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TOPOCHEMICAL DESIGN OF BIOACTIVE PEPTIDES AND PEPTIDOMIMETICS

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Dedicated to the memory of Yuri Ovchinnikov who did so much for bioorganic chemistry and international scientific understanding

For the studies of bioactive peptides, our laboratories have been employed an integrated approach including synthesis, bioassays, and conformational analysis. To obtain highly potent, selective and metabolically stable analogs, peptidomimetics such as peptide backbone modifications (retro-inverso structures), constrained amino acids, and cyclic structures have been incorporated into many bioactive peptide sequences. The conformational studies of the resulting analogs have led to topochemical models for the bioactivities of those peptides. This lecture will be focused on the results of such studies on opioids and somatostatin.

We have synthesized numerous opioid analogs with various peptidomimetics based on three classes: enkephalins, dermorphin-deltorphins, and morphiceptins. Many of these analogs exhibit high potency, selectivity, and metabolic stability. Conformational studies of these analogs have enabled us to define the structural characteristics necessary for bioactivities of morphiceptins, dermorphins, enkephalins, and deltorphins. From these results, we can propose conformational models responsible for bioactivities at the μ- and δ-receptors.

Abbreviations according to IUPAC-IUB Commission (Eur. J. Biochem. 1984. V. 138. P. 9—37) are used throughout. Additional abbreviations: A_2bu — α,γ-diaminobutiric acid, βAc^5c — 2-aminocyclopentane carboxylic acid, (βMe)Phe — β-methylphenylalanine, βNal — β-1-naphthylalanine, (βMe)Trp — β-methyltryptophan, (NMe)Ala — N^{α}-methylalanine, (NMe)Phe — N^{α}-methylphenylalanine, CNS — central nervous system, Pen — β,β-dimethylcysteine, penicillamine, Xaa, Yaa — unspecified amino acid. We are using the following special abbreviations for the partially modified retro-inverso peptides. The standard three letters notation for amino acid residues preceded by letter *g* denotes the *gem*-diaminoalkyl residue prepared from the specified amino acid. Similarly, the prefix m denotes the malonic acid residues which have the same side chain as the amino acid specified by three letter notation.

Our studies of cyclic somatostatin analogs are based on the highly active Merck analog c(-Pro^6-Phe^7-*D*-Trp^8-Lys^9-Thr^{10}-Phe^{11}-) (where the superscripts denote position in native somatostatin). To investigate the topochemical preference of backbone and side chains, unusual amino acids, including β-methylphenylalanine^7 or ^{11}, β-methyltryptophan^8, as well as backbone modifications such as retro-inverso structures have been incorporated. The bioactivity profiles of these peptidomimetic molecules provide much information on the effects of backbone and side chain constraints on bioactivity.

The physiological effects of brain peptides are revealed by binding to their receptors. Most of receptors have their subtypes and each of them has unique biological roles. Naturally occurring peptides usually bind to more than one receptor subtypes. Such poor selectivities create difficulties in physiological studies and clinical applications of these peptides. Thus, ligands which bind to only one receptor subtype have long been desired in all peptide hormone area. In addition, these ligands should be potent to show their effects clearly and stable from enzymatic attacks. Since each receptor requires an unique conformation when peptides bind to them, the conformational features of each peptide have been recognized as one of the most important things to obtain such ideal ligands.

Our laboratories have employed an integrated approach including design, synthesis, bioassays and conformational analysis (Fig. 1). Many novel peptides incorporating peptidomimetics such as backbone modifications (retro-inverso modifications), novel amino acids, cyclic structures, and various groups to interact with receptor environments have been synthesized. Many of these have exhibited high potency, selectivity, and metabolic stability. Our conformational research of these molecules is based on experimental studies using NMR spectroscopy to assess mainchain and side chain conformations. Molecular modeling with and without NMR constraints provides us with key information on the concerted motions and topochemistries necessary for bioactivities of these peptides. From these studies, we have proposed topochemical models required for the bioactivities of several peptides. In this presentation, we will concentrate on aspects of our research dealing with opioids and somatostatins.

OPIOIDS

Naturally occurring peptide opioids are classified into three categories by their origins: enkephalins, dermorphin-deltorphins and morphiceptins. The enkephalin group is isolated from central nervous system (CNS) [1, 2] while the dermorphins and deltorphins are from frog skin [3–5]. The morphiceptin family is obtained from the enzymatic digestions of various proteins [6]. All natural peptide opioids contain an amino terminal tyrosine, a spacer residue(s), and a phenylalanine. Systematic modifications of these residues have shown that the free amine and phenolic groups of tyrosine at the first position and the aryl ring of the third or fourth residue represent the pharmacophoric array [7, 8]. It has been reported that naturally occurring peptide opioids bind to at least three different residues (μ, δ, κ).

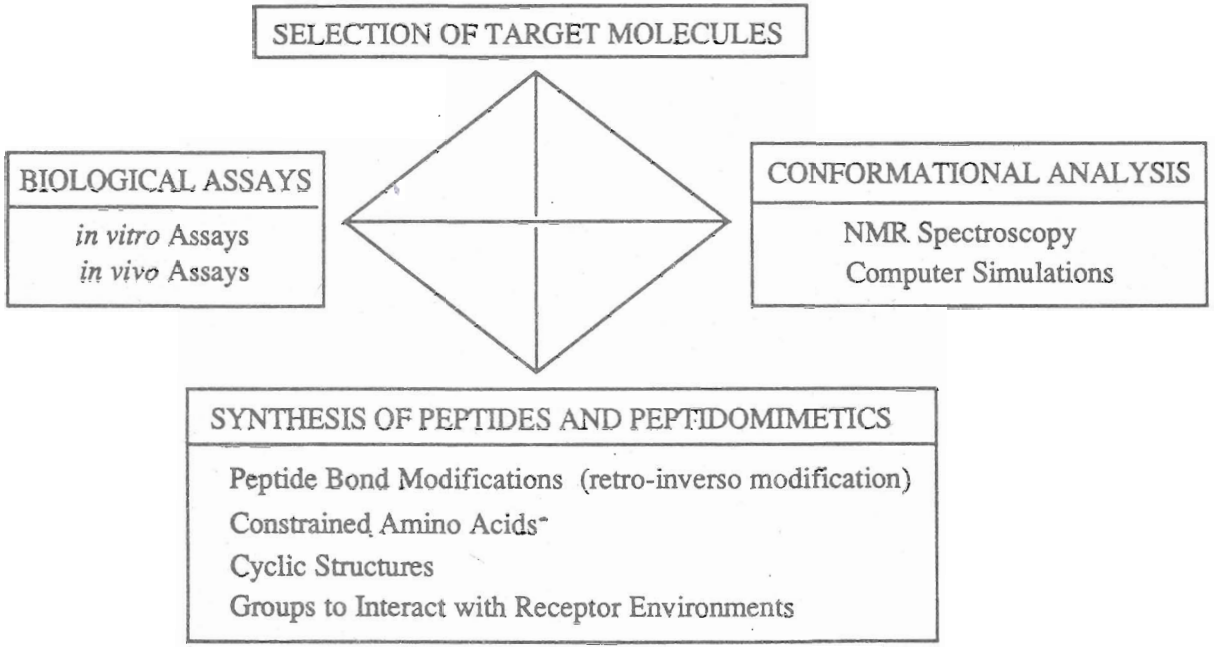

Fig. 1. An integrated approach for bioactive peptides

Since enkephalins (Tyr-Gly-Gly-Phe-Leu/Met-OH) are the first opioid peptides isolated and other CNS opioids contain this sequence at their amino termini, most of early opioid studies involved this family [1, 2]. Enkephalins themselves are highly flexible and can adopt many different conformations which may account for their abilities to bind to the μ- and δ-receptors. Thus, constrained amino acids and/or cyclic structures were employed to reduce their flexibility. Such constraints are also useful for conformational analysis to decrease the number of minimum energy structures. One of the earlest efforts in this area was carried out in our laboratories with the series of 14-membered cyclic enkephalin analogs based on the structure Tyr-c(*D*-A_2bu-Gly-Phe-Leu-) [9–13]. In this series, the retro-inverso modifications [9–11] or unusual amino acids *D*- and *L*-β-naphthylalanine (1) [βNal(1)] [13] were incorporated. The Leu^5 was also replaced with its *D*-chiral counterpart as a part of selected modifications [12]. From this study, we found that the relative orientations of aromatic side chain in Tyr^1 and Phe^4 can determine bioactivity profiles. In Fig. 2, the preferred conformations of these 14-membered cyclic enkephalins at the μ- (Fig. 2A) and δ-receptors (Fig. 2B) are depicted [13]. Similar folded structures are also observed among the preferred conformations for the highly δ-receptor selective Tyr-c(*D*-Pen-Gly-Phe-Pen)-OH [14, 15] and Tyr-*D*-Ser(*t*Bu)-Gly-Phe-Leu-Thr(*t*Bu)-OH [16]. Recently, we also proposed topochemical models to explain the bioactivities of dermorphins, deltorphins and morphiceptins [17].

1. Dermorphins and Deltorphins

Dermorphin (Tyr-*D*-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) [3] and deltorphins (deltorphin, Tyr-*D*-Met-Phe-His-Leu-Met-Asp-NH_2; deltorphin I, Tyr-*D*-Ala-Phe-Asp-Val-Val-Gly-NH_2; and deltorphin II, Tyr-*D*-Ala-Phe-Glu-Val-Val-Gly-NH_2) [4, 5] share the same origin and possess common generalized N-terminal tripeptide sequences Tyr^1-*D*-Xaa-Phe^3 (where *D*-Xaa is either *D*-Ala or *D*-Met). While dermorphin is μ-receptor selective, deltorphins bind only to the δ-receptor [4, 5]. Most of N-terminal tetrapeptides of dermorphin and deltorphins exhibit μ-receptor selective activity. However, when these compounds are cyclized, their bioactivity profiles can be changed.

Most of the reported cyclic dermorphin-deltorphin analogs have been formed by side chain to side chain coupling with two different types of cyclization methods: amide bond formation and disulfide bridge formation [18]. A representative analog of the former type is Tyr-c(*D*-Orn-Phe-Asp)-NH_2. The bioactivity of this compound is similar to those of corresponding linear analogs but its selectivity for the μ-receptor is one of the highest among the cyclic and linear dermorphin analogs. Among a series of disulfide bridged analogs, the compound Tyr-c(*D*-Cys-Phe-Cys)-NH_2 shows μ-selectivity with high activity at the μ-receptor and low activity at the δ-receptor. When we incorporated a *D*-chirality at position 4 and a free C-terminal carboxylic group into this analog, the resulting Tyr-c(*D*-Cys-Phe-*D*-Cys)-OH was highly active at both μ- and δ-receptors and thus was non-selective [19]. Incorporation of penicillamine at the fourth position of this non-selective analog drastically decreases μ-receptor activity without significantly affecting δ-receptor activi-

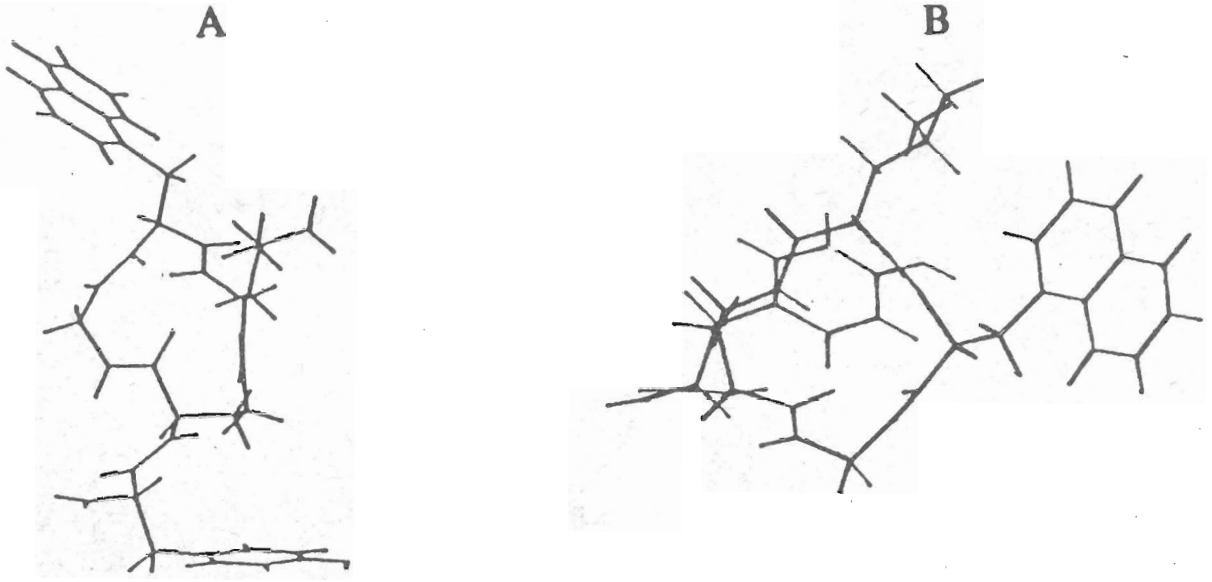

Fig. 2. Bioactive conformations estimated for 14-membered cyclic enkephalin analogs containing β-naphthylalanine (1) as the fourth residue (Tyr-c(D-A^{l}_{2}bu-Gly-βNal-Leu-)) A: at the μ-receptor and B: at the δ-receptor

ty. Thus, the analog Tyr-c(*D*-Cys-Phe-*D*-Pen)-OH shows higher selectivity for the δ- over the μ-receptor [20]. This result strongly suggests that the spatial arrays of three pharmacophoric groups within the N-terminal tripeptide sequence define the bioactivity and selectivity of dermorphin and deltorphin.

Calculations of a series of molecules based on the structure Tyr-c(*D*-Orn-Phe-Asp)-NH_2 proposed that a tilted stacking orientation of the two aromatic side chains at residues 1 and 3 is a structural requirement for high μ-receptor affinity [21]. However, another theoretical study employed various cyclic dermorphin-deltorphin analogs suggested different topologies for μ-receptor recognition, even for the same molecules [22]. Although Tyr-*D*-Ala-Phe-Gly-NH_2 proved too flexible to perform conformational analysis, its proposed bioactive conformation is not in agreement with any of the above topologies [23].

To avoid such ambiguity and to establish a conformational model for dermorphin bioactivity, we have studied 12- and 13-membered cyclic analogs, Tyr-c(*D*-Orn-Phe-Gly-) and Tyr-c(*D*-Orn-Phe-Asp)-NH_2. The *in vitro* bioactivities of these peptides determined from the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays are summarized in Table 1. The 12-membered cyclic analogs has a new cyclic structure formed by the cyclization between a side chain and backbone unlike previous analogs. Its bioactivity profile is extremely similar to dermorphin (Table 1). Although highly μ-receptor selective, it shows bioactivity at the δ-receptor unlike the Tyr-c(*D*-Orn-Phe-Asp)-NH_2 which is active only at the μ-receptor. The analog Tyr-c(*D*-Orn-Phe-Gly-) is more constrained than Tyr-c(*D*-Orn-Phe-Asp)-NH_2 because the cyclization achieved by a side chain-backbone coupling produces a smaller ring.

Conformational analyses were carried out employing NMR spectroscopy and computer simulations [25]. Among the resulting preferred conformations of both analogs, the conformations (Fig. 3A, 3B, 3C) are topologically similar to each other. These topologies contain a common structure in which the aromatic side chain of the Tyr^1 and Phe^3 project away from each other. We therefore propose that dermorphin requires a topochemical structure with this array of aryl groups for μ-receptor recognition. It is worth while mentioning that μ-receptor active conformations of 14-membered cyclic enkephalin analogs are also defined by the same topology [13].

Among the preferred conformations of the Tyr-c(*D*-Orn-Phe-Gly-) analog, a structure with a close proximity of the two aromatic side chains was also observed (Fig. 3D). This structure is topochemically similar to the conformations responsible for δ-receptor recognition of enkephalin analogs (Fig. 2B and [14–16]). This type of molecular array was not observed among the preferred conformations of δ-receptor inactive Tyr-c(*D*-Orn-Phe-Asp)-NH_2. Therefore, the bioactivity of the 12-membered cyclic analog at the μ- and δ-receptor can be explained by the presence of extended and folded forms among the preferred structures.

2. Morphiceptins

The biologically important Tyr^1 and Phe^3 are joined by a constrained amino acid proline in the highly μ-receptor selective morphiceptin (Tyr^1-Pro^2-Phe^3-Pro^4-NH_2) [6]. As a result, the confor-

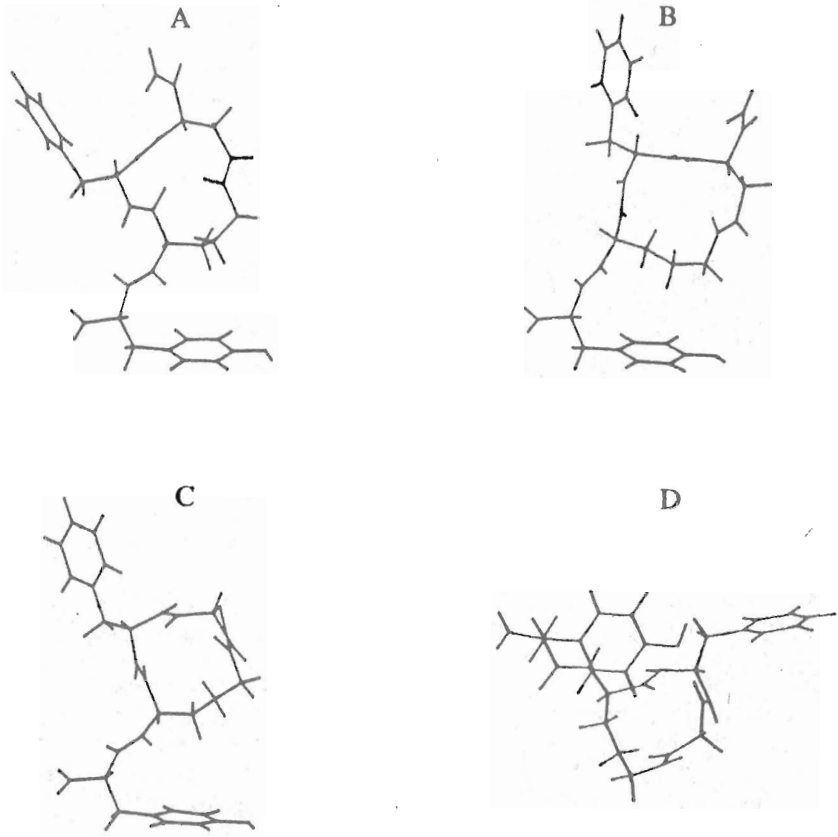

Fig. 3. Preferred conformations of Tyr-c(*D*-Orn-Phe-Asp)-NH_2 (A and B) which is biologically active only at the μ-receptor and Tyr-c(*D*-Orn-Phe-Gly-) (C and D) which is μ-receptor selective but also shows bioactivity at the δ-receptor

Table I

Biological Activities of Cyclic Dermorphin-Deltorphin Analogs*

Analog	GPI IC_{50}, nM	MVD IC_{50}, nM	MVD/GPI IC_{50}-ratio
Tyr-c(*D*-Orn-Phe-Gly-)	8.60±0.78	145±15	16.9
Tyr-c(*D*-Orn-Phe-Asp)-NH_2	36.2±3.7	3880±840	107
Tyr-*D*-Ala-Phe-Gly-NH_2 [24]	45.2±3.19	510±31.82	11.3

*The GPI and MVD activities are measured in Dr. P.W.Schiller's laboratories.

mation and configuration of the second residue must play a significant role in orienting the pharmacophores. Morphiceptin requires an *L*-chirality for Pro^2 and exhibits *cis* and *trans* isomers about the Tyr^1-Pro^2 amide bond in a ratio of 30:70 [26]. The *cis*/*trans* configuration about the Tyr^1-Pro^2 amide bond is particularly significant for the relative orientation of the biologically important Tyr^1 and Phe^3 residues.

In our studies of morphiceptin analogs containing 2-aminocyclopentanecarboxylic acid (βAc^5c) as the second residue in place of proline Tyr-βAc^5c-Phe-Xaa^4-NH_2 [Xaa = Pro or (*L*- and *D*-)Val], the amide bond between residues 1 and 2 adopts completely *trans* structure [27, 28]. Among the four stereoisomers, only the analogs containing (1*S*, 2*R*)-βAc^5c in position 2 show opioid bioactivity. Although the βAc^5c analogs can only adopt a *trans* amide configuration about Tyr-βAc^5c, the bioactive analogs Tyr-(1S, 2R)-βAc^5c-Phe-Xaa^4-NH_2 are topochemically equivalent to morphiceptin with the Tyr-Pro amide bond in a *cis* configuration. On the contrary, the inactive analogs Tyr-(1*R*, 2*S*)-βAc^5c-Phe-Xaa^4-NH_2 share similar preferred conformations with morphiceptin adopting a *trans* configuration at the Tyr-Pro amide bond. We therefore proposed that a *cis* configuration about the Tyr^1-Pro^2 amide bond is required for bioactivity of the morphiceptin related analogs containing a proline as the second residue [28].

This suggestion is ' inconsistent with those proposed from the previous theoretical studies, in which the requirement of *trans* configuration of Tyr-Pro amide bond is implied for the bioactivity of morphiceptin [29, 30]. Thus, systematic conformational studies about the second residue were undertaken to establish the configurational requirements of the Tyr^1-Xaa^2 amide bond of morphiceptin [17]. Such studies can also provide us insight into the "bioactive conformation" of morphiceptin. We have designed and synthesized a series of analogs; Tyr-Xaa^2-Phe^3-*D*-Pro^4-NH_2 (where Xaa = Ala, Pro, and *L*-(NMe)Ala) [17]. The *in vitro* bioactivities of these tetrapeptide analogs are summarized in Table 2.

Since these analogs differ only at the second residue, the differences in bioactivities can be explained in terms of the topochemistry resulting from the effects of changes at residue 2. In order to assess accessible (ϕ, ψ) space for the second residues of the above analogs, energy calculations were carried out for three N-acetylamino acid-N'-methylamide derivatives (Ac-Xaa^2-NHMe; Xaa = Ala, (NMe)Ala, or Pro). The derivatives Ac-Xaa^2-NHMe display the greatest accessible space because they do not have neighboring residues which could restrict geometries for the Xaa^2 residues in a peptide sequence [31].

All the space accessible to the *trans* isomers of Ac-Pro-NHMe and Ac-(NMe)Ala-NHMe are also allowed for Ac-Ala-NHMe. The Tyr-Ala-Phe-*D*-Pro-NH_2 is inactive, while Tyr-Pro-Phe-*D*-Pro-NH_2 and Tyr-(NMe)Ala-Phe-*D*-Pro-NH_2 are active (Table 2). Thus, the Tyr-Xaa^2 amide bond of these two analogs can not be *trans*. We, therefore, examined the conformational (ϕ, ψ) space accessible for the *cis* isomers of Ac-(NMe)Ala-NHMe and Ac-Pro-NHMe, and noted that the two compounds share significant accessible space. Thus, we conclude that the *L*-chirality for the second residue and the *cis* configuration at the Tyr-Xaa^2 amide bond are required for morphiceptin bioactivity [17]. In the 500 MHz ^{1}H-NMR studies, both of the Pro^2 and (NMe)Ala^2 analogs exhibit about 30% of *cis* amide structure for Tyr-Xaa^2 bond

Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assays of Tetrapeptide Related to Morphiceptin

Table 2

Analog	GPI IC_{50}, nM	MVD IC_{50}, nM	MVD/GPI IC_{50}-ratio
Tyr-Ala-Phe-*D*-Pro-NH_2	28100±7500*	Inactive*	-
	26900±7200**	>100000**	-
Tyr-(NMe)Ala-Phe-*D*-Pro-NH_2	32.4±8.05*	1390±1430*	42.9*
	30.6±6.1**	202±28**	6.60**
Tyr-Pro-Phe-*D*-Pro-NH_2	28.7±2.4**	1508±180**	52.5**

*Measured by The NIDA Opiate Compound Testing Program.

**Measured by Dr. P.W.Schiller in the Clinical Research Institute of Montreal.

GPI and MVD Assays* of Morphiceptin Analogs

Table 3

Analog	GPI IC_{50}, nM	MVD IC_{50}, nM	MVD/GPI IC_{50}-ratio
Tyr-Pro-Phe-Pro-NH_2	552±151	3690±740	6.68
Tyr-Pro-Phe-*D*-Pro-NH_2	28.7±2.4	1508±180	52.5
Tyr-Pro-*D*-Phe-Pro-NH_2	109±16	594±77	5.45
Tyr-Pro-*D*-Phe-*D*-Pro-NH_2	557±58	4810±320	8.34
Tyr-Pro-(NMe)Phe-Pro-NH_2	170±25	1790±50	10.5
Tyr-Pro-(NMe)Phe-*D*-Pro-NH_2	20.7±2.4	1250±220	60.4
Tyr-Pro-*D*-(NMe)Phe-Pro-NH_2	>100000	>100000	-
Tyr-Pro-*D*-(NMe)Phe-*D*-Pro-NH_2	2660±120	54800	20.6
Tyr-Gly-Gly-Phe-Leu	246±39	11.4±1.1	0.0463

*The assays were carried out in Dr. Schiller's laboratories.

Binding Assays for Somatostatin Analogs Containing Retro-Inverso Modifications*

Table 4

Analog	IC_{50}, nM
c(-Pro-Phe-*D*-Trp-Lys-Thr-Phe-)	1
c(-gSar-*S*-mPhe-*D*-Trp-Lys-Thr-Phe-)	10
c(-gSar-*R*-mPhe-*D*-Trp-Lys-Thr-Phe-)	50
c(-*R*-mAla-Phe-*D*-Trp-Lys-Thr-gPhe-)	No binding
c(-*S*-mAla-Phe-*D*-Trp-Lys-Thr-gPhe-)	5

*Results were obtained by Dr. T.Reisine, University of Pennsylvania.

while Tyr-Ala-Phe-*D*-Pro-NH_2 is, of course, 100% *trans* about the Tyr-Ala amide bond [17].

A schematic representation of morphiceptin is depicted in Fig. 4. The relative spatial arrangements of the biologically important functional groups, the amine and phenolic groups in the Tyr^1 residue and the aromatic group in the Phe^3 residue, are defined by a set of eight torsion angles ψ^1, χ_1^1 and ω^1 of Tyr^1, ϕ^2, ψ^2 and ω^2 of Pro^2, ϕ^3 and χ_1^3 of Phe^3. Among these eight angles, the ω^1 is 0° because the *cis* configuration of Tyr-Pro amide bond was necessary for bioactivity of morphiceptin analogs. The ϕ^2 angle is restricted to ca. -75° according to the constrained nature of the five-membered pyrrolidine ring of Pro^2 [32]. The ω^2 angle is near 180° (a *trans* configuration) because of the normal amide bond between the Pro^2 and Phe^3 residues.

To identify specific topochemical requirements for the remaining five angles, systematic modifications were carried out for the morphiceptin sequence. The residues both chirality of Phe or (NMe)Phe and Pro were incorporated into position 3 and 4, respectively; Tyr-Pro-Xaa-Yaa-NH_2 (Xaa = *D*- and *L*-Phe and *D*- and *L*-(NMe)Phe. Yaa = *D*- and *L*-Pro). Their bioactivities are summarized in Table 3. Both of the *D*-Phe^3 analogs, Tyr-Pro-*D*-Phe-(*L* and *D*)-Pro-NH_2, are active. Because the two minimum energy conformations of Ac-Pro-NH_2 are different only in ψ angle by about 180°, it can be expected to compensate the chirality change of Phe^3 residue. The N-methylation of the *L*-Phe^3 residues of Tyr-Pro-Phe-(*L*- and *D*-)-Pro-NH_2 maintains the bioactivities of these analogs at the μ-receptor. In contrast, the same modification to the *D*-Phe^3 residues of the Tyr-Pro-*D*-Phe-(*L*- and *D*-)-Pro-NH_2 results in a loss of bioactivity. This result enabled us to reject all preferred conformations of *D*-(NMe)Phe^3 containing analogs in selecting "bioactive conformation" of morphiceptin. As revealed by comparison of bioactivities between Tyr-Pro-Phe-Pro-NH_2 and Tyr-Pro-Phe-*D*-Pro-NH_2 and between Tyr-Pro-*D*-Phe-Pro-NH_2 and Tyr-Pro-*D*-Phe-*D*-Pro-NH_2, the relative potency at the μ-receptor is strongly dependent on the chirality sequence of residues 3 and 4. The analogs with a heterochiral sequence at positions 3 and 4, Tyr-Pro-Phe-*D*-Pro-NH_2 and Tyr-Pro-*D*-Phe-Pro-NH_2, display higher μ-receptor activities than the corresponding analogs with a homochiral sequence at the same positions, Tyr-Pro-Phe-Pro-NH_2 and Tyr-Pro-*D*-Phe-*D*-Pro-NH_2, respectively. Since the Pro^4 residue can affect the side chain conformation of Phe^3, such dependence can give us useful information to determine χ_1^3 of Phe^3.

From the conformational analysis of the above morphiceptin analogs employing ^{1}H-NMR spectroscopy and computer simulations, conformations responsible for their bioactivity were suggested [33]. All of them share essentially the same topologies. The "bioactive conformations" of three representative analogs are depicted in Fig. 5. Since morphiceptin is highly μ-receptor selective, these can be regarded as conformations required for μ-receptor activity. Interestingly, all the conformations suggested for μ-receptor recognition from the studies of enkephalins, dermorphins, and morphiceptins (Fig. 2A, 3A, 3B, 3C, 5A, 5B and 5C) can be defined as topochemically equivalent structures. In these topologies, the two aromatic side chains are project away from each other with distances of more than 11 Å. Thus, we can conclude that enkephalin, dermorphin and morphiceptin can bind to the same type of μ-receptor. In contrast,

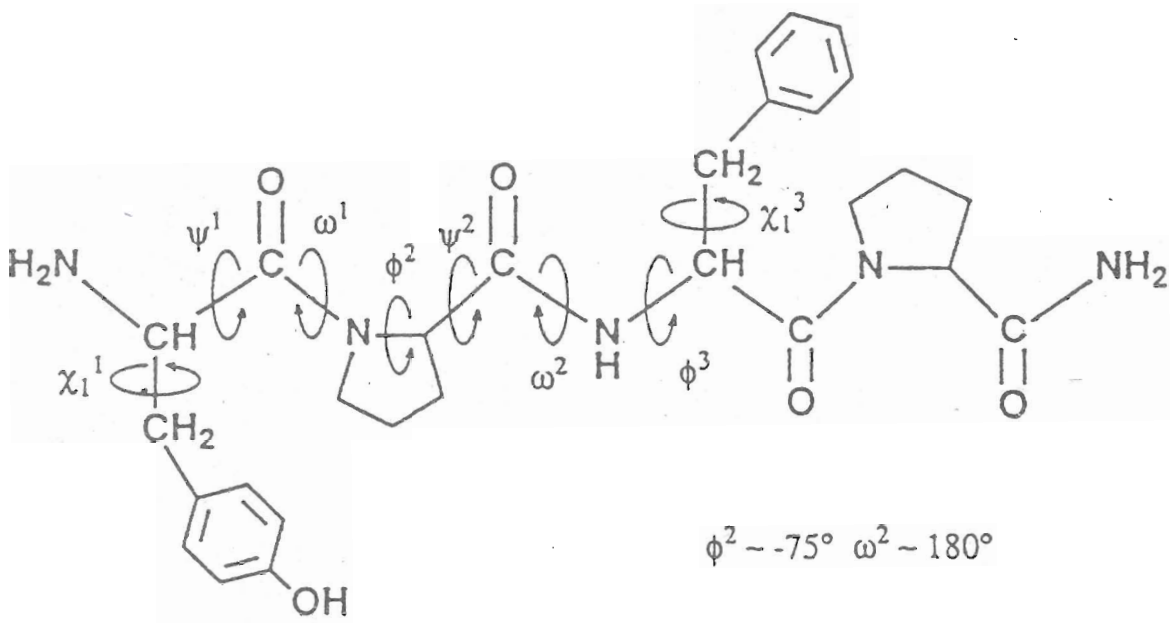

Fig. 4. Torsion angles of morphiceptin to define the array of pharmacophoric groups: phenolic ring and free amine of Tyr^1 and phenyl ring of Phe^3

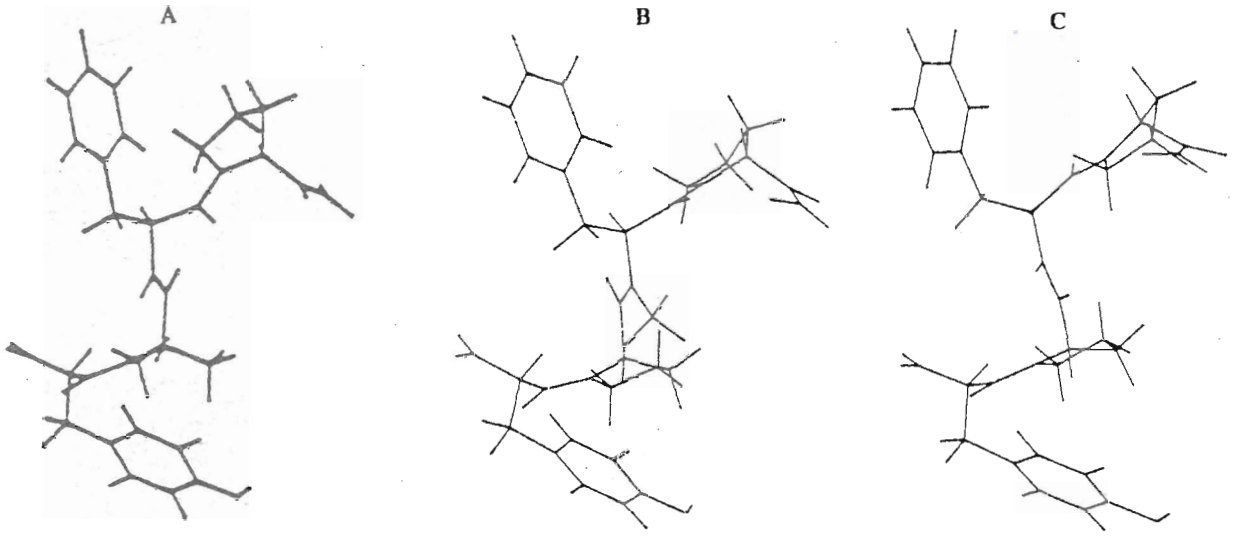

Fig. 5. Bioactive conformations of the representative morphiceptin analogs, A: Tyr-(NMe)Ala-Phe-*D*-Pro-NH_2, B: Tyr-Pro-(NMe)Phe-*D*-Pro-NH_2 and C: Tyr-Pro-*D*-Phe-Pro-NH_2

the folded structures with a close proximity of two aromatic side chains were found among the preferred conformations of all the analogs with δ-receptor bioactivity (Fig. 2B, 3D and [14–16]).

CYCLIC SOMATOSTATIN ANALOGS

In another area of research we have concentrated our approach on constrained cyclic analogs of somatostatin which contain pairwise retro-inverso modifications and unusual amino acid substitutions. Modification of the backbone through inversion of selected amide bonds has been shown to confer high resistance to enzymatic attack along with retention, or even enhancement, of biological activity. Cyclization of these analogs and manipulation of their side chain orientations allow us to investigate those structural features which are necessary for somatostatin bioactivity. Results from binding studies and conformational analysis using ^{1}H-NMR and computer simulations help us understand the effects of these main chain and side chain modifications on overall structure and gain insight into the topochemical basis of biological activity.

Somatostatin, a potent inhibitor of the release of several hormones such as glucagon, growth hormone, insulin and gastrin has been the subject of intense structure-activity studies. Early studies which defined some of the structural requirements for eliciting biological response [34, 35] led to the synthesis of the highly active cyclic hexapeptide c(-Pro^6-Phe^7-*D*-Trp^8-Lys^9-Thr^{10}-Phe^{11}-) (superscript numbers refer to positions in the native tetradecapeptide) by Veber and coworkers (Fig. 6) [36]. An important structural characteristic found in this and most of the active somatostatin analogs is a type II' β turn about the residues Phe^7-*D*-Trp^8-Lys^9-Thr^{10}. This tetrapeptide sequence is conserved in almost all biologically active analogs. In the case of c(-Pro-Phe-*D*-Trp-Lys-Thr-Phe-), it is bridged by Phe^{11}-Pro^6 in a *cis* conformation, which stabilizes the proper orientation of the tetrapeptide (Fig. 6). Although the βII' turn is a required structural feature, it alone is not sufficient for biological activity. Therefore, to obtain more information on the relationship between structure and biological function, we synthesized a series of cyclic hexapeptide analogs, related to Veber's molecule above, which incorporate both backbone and side chain modifications.

Two epimeric analogs in this series, c(-gSar^6-*S*-mPhe^7-*D*-Trp^8-Lys^9-Thr^{10}-Phe^{11}-) and c(-gSar^6-*R*-mPhe^7-*D*-Trp^8-Lys^9-Thr^{10}-Phe^{11}-) incorporate the pairwise retro-inverso modification about positions six and seven. Binding studies (Table 4) show that the *R* isomer displays little or no binding affinity while the *S* isomer shows much higher affinity. Conformational studies of these two analogs showed highly disparate backbone structures. The active *S* isomer contained both a *cis* amide bond in the bridging region and a βII' turn about the tetrapeptide portion, Phe^7-*D*-Trp^8-Lys^9-Thr^{10} (Fig. 7A). The *R* isomer contained a *cis* amide bond in the bridging region, but both ^{1}H-NMR data and computer simulations indicated that a γ turn about the *D*-Trp residue was present (Fig. 7B). Therefore, this pair of analogs demonstrates the importance of the β II' turn in the bioactive region. In addition, it suggests the sensitivity of position seven toward changes in chirality.

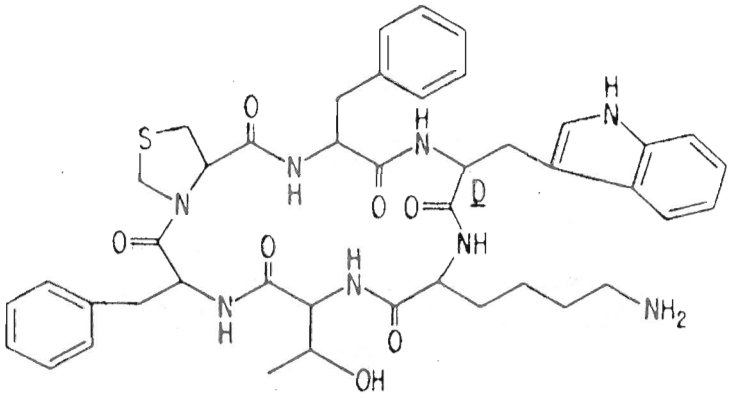

Fig. 6. Structure of the somatostatin analog c(-Pro-Phe-*D*-Trp-Lys-Thr-Phe-)

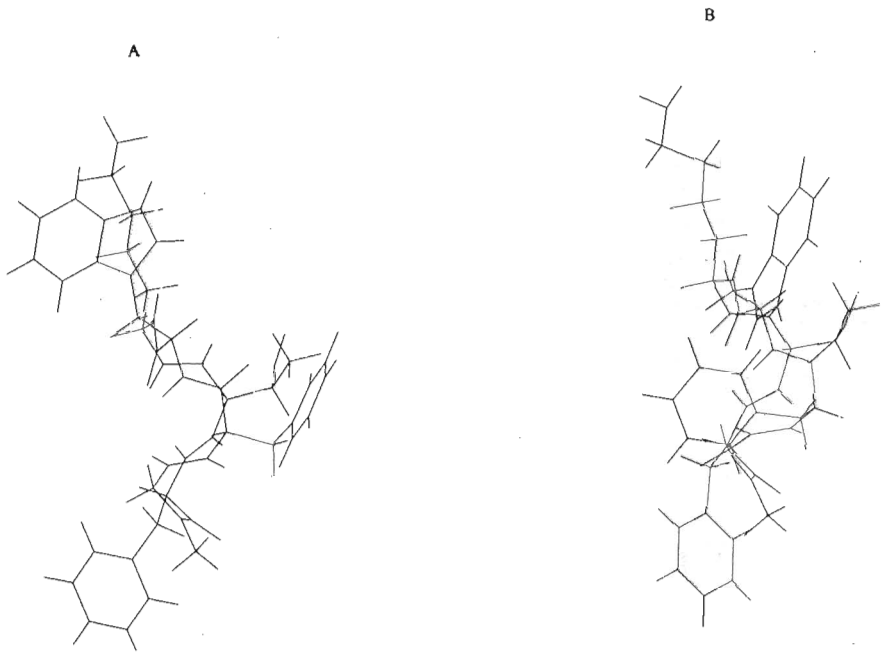

Fig. 7. Preferred conformations of A: c(-gSar-*S*-mPhe-*D*-Trp-Lys-Thr-Phe-) and B: c(-gSar-*R*-mPhe-*D*-Trp-Lys-Thr-Phe-)

The compounds c(-*R*-mAla^6-Phe^7-*D*-Trp^8-Lys^9-Thr^{10}-gPhe^{11}-) and c(-*S*-mAla^6-Phe^7-*D*-Trp^8-Lys^9-Thr^{10}-gPhe^{11}-) incorporate the retro-inverso modification at the bridging region with *R* and *S* chiralities at the malonate of position six. Binding studies of these analogs (Table 4) indicate that the *R* isomer has essentially no affinity for the somatostatin receptor, while the *S* isomer has a substantial affinity. Differences in the conformations of these two isomers were revealed by ^{1}H-NMR analysis. The NOEs of both *R* and *S* containing analogs were consistent with a type β II' arrangement about the tetrapeptide Phe^7-*D*-Trp^8-Lys^9-Thr^{10}. However, while the bridging amide bond between gPhe and *R*-mAla is fully in the *trans* conformation, that between gPhe and *S*-mAla displays a *cis/trans* isomerism with 36% of the *cis* form. The *S* isomer, unlike the *R* isomer, displays a smaller distance between the *D*-Trp and Lys side chains, which is also believed to be important for receptor binding. These conformational differences are attributed to the change in chirality of the C^α of mAla (Fig. 8).

The effect of manipulation of chirality in the side chain was investigated with analogs containing the β methylated residues β-methyl-phenylalanine [(βMe)Phe] [37] and β-methyltryptophan [(βMe)Trp] [38]. These amino acid derivatives were incorporated by solid phase methods into the cyclic compounds c(-Pro^6-(βMe)Phe^7-*D*-Trp^8-Lys^9-Thr^{10}-Phe^{11}-), c(-Pro^6-Phe^7-*D*-Trp^8-Lys^9-Thr^{10}-(βMe)Phe^{11}-) and c(-Pro^6-Phe^7-(βMe)Trp^8-Lys^9-Thr^{10}-Phe^{11}-). A mixture of *R* and *S* stereochemistries at the C^α and C^β centers of the β-methyl containing residues gave four isomers of each analog. Binding studies with these compounds show that affinity for somatostatin receptors is favored by certain chiralities in the side chain. We expected that manipulations at these centers would modify populations of the preferred side chain orientations and thus affect the overall structure of each analog. We are continuing ^{1}H-NMR analysis and computer modeling to learn more specifically the nature of this dependence on C^β chirality.

These results strengthen existing evidence [39] that epimeric analogs can demonstrate profoundly different somatostatin-like ligand-receptor interactions. Of cource, it is not just the stereochemistry of one atom that is the determinant of receptor binding, but the overall structure of the cyclic compound which contains either the *R* or *S* malonate residue. Interestingly, the sensitivity to variation in chirality was noted in atoms of both the main chain and side chain. We expect the analogs containing β-methyl amino acid residues display preferred side chain orientations based on the relative placement of the methyl substituent. Further investigations are being carried out to better assess the effect of chiral variation on overall structure and thus to gain a clearer understanding of the topochemical basis of biological activity of somatostatin.

EXPERIMENTAL SECTION

Synthesis

The syntheses of peptides and peptidomimetics were carried out in solution by standard methods. The N-methylated amino acids were purchased or synthesized in our laboratories. After assembling the partially protected linear peptides with full sequences, cyclization

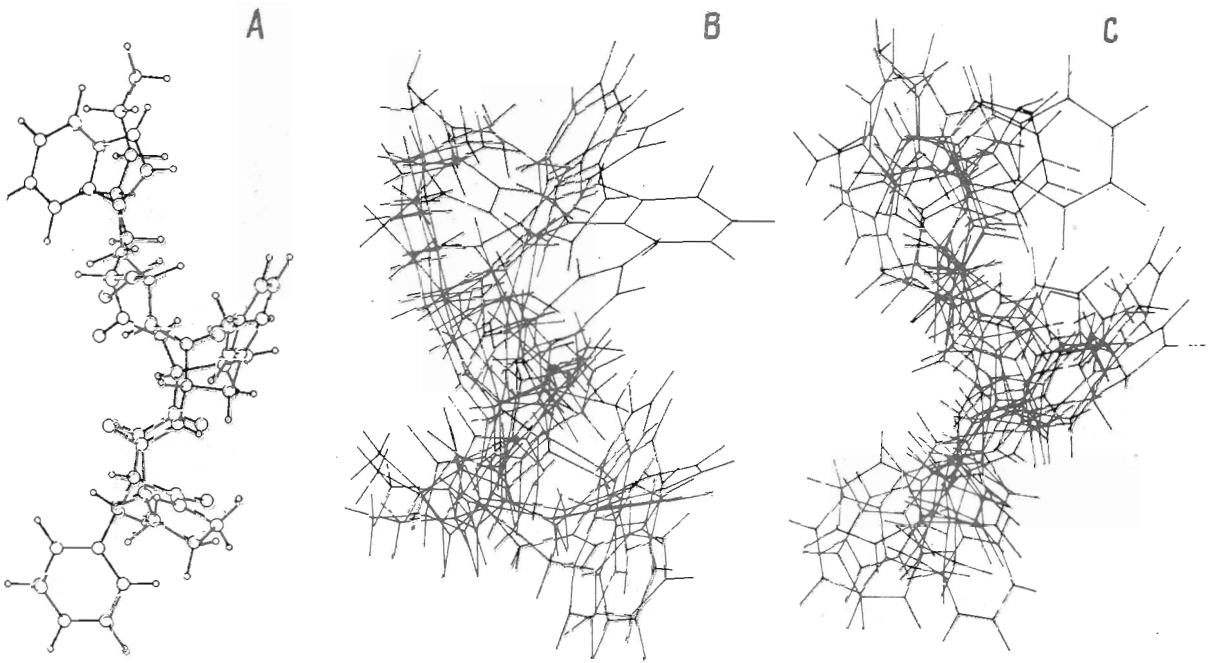

Fig. 8. **A**: preferred conformation of Veber's analog c(-Pro-Phe-*D*-Trp-Lys-Thr-Phe-) and superimposed structures from molecular dynamics of the somatostatin analogs **B**: c(-*R*-mAla-Phe-*D*-Trp-Lys-Thr-gPhe-) and **C**: c(-*S*-mAla-Phe-*D*-Trp-Lys-Thr-gPhe-). Molecular dynamics were carried out for 50 ps in vacuo using CHARMs forcefield program

was carried out with benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP reagent) or diphenylphosphoryl azide (DPPA). The retro-inverso modifications, denoted as a paired *gem*-diamino alkane (i.e. gSar) and alkylmalonate (i.e. mPhe), were incorporated in a stepwise manner. An amino acid amide was converted by oxidative Hofmann rearrangement into the *gem*-diamino residue using iodobenzene[bis(trifluoroacetate)] (IBTFA) [40]. The malonyl residue was incorporated as a racemic alkylated malonic acid derivative. Consequently, two isomers of the product were obtained containing *R* and *S* chirality at the malonyl residue. The *R* and *S* isomers were separated either prior to cyclization, and then cyclized individually, or after the last step of synthesis. After final deprotection, the crude products were purified by HPLC or column chromatographies. The purities of the final products were examined by HPLC (>99%). The structures of them were confirmed by 500 MHz ^{1}H-NMR spectroscopy, thin layer chromatography, amino acid analysis and fast atom bombardment mass spectrometry.

Biological Assays

The *in vitro* activities of opioid analogs were determined by measuring their ability to inhibit the electrically evoked contraction of isolated muscle preparations. The guinea pig ileum (GPI) and mouse vas deferens were used to assess the bioactivity at the μ- and δ-receptor. Receptor affinities of somatostatin analogs were determined by displacement of radioligands from rat brain or pituitary membranes.

^{1}H-NMR Spectroscopy and Molecular Modeling

The ^{1}H-NMR spectra were recorded at 500 MHz on a General Electric GN-500 spectrometer. The samples were prepared in DMSO-d_6 at a concentration of 10 mM. Tetramethylsilane was used as an internal reference for the determination of chemical shifts. All of the proton resonances were assigned using two-dimensional homonuclear Hartman-Hahn experiment (HOHAHA) and rotating frame nuclear Overhauser enhancement spectroscopy (ROESY) experiments. The HOHAHA experiments were carried out using the MLEV17 with a mixing time of 100 ms and time-proportional phase incrementation (TPPI). The ROESY spectra were acquired using a 200 ms mixing time.

Molecular mechanics calculations were carried out employing the DISCOVER force field program. Conformational energies were estimated as the sum of nonbonded van der Waals interactions, coulombic interactions, intrinsic torsional potentials, and energies of deformation of bond lengths and bond angles. Parameters required for the description of the torsional potentials for the internal bond rotation are provided in the DISCOVER program and used without modification. Various force constants defined in the force scheme were adopted as specified in the program.

Molecular dynamics calculations with NOE restraints are performed at a temperature of 300K with a step size of 0.5 fs by using numerical integration of Newton's equation of motion with a fourth-order Gear algorithm. Initial structures for the molecular dynamics calculations are generated by applying a force to torsion

angles ϕ and ψ in order to be consistent with experimentally observed NMR data. The starting conformation was partially minimized for 200 steps of steepest descent to remove any high energy contacts that may a rise from forcing the torsional angles. The structure was then equilibrated at 300K for 2 ps of 4000 steps before the simulation for 20 ps.

Structures taken from the molecular dynamics calculations are used as initial conformations for energy minimization studies without NOE restraints. Conformational energy minimizations are carried out with a quasi Newton-Raphson method using the va09a algorithm until the maximum derivative was less than 0.001 kcal·mol^{-1}Å^{-1}. Initial structures for energy minimizations are also generated by updating selected torsional angles using the MOLEDT utility of the DISCOVER program.

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