Conformational Analysis by NMR and Distance-Geometry Techniques of Deltorphin Analogs

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To identify the peptide conformation that is preferentially recognized by the receptor, we have synthetized by solid-phase method a series of deltorphin I analogs with increasing selectivity for δ- and μ-opioid receptor. Structure-selectivity relationship of these peptides were evaluated on the basis of receptor-binding properties and conformational features, computed by two-dimensional NMR spectra and distance-geometry techniques. These compounds in solution are present with a large number of conformers with no defined secondary structural elements. The analysis of the average properties of these compounds indicate the presence of some distinct conformational preferences that can be related to the observed opioid receptor selectivities. Selectivity for the δ- and μ-opioid receptors can be ascribed to the spatial arrangement of the aromatic moieties. In addition, substitutions in position 2 and 4 are important for the correct arrangement and must be taken into account in the design of δ-opioid receptor-selective ligands.

Introduction

From the examination of small peptides in solution, the identification of the bioactive form (the conformation that binds to the receptor) is a very difficult task. For linear peptides, there are a number of energetically comparable conformations which coexist in equilibrium, conformations which span the full spectrum of possible orientations from fully extended to highly folded structures. Moreover the low-energy conformations of a particular peptide depends greatly on the surrounding environment. This adds a further problem to the identification of the bioactive form, since the most appropriate conditions (e. g., solvent, pH, ionic strength, temperature) to describe the receptor-binding site are not usually known. As a consequence of this molecular plasticity, the bioactive form of the peptide does not need to be the predominate conformation at equilibrium, but one that can dynamically accommodate the conformational requirements of the binding site in an induced fit. However, a dynamically induced form should be accessible with reasonable energy expenditure and therefore must derive from one of the lowest energy conformations observed in the receptor environment.

Many conformational studies have been carried out to explain the selectivity and affinity of opioid peptides for the δ-opioid receptors.[1−27] One of the most frequently used approaches has been the introduction of conformational constraints into the peptide molecule by cyclization in order to reduce the number of accessible conformations and consequently facilitate the theoretical and experimental characterization of the bioactive forms.[28−31] Nevertheless NMR studies have demonstrated that, even though the number of low-energy conformations for a cyclized peptide is smaller than that of linear analogs, this number is still significantly high.[32] Therefore, it is not possible to characterize the receptor-selected conformation from studies of just one analog. Rather, the form of the peptide preferred by each receptor type must be deduced from a comparison of the conformational features of structurally similar analogs, a family or series of ligands with high and low affinities, ranging from nonselective to highly selective compounds for the receptor subtype under investigation.

Deltorphins are naturally occurring peptides with high affinity and selectivity for the δ-opioid receptors.[33][34] Three deltorphins have been isolated from amphibian skin: [met^2]deltorphin or dermenkephalin (Tyr-met-Phe-His-Leu-Met-Asp-NH_2); [ala^2] deltorphin I (Tyr-ala-Phe-Asp-Val-

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Val-Gly-NH_2); and [ala^2]deltorphin II (Tyr-ala-Phe-Glu-Val-Val-Gly-NH_2). To understand the conformation-activity relationships of the deltorphins a knowledge of their conformational preferences in solution is fundamental.

Several NMR studies of deltorphins in DMSO and in cryoprotective (DMSO/water) mixtures have confirmed that these peptides most likely exist in solution as a mixture of different conformers, interconverting quickly on the NMR time scale.[2][35][36] Nevertheless, Tancredi et al.[37] have derived a single [met^2]deltorphin conformation which is in good agreement with the NMR data, in particular with the NOE measurements. On the other hand, Nikiforovich et al.[38][39] have proposed a model for the δ-receptor-bound conformation of an active cyclic analog of [met^2]deltorphin based on energy calculations which has little resemblance to the [met^2]deltorphin solution structure described earlier.[37] Recently, Picone et al.[40] have reported the conformational analysis of deltorphin I analogs containing $C^{\alpha,\alpha}$-dialkylated residues, such as Aib, Ac_3c, Ac_5c, Ac_6c, in position 2, 3 and 4. The NMR analysis combined with MD calculations shows that Xaa^3 analogs are highly structured in DMSO and in DMSO/water at low temperature, and there are several different structures consistent with experimental NOEs. Comparison of the preferred solution conformations of Aib^3 deltorphin-I and Ac_6c^3 deltorphin I with two nonpeptidic δ-selective agonists illustrates very similar shapes and topological orientations.[31].

In order to better map out the pharmacophore and define the importance of each amino acid for receptor recognition, an extensive study of 17 deltorphin-substituted analogs has been carried out.[41] These analogs varied greatly in their affinity and activity at the δ-opioid receptor. It was observed that the residues at positions 2, 4, and 5 were key for opioid receptor affinity and selectivity.[41]

The objective of the present study is to develop a structure-activity relationship between the conformations of these analogs in solution and the observed biological profiles. Analogs incorporating Aib (α-aminoisobutyric acid or α,α-dimethylglycine) residues in place of ala^2 and/or Asp^4 and modifications altering the hydrophobic/hydrophilic character of the peptide have been examined by high resolution NMR measurements and extensive distance geometry (DG) calculations. The Aib residue was chosen based on its ability to induce folding into linear peptides and therefore imparting considerable conformational constraint.[42][43] The comparison of the conformational preferences observed here with the pharmacological profiles will allow for a further understanding of the structural, conformational and topological requirements for δ-opioid selectivity and affinity.

Results and Discussion

Synthesis

We have synthesized nine analogs of [ala^2]deltorphin I (Table I). These peptides can be divided into two series. The first series includes peptide A ([Tyr^5]DELT), peptide B ([Aib^4,Tyr^5]DELT) and peptide C ([Aib^{2,4},Tyr^5]DELT), which all end at the C terminus with Tyr-Val-Gly-NH_2. The second series includes peptide D ([met^2]DELT), peptide E ([Aib^2]DELT), peptide F ([Aib^{2,4}]DELT), peptide G ([Aib^4]-DELT), peptide H ([Gly^4]DELT) and peptide I ([Ala^4]-DELT), which all end at the C terminus with the DELT sequence Val-Val-Gly. The strategy followed in designing the analogs of the first series (peptides A, B, and C) was that of altering the hydrophobicity by substituting Val^5 with Tyr (peptide A) and introducing the helicogenic Aib residue at positions 2 and 4 (peptides B and C). In the second series the substitution of the ala residue at position 2 with a met (peptide D) or Aib (peptide E) residue could also provide information on the conformational role of the amino acid residue located between the two aromatic rings of Tyr^1 and Phe^3. Finally, in peptides F, G, H, and I changes in the hydrophobicity and in the anionic properties of side chains were combined with conformational changes of the peptide backbone by substituting positions 2 and 4 with the helicogenic Aib residue or with the more flexible Gly, Ala residues.

Binding Assay

Table 1 shows that all peptides studied have K_i values for δ-opioid receptor in the nanomolar range, whereas their selectivity for δ-opioid receptors ranged over two orders of magnitude, mainly because of large differences in their affinity for μ-opioid receptors. On the basis of their residual μ-receptor affinity, the peptides could be classified into three types of ligands: ligands scantly recognized by μ-opioid receptors (peptides A, D and E); ligands with moderate μ-receptor affinity (peptides F and C), and nonselective ligands (peptides B, G, H and I) which possess similar affinities for the μ- and δ-opioid receptors. Bioassays on GPI and MVD showed a good agreement with receptor binding results, demonstrating that all peptides are opioid agonists.

NMR Analysis

The solution conformations of seven analogs (peptides A, B, C, D, E, F, G) of [ala^2]DELT were studied by proton NMR in DMSO. For all samples the NH peaks were resolved with the exception of the NH resonances of Tyr^1 and Gly^7. Identification of the complete spin systems for the seven peptides studied were accomplished by homonuclear *J*-correlated 2D techniques such as DQF-COSY and HOHAHA. These techniques readily allowed for the identification of all spin systems but can not determine their position in the sequence. To assign unambiguously AMXX′-type spin systems (Tyr, Phe, Asp) and their position in the peptide sequence, sequential NOE effects between the backbone protons were utilized.[44] Proton-chemical shifts are summarized in Tables 2−4. The β-CH_3 protons of ala in peptides A,B and G and the side-chain protons of met in peptide D are upfield shifted as it was found for the natural

Table 1. Affinity for opioid receptors and biological activity on guinea pig ileum (GPI) and mouse vas deferens (MVD) of deltorphin-like peptides[a]

peptide	MVD	IC_{50} [nM] GPI	δ	μ	K_i [nM] κ	δ/μ
[ala^2]DELT	0.19 ± 0.01	1239 ± 203	0.54 ± 0.04	1985 ±224	5748 ± 480	2.7×10^{-4}
Tyr-ala-Phe-Asp-Val-Val-Gly·NH_2						
A. [Tyr^5]DELT	2.21 ± 0.18	1460 ± 200	4.7 ± 0.59	3135 ± 297	2800	1.5×10^{-3}
B. [Aib^4,Tyr^5]DELT	0.16 ± 0.01	2.58 ± 0.3	2.03 ± 0.21	2.9 ± 0.3	185	7×10^{-1}
C. [Aib^{2,4},Tyr^5]DELT	9.9 ± 0.7	480 ± 27	7.5 ± 1.92	96 ± 10	12300	5.2×10^{-2}
D. [met^2]DELT	173 ± 40	1700 ± 390	170 ± 5.9	3142 ± 175	> 40000	5.4×10^{-2}
E. [Aib^2]DELT	1.75 ± 0.27	> 2000	9.7 ± 0.91	2105 ± 159	> 40000	4.6×10^{-3}
F. [Aib^{2,4}]DELT	2.8 ± 0.17	> 2000	4.8 ± 0.37	269 ± 34	26000	1.78×10^{-2}
G. [Aib^4]DELT	0.3 ± 0.04	18.8 ± 2.5	0.57 ± 0.1	5 ± 0.3	3000	1.14×10^{-1}
H. [Gly^4]DELT	2.62 ± 0.32	22 ± 3	7.35 ± 0.93	11 ± 1.2	2700	6.68×10^{-1}
I. [Ala^4]DELT	0.76 ± 0.08	91 ± 21	1.2 ± 0.21	25 ± 2.7	4200	4.8×10^{-2}

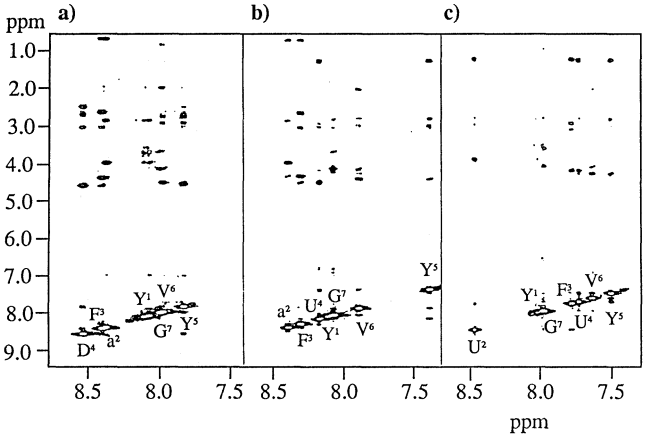
[a] IC_{50}, agonist concentration that produces 50% inhibition of the electrically evoked twitch; K_i, equilibrium dissociation constant of the competing ligand; MVD, mouse vas deferens preparation; GPI, guinea-pig ileum preparation.

deltorphins.[2] This observation strongly indicates that the side chain of the second residue in these peptides is in close proximity to both aromatic ring systems of Tyr^1 and Phe^3,[36] a low-energy conformational arrangement accessible to numerous dermorphin and deltorphin analogs. Values observed for $^3J_{\alpha CH\text{-}NH}$ (Tables 2−4) are consistent with reported values for a random-coil conformation, as one can expect from a mixture of many conformers in fast equilibrium. However, it is worth pointing out that the temperature coefficients of the amide protons (Tables 2−4) are not uniform along the sequence. The temperature coefficient of the Tyr^5 amide proton, in fact, in peptide B of the first series (A, B and C), exhibits a temperature coefficient of zero, suggesting the existence of some folded conformations. In the second series of peptides (D, E, F, G) the NH at position 5 shows a low temperature gradient in peptides F and G, suggesting the involvement of this amide proton in an intramolecular hydrogen bond. The measurement of NOEs provided additional information concerning the preferred conformations of the peptides. All peptides gave weak to medium NOE signals. In particular, some peptides distinctly showed diagnostically valuable inter-residue effects, such as the NH-NH contacts typical of folded structures (Figure 1 and Scheme 1).

The 3−4 NH-NH contact, not outlined for peptides C and F, could not be unambiguously identified since the chemical shifts of these protons are not well resolved. Nevertheless all sequential C^{a}H-NH NOEs, characteristic of extended structures, are observed (Scheme 1).

These data reveal an apparent disagreement between coupling constant results, which are indicative of a mixture of many conformations, and the NH-NH NOE effects, which suggest the existence of rather defined structures. This contradiction, however, can be partially explained by noting that the existence of small amounts of folded conformers, characterized by short interproton distances, can be evidenced, because of the strongly nonlinear dependence of NOEs on distances (i. e., the NOE goes as a function of distance to the inverse sixth power).

Figure 1. 400-MHz expansions of NOESY spectra in DMSO at 298 K of [Tyr^5]DELT (a), [Aib^4,Tyr^5]DELT (b) and [Aib^{2,4},Tyr^5]-DELT (c)



Distance Geometry

In general, the conformational analysis of small peptides by NMR is beset with a number of problems not encountered in the study of other molecules of biological interest (i. e. proteins, nucleic acids, glycosaccharides). Linear peptides are naturally very flexible and can therefore quickly adopt a large number of different conformations. Secondly, the absence of secondary structural elements (i. e., α-helices, β-sheets) and the high percentage of solvent-exposed regions leads to small proton densities and therefore a small number of NOEs are observed.

To obtain structural information on the conformational preferences of deltorphin analogs extensive DG calculations were carried out for peptides A, C, E, F and G. These calculations represent a rough approximation of the complex conformational equilibria existing in solution and in addition allow for a thorough searching of the conformational space consistent with the experimental observations. The NOESY spectra for peptides B and D were poor of significant structural information to apply DG calculations.

Scheme 1. Schematic diagram illustrating d_{NN} and $d_{\alpha N}$ NOE contacts

A. [Tyr^5]DELT	$\mathbf{d_{NN}}$			F^3-D^4	D^4-Y^5	Y^5-V^6	
	$\mathbf{d_{aN}}$	Y^1-a^2	a^2-F^3	F^3-D^4	D^4-Y^5	Y^5-V^6	V^6-G^7
B. [Aib^4,Tyr^5]DELT	$\mathbf{d_{NN}}$			F^3-U^4	U^4-Y^5	Y^5-V^6	V^6-G^7
	$\mathbf{d_{aN}}$	Y^1-a^2	a^2-F^3	F^3-U^4	–	Y^5-V^6	V^6-G^7
C. [Aib^{2,4},Tyr^5]DELT	$\mathbf{d_{NN}}$		U^2-F^3		U^4-Y^5	Y^5-V^6	
	$\mathbf{d_{aN}}$	Y^1-U^2	–	F^3-U^4	–	Y^5-V^6	V^6-G^7
D. [met^2]DELT	$\mathbf{d_{NN}}$			F^3-D^4	D^4-V^5	V^5-V^6	
	$\mathbf{d_{aN}}$	Y^1-m^2	m^2-F^3	F^3-D^4	D^4-V^5	V^5-V^6	V^6-G^7
E. [Aib^2]DELT	$\mathbf{d_{NN}}$		U^2-F^3	F^3-D^4	D^4-V^5	V^5-V^6	
	$\mathbf{d_{aN}}$	Y^1-U^2	–		D^4-V^5	V^5-V^6	V^6-G^7
F. [Aib^{2,4}]DELT	$\mathbf{d_{NN}}$		U^2-F^3		U^4-V^5	V^5-V^6	V^6-G^7
	$\mathbf{d_{aN}}$	Y^1-U^2	–	F^3-U^4	–	V^5-V^6	V^6-G^7
G. [Aib^4]DELT	$\mathbf{d_{NN}}$			F^3-U^4	U^4-V^5	V^5-V^6	V^6-G^7
	$\mathbf{d_{aN}}$	Y^1-a^2	a^2-F^3	F^3-U^4	–	V^5-V^6	V^6-G^7

Table 2. Proton chemical shifts [ppm] at 298 K for peptides [Tyr^5]-DELT, [Aib^4,Tyr^5]DELT and [Aib^{2,4},Tyr^5]DELT

[Tyr^5]DELT, peptide A

AA	NH	αCH	βCH	γCH	others
Tyr^1	8.08	3.95	2.72		(2,6)6.98-(3,5)6.67-OH 9.30
ala^2	8.36	4.38	0.65		
Phe^3	8.39	4.55	2.90−2.60		(2,6,3,5)7.24-(4)7.18
Asp^4	8.52	4.52	2.68−2.45		
Tyr^5	7.82	4.50	2.90−2.75		(2,6)6.99-(3,5)6.62-OH 9.14
Val^6	7.96	4.12	1.98	0.86−0.82	
Gly^7	7.99	3.65			NHt 7.21−7.02

[Aib^4,Tyr^5]DELT, peptide B

AA	NH	αCH	βCH	γCH	others
Tyr^1	8.09	3.96	2.82		(2,6)6.99-(3,5)6.67-OH9.30
ala^2	8.37	4.32	0.70		
Phe^3	8.28	4.50	3.03−2.64		(2,6,3,5)7.22-(4)7.18
Aib^4	8.15	–	1.28−1.24		
Tyr^5	7.38	4.41	2.97−2.79		(2,6)6.94-(3,5)6.60-OH 9.10
Val^6	7.87	4.13	2.00	0.86	
Gly^7	8.05	3.65			NHt 7.19−7.04

[Aib^{2,4},Tyr^5]DELT, peptide C

AA	NH	αCH	βCH	γCH	others
Tyr^1	7.98	3.88	2.77−2.97		(2,6)7.04-(3,5)6.72-OH 9.36
Aib^2	8.45	–	1.23−1.20		
Phe^3	7.76	4.19	3.09−2.91		(2,6,4)7.18-(3,5)7.25
Aib^4	7.72	–	1.28−1.24		
Tyr^5	7.49	4.27	3.01−2.82		(2,6)6.98-(3,5)6.60-OH 9.11
Val^6	7.62	4.09	2.03	0.85	
Gly^7	7.96	3.58−3.65			NHt 7.16−7.02

Table 3. Proton chemical shifts [ppm] at 298 K for peptides [met^2]-DELT, [Aib^2]DELT, [Aib^{2,4}]DELT and [Aib^4]DELT

[met^2]DELT, peptide D

AA	NH	αCH	βCH	γCH	others
Tyr^1	8.07	3.98	3.33−2.83		(2,6)7.00-(3,5)6.67-OH 9.31
met^2	8.48	4.40	1.37−1.26	1.79	
Phe^3	8.54	4.63	3.04−2.63		(2,6,3,5)7.27-(4)7.20
Asp^4	8.64	4.60	2.70−2.51		
Val^5	7.71	4.25	1.97	0.81	
Val^6	7.89	4.10	1.95	0.84	
Gly^7	8.06	3.63			NHt 7.20−7.01

[Aib^2]DELT, peptide

AA	NH	αCH	βCH	γCH	others
Tyr^1	8.00	3.91	2.97−2.77		(2,6)7.05-(3,5)6.70-OH 9.35
Aib^2	8.31	–	1.26−1.19		
Phe^3	7.50	4.50	3.08−2.86		(2,6,3,5)7.22-(4)7.17
Asp^4	8.30	4.60	2.73−2.54		
Val^5	7.70	4.23	1.99	0.83−0.80	
Val^6	7.86	4.09	1.97	0.85	
Gly^7	8.07	3.67−3.60			NHt 7.21−7.03

[Aib^{2,4}]DELT, peptide F

AA	NH	αCH	βCH	γCH	others
Tyr^1	8.00	3.89	2.96−2.80		(2,6)7.04-(3,5)6.71-OH 9.36
Aib^2	8.45	–	1.27−1.20		
Phe^3	7.76	4.23	3.09−2.94		(2,6,4)7.19-(3,5)7.26
Aib^4	7.77	–	1.33−1.30		
Val^5	7.17	4.10	2.05	0.84	
Val^6	7.65	4.11	1.98	0.83	
Gly^7	8.02	3.61			NHt 7.18−7.01

[Aib^4]DELT, peptide G

AA	NH	αCH	βCH	γCH	others
Tyr^1	8.05	3.95	2.84		(2,6)6.99-(3,5)6.67-OH 9.30
ala^2	8.35	4.30	0.74		
Phe^3	8.30	4.55	3.07−2.69		(2,3,5,6)7.25-(4)7.19
Aib^4	8.19	–	1.35		
Val^5	7.16	4.18	1.99	0.83−0.77	
Val^6	7.88	4.11	1.99	0.86	
Gly^7	8.04	3.63			NHt 7.19−7.01

In Tables 5 the comparison between relevant NOE-derived distances and the corresponding average calculated distances derived from the DG calculations is reported. In Figures 2−6 40 structures refined with the DADD procedure calculations of the various peptides superimposed with the backbone atoms N, C^α, C, and O are shown. The analysis of the structures obtained for all peptides reveals that no definite regular secondary structures can be identified and a large flexibility in the C-and N-terminal parts is found. To obtain structural information we have computed from the DG structures some average properties, that can be used to explain the δ/μ selectivity for this class of compounds. In Table 6 the average distance between the aromatic rings in position 1 and 3 and the average distance between the NH in position 1 and the aromatic ring in position 3 for all compounds analyzed with DG techniques are reported.

The results confirm that the structural elements of [ala^2]-deltorphin I (i. e., ala^2, Asp^4 and Val^5) are all necessary for

Table 4. Temperature coefficients [ppb/K] and $^3J_{NH-\alpha CH}$ [Hz] for peptides [Tyr^5]DELT, [Aib^4,Tyr^5]DELT, [Aib^{2,4},Tyr^5]DELT, [met^2]-DELT, [Aib^2]DELT, [Aib^{2,4}]DELT and [Aib^4]DELT

[Tyr^5]DELT, peptide A			[met^2]DELT, peptide D		
AA	**Δδ/Δ*T***	**^3J(NH-αCH)**	**AA**	**Δδ/Δ*T***	**^3J(NH-αCH)**
Tyr^1			**Tyr**^{1}		
ala^2	−3.4	8.2	**met**^{2}	−3.2	8.4
Phe^3	−5.3	9.9	**Phe**^{3}	−5.1	8.9
Asp^4	−4.7	7.9	**Asp**^{4}	−3.8	7.6
Tyr^5	−2.5	7.8	**Val**^{5}	−3.1	9.1
Val^6	−5.1	8.3	**Val**^{6}	−3.7	8.5
Gly^7	−4.7		**Gly**^{7}		5.3
[Aib^4,Tyr^5]DELT, peptide B			[Aib^2]DELT, peptide E		
AA	**Δδ/Δ*T***	**^3J(NH-αCH)**	**AA**	**Δδ/Δ*T***	**^3J(NH-αCH)**
Tyr^1			**Tyr**^{1}		
ala^2	−3.2	7.7	**Aib**^{2}	−2.7	
Phe^3	−4.3	8.5	**Phe**^{3}	−3.0	8.3
Aib^4	−5.3		**Asp**^{4}	−4.8	8.3
Tyr^5	0	7.3	**Val**^{5}	−3.2	9.0
Val^6	−5.0	8.5	**Val**^{6}	−3.9	8.3
Gly^7	−4.6		**Gly**^{7}	−5.0	5.6
[Aib^{2,4},Tyr^5]DELT, Peptide C			[Aib^{2,4}]DELT, Peptide F		
AA	**Δδ/Δ*T***	**^3J(NH-αCH)**	**AA**	**Δδ/Δ*T***	**^3J(NH-αCH)**
Tyr^1			**Tyr**^{1}		
Aib^2	−4.2		**Aib**^{2}	−3.6	
Phe^3	−6.0	6.6	**Phe**^{3}	−5.3	7.1
Aib^4	−2.2		**Aib**^{4}	−2.5	
Tyr^5	−2.2	8.2	**Val**^{5}	−0.7	8.3
Val^6	−2.4	8.2	**Val**^{6}	−3.0	8.1
Gly^7	−5.0	5.8	**Gly**^{7}	−5.1	5.7

[Aib^4]DELT, peptide G		
AA	**Δδ/Δ*T***	**^3J(NH-αCH)**
Tyr^1		
ala^2	−4.0	7.7
Phe^3	−5.5	8.6
Aib^4	−6.4	
Val^5	0	8.6
Val^6	−5.0	8.3
Gly^7	−5.3	5.8

full retention of the biological activity. In comparison with the parent peptide, substitution of Val^5 by a Tyr residue reduces δ-selectivity by a factor of 10 and replacement of ala^2 with both met or Aib residues further decreases the affinity for δ-opioid receptors by a factor of 20 (for peptides D and E). In contrast, substitution of Asp^4 by an Aib residue or, to a lesser extent, with a Gly or Ala residue produces more pronounced changes in receptor selectivity, as seen in peptides B, C, F, G, H and I, which show an appreciable increase in affinity toward μ-opioid receptors. The elimination of the side-chain carboxylic acid group, which is supposedly involved in intramolecular hydrogen bonds with the N-terminal amino group, and substitution of Asp^4 residue with an Aib residue, which is known to induce folded or helical structures, or with more flexible residues (Gly or Ala), must be considered responsible for the sharp decrease in δ-opioid receptor selectivity.

The conformational analysis reveals that the C-terminal tripeptide fragment appears far more flexible than the N-

Table 5. Relevant NOE-derived distances [Å] and corresponding average calculated distances derived from DG

[Tyr^5]DELT, peptide A		measured	calculated
Tyr5 Hα	Val6 NH	2.32	2.22
Tyr1 Hα	Ala2 NH	2.32	2.53
Asp4 NH	Tyr5 NH	2.50	2.56
Phe3 Hα	Tyr5 NH	2.50	2.92
Val6 NH	Val6 Hβ	2.50	2.64
Tyr1 Hβ2	ala2 NH	2.50	2.21
Tyr1 Hβ1	ala2 NH	2.50	2.23
Phe3 NH	Phe3 Hβ1	2.50	2.50
ala2 Hα	Phe3 NH	2.57	2.45
Tyr5 NH	Val6 NH	3.00	3.34
Phe3 Hβ2	Asp4 NH	3.00	2.78
Tyr5 Hβ1	Val6 NH	3.00	3.46
Gly7 NH	Gly7 Hα1	2.74	2.75
Gly7 NH	Gly7 Hα2	2.74	2.74
Tyr5 NH	Tyr5 Hβ1	3.00	3.14
Tyr5 Hβ2	Val6 NH	3.00	2.96
Phe3 NH	Phe3 Hβ2	3.00	3.29
Phe3 NH	Asp4 NH	4.00	4.20
Tyr1 Hδ1	ala2 NH	3.32	3.29
Tyr1 Hδ2	ala2 NH	3.40	3.34

[Aib^{2,4},Tyr^5]DELT, peptide C		measured	calculated
Phe3 Hα	Aib4 NH	2.39	2.40
Aib4 NH	Aib4 βCH3	2.68	2.68
Tyr1 Hα	Aib2 NH	2.50	2.28
Aib2 NH	Aib2 βCH3	2.35	2.68
Aib4 NH	Tyr5 NH	2.52	2.59
Tyr5 Hα	Val6 NH	2.60	2.47
Aib2 βCH3	Phe3 NH	3.35	3.35
Aib2 NH	Phe3 NH	2.94	2.73
Tyr5 NH	Val6 NH	3.00	3.02
Tyr5 NH	Tyr5 Hβ1	3.00	3.07
Aib4 βCH3	Tyr5 NH	3.35	3.36
Val6 Hα	Gly7 NH	2.56	2.44
Val6 NH	Val6 Hα	2.74	2.81

[Aib^2]DELT, peptide E		measured	calculated
Aib2 NH	Phe3 NH	3.03	2.98
Asp4 Hα	Val5 NH	2.48	2.43
Asp4 NH	Asp4 Hα	2.62	2.64
Phe3 Hα	Asp4 NH	2.76	2.40
Tyr1 Hα	Aib2 NH	2.36	2.31
Val6 Hα	Gly7 NH	2.48	2.45
Tyr1 NH	Tyr1 Hα	2.52	2.55
Val5 Hα	Val6 NH	2.36	2.35
Val6 NH	Val6 Hα	2.80	2.63
Val5 NH	Val5 Hα	2.76	2.62
Phe3 NH	Phe3 Hα	2.79	2.75
Val6 NH	Val6 Hβ	3.02	2.85
Asp4 NH	Val5 NH	2.98	2.86
Asp4 NH	Gly7 NH	3.20	3.41

[Aib^{2,4}]DELT, peptide F		measured	calculated
Aib2 NH	Aib4 NH	3.15	3.41
Aib4 NH	Val5 NH	2.32	2.41
Val5 NH	Val6 NH	3.85	2.95
Val6 NH	Gly7 NH	2.83	2.71
Tyr1 Hα	Aib2 NH	2.72	2.64
Val6 Hα	Gly7 NH	3.03	3.03
Phe3 Hα	Aib4 NH	2.53	2.53
Val6 NH	Val6 Hα	2.87	2.74
Aib4 NH	Aib4 βCH3	2.77	2.78
Aib2 NH	Aib2 βCH3	2.80	2.82

Table 5. *(Continued)*

Val5 NH	Val5 Hα	2.71	2.72
Val5 NH	Val5 Hβ	3.03	3.06
Val5 Hα	Val6 NH	2.63	2.56
	[Aib[4]]DELT, peptide G	measured	calculated
Tyr1 Hδ1	ala2 NH	3.41	3.42
Tyr1 Hδ2	ala2 NH	3.42	3.40
Aib4 NH	Val5 NH	2.54	2.48
Val5 NH	Val6 NH	3.12	2.89
Val6 NH	Gly7 NH	3.37	3.45
Tyr1 Hα	ala2 NH	2.41	2.51
Tyr1 ββ′CH2	ala2 NH	3.76	3.74
ala2 βCH3	Phe3 NH	3.83	3.86
Phe3 NH	Phe3 Hβ2	2.31	2.33
Phe3 NH	Phe3 Hα	2.73	2.54
Phe3 NH	Phe3 Hα	2.53	2.54
ala2 NH	ala2 Hα	2.83	2.79
Phe3 Hα	Aib4 NH	2.54	2.58
Phe3 Hβ1	Aib4 NH	3.45	3.25
Aib4 NH	Aib4 βCH3	2.97	3.01
Phe3 Hβ2	Aib4 NH	3.85	3.91
Val6 Hα	Gly7 NH	2.33	2.21
Gly7 NH	Gly7 Hαα′	2.65	2.64
Val6 Hβ	Gly7 NH	3.18	3.32
Tyr1 NH	Aib4 βCH3	3.65	3.70
Val5 Hα	Val6 NH	2.53	2.61
Val6 NH	Val6 Hα	2.99	2.90
Val5 NH	Val5 Hα	2.79	2.69
Aib4 βCH3	Val5 Hα	4.98	5.12
Aib4 β′CH3	Val6 Hβ	4.65	4.69

Table 6. Average distance between the aromatic rings in position 1 and 3 (R1−R3) and between the NH in position 1 and the aromatic ring in position 3 (N_1H−R3) for all compounds analyzed with DG techniques

peptide sequence	distance [Å] R1−R3	distance [Å] N_1H−R3
A. [Tyr[5]]DELT	12	10
C. [Aib[2,4],Tyr[5]]DELT	< 9	7.6
E. [Aib[2]]DELT	10−11	9
F. [Aib[2,4]]DELT	10−11	7
G. [Aib[4]]DELT	8	5

terminal sequence. The low-temperature coefficient of the NH of residue 5 in both peptides F and G can be explained on the basis of the DG analysis because of the NH interaction with acceptor groups. The structures of the peptides examined by DG calculations (Peptides A, C, E, F and G) can be categorized in terms of topological features into three different families. The first family of structures comprising peptides A and E (Figures 2 and 3) is characterized by a bent conformation around the residue at position 2. As a consequence, the two aromatic rings are at a distance of about 11 Å, and the terminal N_1H and the Phe[3] are about 10 Å apart. Substitution of ala[2] with Aib leads to a reduction in δ-opioid receptor affinity, showing that an optimum size is achieved with a single methyl group of the chiral residue, whereas bulkier groups tend to interfere with the onset of the required topology for δ-opioid receptor affinity. All these molecules are not recognized by μ-opioid receptors (Table 1). The slightly less selective C and F ligands belong to the second family of structures in which the aromatic rings of Tyr[1] and Phe[3] are still in close proximity (approximately 9 Å apart), but the methyl groups of the Aib[2] residue do not interact with the aromatic rings and the distance between the N_1H and the Phe[3] is about 7 Å. The topology of these rings grossly resembles that of the first family of structures and consequently these ligands

Figure 2. The 40 structures refined with the DADD procedure calculations superimposed with the backbone atoms N, C^{α}, C and O of [Tyr [5]]DELT (Peptide A)

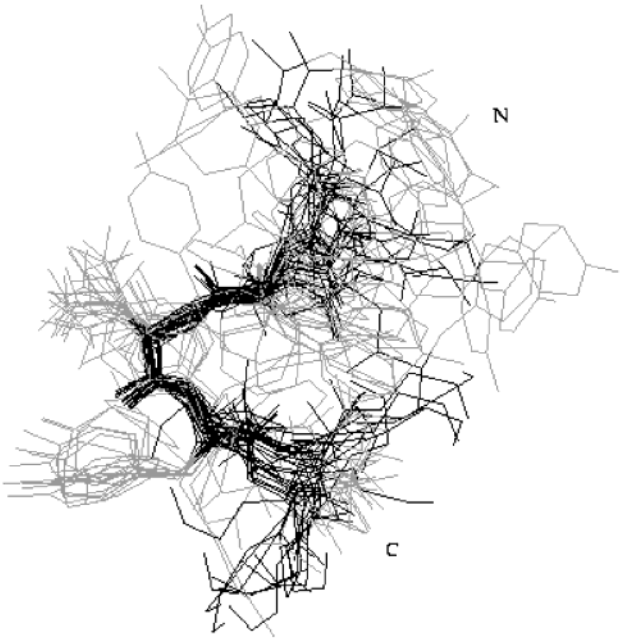


Figure 3. The 40 structures refined with the DADD procedure calculations superimposed with the backbone atoms N, C^{α}, C and O of [Aib [2]]DELT (Peptide E)

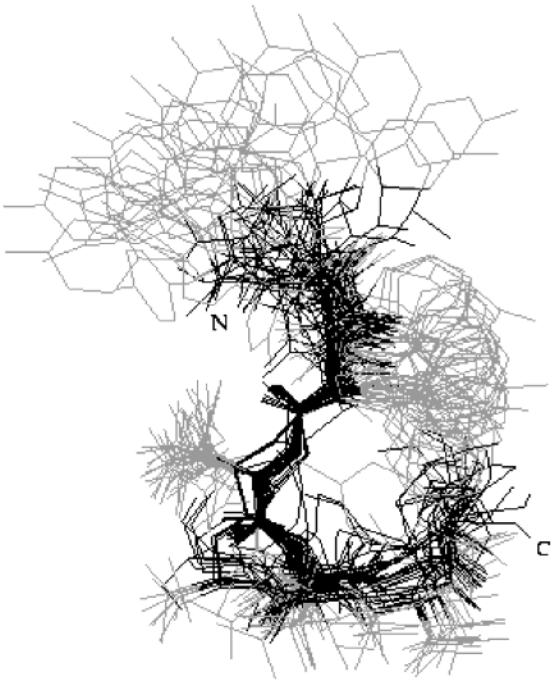


Figure 4. The 40 structures refined with the DADD procedure calculations superimposed with the backbone atoms N, C^{α}, C and O of [Aib ^{2,4},Tyr ^{5}]DELT (Peptide C)

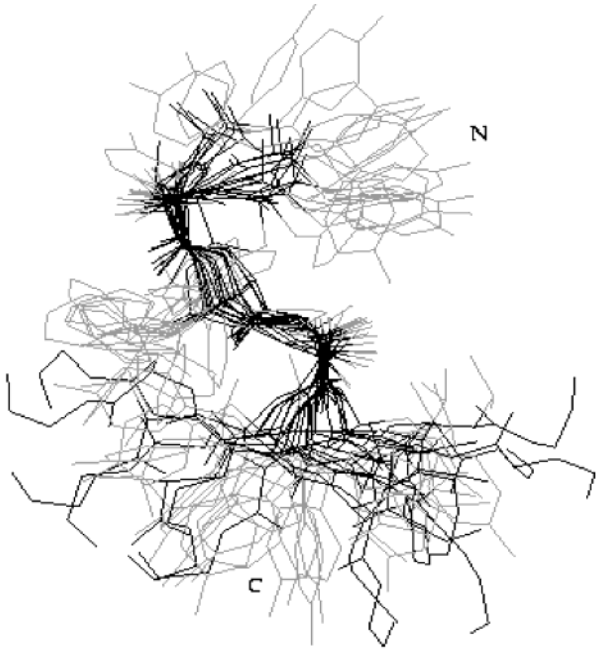
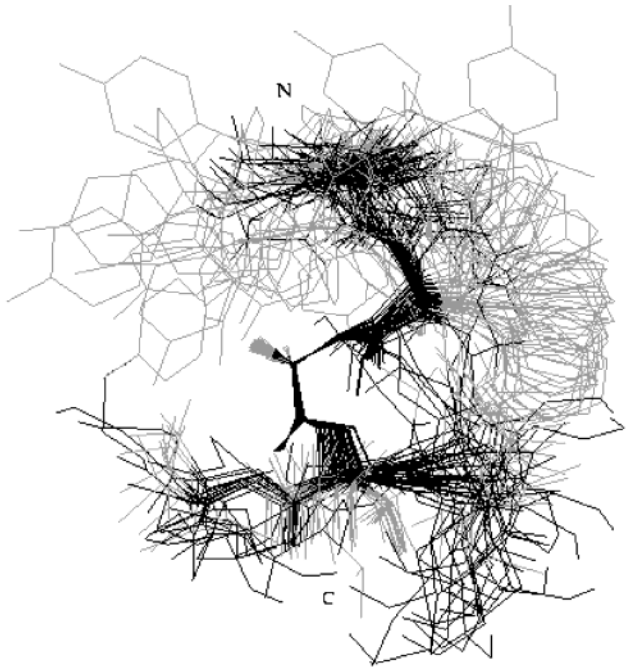


Figure 5. The 40 structures refined with the DADD procedure calculations superimposed with the backbone atoms N, C^{α}, C and O of [Aib ^{2,4}]DELT (Peptide F)



continue, even if to a reduced degree, to show selectivity for the δ-opioid receptor. The analysis of the structures reveals two bends centered about the Aib residues in position 2 and 4 giving rise to an S-shaped structure (Figures 4 and 5). Finally in the nonselective G ligand, the topological disposition of the two aromatic rings of Tyr^1 and Phe^3 nearly 8 Å is combined with a short average distance (about 5 Å) of

Figure 6. The 40 structures refined with the DADD procedure calculations superimposed with the backbone atoms N, C^{α}, C and O of [Aib ^{4}]DELT (Peptide G)

the N_1H and the Phe^3. The structures are characterized by one bend in the N-terminal part (Figure 6).

These structural findings are in agreement with the binding assays and the emerging topologies can explain the observed receptor affinities. In line with other reports on opioid peptide conformations,[45][46][47] our data can be interpreted in terms of a simple model for δ/μ-opioid receptor selectivity. Recognition at the δ-opioid receptor is achieved by peptide conformations in which the distance of the two aromatic rings of Tyr^1 and Phe^3 is at about 8 Å and the molecule assumes an overall flat ellipsoid shape. On the contrary, μ-receptor recognition is attained by topologies in which the aromatic rings of Tyr^1 and Phe^3 are at a distance larger than 12−13 Å and in which the bulk of the hydrophobic C-terminal domain extends away from the average plane of the rest of the molecule. In the series of peptides under investigation these two topologies have been obtained and to a certain extent modulated by the onset of specific secondary structures, stabilized by the substitution of constrained or in an opposite manner, flexible, residues at position 4.

Conclusion

We have studied a series of deltorphin I analogs designed to investigate the influence of substitutions in positions 2 and/or 4 in solution by NMR and DG techniques. We found that these compounds in solution are present with

a large number of conformers with no defined secondary structural elements. Nevertheless, the careful analysis of the average properties of these compounds indicate the presence of some distinct conformational preferences that can be related to the observed opioid receptor selectivities. Our experimental results are in good agreement with the model for the δ-opioid receptor-bound conformation proposed by Nikiforovich et al.[38][39] based only on energy calculations. Selectivity for the δ- and μ-opioid receptors can therefore be ascribed to the spatial arrangement of the aromatic moieties. It must be noted that substitutions in position 2 and 4 are important for the correct arrangement and must be taken into account in the design of δ-opioid receptor selective ligands.

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Experimental Section

Synthesis of Peptides: Nine deltorphin analogs were synthesized by the solid-phase method with an Applied Biosystems 430A automated peptide synthesizer (0.5-mmol scale, Fostercity, CA, USA), using *t*Boc chemistry on *p*-methyl-BHA resin. Amino acid derivatives, resins, all solvents and reagents used in solid-phase peptide synthesis were purchased from Applied Biosystems (Fostercity, CA, USA) with exception of *t*Boc-ala-OH, *t*Boc-met-OH and H-Aib-OH that were Bachem (Bubendorf, Switzerland) and Fluka (Buchs, Switzerland) products, respectively. *t*Boc-Aib-OH was synthesized as described.[48] The syntheses were performed with the classical protocols for the N-α-*t*Boc strategy. The peptides were cleaved from the resin with anhydrous HF (10 ml) and anisole (1 ml) as scavanger, for 30 min, at 0°C. HF was then removed by vacuum evaporation and the peptide resin completely dried under high vacuum on a KOH trap. The residue was washed in diethyl ether and extracted with 50% acetic acid. The solvent was removed in vacuum and the crude peptides were lyophilized from water.

Peptide Purification: The crude peptides were purified by reversed-phase HPLC as previously described.[34] H_2O and CH_3CN used for purification were HPLC grade and were supplied by Lab-Scan Analytica (Dublin, Ireland). − FAB mass spectrometry, performed with a VG ZAB 2SE double-focusing mass spectrometer (Manchester, U. K.), equipped with a cesium gun operating at 25 kV (2 μA), confirmed molecular weight, and the fragment analysis revealed the desired sequence. − Capillary electrophoresis was also performed with a Beckman System 2000 (San Ramon, CA, USA).

NMR Measurements: Samples for NMR measurements were prepared by dissolving 6−8 mg of each peptide in 1 ml of neat 99.98% [D_6]DMSO obtained from Aldrich (Milwaukee, Wi, USA). All spectra were acquired at 400 MHz with a VARIAN UNITY 400 spectrometer and processed with a SUN SPARC-STATION 330 using the standard VARIAN processing software. For 1D spectra 32−64 scans were acquired with 16384 data points and processed with 32768. All measurements were run at 298 K and all chemical shifts refer to internal tetramethylsilane. Double quantum filtered COSY,[49] NOESY[44] and HOHAHA[50] spectra were acquired in phase-sensitive mode using the States-Haberkorn method.[51]. − For all spectra 512 FIDs, each of 2048 complex data points, were collected. For each t_1 increment 32−64 transients were recorded. Zero-filling to yield a 2048 times 1024 real data matrix and multiplication with Gaussian function were done before transformation. NOESY spectra were acquired using a 300-ms mixing time. HOHAHA spectra were acquired using a 70-ms mixing time, the oscillatory coherence transfer was obtained by MLEV-17 pulse sequence in low-power transmitter mode.

Distance Geometry: The distance-geometry calculations were carried out with a modified version of the DGII program[52][53] following published procedures.[54][55][56][57] Experimentally determined distances (calculated using the isolated two-spin approximation), which were more restrictive than the geometric distance bounds (holonomic restraints),[54] were added to create a distance matrix. Distances between the upper and lower bounds, which also satisfy the triangular inequality law, were then chosen randomly using the random metrization procedure.[55] The structures were first embedded in four dimensions and then partially minimized using conjugate gradients followed by distance- and angle-driven dynamics (DADD),[58][59] the DADD simulation was carried out at 1000 K for 50 ps and then there was a gradual reduction in temperature over the next 30 ps. The DADD procedure utilizes the holonomic and experimental distance constraints plus a chiral penalty function for the generation of the violation "energy" and forces. A distance matrix was then calculated from each structure and the EMBED algorithm[53] used to calculate coordinates in 3D. The optimization and DADD procedure were then repeated. The metrization and refinement of 100 structures required approximately 12 h of cpu using a single processor of an SGI Indigo2 (R4400) at 180 MHz. Interactive modelings were performed using the Insight II program from Biosym Technologies.

Binding Assays: Binding to δ-, μ-, and κ-opioid sites was assayed in crude membranes preparations from rat (μ and δ) and guinea pig (κ) brain at pH = 7.4 in 50 mM Tris-HCl buffer as previously described.[60] The μ-binding site was selectively labeled with [^{3}H][ala^2,Phe-(Me)^4,Gly-ol^5]enkephalin ([3H]DAGO, Amersham, UK, 0.5 nM); the δ-binding site with (3,5-^{3}H-tyrosyl)-ala-Phe-Asp-Val-Val-Gly-NH_2, ([^{3}H][ala^2]deltorphin I, 0.3 nM); and the κ-binding site with (5a,7a,8b)-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-(3,4-^{3}H)benzeneacetamide, [^{3}H]U-69,593 (du Pont de Nemour, NEN Division, Dreiech, Germany, 1 nM). Competition curves were determined in triplicate and were calculated by fitting the displacement curves with the nonlinear regression programme LIGAND,[61] using one-site or two-site models. The results are given as means ± S. E. of at least six separate determinations.

Bioassays on Isolated Preparations: Preparations of the myenteric plexus-longitudinal muscle obtained from the small intestine of male guinea pigs (400−500 g) and preparations of vasa deferentia of mice were used for field stimulation with bipolar rectangular pulses of supramaximal voltage, as described previously.[62]

[1] C. Keys, P. Payane, P. Amsterdam, L. Toll, G. Loew, *Mol. Pharmacol.* **1988**, *33*, 528−536.
[2] P. A. Temussi, D. Picone, T. Tancredi, R. Tomatis, S. Salvadori, M. Marastoni, G. Balboni, *FEBS Lett.* **1989**, *247*, 283−288.
[3] H. O. C. ChewVillar, G. H. Loew, *Biopolymers* **1993**, *33*, 647−657.
[4] P. W. Schiller, G. Weltrowsha, T. M.-D. Nguyen, B. C. Wilkes, N. N. Chung, C. Lemieux, *J. Med. Chem.* **1992**, *35*, 3956−3961.
[5] M. Goodman, D. F. Mierke, *J. Am. Chem. Soc.* **1989**, *111*, 3489−3496.
[6] W. M. Kazmierski, R. D. Ferguson, A. W. Lipkowski, V. J. Hruby, *Int. J. Pept. Protein Res.* **1995**, *46*, 265−278.
[7] N. G. J. Delaet, P. M. F. Verheyden, D. Tourwe, G. Van Binst, P. Davis, T. F. Burks, *Biopolymers* **1992**, *32*, 957−969.
[8] M. A. Castiglione-Morelli, G. Saviano, P. A. Temussi, G. Bal-

doni, S. Salvadori, R. Tomatis *Biopolymers* **1989**, *28*, 129−138.
[9] Y. Ohno, M. Segawa, H. Ohishi, M. Doi, K. Kitamura, T. Ishida, M. Inoue, T. Iwashita, *Eur. J. Biochem.* **1993**, *212*, 185−191.
[10] L. J. Penkler, P. H. Van Rooyen, P. L. Wessels, *Int. J. Pept. Protein Res* **1993**, *41*, 261−274.
[11] P. W. Schiller, T. M.-D. Nguyen, N. N. Chung, C. Lemieux, *J. Med. Chem*. **1989**, *32*, 698−703.
[12] M. R. Ciajolo, G. Balboni, D. Picone, S. Salvadori, T. Tancredi, P. A. Temusssi, A. Tuzi, *Int. J. Pept. Protein Res.* **1995**, *46*, 134−138.
[13] P. S. Portoghese, M. Sultana, W. L. Nelson, P. Klein, A. E. Takemori, *J. Med. Chem*. **1992**. *35*, 4086−4091.
[14] M. A. Castiglione-Morelli, F. Lelj, A. Pastore, S. Salvadori, T. Tancredi, R. Tomatis, E. Trivellone, P. A. Temussi, *J. Med. Chem*. **1987**, *30*, 2067−2073.
[15] Y. Sasaki, A. Ambo, K. Suzuki, *Biochem. Biophys. Res. Comm*. **1991**, *180*, 822−827.
[16] L. H. Lazarus, S. Salvadori, P. Grieco, W. E. Wilson, R. Tomatis, *Eur. J. Med. Chem*. **1992**, *27*, 791−797.
[17] S. D. Bryant, S. Salvadori, M. Attila, L. H. Lazarus, *J. Am. Chem. Soc*. **1993**, *115*, 8503−8504.
[18] A. Misicka, A. W. Lipkowski, R. Horvath, P. Davis, T. H. Kramer, H. I. Yamamura, V. J. Hrubry, *Life Sci*. **1992**, *51*, 1025−1032.
[19] X. Qian, K. E. Kover, M. D. Shenderovich, B. S. Lou, A. Misicka, T. Zalewska, R. Horvath, P. Davis, E. J. Bilsky, F. Porreca, *J. Med. Chem*. **1994**, *37*, 1746−1757.
[20] M. Segawa, Y. Ohno, M. Doi, M. Inoue, T. Ishida, T. Iwashita, *Int. J. Pept. Protein Res.* **1994**, *44*, 295−304.
[21] S. D. Bryant, M. Attila, S. Salvadori, R. Guerrini, L. H. Lazarus, *Pept. Res.* **1994**, 7, 175−184.
[22] R. Schmidt, A. Kalman, N. N. Chung, C. Lemieux, C. Horvard, P. W. Schiller, *Int. J. Pept. Protein Res.* **1995**, *46*, 47−55.
[23] H. Ji, R. Zhang, X. Hu, L. Lai, X. Xu, *Biochem. Biophys. Res. Commun*. **1995**, *207*, 99−104.
[24] Y. Sasaki, T. Chiba, *J. Med. Chem*. **1995**, *38*, 3995−3999.
[25] M Segawa, Y. Ohno, M. Doi, T. Ishida, T. Iwashita, *Int. J. Pept. Protein Res.* **1995**, *46*, 37−46.
[26] K. Befort, L. Tabbara, D. Kling, B. Maigret, B. L. Kieffer, *J. Biol. Chem*. **1996**, *271*, 10161−10168.
[27] M. Naim, P. Nicolas, D. Baron, *Int. J. Pept. Protein Res.* **1996**, *47*, 353−360.
[28] V. J. Hruby, L. F. Kao, B. M. Pettitt, M. Karplus, *J. Am. Chem. Soc.* **1988**, *110*, 3351−3353.
[29] M. Froimowitz, *Biopolymers* **1990**, *30*, 1011−1025.
[30] T. Yamazaki, O. E. Said-Nejad, P. W. Schiller, M. Goodman, *Biopolymers* **1991**, *31*, 877−879.
[31] O. Crescenzi, P. Amodeo, G. Cavicchioni, R. Guerrini, D. Picone, S. Salvadori, T. Tancredi, P. A. Temussi, *J. Pept. Sci.* **1996**, *2*, 209−308.
[32] C. Chew, H. O. Villar, G. H. Loew, *Mol. Pharmacol.* **1991**, *39*, 502−510.
[33] G. Kreil, D. Barra, M. Simmaco, V. Erspamer, G. Falconieri-Erspamer, P. Melchiorri, L. Negri, C. Severini, R. Corsi, *Eur. J. Pharmacol.* **1989**, *162*, 123−128.
[34] V. Erspamer, P. Melchiorri, G. Falconieri-Erspamer, L. Negri, R. Corsi, C. Severini, D. Barra, M. Simmaco, G. Kreil, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 5188−5192.
[35] G. Balboni, M. Marastoni, D. Picone, S. Salvadori, T. Tancredi, P. A. Temussi, R. Tomatis, *Biochem. Biophys. Res. Commun.* **1990**, *169*, 617−622.
[36] P. Amodeo, A. Motta, T. Tancredi, S. Salvadori, R. Tomatis, D. Picone, G. Saviano, P. A. Temussi, *Pept. Res.* **1992**, *5*, 48−55.
[37] T. Tancredi, P. A. Temussi, D. Picone, P. Amodeo, R. Tomatis, S. Salvadori, M. Marastoni, V. Santagada, G. Balboni, *Biopolymers* **1991**, *31*, 751−760.
[38] G. H. Nikiforovich, V. J. Hruby, *Biochim. Biophys. Res. Commun.* **1990**, *173*, 521−527.
[39] G. H. Nikiforovich, V. J. Hruby, O. Parakash, C. A. Gehrig, *Biopolymers* **1991**, *31*, 941−955.
[40] D. Picone, P. Amodeo, O. Crescenzi, P. A. Temussi, S. Salvadori, G. Cavicchioni, L. H. Lazarus, T. Tancredi, in: *Peptides 1994, Proceedings of the 23rd European Peptide Symposium* (Ed.: H. L. S. Maia), ESCOM, Leiden, **1995**, pp. 630−631.
[41] P. Melchiorri, L. Negri, G. Falconieri-Erspamer, C. Severini, R. Corsi, M. Soaje, V. Erspamer, D. Barra, *Eur. J. Pharmacol.* **1991**, *195*, 201−207.
[42] I. L. Karle, M. Sukumar, P. Balaram, *Proc. Natl. Acad. Sci. USA* **1986**, 83, 9284−9288.
[43] C. Toniolo, E. Benedetti, *Macromolecules* **1991**, *24*, 4004−4009.
[44] D. Neuhaus, M. Williamson, *The Nuclear Overhauser Effect*, VCH Publishers Inc., New York, **1989**.
[45] M. Goodman, S. Ro, T. Yamazaki, G. Osapay, A. Toy-Palmer, Q. Zhu, A. Polinsky, in *Peptide Chemistry 1992* (Ed.: N. Yanaihara), ESCOM, Leiden **1993**, pp. 239−242.
[46] D. Duchesne, M. Naim, P Nicolas, D. Baron, *Biochem. Biophys. Res. Commun.* **1993**, *195*, 630−636.
[47] S. D. Bryant, S. Salvadori, M. Attila, L. H. Lazarus, *J. Am. Chem. Soc*. **1993**, *115*, 8503−8504.
[48] M. Bodanszky, A. Bodanszky, *The Practice of Peptide Synthesis*, Springer-Verlag, Berlin, **1984**, *21*, 20.
[49] U. Piantini, O. W. Sorensen, R. R. Ernst, *J. Am. Chem. Soc.* **1982**, *104*, 6800−6801.
[50] A. Bax, D. G. Davis, *J. Magn. Reson.* **1985**, *65*, 355−360.
[51] D. J. States, R. A. Haberkorn, D. J. Ruben, *J. Magn. Reson.* **1982**, *48*, 286−292.
[52] T. F. Havel, DISGEO, Quantum Chemistry Exchange Program Exchange No. 507, Indiana University, **1986**.
[53] G. M. Crippen, T. F. Havel, Distance Geometry and Molecular Conformation, Research Studies Press LTD., Somerset, England; John Wiley, New York, **1988**.
[54] T. F. Havel, *Prog. Biophys. Molec. Biol.* **1991**, *56*, 43−78.
[55] N. A. J. van Nuland, J. Grötzinger, K. Dijkstra, R. M. Scheek, G. T. Robillard, *Eur. J. Biochem.* **1992**, *210*, 881−891.
[56] R. M. Scheek, A. E. Torda, J. Kenmink, W. F. van Gunsteren in *Computational Aspects of the Study of Biological Macromolecules by Nuclear Magnetic Resonance*, Plenum Press, New York, **1991**, pp. 209−217.
[57] J. Kenmink, C. P. M. van Mierlo, R. M. Scheek, T. E. Creighton, *J. Mol. Biol.* **1993**, *230*, 312−322.
[58] R. Kaptein, R. Boelens, R. M. Scheek, W. F. van Gunsteren, *Biochemistry* **1988**, *27*, 5389−5395
[59] R. M. Scheek, W. F. van Gunsteren, R. Kaptein, *Methods in Enzymology*, vol. 177 (Eds.: N. J. Oppenheimer, T. L. James), Academic Press, New York, **1989**, pp. 204−218
[60] L. Negri, G. Falconieri-Erspamer, C. Severini, P. Melchiorri, V. Erspamer, *Proc. Natl. Acad. Sci.* **1992**, *89*, 7203−7207.
[61] P. J. Munson, D. Rodbard, *Anal. Biochem.* **1980**, *107*, 220−239.
[62] M. Broccardo, V. Erspamer, G. Falconieri-Erspamer, G. Improta, G. Linari, P. Melchiorri, P. C. Montecucchi, *Br. J. Pharmacol.* **1981**, *73*, 625−631.

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