Structural Consequences of Metal Complexation of *cyclo*[Pro-Phe-Phe-Ala-Xaa]$_2$ Decapeptides**

Gabriella Saviano,[a] Filomena Rossi,[b] Ettore Benedetti,[b] Carlo Pedone,[b] Dale F. Mierke,[c] Annamaria Maione,[d] Giancarlo Zanotti,[d] Teodorico Tancredi,[e] and Michele Saviano*[b]

Abstract: The conformational features of both free and Ca^{2+}-complexed *cyclo*-[Pro-Phe-Phe-Ala-Xaa]$_2$ (with Xaa = Glu(OtBu), Lys(ClZ), Leu, and Ala) in solution have been determined by NMR spectroscopy and extensive distance–geometry calculations. The decapeptides are conformationally homogeneous in solution and show common structural features in their free and complexed forms. The structures of the free form contain only *trans* peptide bonds and are topologically similar to the structure of gramicidin-S, folded up in two antiparallel extended structures, stabilized by interstrand hydrogen bonds, and closed at both ends by two β-turns. In contrast, the Ca^{2+}-complexed peptides present two *cis* peptide bonds and are generally similar to those observed for the metal-complexed forms of antamanide and related analogues, folded into a saddle shape with two β-turns. The Glu(OtBu)-, Leu-, and Lys(ClZ)-containing peptides examined here maintain the biological activity of the cyclolinopeptide A in their ability to competitively inhibit cholate uptake. The natural antamanide and cyclolinopeptide A are both able to inhibit the uptake of bile salts into hepatocytes. They share the same postulated active sequence Pro-Phe-Phe. Based on our structural results, we conclude that the ability to adopt a global conformation, characterized by a clear amphipathic separation of hydrophobic and hydrophilic surfaces, is an important feature for the functioning of this class of peptides.

Keywords: antamanide · cyclolinopeptide A · ion binding · NMR spectroscopy · peptides

Introduction

Transport of metal ions in biological systems represents an important function in life. One way of transporting charged atoms through lipophilic systems, such as membranes, is by encapsulation of the metal ion into a peptide molecule that has external lipophilic properties. Peptides are well suited for this task since they can fold and twist around the metal n such a manner as to provide carbonyl oxygen atoms in a proper orientation for the coordination of the metal ion. This property is attributed to the flexibility of the peptide molecule about the N–C^{α} and C^{α}–C′ bonds in the backbone (ϕ and ψ dihedral angles), as well as bonds in the side chains.

The ability of peptides to interact with metals can be enhanced by cyclization, a commonly employed method for reducing the conformational freedom of the molecule. Cyclizing the peptide in the appropriate manner can result in the shape required to complex metal ions, either by a partial or a complete ion encapsulation.[1–3] Because of this, cyclic peptides are considered good candidates to act as bioregulators in the transport of ions across biological membranes. Characterization of the conformational changes in cyclic peptides induced upon complexation should provide the necessary insight to design molecules with improved ion affinity and selectivity and therefore enhanced biological function.

The natural antamanides, (AA, *cyclo*[Val-Pro-Pro-Phe-Phe-Val-Pro-Pro-Phe-Phe])[4] and cyclolinopeptide A (CLA,

[a] Dr. G. Savianoa
Facoltà di Scienze MM.FF.NN., Università degli Studi del Molise
Via Mazzini 8, 86170 Isernia (Italy)

[b] Dr. M. Saviano, Dr. F. Rossi, Prof. E. Benedetti, Prof. C. Pedone
Centro di Studio di Biocristallografia del C.N.R.
Dipartimento di Chimica, Università di Napoli "Federico II"
Via Mezzocannone 4, 80134 Napoli (Italy)
Fax: (+39) 081-5514305
E-mail: saviano@chemistry.unina.it

[c] Prof. D. F. Mierke
Department of Molecular Pharmacology
Division of Biology & Medicine
Department of Chemistry, Brown University
Providence, RI 02912 (USA)

[d] Dr. A. Maione, Dr. G. Zanotti
Centro di Chimica del Farmaco del CNR
Università di Roma "La Sapienza"
Piazzale A.Moro, Roma (Italy)

[e] Dr. T. Tancredi
Istituto Chimica MIB del CNR
Via Toiano 6, 80072 Arco Felice (Italy)

[**] Amino acids are in L configuration unless indicated as D.

0947-6539/01/0706-1176 $ 17.50+.50/0

cyclo[Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val])[5] are both able to inhibit the uptake of bile salts into hepatocytes.[6] CLA and AA share the same postulated active sequence Pro-Phe-Phe.[7] This feature is thought to represent the minimum necessary prerequisite for bioactivity[8–11] (an additional proline, preceding this triplet of residues, increases the biological activity of both CLA and AA).

Studies of AA and related analogues have illustrated a strong correlation between bioactivity and ability to complex ions.[12, 13] This capability could reflect the readiness of the molecule to assume the proper conformation for binding of the substrate or, as suggested by Karle et al.,[14] the metal ion could induce the AA molecule to adopt the proper shape. The differences in the conformations between the complexed and uncomplexed forms of AA and related molecules have been studied in the solid state by X-ray structure analysis[15] and in solution by NMR spectroscopy.[16] For the uncomplexed state in crystals, only one conformation has been observed which is characterized by two *cis* Pro–Pro peptide bonds. The uncomplexed conformation is quite distinct from the Li^+- or Na^+-complexed structure, although the two *cis* peptide bonds are preserved. The major difference is the change from the elongated, relatively planar backbone ring in the uncomplexed molecule to the folded ring in the alkali complex brought about by the rearrangement of the carbonyl groups of the peptide backbone to form the metal complex. This arrangement gives rise to different intramolecular hydrogen bonding schemes: a pair of the $4 \rightarrow 1$-type hydrogen bonds in the metal AA complexes and a pair of $5 \rightarrow 1$-intramolecular hydrogen bonds in the uncomplexed structures.

Cyclolinopeptide A is an homodetic nonapeptide, isolated from linseed,[17] which has been characterized in solution[18, 19] and in the solid state.[20] In solution the high degree of flexibility of CLA and the use of polar solvents always prevented the observation of a single conformer. Only in chloroform at 214 K was it possible to solve the solution structure of CLA by NMR spectroscopy.[20] Under these conditions, the structural features of CLA are practically identical to the solid-state form. This conformation, however, cannot be regarded as the bioactive one since it corresponds to that exhibited, both in the solid state and in solution at room temperature, by the less active *cyclo*[Pro-Pro-Phe-Phe-Aib-Aib-Ile-D-Ala-Val].[21] Addition of bivalent metal ions to the CLA solution induces drastic structural changes. In particular, Ba^{2+} ions stabilize a well-defined equimolar cation/peptide complex. The complexed peptide structure is characterized by all-*trans* peptide bonds and two clearly distinct surfaces, a polar one hosting the Ba^{2+} ion and a predominantly apolar face.[22]

Recently we reported the structural analysis, both in the solid state and in solution,[23] of the homodetic cyclopeptide *cyclo*[Pro-Phe-Phe-Ala-Glu(O*t*Bu)]$_2$ (*c*DECA), a synthetic analogue of CLA. Despite both CLA and *c*DECA exhibiting similar CD_{50} (peptide concentration required to inhibit the cholate uptake in hepatocytes by 50%) values of 0.8 and 0.6 mM, respectively (K. Ziegler, Institut für Pharmakologie und Toxikologie, Giessen, Germany, private comunication), their conformational preferences were observed to be very different. The structure of *c*DECA was found to be much more rigid, with respect to CLA, with almost no changes observed between the conformation in solution and that in the solid state.

To study how metal ions can modulate the conformation of cDECA, we have undertaken the structural characterization of this decapeptide upon complexation with metal ions by employing high-resolution NMR spectroscopy and distance–geometry calculations. Moreover, we report results from the conformational study of the free and calcium-complexed forms of three new analogues of *c*DECA, in which the Glu(OtBu) group is replaced by Lys(ClZ), Leu, and Ala, respectively. These modifications all lead to a reduction in biological activity, with respect to *c*DECA: CD_{50} values of 1.8 mM, 2.2 mM, and 14 mM, for the Lys(ClZ)-, Leu-, and Ala-containing compounds, respectively (K. Ziegler, private comunication).

Results and Discussion

NMR analysis: Each of the peptides examined here, vary in terms of the size and nature of the amino acid at the Glu(OtBu) position in the parent decapeptide (peptide **1**), *cyclo*[ProPhePheAlaGlu(O*t*Bu)]$_2$: Lys(ClZ) (peptide **2**), Leu (peptide **3**), and Ala (peptide **4**). The structural features of the molecules were determined from analysis of the chemical shift values, quantitative evaluation of $^3J_{NH,CH}$ vicinal coupling constants, temperature coefficients of the NH resonances, and the proton–proton distances based on the NOEs. The examination of the free form of the peptides was carried out at 300 K, while for the metal-complexed forms, a range of temperatures (245–300 K) was employed. All spin systems were identified by means of total correlation spectroscopy (TOCSY)[24] and double quantum-filtered correlated spectroscopy (DQF-COSY)[25] experiments. Rotating frame nuclear Overhauser enhancement spectroscopy (ROESY)[26] and nuclear Overhauser enhancement spectroscopy (NOESY)[27] spectra were used to sequentially assign the resonances of identical residues. Under the experimental conditions utilized and at 500 MHz, the decapeptides are in the extreme narrowing limit, displaying positive NOEs in the NOESY spectra.

Table 1, Table 2, and Table 3 contain $^3J_{NH,CH}$, NH temperature coefficients, and interproton distances measured for the free forms of the three new decapeptides, respectively. These data allowed us to propose common structural features characterizing all four peptides (including the parent com-

Table 1. ^3J(NH,CH) values [Hz] of the uncomplexed *cyclo*[Pro^1-Phe^2-Phe^3-Ala^4-Xaa^5]$_2$ peptide in acetonitrile at room temperature (Xaa = Glu(OtBu) (**1**), Lys(ClZ) (**2**), Leu (**3**), Ala (**4**)).

Residue	Peptide number				ϕ
	1	**2**	**3**	**4**	[°]
Phe^2	6.1	6.4	6.5	7.4	−80 to −60
Phe^3	10.8	11.1	10.6	10.6	−120
Ala^4	7.5	7.4	7.9	7.8	−160 to −140, −80
Xaa^5	11.2	11.0	10.6	11.0	−120

  0947-6539/01/0706-1177 $ 17.50+.50/0

Table 2. NH Temperature coefficients [$\Delta\delta/\Delta T$ (ppb/K)] of the uncomplexed *cyclo*[Pro[1]-Phe[2]-Phe[3]-Ala[4]-Xaa[5]]$_2$(Xaa = Glu(OtBu) (**1**), Lys(ClZ) (**2**), Leu (**3**), Ala (**4**)).

Residue	Peptide number			
	1	**2**	**3**	**4**
Phe[2]	−4.6	−4.4	−5.0	−6.2
Phe[3]	−0.6	−1.0	−0.6	−1.7
Ala[4]	−2.4	−2.8	−3.7	−3.1
Xaa[5]	−0.6	−2.0	−1.0	−1.8

Table 3. Interproton distances of the uncomplexed *cyclo*[Pro[1]-Phe[2]-Phe[3]-Ala[4]-Xaa[5]]$_2$ (Xaa = Glu(OtBu) (**1**), Lys(ClZ) (**2**), Leu (**3**), Ala (**4**); from NOESY spectra in acetonitrile, 300 K, τ_m = 200 ms).

Distance[a]	Peptide number			
	1	**2**	**3**	**4**
d[NHPhe[3]–NHAla[4]]	2.6	2.6	2.5	2.6
d[αPhe[3]–NHAla[4]]	3.1	3.2	–	3.2
d[αAla[4]–NHAla[4]]	2.3	2.3	2.2	2.3
d[αAla[4]–NH Xaa[5]]	2.5	2.5	2.7	2.6
d[αGlu[5]–NH Xaa[5]]	3.2	2.9	3.0	3.0
d[δPro[1]–NHPhe[2]]	3.0	[b]	–	2.9
d[αPhe[2]–NHPhe[2]]	2.9	2.9	2.9	[b]
d[βPhe[2]–NHPhe[2]]	3.2	3.0	–	[b]
d[β'Phe[2]–NHPhe[2]]	2.7	2.6	2.6	2.6
d[αPhe[3]–NHPhe[3]]	3.1	3	3.1	3.0
d[β'Phe[3]–NHPhe[3]]	2.6	2.6	2.7	2.6
d[αGlu[5]–δPro[1]]	2.3	2.2	2.3	2.2
d[αGlu[5]–δ'Pro[1]]	3.2	3	3.2	3.3
d[βPhe[3]–β'Phe[3]]	**1.8**	**1.8**	**1.8**	**1.8**
d[αPro[1]–NHPhe[2]]	3.7	–	–	[b]
d[NHPhe[3]–NHXaa[5]]	3.4	–	–	–
d[NHAla[4]–NHXaa[5]]	–	3.2	3.0	2.7
d[αPhe[2]–βPhe[2]]	2.7	2.7	2.5	2.6
d[αPhe[2]–β'Phe[2]]	3	2.9	2.7	2.9
d[αPhe[3]–βPhe[3]]	2.6	2.6	2.7	2.5
d[αPhe[3]–β'Phe[3]]	3	3	2.7	2.9
d[NHPhe[2]–NHPhe[3]]	[b]	[b]	2.8	[b]
d[αPhe[2]–NHPhe[3]]	–	–	2.8	[b]

[a] Reference distance *d*[βPhe[3]-β'Phe[3]] = 1.8. [b] Peaks superimposed

pound previously investigated).[23] The essential features include: 1) a complete symmetry of the two pentameric moieties, 2) a backbone with all peptide bonds adopting a *trans* conformation, 3) two antiparallel extended structures, stabilized by interstrand Xaa → Phe hydrogen bonds, closed at both ends by two β-turns of type I centered on the two -Xaa-Pro-Phe-Phe- moieties.

The topological features of the three anologues are almost identical to that of the Glu(O*t*Bu) parent peptide[23] and are characterized by two well-separated surfaces, a hydrophilic one consisting of the peptide backbone and a hydrophobic one above the peptide backbone plane, formed by the protruding side chains of all of the residues. We have previously reported a similar amphipathic separation for the barium-complexed form of cyclolinopeptide A.[22]

The ability of the four cyclic peptides to form complexes with sodium and calcium ions was then examined. The addition of sodium ions does not alter the appearance of the NMR spectra of all four peptides and therefore was taken to indicate that no sodium complex was formed. Addition of calcium ions, on the contrary, enormously influences all spectra, and it was possible to monitor the formation of equimolar cation/peptide complexes. Figure 1 shows the

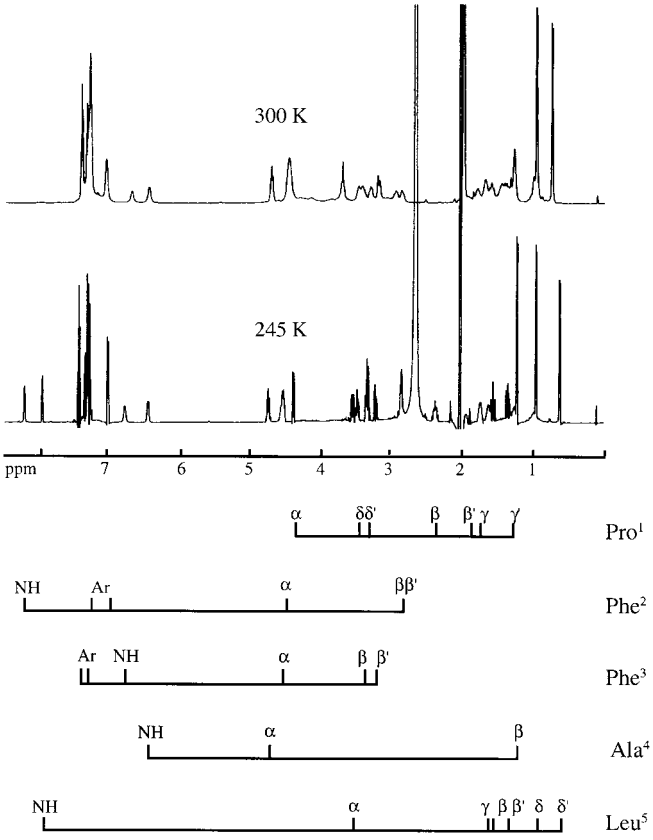

Figure 1. 500 MHz ^{1}H NMR spectra of Ca^{2+}-complexed *cyclo*[Pro-Phe-Phe-Ala-Leu]$_2$ at 300 and 245 K. The subspectra of all amino acid residues are referred to the low-temperature spectrum. Greek letters identify different types of protons of each residue.

spectra that result upon addition of calcium to peptide **3** (Xaa = Leu). The room-temperature spectrum shows resonances lacking fine structure and the NH groups of residues 2 and 5 merge into the noise. Lowering the temperature to 245 K freezes the molecular conformation and/or the exchange with solvent molecules, leading to a finely structured spectrum. This residual conformational freedom does not imply any *cis*–*trans* isomerization of Pro residues. In fact, NOESY spectra at 300 and 245 K show that all Pro residues are in the same conformation (see above). Similar trends are observed for all four calcium-complexed peptide analogues.

As for the free peptides, the Ca^{2+}-complexed forms of the cyclodecapeptides in acetonitrile adopt symmetric conformations, as indicated by the appearance of only one set of sharp signals (see Figure 1). In particular, in the low-field region of the spectra, it is possible to distinguish only four doublets attributable to amide protons (see Table 4). This behavior is confirmed by the remaining regions of the spectra, indicating that the structure of the cyclic dimers in solution is perfectly symmetric, only one set of resonances is obtained for the protons of the *i* and the *i*+*5* residues. Since the amino acids are indistinguishable, in the NMR analysis section of this paper

0947-6539/01/0706-1178 $ 17.50+.50/0 *Chem. Eur. J.* **2001**, *7*, No. 6

Table 4. Chemical shifts of the Ca^{2+} complex of *cyclo*[Pro-Phe-Phe-Ala-Glu(O*t*Bu)]$_2$ in acetonitrile at 245 K (^{13}C values in parentheses).

	Pro[1]	Phe[2]	Phe[3]	Ala[4]	Glu[5]
NH	–	8.60	7.0	6.35	8.20
αCH	5.16	4.24	4.45	4.80	3.86
	(59.3)	(59.4)	(55.5)	(47.0)	(53.0)
βCH	2.44–2.23	3.03–2.61	3.28–3.16	1.24	2.10–1.75
	(32.7)	(37.0)	(35.2)	(19.3)	(24.0)
γCH	1.85–1.34	–	–	–	2.50–2.36
	(21.9)	–	–	–	(30.1)
δCH	3.51–3.39				
	(47.3)				
others	–	H2,6 6.5	H2,6 7.35	–	1.49(O*t*Bu)
		H3,5 7.17	H3,5 7.48		
		H4 7.25	H4 7.39		

we use the abbreviation Pro[1,6] to indicate both Pro[1] and Pro[6] residues, and similarly for the other amino acids. Interestingly, all diastereotopic proton pairs exhibit distinct chemical shifts (Table 4) as Ca^{2+} complexes.

Upon formation of the ion complex, some of the proton resonances undergo large variations. For example, in all of the free peptides, Ala[4] NH is the resonance at lowest field, while in the calcium-complexed spectra it is the amide at highest field. All Ala[4] CαH groups, on the other hand, shift in exactly the opposite direction. Phe[2] NH, which is the highest field NH resonance in all of the free peptides, becomes the lowest field resonance in the complexed forms. Even the CαH protons of Pro[1] and Xaa[5] change chemical shifts considerably upon complexation, with Pro[1] shifting 1 ppm to lower field, while Xaa[5] shifts 1.2 ppm to higher field. These variations in chemical shift suggest considerable structural modification of the peptides upon calcium ion complexation.

In addition to the chemical shift changes, a number of NOEs are found to be unique to the complexed forms of the peptides. The first relevant structural effect of the calcium complexation concerns the backbone conformation: the two Xaa[10]–Pro[1] and Xaa[5]–Pro[6] peptide bonds, which are *trans* in the free cyclodecapeptides, adopt a *cis* orientation upon cation complexation. A comparison of the ROESY spectra in the $\alpha-\delta$ region of peptide **4** in its free and complexed forms at 245 K is shown in Figure 2. The Ala[5]–Pro[6] and Ala[10]–Pro[1] $d_{\alpha\delta}$ and $d_{\alpha\delta'}$, indicative of *trans* peptide bonds, characterize the free peptide; in contrast, Ala[5]–Pro[6] and Ala[10]–Pro[1] $d_{\alpha\alpha}$ confirm a *cis* arrangement of this bond in the complexed peptide. In fact, the Xaa–Pro peptide bonds of all four analogues were found to be *cis*, as indicated by strong Xaa–Pro $d_{\alpha\alpha}$ NOEs and the observation of a large difference (ca. 10 ppm) in the ^{13}C chemical shift between β- and γ-carbon atoms of the proline residues.[28]

The NH–CαH coupling constants were measured both directly from the 1D and DQF-COSY spectra. The experimental $^3J_{NH,C\alpha H}$ values measured for the complexed form of the peptides, with the corresponding allowed ϕ ranges[29] are given in Table 5. The $^3J_{NH,C\alpha H}$ value for the Ala[4,9] residues is large enough to be compatible with a narrow set of ϕ angle values, centered around -120°, corresponding to a *trans* arrangement of the HN-CαH moiety. All $^3J_{NH,C\alpha H}$ values of

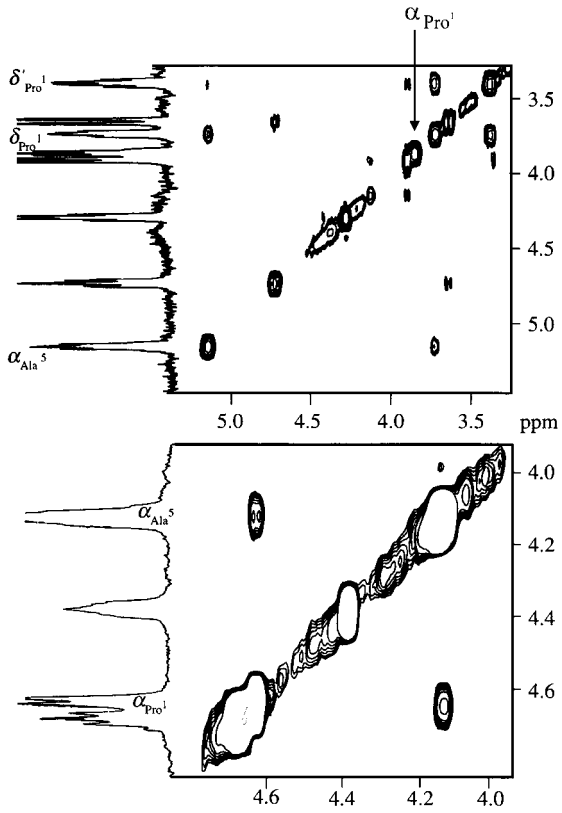

Figure 2. Comparison of the α-δ region of the ROESY spectra at 245 K of *cyclo*[Pro-Phe-Phe-Ala-Ala]$_2$ in its free (top) and complexed forms (bottom). The free peptide spectrum clearly shows the Ala[5]–Pro[6] and Ala[10]–Pro[1] $d_{\alpha\delta}$ and $d_{\alpha\delta'}$, indicative of *trans* peptide bonds. In the calcium-complexed peptide spectrum the presence of Ala[5]–Pro[6] and Ala[10]–Pro[1] $d_{\alpha\alpha}$ indicates *cis* peptide bonds.

Table 5. $^3J_{NH,CH}$ values [Hz] of the Ca^{2+} complex of *cyclo*[Pro-Phe-Phe-Ala-Glu(O*t*Bu)]$_2$ in acetonitrile at 245 K.

Residue	Peptide number				ϕ
	1	**2**	**3**	**4**	[°]
Phe[2]	3.0	3.0	3.3	3.0	$-60, 0, 120$
Phe[3]	8.4	8.3	4.7	7.9	$-140, -80$
Ala[4]	9.8	8.9	8.4	9.1	-120
Xaa[5]	5.2	6.5	4.7	7.9	$-160, -80, -60$

Phe[2,7] and Phe[3,8], with the exception of that of Phe[3,8] residue in peptide **3** (Xaa = Leu), are compatible with ϕ values characteristic of residues in the *i+1* and *i+2* position of a type I β-turn (-60° and -90°).

Interproton distances were calculated from NOESY spectra collected with mixing times of 200 and 350 ms. The values for the parent compound **1**, are given in Table 6. Short distances between the NH protons of Phe[2] and Phe[3] and between the NH protons of Phe[3] and Ala[4] confirm the presence of two type-I β-turns ranging from Pro[1,6] to Ala[4,9]. Similar NOEs were found for complexes **2** and **3**; complex **4**, on the the other hand, shows slightly longer distances for the same pairs of protons.

Combining all of the structural parameters derived from the NMR analysis allowed the development of a preliminary

Table 6. Relevant NOE-derived distances [Å] for the Ca^{2+} complex of *cyclo*[Pro-Phe-Phe-Ala-Glu(O*t*Bu)]$_2$ in acetonitrile at 245 K, and corresponding average calculated distances derived from distance–geometry calculations.

NOEs	Measured	Calculated	NOEs	Measured	Calculated
NHPhe^{2,7}–NHPhe^{3,8}	2.63	2.82	NHPhe^{3,8}-NHAla^{4,9}	2.53	2.53
αPro^{1,6}–NHPhe^{2,7}	2.23	2.08	αPhe^{2,7}–NHPhe^{2,7}	2.61	2.80
βPhe^{2,7}–NHPhe^{2,7}	2.35	2.50	β'Phe^{2,7}–NHPhe^{2,7}	2.8	2.92
βPro^{1,6}–NHPhe^{2,7}	3.2	3.29	β'Pro^{1,6}–NHPhe^{2,7}	2.5	2.54
αAla^{4,9}–NHGlu^{5,10}	2.1	2.16	αGlu^{5,10}–NHGlu^{5,10}	2.68	2.63
γGlu^{5,10}–NHGlu^{5,10}	2.82	2.82	γ'Glu^{5,10}–NHGlu^{5,10}	2.56	2.56
αPhe^{3,8}–NHPhe^{3,8}	2.63	2.84	αPhe^{2,7}–NHPhe^{3,8}	3.15	3.06
βPhe^{3,8}–NHPhe^{3,8}	2.78	2.84	β'Phe^{3,8}–NHPhe^{3,8}	2.51	2.54
αAla^{4,9}–NHAla^{4,9}	2.68	2.90	αPhe^{3,8}–NHAla^{4,9}	3.1	3.11
αGlu^{5,10}–αPro^{1,6}	2.08	2.23	βPro^{1,6}–αPro^{1,6}	2.28	2.34
β'Pro^{1,6}–αPro^{1,6}	2.53	2.75	βPhe^{2,7}–αPhe^{2,7}	2.4	2.43
βPhe^{2,7}–αPhe^{2,7}	2.95	3.04	βPhe^{3,8}–αPhe^{3,8}	2.48	2.42
β'Phe^{3,8}–αPhe^{3,8}	2.59	2.99			

model of the peptide structures for the Ca^{2+}/cyclodecapeptide complexes. The model is characterized by two Xaa–Pro *cis* peptide bonds and two type-I β-turns stabilized by two intramolecular hydrogen bonds between the NH group of Ala^4 and the C=O group of Pro^1, and between the NH group of Ala^9 and the C=O group of Pro^6. The conformation of the Ca^{2+}/peptide complex differs from both the solid-state and solution structures of the free decapeptide,[23] which presents all-*trans* peptide bonds and an intramolecular hydrogen-bonding pattern similar to that observed for gramicidin S.[30]

Computational results: The distance–geometry calculations, used to build the three-dimensional structure of the *c*DECA complex matching all of the experimental data, illustrated that a single conformer could satisfy all of the NMR-derived structural parameters (Figure 3). This structure exhibits a twofold symmetry with two repeating sequences Pro^1-Phe^2-Phe^3-Ala^4-Glu(O*t*Bu)^5 linked together by turns with the Phe-Phe residues in the corners. This conformer is consistent with the distance restraints, temperature coefficients, and coupling constants. A comparison of the average distance–geometry structural parameters (i.e. the corresponding dihedral angles) with the corresponding values derived from the NMR experiments (the experimental interproton NOE distances) shows a good agreement (see Table 6 and 7).

A stereoview of the average structure of Ca^{2+}/*c*DECA, as obtained from the distance–geometry calculations, which illustrates all of the intramolecular hydrogen bonds, is presented in Figure 4b. As predicted, the structure possess two *cis* peptide bonds (between Glu(O*t*Bu)^{5,10}–Pro^{1,6}) two type I β-turns with the Phe-Phe residues in the $i+1$ and $i+2$ position, respectively. In addition, the carbonyl oxygen atoms of Pro^{1,6}

Table 7. Torsional angles [°] of the Ca^{2+}-complexed *cyclo*[Pro-Phe-Phe-Ala-Glu(O*t*Bu)]$_2$ as obtained from distance–geometry calculations.

Residue	ϕ	ψ	ω
Pro^{1,6}	– 79.8	164.2	– 177.5
Phe^{2,7}	– 56.6	– 19.9	– 179.6
Phe^{3,8}	– 91.8	– 16.1	– 179.7
Ala^{4,9}	– 124.2	– 162.1	170.9
Glu(O*t*Bu)^{5,10}	– 44.5	134.4	– 26.7

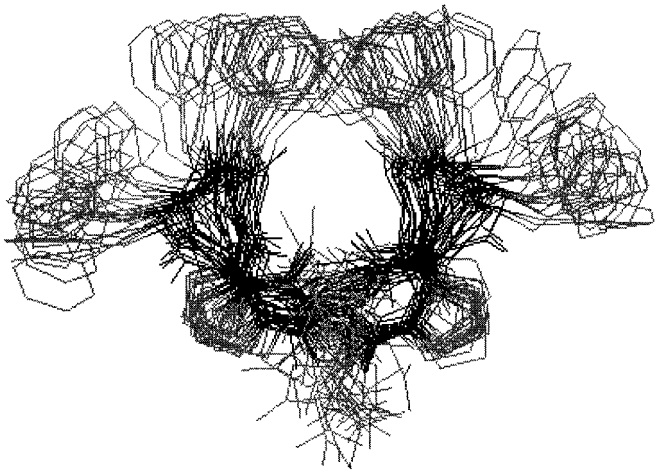

Figure 3. The 80 structures refined with the distance- and angle-driven dynamics (DADD) procedure superimposed with the backbone atoms N, C$^\alpha$, C, and O of the Ca^{2+}-complexed *cyclo*[Pro-Phe-Phe-Ala-Glu(O*t*Bu)]$_2$ as obtained from distance–geometry analysis

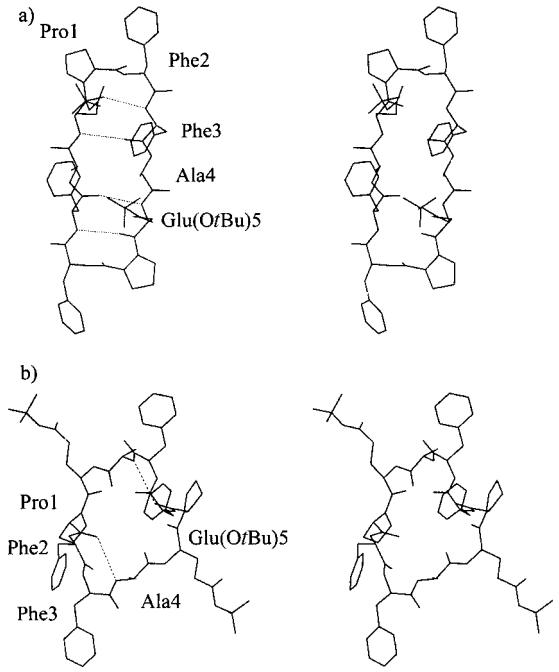

Figure 4. Stereoview of a) the metal-free *c*DECA structure as obtained by X-ray structure analysis[23] and b) average solution structure of the Ca^{2+}-complexed *cyclo*[Pro-Phe-Phe-Ala-Glu(O*t*Bu)]$_2$ as obtained from distance–geometry analysis.

0947-6539/01/0706-1180 $ 17.50+.50/0 *Chem. Eur. J.* **2001**, *7*, No. 6

and Ala[4,9] are arranged in an ideal geometry for complexation with the metal ion.

For comparison purposes, the X-ray structure of the metal-free *c*DECA (equivalent to the structure observed in solution) is depicted in Figure 4a. The major differences with the structure of the complexed peptide, namely the two *cis* peptide bonds (Table 8) and a remarkably different intra-

Table 8. Torsional angles [°] of *cyclo*[Pro-Phe-Phe-Ala-Glu(O*t*Bu)]$_2$ as obtained from X-ray structure analysis.

Residue	ϕ	ψ	ω	$\chi^{1,1}$
Pro[1]	– 58	127	172	
Phe[2]	65	18	179	– 51
Phe[3]	– 156	123	180	– 169
Ala[4]	– 133	138	– 179	
Glu(O*t*Bu)[5]	– 149	176	– 175	65
Pro[6]	– 61	– 32	– 172	
Phe[7]	– 94	– 15	– 178	– 77
Phe[8]	– 166	162	170	58
Ala[9]	– 128	143	176	
Glu(*O*tBu)[10]	– 147	177	– 179	69

molecular hysrogen-bonding scheme, are clearly visible. In the X-ray structure of *c*DECA, the central amide bonds of the β-turns are oriented upward and downward. Upon complexation, the orientation of the amides rearranges to accomodate the ion. Interestingly, the structure of the free peptide is characterized by a much more compact shape, in which most of the oxygen atoms used to bind Ca^{2+} in the complex are now involved in intramolecular hydrogen bonds. This rearrangement gives rise to two clearly distinct surfaces, a polar one hosting the Ca^{2+} ion and a predominantly apolar one (Figure 5); the overall shape of the molecule is similar to that observed for the Ba^{2+}–cyclolinopeptide A complex (Figure 6).

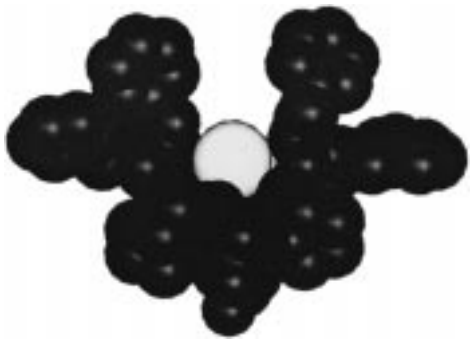

Figure 5. Molecular model of the Ca^{2+}-complexed *cyclo*[Pro-Phe-Phe-Ala-Glu(O*t*Bu)]$_2$. The metal ion is represented as a gray sphere.

Mapping out the surface exposed region of the final conformation indicates a good agreement with an aqueous environment. Therefore, the results in acetonotrile have biological relevance, and in fact, we believe that the observations reported here will be more strongly borne out in aqueous solution, in which a stronger tendency for a hydrophobic collapse can be supposed.

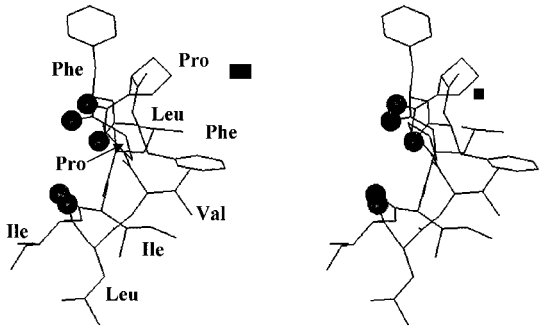

Figure 6. Stereodrawing of the solution structure of Ba^{2+}-complexed cyclolinopeptide A.[22] The oxygen atoms probably involved in the metal coordination are represented as black spheres.

Finally, it is important to note that the solution structures of the Ca^{2+}-*c*DECA complex and the crystal structures of Li^+ complexes of [Val[8]-Ala[9]]-AA[2a] (Figure 7) and perhydro-AA[2b] are structurally similar. The root mean square (rms) deviation of the backbone atom superposition is 0.69 and 0.74 Å for [Val[8]-Ala[9]]-AA and perhydro-AA Li^+ complexes, respectively. In addition, the structures present the same intramolecular hydrogen-bonding scheme.

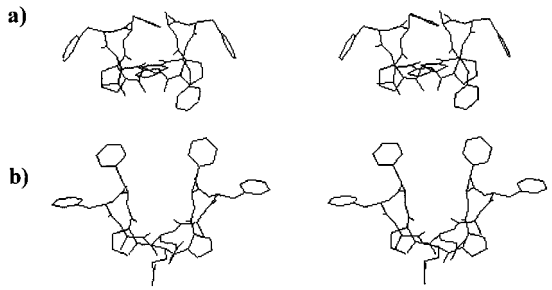

Figure 7. Stereodrawing of a) the crystal structure of the Li^+ complex of [Val[8]-Ala[9]] antamanide[2a] and of b) the average structure of Ca^{2+}-complexed *cyclo*[Pro-Phe-Phe-Ala-Glu(O*t*Bu)]$_2$

Notably, the orientation of the Phe side chains in the solution structure was not determined except by distance–geometry procedure. Then, they should be folded against the folded backbone to form a hydrophobic envelope around the polar interior as found for the Li^+ complex of [Val[8]-Ala[9]]-AA[2a], in order for the complex to be compatible with the membrane environments. In fact, the crystal structure of the only inactive metal complex (Li^+-perhydroAA),[2b] in which Phe residues are replaced with Cha (cyclohexylalanyl) residues, shows the Cha side chains extended away from the backbone.

Conclusion

The conformational behavior of the calcium-complexed cyclodecapeptide *cyclo*[Pro-Phe-Phe-Ala-Glu(O*t*Bu)]$_2$ (*c*DECA), a biologically active synthetic analogue of CLA with similar CD_{50} values, has been investigated by NMR spectroscopy and distance–geometry calculations. We have also analyzed the free and calcium-complexed forms of three new

analogues of *c*DECA, in which the Glu(O*t*Bu) residue is replaced by Lys(ClZ), Leu, and Ala residues, respectively. These analogues, with the exception of the Ala-containing peptide, have higher CD_{50} values similar to that of *c*DECA.

The *c*DECA is characterized by different conformational behavior in its free and complexed forms in solution. In particular, the structure of the complexed peptide presents two *cis* peptide bonds, while the free peptide shows all-*trans* peptide bonds. Both the solid-state and solution structures of ion-free *c*DECA show two C_{10} ring structures involving residues Glu(O*t*Bu)^{10}–Phe^{3} and Glu(O*t*Bu)^{5}–Phe^{8} interconnected by two nearly extended strands.[23] This structure is topologically similar to that of gramicidin-S[30] but differs from those of antamanide and related analogues, both in their free[31] and metal-complexed forms.[2, 3, 16]

Notably, all decapeptides examined here are conformationally homogeneous in solution and show common structural features in their free and complexed forms. All cyclodecapeptides, with the exception of *cyclo*[Pro-Phe-Phe-Ala-Ala]$_2$, exhibit CD_{50} values close to that of the parent natural compound CLA. Considering the chemical composition of these peptides it appears evident that the overall hydrophobic character of the molecules plays a role in their activity. In fact, the long and hydrophobic side chains enhance bioactivity, as the corresponding CD_{50} values clearly show.

From these results, it seems possible to infer that metal ions are able to modulate the conformation of this class of compounds. Moreover, these data further support the hypothesis that the biological activity of this class of peptides (as competitive inhibitors of cholate uptake) is related to their ability to adopt a global shape characterized by a clear separation of hydrophobic and hydrophilic surfaces upon metal-ion complexation.

Experimental Section

Synthesis: The protected linear pentapeptides Boc-Lys(ClZ)-Pro-Phe-Phe-Ala-O*t*Bu, Boc-Leu-Pro-Phe-Phe-Ala-O*t*Bu, and Boc-Ala-Pro-Phe-Phe-Ala-O*t*Bu (Boc = *tert*-butyloxycarbonyl) were prepared in solution by adopting the mixed anhydride coupling method. The removal of Boc and O*t*Bu groups was achieved by treatment of the protected pentapeptides (1 mmol) with trifluoroacetic acid (TFA; 20 mL) for 1 h at room temperature under stirring. The solvent was evaporated in vacuo and the residue thoroughly treated with diethyl ether.

Cyclization reaction, general procedure: The deprotected linear pentapeptide trifluoroacetates (1 mmol) were dissolved in a mixture of DMF (120 mL) and THF (100 mL) and treated with isobutylchloroformate (136 mg, 1 mmol) at $-10\,^{\circ}C$ under stirring. After 10 min stirring at $-10\,^{\circ}C$ a precooled solution of *N*-methylmorpholine (0.22 mL) in DMF (450 mL) and THF (250 mL) was added, and the reaction mixture stirred for 1 h at $0\,^{\circ}C$ and then overnight at room temperature. The organic solvent was evaporated under reduced pressure and the residue was taken up in CH_2Cl_2 (100 mL). It was then washed with saturated $NaHCO_3$, $KHSO_4$ (0.5 M), and water, dried over Na_2SO_4, and evaporated. The residues obtained were chromatographed on a silica gel column (100×25 cm) in $CHCl_3$–CH_3OH (95:5 v/v) as eluant. The fractions containing the cyclic compounds were collected and the products further purified by preparative HPLC using a C_{18} reverse-phase column (2.2×25 cm) eluted by linear gardient H_2O 0.1% TFA and CH_3CN 0.1% TFA.

From linear precursor NH_2-Lys(ClZ)-Pro-Phe-Phe-Ala-OH both monomeric cyclopentapeptide and dimeric cyclodecapeptide were obtained in 3.0% and 20% yield, respectively. $R_f = 0.40$ in $CHCl_3$–CH_3OH (95:5 v/v); FAB-MS: *m*/*z* 579 for *cyclo*[Pro-Phe-Phe-Ala- Lys(ClZ)]. $R_f = 0.45$ in $CHCl_3$–CH_3OH (95:5 v/v); FAB-MS: *m*/*z* 1518 as expected for *cyclo*[Pro-Phe-Phe-Ala-Lys(ClZ)]$_2$ (peptide **2**).

From linear precursors NH_2-Leu-Pro-Phe-Phe-Ala-OH and NH_2-Ala-Pro-Phe-Phe-Ala-OH only the corresponding dimeric cyclopeptides were obtained in 12% and 20% yields respectively.

For *cyclo*[Pro-Phe-Phe-Ala-Leu]$_2$ (peptide **3**) $R_f = 0.35$ in $CHCl_3$–CH_3OH (95:5 v/v); FAB-MS: *m*/*z* 1150.

For *cyclo*[Pro-Phe-Phe-Ala-Ala]$_2$ (peptide **4**) $R_f = 0.28$ in $CHCl_3$–-CH_3OH (95:5 v/v); FAB-MS: *m*/*z* 1066.

The amino acid analysis was satisfactory, the use of chiral stationary phases for determination of the optical purity of the amino acids showed the absence of racemization during the peptide synthesis.

NMR measurements: All samples were prepared by dissolving the appropriate amount of peptide in deuterated acetonitrile (99.98% Carlo Erba, Milano Italy) up to a final concentration of 2 mM. For ion complexation, the peptides were titrated with hexahydrate sodium and calcium perchlorate (Carlo Erba Milano Italy). Acetonitrile was used because of the high solubility of the peptides in this solvent and in order to compare our results with literature data for similar compounds.

NMR spectra were recorded on a Bruker AMX-500 spectrometer in the 245–300 K range. All chemical shifts, in parts per million (ppm), are referred to the methyl resonance of acetonitrile ($\delta = 2.0$ ppm for ^1H spectra; $\delta = 1.5$ ppm for ^{13}C spectra). One-dimensional (1D) NMR spectra were typically acquired using 32–48 scans with 32,768 data size. Pulse programs of the standard Bruker software library were used for the two-dimentional (2D) experiments. All 2D spectra were acquired in the phase-sensitive mode, with quadrature detection in both dimensions, by use of the time proportional phase increment (TPPI).[32] Typically 256 experiments of 48 scans each were performed: relaxation delay, 1 s; size: 2048; 6024 Hz spectral width in F2; zero-filling to 1024 in F1; squared cosine or Gaussian multiplication were used in both dimensions before the Fourier transformation. Mixing times of 70 and 150 ms were used for TOCSY[24] and ROESY,[26] respectively. NOESY[27] experiments were run at mixing times from 150 to 350 ms. Nuclear Overhauser effects (NOEs) of potential diagnostic value were measured at 200 and 350 ms. Interatomic distances were calculated by the method of Esposito and Pastore,[33] using the distance between the two β protons of Phe^2 (1.80 Å) for calibration. Temperature coefficients of the amide protons were measured in the 280–310 K range.

Distance–geometry calculations: The distance–geometry calculations were carried out by a modified version of the DGII program[34, 35] according to published procedures.[36–39] Experimentally determined distances (calculated using the isolated two-spin approximation) that were more restrictive than the geometric distance bounds (holonomic restraints)[36] were merged 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.[37] The structures were first embedded in four dimensions and then partially minimized using conjugate gradients followed by distance and angle driven dynamics (DADD).[40, 41] 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[35] used to calculate coordinates in three dimensions. The optimization and DADD procedure were then repeated. The metrization and refinement of 100 structures required approximately 12 h of cpu time using a single processor on a SGI Indigo2 (R4400) at 180 MHz. Interactive modeling was performed using the Insight II program from Molecular Simulations, Inc.

Acknowledgement

We gratefully acknowledge M.U.R.S.T., the Ministry of University and Scientific and Technological Research, and the National Research Council of Italy, C.N.R. for their continuous and generous support. D.F.M. thanks the Research Corporation for support through the Cottrell Scholars Program. Mr. Giuseppe Perretta is also acknowledged for technical assistance.

  0947-6539/01/0706-1182 $ 17.50+.50/0 *Chem. Eur. J.* **2001**, *7*, No. 6

[1] I. L. Karle, J. Karle, T. Wieland, W. Burgermeister, H. Faulstich, B. Witkop, *Proc. Nat. Acad. Sci. USA* **1973**, *70*, 1836 – 1840.
[2] a) I. L. Karle, *J. Am. Chem. Soc.* **1974**, *96*, 4000 – 4006;b) I. L. Karle, *Proc. Natl Acad Sci. USA* **1985**, *82*, 7155 – 7159.
[3] I. L. Karle, *Biochemistry* **1974**, *13*, 2155 – 2162.
[4] T. Wieland in *Peptides of Poisonous Amanita Mushrooms*, Springer, New York, **1986**.
[5] H. P. Kaufmann, A. Tobshirbel, *Chem. Ber.* **1959**, *92*, 2805 – 2809.
[6] H. Kessler, M. Gehrke, A. Haupt, M. Klein, A. Müller, K. Wagner, *Klin. Wochenschr.* **1986**, *64*, 74 – 78.
[7] K. Ziegler, M. Frimmer, H. Kessler, A. Haupt, *Biochim. BioPhys. Acta* **1988**, *945*, 263 – 272.
[8] T. Wieland, M. Nassal, W. Kramer, G. Fricker, V. Bichel, G. Kurz, *Proc. Nat. Acad. Sci. USA* **1984**, *81*, 5232 – 5236.
[9] T. Wieland in *Chemistry and Biology of Peptides* (Ed.: J. Meienhofer), Ann Arbor Science, Ann Arbor MI, **1972**, pp. 377 – 396.
[10] I. L. Karle, T. Wieland, *Int. J. Peptide Protein Res.* **1987**, *29*, 596 – 603.
[11] H. Kessler, J. W. Bats, C. Griesinger, S. Koll, M. Will, K. Wagner, *J. Am. Chem. Soc.* **1986**, *110*, 1033 – 1049.
[12] T. Wieland, L. Lapatsanis, J. Fasel, W. Kurz, *Liebigs Ann. Chem.* **1971**, *747*, 194 – 206.
[13] T. Wieland, H. Faulstich, W. Burgermeisten, *Biochem. Biophys. Res. Comm.* **1972**, *47*, 984 – 992.
[14] I. L. Karle, J. L. Flippen-Anderson, T. Wieland, *Int. J. Peptide Protein Res.* **1989**, *33*, 422 – 427.
[15] I. L. Karle, E. Duesler, *Proc. Nat. Acad. Sci. USA* **1977**, *74*, 2602 – 2606.
[16] P. Amodeo, G. Saviano, G. Borin, A. Calderan, P. Ruzza, T. Tancredi, *J. Peptide Res.* **1998**, *51*, 180 – 187.
[17] H. P. Kaufmann, A. Tobshirbel, *Chem. Ber.* **1959**, *92*, 2805 – 2809.
[18] T. Tancredi, G. Zanotti, F. Rossi, E. Benedetti, C. Pedone, P. A. Temussi, *Biopolymers* **1989**, *28*, 513 – 523.
[19] M. Saviano, M. Aida, G. Corongiu, *Biopolymers* **1991**, *31*, 1017 – 1024.
[20] B. Di Blasio, F. Rossi, E. Benedetti, V. Pavone, C. Pedone, P. A. Temussi, G. Zanotti, T. Tancredi, *J. Am. Chem. Soc.* **1989**, *111*, 9089 – 9098.
[21] B. Di Blasio, F. Rossi, E. Benedetti, V. Pavone, M. Saviano, C. Pedone, G. Zanotti, T. Tancredi, *J. Am. Chem. Soc.* **1992**, *114*, 8277 – 8283.
[22] T. Tancredi, E. Benedetti, M. Grimaldi, C. Pedone, F. Rossi, M. Saviano, P. A. Temussi, G. Zanotti, *Biopolymers* **1991**, *31*, 761 – 767.
[23] G. Zanotti, F. Rossi, M. Saviano, T. Tancredi, G. Saviano, A. Maione, M. Filizola, B. Di Blasio, C. Pedone, *J. Am. Chem. Soc.* **1995**, *117*, 8651 – 8658.
[24] A. Bax, D. G. Davis, *J. Magn. Reson.* **1985**, *65*, 355 – 360.
[25] M. Rance, O. W. Sørensen, G. Bodenhausen, K. Wagner, R. R. Ernst, K. Wütrich, *Biochem. Biophys. Res. Commun.* **1983**, *116*, 479 – 485.
[26] A. A. Bothner-By, R. L. Stephens, J. Lee, C. D. Warren, R. W. Jeanloz, *J. Am. Chem. Soc.* **1984**, *106*, 811 – 813.
[27] J. Jeener, B. H. Meyer, P. Bachman, R. R. Ernst, *J. Chem. Phys.* **1979**, *71*, 4546 – 4553.
[28] E. D. Dorman, F. A. Bovey, *J. Org. Chem.* **1973**, *38*, 2379 – 2383.
[29] V. F. Bystrov, *Prog. NMR Spectroscopy* **1976**, *10*, 41 – 81.
[30] S. E. Hull, R. Karlsson, P. Main, M. M. Woolfson, E. J. Dodson, *Nature* **1978**, 206 – 207.
[31] R. Brüschweiler, M. Blackledge, R. R. Ernst, *J. Biomol. NMR* **1991**, 3 – 11.
[32] D. Marion, K. Wuthrich, *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967 – 971.
[33] G. Esposito, A. Pastore, *J. Magn. Reson*. **1988**, *76*, 331 – 336.
[34] T. F. Havel, DISGEO, *Quantum Chemistry Exchange Program Exchange N° 507*, Indiana University, **1986**.
[35] G. M. Crippen, T. F. Havel, *Distance Geometry and Molecular Conformation*, Wiley, New York, **1988**.
[36] T. F. Havel, *Prog. Biophys. Molec. Biol.* **1991**, *56*, 43 – 78.
[37] N. A. J. van Nuland, J. Grotzinger, K. Dijkstra, R. M. Scheek, G. T. Robillard, *Eur. J. Biochem.* **1992**, *210*, 881 – 891.
[38] 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.
[39] J. Kenmink, C. P. M. van Mierlo, R. M. Scheek, T. E. Creighton, *J. Mol. Biol.* **1993**, *230*, 312 – 322.
[40] R. Kaptein, R. Boelens, R. M. Scheek, W. F. van Gunsteren, *Biochemistry* **1988**, *27*, 5389 – 5395.
[41] 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.

Received: July 27, 2000 [F2631]