close contacts with other residues different from those already present in the corresponding conformers of [Aib^3]DT I.

It may also be interesting to compare the final structures of [Aib^3]DT I with the shapes of other δ selective agonists, notably those of DPDPE and JOM-13, two cyclic peptides that have attracted considerable interest during the last few years. The models of DPDPE and JOM-13 were derived from their X-ray structures [44,45]. Figure 7 shows the overlays of conformer **9**, the one that showed the best fit with SIOM, with the models of DPDPE and JOM-13; the same figure also shows the overlays with SIOM. It is

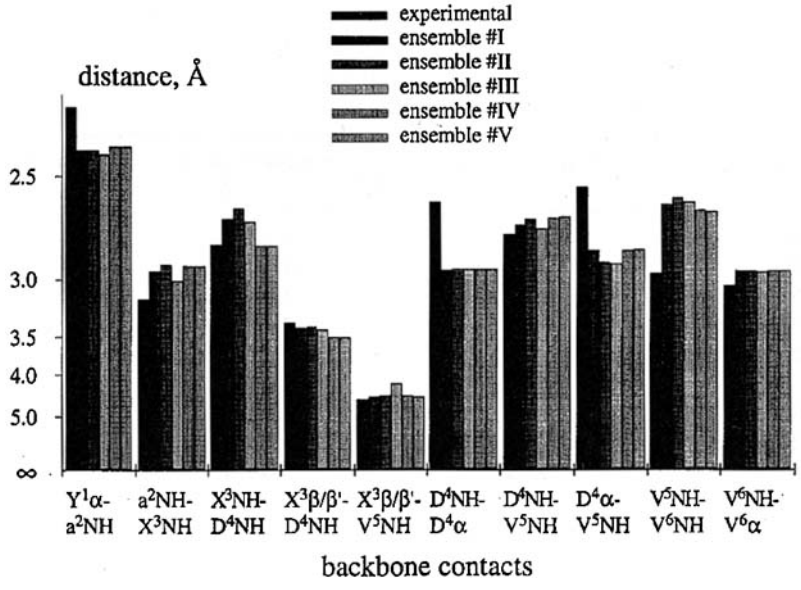

Figure 3 Histograms illustrating the comparison between backbone distances, derived from experimental NOEs, and the corresponding distances averaged over different structures of the five best ensembles (nos I, II, III, IV, V) calculated for [Àib^3]DT I in solution.

clear that a good overlay with the structures derived from the solid-state work is difficult, mainly because the orientation of the two aromatic rings reflects the forces of crystal packing rather than intrinsic conformational tendencies of the two molecules. It can be noted, however, that the separation of the two rings is consistent with that observed both in SIOM and in our peptides so that a modest conformational rearrangement can lead to a satisfactory overlap with the corresponding rings of SIOM. Recently, Mosberg *et al.* [46,47] were able to restrict the number of accessible side-chain conformations for the two aromatic rings of JOM-13. The resulting proposed bioactive conformation is closer to the shape of rigid alkaloids than the quoted X-ray structure.

CONCLUSION

The conformational analysis of [Aib^3]DT I and [Ac$_6$c^3]DT I shows that when the NMR data are inconsistent with the presence of a single minimum energy conformer in solution, it may still be possible to interpret the spectroscopic data of a flexible linear peptide with good accuracy in terms of conformational mixtures. However, in spite of the excellent agreement obtained between experimental and calculated NMR parameters, we cannot possibly describe the conformational state of these two peptides as a unique mixture of conformers, e.g. those of ensemble no. I, since the slightly different mixtures of the other ensembles explain the experimental data in a satisfactory way. Besides, owing to the dimensions of conformational space, it is difficult to say whether we have employed, in our averaging procedure, all possible conformers. In other words, it is difficult to describe the complete composition of the conformational mixture of these linear peptides in solution. This statement should not be taken as a negative conclusion since it must be remembered that the main goal of these studies remains the identification of likely bioactive conformations. This task is so difficult that has been called an elusive goal [48], mainly owing to the difficulty of exploring the multidimensional conformational space with a fine enough mesh in a reasonable time. In our case, we are confident that most, if not all, likely candidates (for the bioactive conformation) present in solution have been identified. The combination of (i) cryoprotective solutions at low temperatures, (ii) MM and MD calculations, (iii) advanced methods for averaging NMR parameters and (iv) appropriate comparisons with rigid non-peptidic compounds represents a method of sampling conformational space, in the search of bioactive conformations, more powerful than those based on computations alone. It amounts

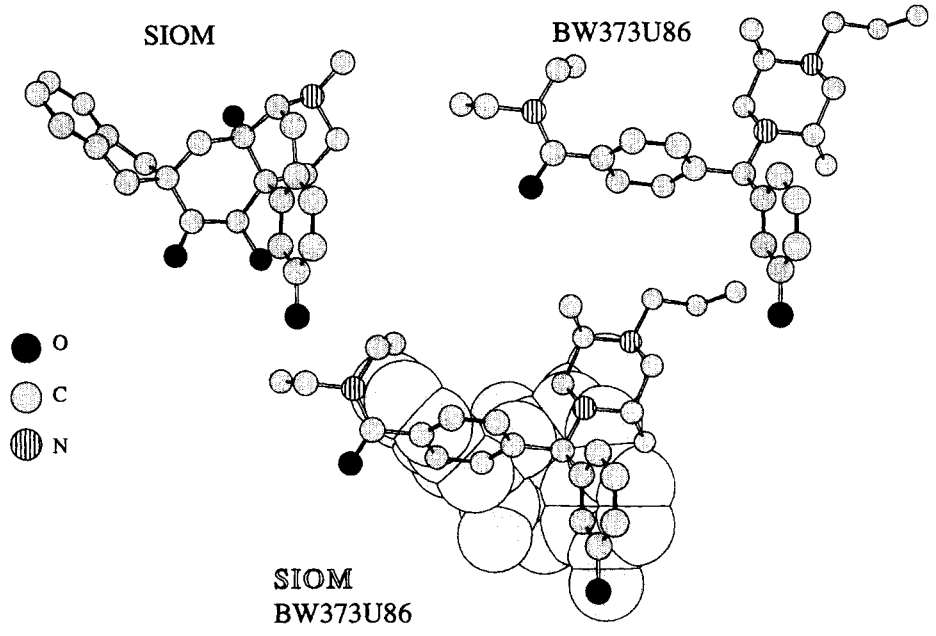

Figure 4 Molecular models of SIOM and BW373U86 and overlay of the two models (SIOM is represented as a space-filling model in the overlay).

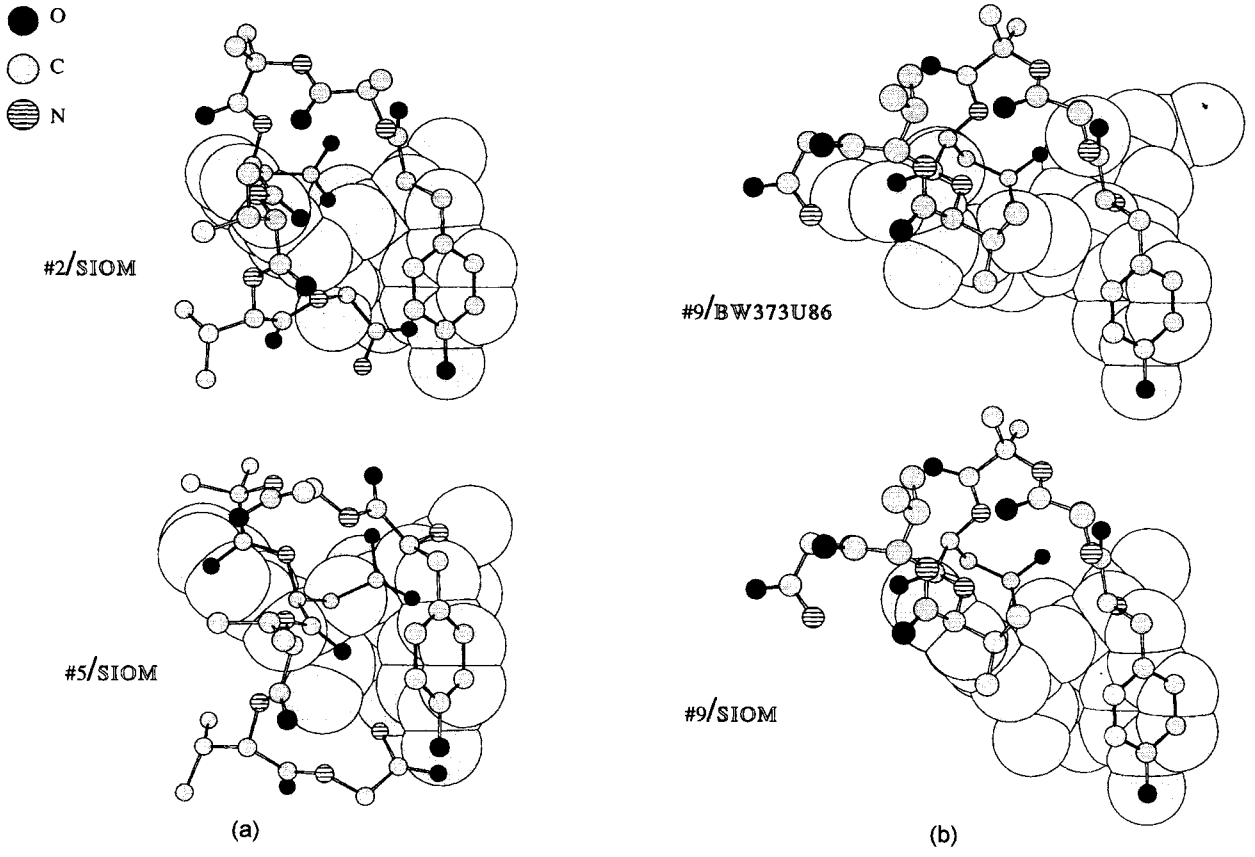

Figure 5 Overlays of the models of conformers **2**, **5** and **9** of [Aib^3]DT I with the model of SIOM. Conformer **9** is also compared with the model of BW373U86. (SIOM and BW373U86 are represented as space-filling models in the overlays.)

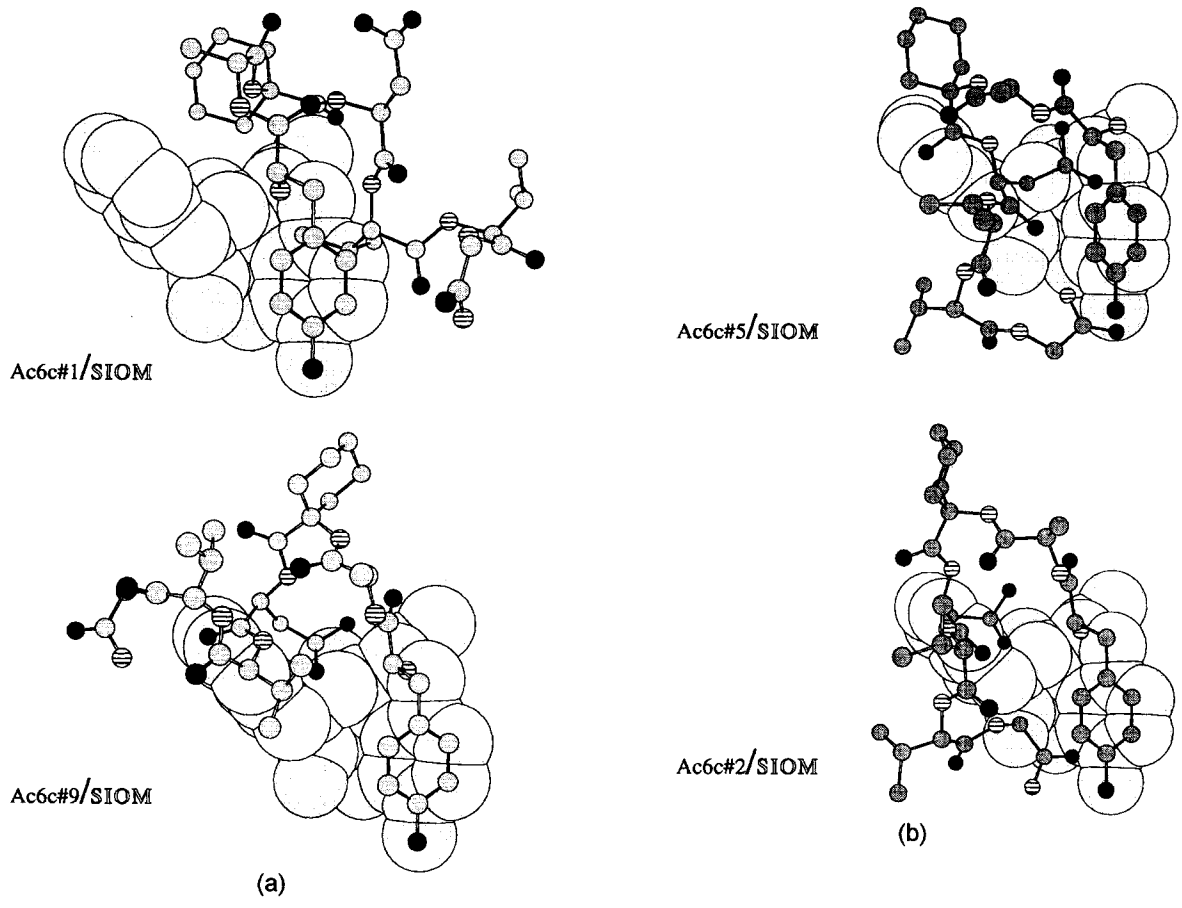

Figure 6 Overlays of the models of conformers **1**, **2**, **5**, and **9** of [Ac_6c^3]DT I with the model of SIOM (space-filling model).

to sampling conformational space by a very fine mesh since all energetically accessible conformations are effectively present in solution and, consequently, are sampled, with the only limitations that the energy scale is influenced by the particular environment used in the solution study. In most cases, however, this environment can be made closer to the biological environment than those reproduced in purely theoretical calculations.

The most important result of the present work, however, is the novel insight in the shape of δ-selective agonists and its relationship with the so-called message/address concept.

When the concepts of message domain and address domain were introduced [6] they were complementary to the specificity of the constitution of the peptide with respect to receptor subtypes. In other words, it is perfectly possible that each receptor (μ, δ and κ) has different requirements for the recognition site of the peptide (message domain), although selectivity may be greatly enhanced (or changed) by the address domain. The very concept of address was mainly linked to the so-called membrane-catalysed receptor selection. The distinguishing feature of the address, according to Schwyzer [6], is the presence of charges rather than particular conformational preferences: thus the μ address ought to be positively charged, the δ address ought to be negatively charged, but the κ address ought to be positively charged, as the μ address, but much longer and able to assume a helical conformation that can favour insertion into the membrane at a very specific angle.

In the case of flexible peptides it is obvious not only that the message can be different in constitution for different selective agonists (see above) but also that the address can influence the message conformation. However, in all subsequent utilizations (by researchers other than Schwyzer) of the message/address concept the two domains have been simply identified with given partial sequences; Tyr-Gly-Gly-Phe-, Tyr-D-Ala-Phe, Tyr-Pro-Phe, etc. This view was

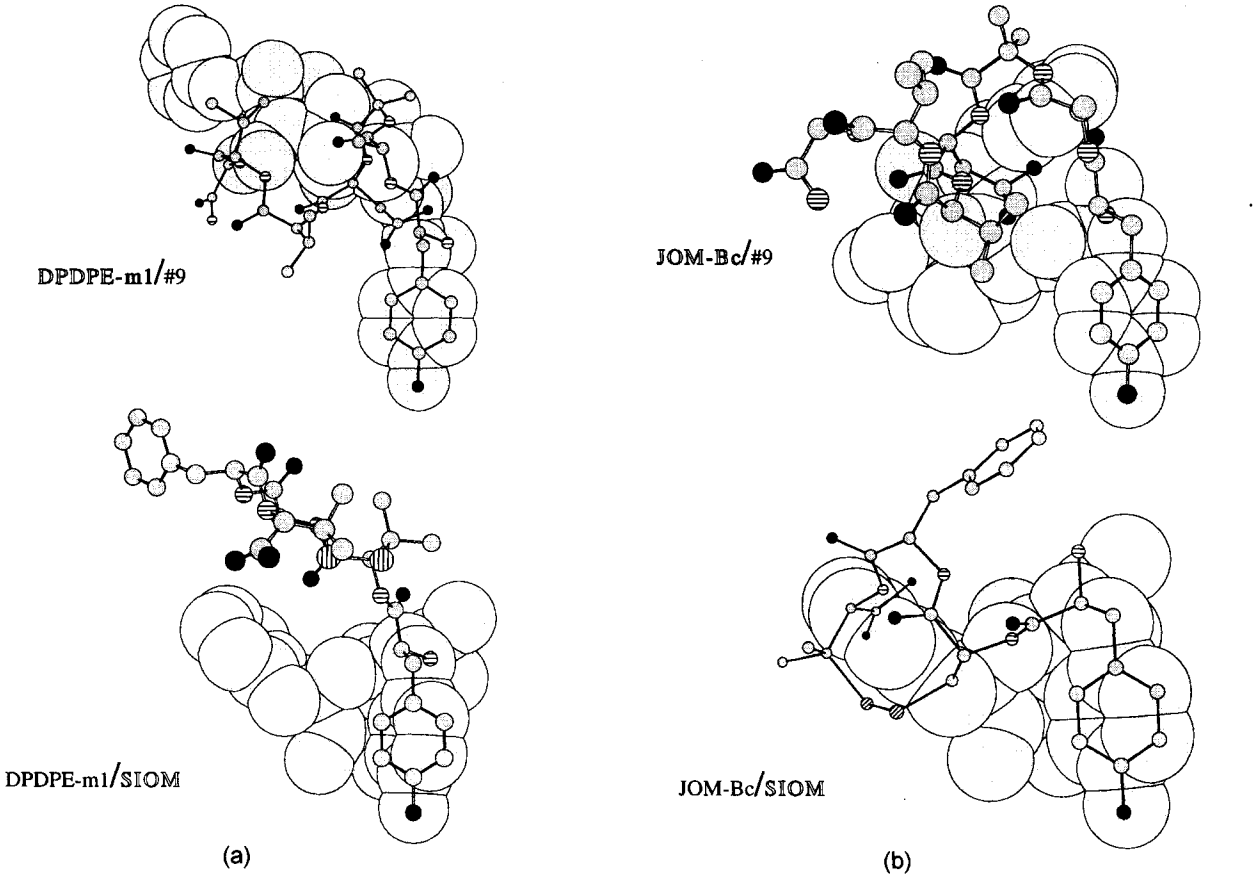

Figure 7 Overlays of conformer **9** of [Aib^3]DT I with the models of DPDPE and JOM-13. The same figure shows also the overlays of DPDPE and JOM-13 with SIOM.

apparently substantiated by the design of chimeric δ-selective dermorphin and μ-selective deltorphin obtained by simply swapping the C-terminal moieties of these peptides and keeping the common Tyr-D-Ala-Phe- message [49].

A rigid identification of a 'domain' with a moiety of fixed constitution is obviously possible in the case of alkaloids, a field where the message/address concept has been recently transferred. Also in this case however, it is not obvious whether one can consider the tyramine moiety as 'the message' since there is no instance of potent, non-selective opioid containing only this moiety (and lacking the second aromatic ring); morphine, the prototype alkaloid opioid, in fact does not contain the second aromatic ring but is clearly a μ-selective agonist.

It is clear that a rigid division of the sequences of peptides in message and address or even worse of the (compact) structure of alkaloids in two moieties corresponding to message and address is unlikely. It seems more realistic to think of message and address domains in conformational terms, i.e. they represent different portions of the whole molecule in a given conformation and may be composed of different parts of the molecule, a behaviour normally found in longer peptide hormones, e.g. insulin [50]. This view is strongly supported by the recent finding [51] that the bioactive 'shape' of a μ agonist can be simulated by the side chains of a peptide composed solely of D residues and lacking Tyr.

The results of the conformational analysis on our peptides demonstrate that the message domain must be identified with a 3D shape and not with a given constitution. It is clear that Phe, in either third or fourth position, is not a necessary part of either the message or the address domain of opioid peptides. Substitution, in the sequence of deltorphin I, of the natural third residue (Phe^3) with Ac$_x$c residues and *a fortiori* with Aib, whose side chains have a much smaller steric encumbrance than that of Phe^3, hints

that these side chains do not substitute the aromatic ring of Phe^3 in a similar backbone conformation. This view is supported by the finding that, in the putative bioactive conformers (**2**, **5**, **9**), the side chains of both Aib and Ac_6c occupy a region of space quite distant from that occupied by the second aromatic ring of naltrindoles.

We can conclude that the message domain of our peptides cannot be inferred in an obvious way from the sequence: it is defined by the backbone conformation and contains the side chain of Tyr, as expected, but also those of Val^5 and Val^6, i.e. two residues normally attributed to the address domain.

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