Bioactive Peptides: X-ray and NMR Conformational Study of [Aib^{5,6}-D-Ala^{8}]cyclolinopeptide A

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Abstract: The conformational analysis of [Aib^{5,6}-D-Ala^{8}]cyclolinopeptide A, in the solid state and solution, has been carried out by X-ray diffraction and NMR spectroscopy. The structure of the orthorhombic form, obtained from methanol–water mixture [a = 29.92 (3) Å, b = 19.85 (3) Å, c = 9.90 (1) Å, space group $P2_12_12_1$, Z = 4], shows the presence of five intramolecular N–H···O=C hydrogen bonds, with formation of one C_{17} ring structure, one α-turn (C_{13}), one γ-turn (C_7), and two β-turns (C_{10}, one of type III and one of type I). The Pro^1-Pro^2 peptide unit is cis (ω = 9°), all others are trans. The conformational study in solution by NMR spectroscopy indicates that, even at room temperature, the peptide is conformationally homogeneous; the structure determined is almost identical to that observed in the solid state. The solution study reveals, also, that the constraints imposed by the two Aib and D-Ala residues are particularly strong, because the NMR conformational parameters are only slightly affected by wide temperature variations and salt addition.

Cyclolinopeptide A (CLA), a homodetic cyclic nonapeptide of sequence cyclo(Pro^1-Pro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9), isolated from linseed,[1] belongs to a class of natural peptides endowed with cytoprotective properties against toxic agents such as phalloidine.[2] There are evidences that its ability to inhibit the uptake of cholate by hepatocytes is linked to well defined sequential features included in the -Pro^1-Pro^2-Phe^3-Phe^4- moiety.[3,4] Recently we have reported a detailed study of the CLA structure, both in the solid state and in apolar solution at 214 K.[5] In addition, other crystalline modifications of CLA have shown that, regardless of the different cocrystallized solvent molecules, the overall struture, including the side chains, is maintained.[6] The results of these studies show that the -Leu^5-Ile^6-Ile^7-Leu^8- sequence is characterized by ϕ and ψ values appropriate for residues occupying corner positions of type III and type I β-turns; in addition, the Leu^8 residue shows ϕ and ψ values characteristic of a left-handed α-helix, typically allowed to D residues. On the basis of these observations, we have designed a CLA analogue with a modified sequence in the portion of the molecule not directly involved in the interaction with the receptor,[7] by substituting two Aib residues at positions 5 and 6 and a D-Ala residue at position 8. These changes in the sequence should act in making the peptide backbone more rigid than that of CLA.

The following are the results of the conformational analysis of the peptide cyclo(Pro^1-Pro^2-Phe^3-Phe^4-Aib^5-Aib^6-Ile^7-D-Ala^8-Val^9), henceforth called CLAIB, carried out at room temperature, both in solution by NMR and in the solid state by X-ray diffraction.

Experimental Section

Synthesis. Amino acid precursors were obtained by NOVA Biochem and Fluka. Z-Aib has been prepared according to ref 8. Solvents were reagent grade; other organic reagents, commercially available, were used without further purification. Amino acid couplings were carried out either by the mixed anhydride method (MA) or by the dicyclohexylcarbodiimide method (DCCI).

Benzyloxycarbonyl group (Z) was used to protect the amino terminus, and a methyl ester (OMe) or a *tert*-butyl ester (OtBu) were used to protect the carboxyl terminus.

Removal of the Z group was achieved with H_2 over a palladium/carbon catalyst, while removals of the OMe and OtBu groups were achieved with aqueous sodium hydroxide in methanolic solution and with 1 N HCl in acetic acid, respectively.

Identity and purity of intermediates were checked with thin-layer chromatography (TLC) using silica gel plates from Merck, mass spectrometry, and nuclear magnetic resonance spectroscopy.

A detailed description of the synthesis of the cyclic nonapeptide is given below starting with the protected linear nonapeptide precursor obtained by condensation of an hexapeptide (Z-D-Ala-L-Val-L-Pro-L-Pro-L-Phe-L-Phe-OH) and a tripeptide (H-Aib-Aib-L-Ile-OtBu).

Z-D-Ala-L-Val-L-Pro-L-Pro-L-Phe-L-Phe-Aib-Aib-L-Ile-OtBu. To a stirred solution of 1.17 g of Z-D-Ala-L-Val-L-Pro-L-Pro-L-Phe-L-Phe-OH (1.44 mmol) in 50 mL of methylene chloride were added at 0 °C 1 equiv of dicyclohexylcarbodiimide, 2 equiv of hydroxybenzotriazole, and 0.417 g of H-Aib-Aib-L-Ile-OtBu (1.44 mmol), dissolved in 15 mL of methylene chloride.

After 1 h stirring at 0 °C the reaction mixture was left overnight at room temperature. The precipitated dicyclohexylurea (DCU) was filtered, and the solution was extracted with 0.5 M $KHSO_4$, then with 5% $NaHCO_3$, and finally with water. The organic phase was dried over Na_2SO_4 and evaporated in vacuo. The residue was chromatographed on 70 × 3 cm column of silica gel by eluting it with a chloroform:methanol (95:5 v/v) mixture. Mass spectrum measurement (FAB) of $(M - H)^+$ gave a mass of 1096 uma, as expected. The amino acids analysis was satisfactory.

Tfa·D-Ala-L-Val-L-Pro-L-Pro-L-Phe-L-Phe-Aib-Aib-L-Ile-OH. To remove the Z group, 1.04 g (0.907 mmol) of protected nonapeptide were hydrogenated in 50 mL of methanol over 0.2 g of 10% Pd on charcoal. Hydrogenation was completed after 4 h at room temperature. The catalyst was filtered off and the solution was evaporated. The residue was dissolved in 10 mL of trifluoroacetic acid to remove the OtBu group. After 2 h at room temperature the solution was evaporated in vacuo to dryness and the residue was triturated with ether. The precipitate was filtered, washed with ether and dried in vacuo over KOH pellets to give 0.924 g of pure Tfa salt, as shown by TLC: yield 95%; R_f 0.3 in *n*-butanol–acetic acid–water (4:1:1 v/v).

Cyclo(L-Pro-L-Pro-L-Phe-L-Phe-Aib-Aib-L-Ile-D-Ala-L-Val). To a stirred solution of 0.750 g (0.7 mmol) of the Tfa salt of the deprotected nonapeptide in 85 mL of dimethylformamide and 55 mL of tetrahydro-

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Table I. Crystallographic Data for CLAIB

molecular formula	$C_{50}H_{71}N_9O_9 \cdot 2CH_3OH \cdot 2H_2O$
molecular wt, amu	1042.2
size, mm	0.20 × 0.17 × 0.56
crystal system	orthorhombic
space group	$P2_12_12_1$
Z, molecules/unit cell	4
a, Å	29.92 (3)
b, Å	19.85 (3)
c, Å	9.90 (1)
V, Å^3	5879.7
μ_r, cm^{-1}	6.6
d_{cal}, g/cm^3	1.178
d_{expt}, g/cm^3	1.17
radiation, Å	CuKα (λ = 1.5418)
measd reflections	6221
reflections with $I > 3.0\sigma(I)$	5509
final R value	0.063
temp, °C	ambient

furan was added at −20 °C 96 mg (0.7 mmol) of isobutyl chloroformate. After 10 min a precooled solution of 0.15 mL of *N*-methylmorpholine in 220 mL of dimethylformamide and 110 mL of tetrahydrofuran was added.

The reaction mixture was stirred for 1 h at 0 °C, left overnight at room temperature, and then it was evaporated under vacuo, and the residue, dissolved in chloroform, was extracted with 0.5 M $KHSO_4$, then with 5% $NaHCO_3$, and finally with water. The organic phase was dried over Na_2SO_4 and evaporated in vacuo to give a residue which was chromatographed on 100 × 3 cm column of silica gel, using a chloroform:methanol (95:5 v/v) mixture as eluant. Fractions (20 mL) 25–32 afforded 112 mg (17% yield) of TLC pure material which was then crystallized from methanol solution: R_f 0.25 in chloroform:methanol (95:5 v/v). Mass spectrum measurement (FAB) of $(M - H)^+$ gave a mass of 942 uma, as expected. The amino acids analysis was satisfactory.

X-ray Diffraction Analysis. Single crystals of CLAIB were grown from methanol–water mixture as colorless parallelepipeds by slow evaporation at room temperature.

Accurate cell parameters, orientation matrix, intensity data, structure determination, and refinement were carried out on a Nonius CAD 4 diffractometer equipped with a Microvax II Digital computer at the Centro di Metodologie Chimico Fisiche of the University of Naples.

A summary of the crystallographic data is given in Table I.

Intensity data were collected in the ω–2θ scan mode, using monochromatic Ni-filtered Cu Kα radiation, with a scan angle $\Delta\omega = (1{,}1 + 0{,}16 \tan\theta)°$; background counts were taken in an additional area of $\Delta\omega/4$, on both sides of the main scan, using the same scan speed. Prescan runs were made with a speed of 20.17°/5 min. Reflections with a net intensity $I > 0.5\sigma(I)$ were measured at lower speed, depending on the $\sigma(I)/I$ value, in the range 1 + 4°/min.

The stability of the crystal and of the electronic apparatus was monitored by collecting two standard reflections every 60 min of X-ray exposure time; no significant change in their intensity was observed during data collection.

In the range 1–65° of θ 6211 reflections were collected; 5509 of which, having a net intensity greater than $3.0\sigma(I)$, were considered observed and used for further calculations.

Direct application of the phase determination procedures as programmed in SHELX84,[9] SHELX76,[10] and MULTAN[11] failed to give sensible interpretations of E-maps. The phase problem of the cyclic peptide CLAIB was overcome applying the molecular replacement technique by using a molecular fragment of known structure. In fact, the coordinates of the Pro-Pro-Phe-Phe fragment, as determined previously in the structure of the cyclic peptide CLA,[5] were used as a starting model for the vector search procedure on the set of diffraction data collected for CLAIB; the rotation function, part of the crystallographic computing program DIRDIF,[12] successfully led to a sensible stepwise extension of the starting fragment.

Table II. Positional Parameters and Equivalent Thermal Factor with Their Estimated Standard Deviations

atom	x	y	z	B_{eq}
N_1	0.1430 (2)	0.3122 (3)	0.7972 (6)	3.3 (1)
C^{α}_1	0.1646 (2)	0.3648 (4)	0.7181 (8)	3.5 (1)
C^{β}_1	0.1434 (3)	0.4303 (4)	0.7774 (9)	4.8 (2)
C^{γ}_1	0.1275 (4)	0.4097 (5)	0.916 (1)	8.0 (3)
C^{δ}_1	0.1146 (3)	0.3398 (4)	0.9052 (9)	4.4 (2)
C'_1	0.2153 (2)	0.3633 (3)	0.7409 (8)	3.5 (1)
O_1	0.2317 (2)	0.3356 (3)	0.8389 (6)	4.6 (1)
N_2	0.2409 (2)	0.3958 (3)	0.6500 (7)	3.8 (1)
C^{α}_2	0.2252 (2)	0.4403 (3)	0.5400 (8)	3.7 (1)
C^{β}_2	0.2634 (3)	0.4903 (4)	0.5272 (9)	5.1 (2)
C^{γ}_2	0.3048 (3)	0.4482 (5)	0.559 (1)	5.6 (2)
C^{δ}_2	0.2898 (2)	0.4041 (4)	0.673 (1)	4.7 (2)
C'_2	0.2155 (2)	0.4052 (4)	0.4059 (8)	3.8 (1)
O_2	0.1943 (2)	0.4355 (3)	0.3203 (7)	6.1 (1)
N_3	0.2321 (2)	0.3432 (3)	0.3855 (6)	3.2 (1)
C^{α}_3	0.2262 (2)	0.3099 (4)	0.2551 (7)	3.7 (1)
C^{β}_3	0.2689 (3)	0.2688 (4)	0.2182 (9)	4.4 (2)
C^{γ}_3	0.3090 (2)	0.3148 (4)	0.1994 (8)	4.1 (2)
$C^{\delta 1}_3$	0.3136 (3)	0.3549 (4)	0.0867 (9)	4.7 (2)
$C^{\epsilon 1}_3$	0.3505 (3)	0.3972 (5)	0.071 (1)	5.8 (2)
C^{ζ}_3	0.3837 (3)	0.3978 (5)	0.168 (1)	6.9 (2)
$C^{\epsilon 2}_3$	0.3795 (3)	0.3584 (6)	0.283 (1)	6.7 (2)
$C^{\delta 2}_3$	0.3425 (3)	0.3166 (5)	0.296 (1)	6.2 (2)
C'_3	0.1869 (2)	0.2624 (4)	0.2530 (7)	3.5 (1)
O_3	0.1615 (2)	0.2604 (3)	0.1547 (5)	5.2 (1)
N_4	0.1807 (2)	0.2223 (3)	0.3614 (6)	3.3 (1)
C^{α}_4	0.1476 (2)	0.1681 (3)	0.3614 (7)	3.1 (1)
C^{β}_4	0.1658 (2)	0.1067 (4)	0.4349 (8)	3.9 (2)
C^{γ}_4	0.2010 (3)	0.0712 (4)	0.3567 (8)	4.1 (2)
$C_{\delta 14}$	0.2449 (3)	0.0907 (5)	0.360 (1)	5.3 (2)
$C^{\epsilon 1}_4$	0.2771 (3)	0.0602 (6)	0.277 (1)	7.4 (3)
C^{ζ}_4	0.2641 (4)	0.0084 (6)	0.194 (1)	8.2 (3)
$C^{\epsilon 2}_4$	0.2210 (4)	−0.0137 (6)	0.189 (1)	7.6 (3)
$C^{\delta 2}_4$	0.1886 (3)	0.0172 (5)	0.272 (1)	6.0 (2)
C'_4	0.1026 (2)	0.1922 (3)	0.4137 (7)	3.2 (1)
O_4	0.0827 (2)	0.1648 (2)	0.5081 (5)	3.7 (1)
N_5	0.0853 (2)	0.2434 (3)	0.3437 (6)	3.6 (1)
C^{α}_5	0.0427 (2)	0.2751 (4)	0.3784 (7)	3.5 (1)
$C^{\beta 1}_5$	0.0304 (3)	0.3234 (4)	0.2620 (9)	5.0 (2)
$C^{\beta 2}_5$	0.0477 (3)	0.3143 (4)	0.5126 (9)	5.2 (2)
C'_5	0.0063 (2)	0.2214 (4)	0.3874 (7)	3.6 (1)
O_5	−0.0235 (2)	0.2241 (3)	0.4769 (5)	4.1 (1)
N_6	0.0068 (2)	0.1730 (3)	0.2934 (6)	3.6 (1)
C^{α}_6	−0.0277 (3)	0.1219 (4)	0.2766 (8)	4.3 (2)
$C^{\beta 1}_6$	−0.0081 (3)	0.0674 (5)	0.1756 (8)	5.5 (2)
$C^{\beta 2}_6$	−0.0711 (3)	0.1533 (6)	0.225 (1)	6.6 (2)
C'_6	−0.0365 (2)	0.0821 (4)	0.4088 (7)	3.6 (1)
O_6	−0.0726 (2)	0.0559 (3)	0.4262 (6)	5.4 (1)
N_7	−0.011 (2)	0.0747 (3)	0.4893 (5)	3.2 (1)
C^{α}_7	−0.0036 (2)	0.0330 (3)	0.6095 (7)	3.3 (1)
C^{β}_7	0.0267 (3)	−0.0302 (4)	0.6015 (8)	4.1 (2)
$C^{\gamma 1}_7$	0.0102 (3)	−0.0753 (4)	0.487 (1)	5.6 (2)
$C^{\gamma 2}_7$	0.0750 (3)	−0.0104 (4)	0.581 (1)	5.2 (2)
$C^{\delta 1}_7$	0.1074 (4)	−0.0686 (7)	0.596 (2)	9.4 (4)
C'_7	0.0043 (2)	0.0737 (4)	0.7383 (7)	3.6 (1)
O_7	0.0125 (2)	0.0447 (3)	0.8464 (5)	5.1 (1)
N_8	0.0008 (2)	0.1404 (3)	0.7295 (5)	3.2 (1)
C^{α}_8	0.0021 (2)	0.1811 (4)	0.8533 (7)	3.7 (1)
C^{β}_8	−0.0184 (3)	0.2495 (5)	0.8293 (9)	5.3 (2)
C'_8	0.0498 (2)	0.1888 (4)	0.9076 (7)	3.5 (1)
O_8	0.0549 (2)	0.2008 (3)	1.0306 (5)	4.5 (1)
N_9	0.0835 (2)	0.1858 (3)	0.8202 (5)	3.0 (1)
C^{α}_9	0.1305 (2)	0.1937 (3)	0.8610 (7)	3.2 (1)
C^{β}_9	0.1567 (3)	0.1279 (4)	0.8586 (8)	3.9 (2)
$C^{\gamma 1}_9$	0.1317 (3)	0.0733 (4)	0.9402 (9)	5.2 (2)
$C^{\gamma 2}_9$	0.2031 (3)	0.1395 (5)	0.916 (1)	5.8 (2)
C'_9	0.1510 (2)	0.2477 (3)	0.7686 (7)	3.0 (1)
O_9	0.1745 (2)	0.2316 (2)	0.6697 (5)	3.9 (1)
O(W1)	0.2604 (2)	0.2359 (3)	0.5698 (6)	5.3 (1)[a]
O(W2)	0.1042 (3)	0.4633 (4)	0.292 (1)	11.8 (2)[a]
OM1	0.4101 (4)	0.1750 (6)	0.542 (1)	10.7 (3)[a]
CM1	0.4210 (5)	0.1007 (8)	0.503 (2)	18.8 (5)[a]
OM2	0.3358 (4)	0.2037 (1)	0.684 (1)	11.7 (4)[a]
CM2	0.3374 (6)	0.207 (1)	0.842 (2)	10.3 (5)[a]

[a] These atoms were refined isotropically. Anisotropically refined atoms are given in the form of the isotropic equivalent displacement parameter defined as $(^4/_3)[a^2B(1,1) + b^2B(2,2) + c^2B(3,3) + ab(\cos\gamma)B(1,2) + ac(\cos\beta)B(1,3) + bc(\cos\alpha)B(2,3)]$.

From the initial model, containing 34 atoms correctly oriented and positioned, the direct phase expansion procedure by the tangent formula

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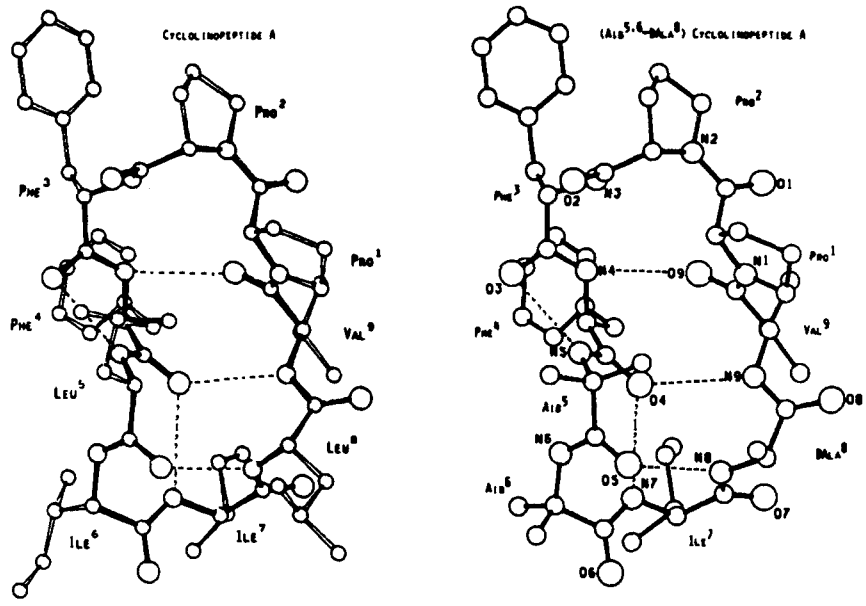

Figure 1. Molecular model of CLAIB (right) as compared with that of CLA (left). The numbering of each residue is reported. Intramolecular hydrogen bonds are indicated as dashed lines.

led to a molecular fragment containing 48 atoms, which eventually could be expanded to the complete molecular structure by Fourier techniques.

The positional and thermal parameters were refined by a full matrix least-squares procedure.[10] The quantity minimized was $\sum w(F_o^2 - F_c^2)^2$, with weights w equal to $1/\sigma^2(F_o)$. In the course of the refinement the analysis of the difference Fourier map revealed the positions of four solvent molecules, corresponding to two water molecules and two methanol molecules for each peptide molecule, which were included in the successive cycles of refinement. Hydrogen atoms were introduced in the final refinement in their stereochemically expected positions, with isotropic temperature factors equal to the equivalent thermal factor of the atom to which each of them is linked.

The refinement with isotropic thermal parameters for the solvent molecules and anisotropic thermal parameters for all C, N, and O atoms of the peptide molecule converged to an R value of 0.063 (Rw = 0.064). The final positional parameters are listed in Table II.

Solution Conformational Analysis. Spectrophotometric grade acetonitrile (99%, Jansen), refluxed and distilled in the presence of calcium hydride, was used for CD measurements. Hexahydrate calcium perchlorate, magnesium, barium and sodium perchlorates, and potassium chloride were supplied by Aldrich, Milwaukee, WI.

Tubes (5-mm) (Wilmad, Buena, NJ), deuterated acetonitrile, and chloroform (99.98% isotopically pure, Aldrich, Milwakee, WI) were used for NMR spectra.

CD spectra were recorded with a Jasco J-500A dichrograph, using a cylindrical silica cell with path lengths of 0.1 and 1.0 cm, at room temperature.

NMR spectra were recorded on Bruker spectrometers (AM 400 and WM 500). The peptide concentration was 6 mM. All chemical shifts in part per millions (ppm) are referred to internal tetramethylsilane (TMS). One-dimensional (1D) spectra have been acquired using typically 16–32 scans with 16 k data size. For the two-dimensional (2D) experiments, pulse programs of the standard Bruker software library were used.

All 2D experiments have been acquired in the phase sensitive mode, with quadrature detection in both dimensions, by use of the time proportional phase increment (TPPI).[13] Typically 256 experiments of 32–96 scans each were performed: relaxation delay, 1 s; size 2 k; 10 ppm spectral width in F2; zero filling to 1 k in F1; apodization in both dimensions with Lorentz to Gauss[14] multiplication (GB = 0.08; LB = −3). NOESY spectra have been acquired with mixing times in the interval 300 to 800 ms, ROESY spectra from 150 to 300 ms.

Results and Discussion

Solid State Structure and Conformation. A perspective view of the cyclic nonapeptide CLAIB, together with numbering of residues and intramolecular hydrogen bonds, is reported in Figure 1. In Figure 1 a view of the CLA molecule, with the same orientation, is also represented: the backbone atoms as well as most of the side chain atoms are almost superimposable.

The geometric parameters of all residues in the CLAIB structure are unexceptional. On the average, bond distances and average bond angles (estimated standard deviation, approximately 0.01 Å for bonds and 0.5° for angles) involving both the backbone and side chain atoms, compare well with literature values for other peptides of similar size.[15,16] This indicates that no crystallographic artefacts or abnormal intracyclic, solvent interaction or crystal packing strain occur in the crystal. The backbone and side chain conformational parameters are listed in Table III.

Eight out of the nine peptide bonds assume the trans conformation; while only the Pro^1-Pro^2 peptide bond is cis (ω = 9°). The average ω torsion angle is 175°, only 5° from the ideal (ω = 180°) trans conformation; individual deviations range from 180° − 9.6° to 180° + 7.8°, with six of them falling into the range 180° ± 4°. Nonplanar deviations of the peptide bond up to $\Delta\omega$ = 15° are not unusual:[15,16] the corresponding increase in energy is of the order of only 0.5 Kcal/mol.

The conformation observed for CLAIB shows a peptide backbone folding almost identical to that found in the three differently solvated crystalline forms reported for CLA. Furthermore, the substitution of Leu^5, Ile^6, and Leu^8 residues with two conformationally constrained Aib residues and a D-Ala residue, respectively, does not perturb the backbone and side chain conformations and actually increases the molecular rigidity. The conformational angles of CLAIB, shown in Table III, are very close (within 12°) to those found for the crystalline orthorhombic form of CLA, with the exception of those of the D-Ala^8 residue, for which both ϕ and ψ present deviations of 22°.

The observed conformational ϕ,ψ values of the backbone ring of CLAIB fall within the allowed region of the Ramachandran map. In particular the conformational parameters, φ, ψ of Aib residues at positions 5 and 6 are characteristic of the "3_{10}-helix" region: in the cyclic peptide they generate an incipient helical stretch.

The intramolecular hydrogen bond pattern in CLAIB, shown in Table IV, is the same as that described for three crystalline

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Table III. Conformational Parameters for CLAIB[a]

angle, deg	Pro[1]	Pro[2]	Phe[3]	Phe[4]	Aib[5]	Aib[6]	Ile[7]	D-Ala[8]	Val_9
$\varphi_i(N_i-C_{\alpha i})$	−65	−90	−97	−91	−54	−55	−118	77	−128
$\psi_i(C_{\alpha i}-C'_i)$	163	−17	−43	58	−41	−31	17	28	80
$\omega_i(C'_i-N_{i+1})$	9	−176	−171	−179	−173	−174	172	179	176
$\chi^{1,1}$	23	34	−64	−72			−64		−174
$\chi^{1,2}$							59		−52
$\chi^{2,1}$			−74	85			170		
$\chi^{2,2}$			107	−93					

[a] Estimated standard deviations for torsion angles are ~0.1°.

Table IV. Hydrogen Bonds

type	donor D–H	acceptor A	length[a] (Å) D–A	angle[b] (deg) N–O=C′	symmetry operation
			Intramolecular		
C7	N5	O3	2.97	89	
C_{10}	N_7	O_4	3.09	127	
C_{10}	N_8	O_5	3.09	112	
C_{13}	N_4	O_9	3.06	146	
C_{17}	N_9	O_4	3.12	133	
			Intermolecular		
	N_6	O_8	3.03	138	$(x, y, z-1)$
			Peptide–Solvent		
	N_3	Ow_1	2.93		(x, y, z)
	Ow_1	O_9	2.75	143	(x, y, z)
	Ow_2	O_6	2.99	116	$(-x, {}^1/_2+y, {}^1/_2-z)$
	Ow_2	O_2	2.77	133	(x, y, z)
	OM_1	O_5	2.83	119	$({}^1/_2+x, {}^1/_2-y, 1-z)$
			Solvent–Solvent		
	Ow_1	OM_2	2.60	117	(x, y, z)
	OM_2	OM_1	2.69	120	(x, y, z)

[a] Estimated Standard deviation 0.02 Å. [b] Estimated standard deviation 1°.

forms previously reported for CLA.[5,6] The 27-membered ring is conformationally stabilized by five transannular hydrogen bonds, one of the 1 ← 5 type, two of the 1 ← 4 type, one of the 1 ← 3 type, and a C_{17} structure. The 1 ← 5 hydrogen bond, forming a C_{13} ring structure or α-turn, occurring between the Phe[4] NH and the Val[9] C′O, contains the cis peptide unit. The conformational parameters of Pro[2] and Phe[3] (Pro[2] $\varphi_2 = -90°$, $\psi_2 = -17°$; Phe[3] $\varphi_3 = -97°$, $\psi_3 = -43°$), central residues of the α-turn, are very close to those pertaining to allowed helical region in the Ramachandran map.

The two 1 ← 4 hydrogen bonds are consecutive along the sequence, giving rise to two β-turns: the first, of type III, occurs between the Ile[7] NH and the Phe[4] C′O and the second, of type I, occurs between the Ala[8] NH and the Aib[5] C′O. The intramolecular 1 ← 3 hydrogen bond (γ-turn) involves the Aib[5] NH and the Phe[3] C′O. An additional hydrogen bond, occurring between the Val[9] NH and the Phe[4] C′O, stabilizes the C_{17} ring structure which includes the α-turn previously described.

Figure 2 shows the mode of packing of the CLAIB molecules as viewed along the *c* axis. The peptide molecules in the crystal are held together by a network of intermolecular hydrogen bonds in which the two H-bond donor N_3–H and N_6–H groups, not involved in the intramolecular H-bonding scheme, and the four solvent molecules participate. Hydrogen bond parameters are reported in Table IV. Each peptide molecule packs one on top of the other by one intermolecular N–H···O=C′ H-bond (N_6–H···O_8=C_8', 3.03 Å) which involves molecules translated along the *c* axis. Long rows of H-bonded peptide molecules are then formed. These rows pack with each other in a hexagonal fashion with similar adjacent rows. The rows are bridged together by the cocrystallized solvent molecules, as represented in Figure 2. Van der Waals interactions between hydrophobic groups further stabilize the layers. Along the *c* axis the solvent molecules are aligned one on top of the other as being part of a channel, similarly to what observed in other cyclic peptide structures.[17]

Solution Conformational Analysis. Previously we have been able to show, by an NMR conformational study,[5] that in chloroform solution it is possible to freeze, at low temperature (214 K), a unique conformation of CLA; this result has been confirmed by a distance geometry and molecular dynamics study[18] in vacuo and apolar solvent. A theoretical study on CLA in presence of water molecules showed the stabilization of a different conformation[19] with lower energy. In order to compare the solution conformational behavior of CLAIB with that of its parent peptide, even the conformational analysis of CLAIB has been carried out in chloroform solution. All NMR parameters used in the present study have been derived from 2D spectra performed at 295 K, with 6 mM sample concentration. Control ROESY[20] and NOESY[21] spectra at different concentration (2 mM) and temperatures (270 and 315 K) have been performed, with almost identical results. Figure 3 shows the comparison between the 1D spectra of CLA at 214 K and CLAIB at 295 K. The great similarity of the two spectra suggests that the low temperature structure of CLA and the room temperature structure of CLAIB should be characterized by common structural features. Moreover, the spectrum of CLAIB at 295 K is characteristic of the existence in solution of only one conformer, as indicated by the spreading of the NH resonances over 2 ppm, the nonequivalence of the aromatic resonances of the two Phe residues, the spread of αCH and βCH_2 resonances, and the high field shift of a single aliphatic peak at 0.5 ppm.

All spin systems have been identified by means of TOCSY[22] and DQF COSY.[23] The sequential assignments of the resonances of the three pairs of identical residues, i.e., Phe, Aib, and Pro, rely on NOESY and ROESY spectra. In the experimental conditions chosen, at room temperature, the 500 MHz NOESY spectra do show positive NOEs, i.e., we are in the extreme narrowing limit. ROESY spectra, performed on the Bruker AM 400 spectrometer, have been largely used in the present analysis, due to their less pronounced T1 and T2 distortions.

Pro[1] spin system has been assigned via a Val[9]-Pro[1] $d_{\alpha\delta}$; Phe[3] resonances have been identified through Pro[2]–Phe[3] $d_{\delta N}$, $d_{\gamma N}$, and $d_{\gamma\delta}$. Finally, a $d_{N\alpha}$ connecting Aib[5] and Phe[4] made it possible to label the two spin systems of the two Aib residues. Table V summarizes all chemical shift values.

The conformational analysis of the backbone conformation of CLAIB in chloroform solution at room temperature has been performed taking into account the chemical shift values, the temperature coefficients of the NH resonances, the quantitative evaluation of J_{NHCH} values, and ROEs. As already mentioned, for CLAIB at room temperature we can confidently speak of conformational homogeneity. In fact, we observe only one set of sharp resonances, characterized by fairly distinct δ values for chemically homogeneous protons, both in the peptide skeleton and in the side chains: NHs exhibit the maximum spread of δ, with a Δδ value of 2.03 ppm between Phe[3] and Phe[4]. As far as the

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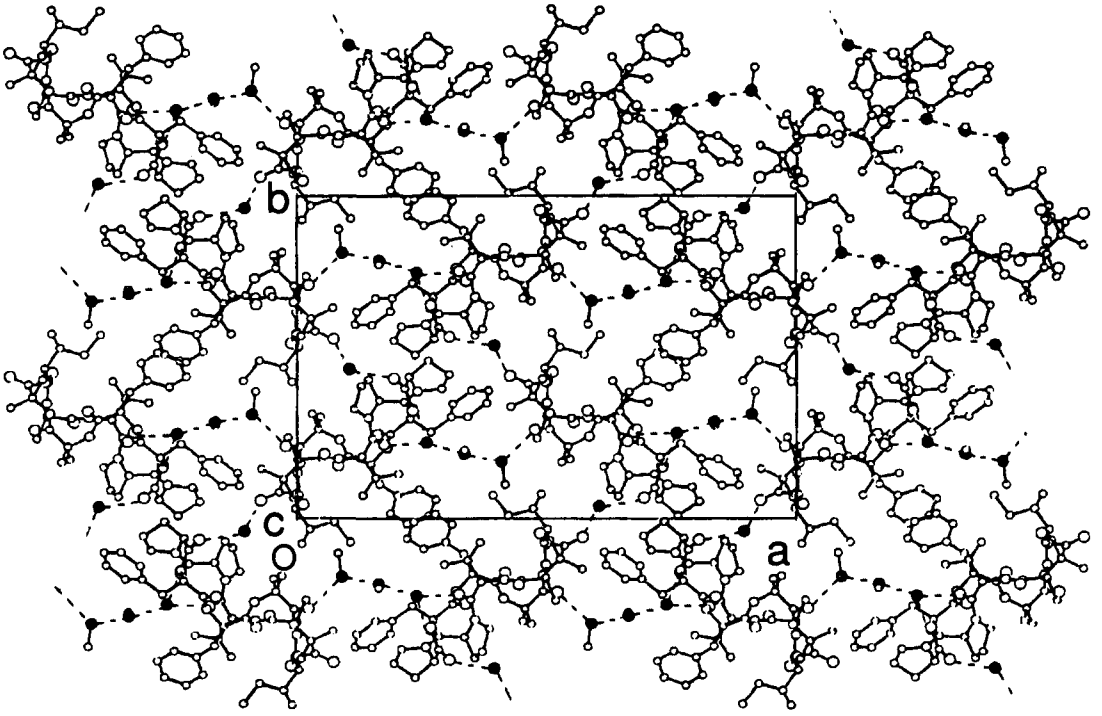

Figure 2. Mode of packing of the CLAIB molecules as view along the *c* axis. Intramolecular hydrogen bonds between peptide and solvent molecules are indicated as dashed lines. The peptide to peptide intermolecular hydrogen bond of the N–H···O=C′ type occurring along the *c* axis is not shown.

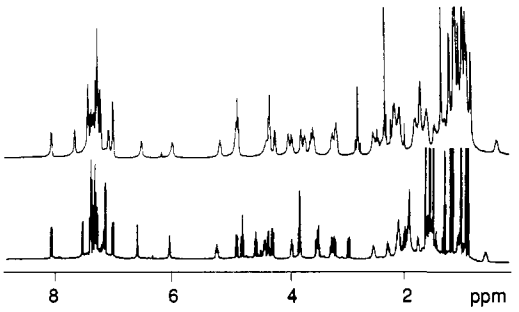

Figure 3. 500 MHz ^{1}H NMR spectrum of CLAIB in $CDCl_3$ at room temperature (bottom trace) as compared with 500 MHz ^{1}H NMR spectrum of CLA in $CDCl_3$ at 214 K (top trace).

prolines are concerned, we observe a maximum $\Delta\delta$ value of 0.9 ppm for γ,γ protons for Pro^2 and a 0.15 ppm value of the corresponding pair of Pro^1; the γ proton of Pro^2 experiences a high field shift up to δ = 0.55 ppm. As far as the β protons of Phe^3 and Phe^4 are concerned, they show $\Delta\delta$ values of 0.96 and 0.32 ppm, respectively. The complete lacking of degeneracy is pinpointed by the chemical shift values of all protons, listed in Table V. A careful comparison of these values with the corresponding ones for CLA (Table V of ref 5) indicates a striking similarity of the solution structures of the two peptides. Further support to this observation comes from the analysis of the J_{NHCH} values, NH temperature coefficients, and NOEs.

Table VI shows all experimental J_{NHCH} values, corrected for electronegativity, with the corresponding allowed φ ranges;[24] for sake of comparison, the values previously found for CLA at 214 K[5] are also reported. The values of Phe^4, Ile^7, and Val^9 are typical of $C_\alpha H$–NH moieties in trans arrangement. In the case of CLAIB, no information on φ angles for residues in position five and six can obviously be obtained by J_{NHCH} data, due to the absence of $C_\alpha H$ in Aib residues. The solution φ values correspond fairly well to the solid state ones.

The temperature coefficients of the NHs can provide information on the intramolecular hydrogen bond pattern. Table VII shows the NH $\Delta\delta/\Delta T$ values measured in chloroform solution of CLAIB in the range 295–325 K. In this range the dependence of all NH chemical shifts on temperature is linear, indicating that no conformational transition is taking place. Four out of seven NHs, namely the Phe^4, Ile^7, D-Ala^8, and Val^9 ones, exhibit fairly low temperature coefficients, suggesting that they remain hydrogen bonded in the whole temperature range examined. The values for Phe^3 and Aib^6 are −2.3 and −2.5 ppb/K, respectively, i.e., values very close to −2.4 ppb/K, the typical value pertaining to amide protons freely exposed to the solvent,[25] so that they can be regarded as solvent exposed. The value of −3.0 ppb/K for Aib^5 NH is suggestive of an amide proton passing from a bound state to an unbound one, i.e., of a somehow labile intramolecular hydrogen bond. These hydrogen bond patterns found in solution is in agreement with the solid state structure; in solution, at higher temperature, the hydrogen bond between Aib^5 NH and Phe^3 CO, closing the γ turn, is relaxed.

The analysis of ROEs, combined with the parameters already discussed, allows us to define the solution structure of CLAIB. Table VIII lists the prominent intraresidue ROEs observed for CLAIB in chloroform at room temperature. In practice, all ROE contacts listed coincide with NOEs found for CLA in chloroform at 214 K,[5] taking into account the residue substitutions in the sequence. As far as the stereochemistry of the two Xxx-Pro peptide bonds is concerned, the cis nature of the Pro^1-Pro^2 bond is indicated by the three contacts $d_{\alpha a}$, $d_{\beta\alpha}$, and $d_{\beta'\alpha}$. The strong $d_{\alpha\delta}$ and $d_{\alpha\delta'}$ between Val^9-Pro^1 are characteristic of a trans arrangement of this peptide bond.

All ROEs observed are consistent with the global structure of CLAIB as determined from the X-ray diffraction analysis. Apart from the already mentioned ROE contacts involving the Pro^1-Pro^2 and the Pro^2-Val^9 protons, others interresidue ROEs are in agreement with the α-turn conformation of the Val^9-Pro^1-Pro^2-Phe^3-Phe^4 moiety: the Phe^3-Phe^4 d_{NN}, the Pro^2-Phe^3 $d_{\gamma A r}$ and $d_{\beta N}$, the $d_{\alpha N}$ occurring between Pro^1 and Phe^3, the intraresidue ROE between the NH and the 2,6 aromatic protons of Phe^3, and the $d_{\alpha N}$ between Pro^1 and Phe^4. The correspondence with the solid state conformation of the central part of the molecule, involving

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Table V. Chemical Shifts, in ppm from TMS, of CLAIB in Chloroform at 295 K

	Pro[1]	Pro[2]	Phe[3]	Phe[4]	Aib[5]	Aib[6]	Ile[7]	D-Ala[8]	Val[9]
NH			5.95	7.98	6.51	7.08	7.19	7.44	6.93
αCH	4.26	4.19	4.32	5.14	1.44	1.57	4.80	4.47	4.71
α'CH					1.15	1.44			
βCH	2.06	2.03	2.88	3.43			2.46	1.50	2.20
β'CH	1.70	1.54	1.92	3.12					
γCH	2.00	1.47					1.54		
γCH	1.85	0.55					1.02		
δCH	3.85	3.40							
δ'CH	3.71	3.18							
γMe							0.89		1.11
									0.97
δMe							0.85		
Ar 2,6			7.05	7.26					
Ar 3,4,5			7.25	7.30					

Table VI. Vicinal $^3J_{NHC\alpha H}$ of CLAIB in Chloroform at 295 K with the Corresponding φ Angles[a]

residue	$^3J_{NHC\alpha H}$, Hz	φ, deg
Phe[3]	6.4 (8.8)	−170, −80, 30, 90
Phe[4]	11.2 (10.0)	−110 ÷ −125
Ile[7]	11.2 (11.0)	−110 ÷ −127
D-Ala[8](Leu[8])	6.4 (7.8)	170, 80, −30, −90
Val[9]	11.2 (11.2)	−110 ÷ −125

[a] Data in parentheses refer to the chloroform CLA solution at 214 K.[5]

Table VII. Temperature Dependence of the NH Chemical Shifts ($-\Delta\delta/\Delta T$, ppb/K) of CLAIB in Chloroform, in the Range 295–325 K

Phe[3]	Phe[4]	Aib[5]	Aib[6]	Ile[7]	D-Ala[8]	Val[9]
2.3	0.5	3.0	2.5	0.6	1.5	0.5

Table VIII. Prominent Interresidue ROEs in CLAIB in Chloroform Solution at 295 K

atom(s) 1	atom(s) 2	atom(s) 1	atom(s) 2
Pro[1] H_α	Pro[2] H_α	Aib[5] H_α'	D-Ala[8] H_β
Pro[1] H_α	Phe[4] HN	Aib[5] H_α'	Pro[1] H_δ'
Pro[1] H_α	Aib[5] H_α'	Aib[6] H_α	Ile[7] H_δ
Pro[2] H_α	Pro[1] H_β	Ile[7] HN	D-Ala[8] H_α
Pro[2] H_α	Pro[1] H_β'	D-Ala[8] HN	Val_9 HN
Pro[2] H_δ'	D-Ala[8] H_β	D-Ala[8] HN	Ile[7] H_α
Pro[2] H_δ'	Phe[3] HN	D-Ala[8] H_β	Val_9 HN
Phe[3] HN	Phe[4] HN	Val[9] HN	Aib[5] H_α'
Phe[3] HN	Pro[1] H_α	Val[9] H_α	Pro[1] H_δ
Phe[3] Ar	Pro[1] H_γ'	Val_9 H_α	Pro[1] H_δ'
Phe[4] HN	Pro[1] H_α	Val_9 H_β	Phe[4] H_β
Phe[4] H_β'	Ile[7] H_δ	Val[9] H_β	Phe[4] H_β'
Aib[5] HN	Phe[4] H_α		

the residues Phe[4], Aib[5], D-Ala[8], and Val[9], inferred from the φ values and the NH temperature coefficients, is confirmed by the $d_{\alpha\alpha'}$ and $d_{\beta'\alpha'}$ between Pro[1] and the high field methyl of Aib[5], $d_{\beta\beta}$ and $d_{\beta\beta'}$ Val_9-Phe[4], and the $d_{N\alpha'}$ Val_9-Aib[5]. The same is true for the sequence Aib[5]-Aib[6]-Ile[7]-D-Ala[8]-Val[9]; the $d_{N\beta}$ Val_9-D-Ala[8], the $d_{\alpha'\beta}$ Aib[5]-D-Ala[8], the intraresidue D-Ala[8] $d_{N\beta}$, the $d_{\alpha\delta}$ Aib[6]-Ile[7], the $d_{\beta'\delta}$ Phe[4]-Ile[7], and the $d_{\beta'\beta}$ Pro[2]-D-Ala[8], combined with the φ values of Table VI and the temperature coefficients of Val[9], D-Ala[8], and Ile[7] NHs reproduce the X-ray structure of this part of the molecule. As a side effect of ROE pattern, it is also possible to assign stereochemically the α-methyls of the two Aib in position 5 and 6: for both residues, the low field resonances (labeled "α" in Table V) are to be assigned to the *pro-R* form.

In a recently published work,[26] we have shown, by means of CD and NMR, that CLA is able to complex positive ions; the comprehensive conformational analysis of the equimolar complex CLA:Ba^{2+} in solution, based on CD, NMR, and molecular mechanics studies, demonstrated that the structure of CLA, upon complexation, is largely modified both in the backbone, containing all trans peptide bonds, and in the side chains, assuming a bowl-shaped form with two well-defined surfaces, a concave polar one hosting the cation and a convex apolar one.

We investigated the interaction of CLAIB with mono- and divalent cations in acetonitrile solution, by means of CD and NMR studies, up to cation:peptide molar ratio of 3. The CD spectra are unaffected by the salt addition, indicating that no conformational rearrangement is taking place. The CD results are confirmed by the NMR study: the perturbations induced in the CLAIB spectrum by the addition of Na^+, K^+, Ca^{2+}, or Ba^{2+} ions are absolutely insignificant and easily ascribable to the change in ionic strength of the solution. We can conclude that mono- and divalent cations, able to complex and to modify the structure of CLA, are instead unable both to complex and to modify the CLAIB structure.

Conclusions

The solid state conformation of CLAIB is stabilized by five transannular N–H···O=C′ hydrogen bonds, with formation of one C_{17} ring structure, one α-turn (C_{13}, containing the only cis Pro[1]-Pro[2] peptide bond), one γ-turn (C7), and two β-turns (C_{10}, one of type III and one of type I). The overall solid state CLAIB structure is very similar to that found for three CLA crystalline modifications.[5,6] The results of the conformational analysis is chloroform solution by NMR spectroscopy indicate that, at room temperature, solution and solid state conformations are essentially the same. The present study demonstrates that insertion, in the CLA sequence, of constrained residues such as Aib and D-Ala, effectively quenches the very high flexibility[5] of this peptide. The extent of the rigidity of the CLAIB structure can be inferred by the similarity of ROESY spectra at three different temperatures, 270, 295, and 315 K; moreover, cations, able to induce dramatic changes in the spectrum, and hence in the structure, of CLA[5], are completely ineffective in inducing significant modification of CLAIB conformation. This conformational rigidity could be perhaps one of the reasons of the lowering of biological activity of CLAIB when compared to that of its native peptide. In fact, the peptide concentration required to inhibit the cholate uptake

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in hepatocytes by 50% (CD_{50}) increases from a value of 0.84 μM for CLA[27] to a CD_{50} = 30 μM for CLAIB (Ziegler, K., private communication). It seems likely that flexibility plays a role in the explication of the bioactive function of cyclic peptides of this class. This is not the first example of activity diminishing in a peptide agonist as a consequence of the introduction of conformational constraints. In conformation activity relationship studies aiming to determine the bioactive conformation of peptides, the synthesis of conformationally restricted analogues has become a common practice. Successful examples of this practice are, inter alia, somatostatin[28,29] and enkephalin analogues.[30,31] Negative examples are the cyclic analogues of thymopentin,[32,33] where the conformation restriction introduced in order to reproduce the most probable conformation of thymopentin, as derived by NMR and energy minimization studies, led to a completely inactive analog.

Registry No. Cyclo(Pro-Pro-Phe-Phe-Aib-Aib-Ile-D-Ala-Val), 141666-94-6; cyclo(Pro-Pro-Phe-Phe-Aib-Aib-Ile-D-Ala-Val)·2MeOH·$2H_2O$, 141781-15-9; Z-D-Ala-Val-Pro-Pro-Phe-Phe-OH, 141666-96-8; H-Aib-Aib-Ile-O*t*Bu, 141666-95-7; Z-D-Ala-Val-Pro-Pro-Phe-Phe-Aib-Aib-Ile-O*t*Bu, 141666-97-9; H-D-Ala-Val-Pro-Pro-Phe-Phe-Aib-Aib-Ile-OH·TFA, 141666-99-1.

Communications to the Editor

Use of One-Bond C^{α}–H^{α} Coupling Constants as Restraints in MD Simulations

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Restraints within molecular dynamics (MD) simulations derived from coupling constants using the homonuclear $^3J_{HN\text{-}H^{\alpha}}$[1] coupling and numerous homo- and heteronuclear couplings about a single torsion[2] have been proposed and applied to peptides and proteins. This idea shows promise as these couplings have become more facile to measure, even in natural abundance, with increasing accuracy.[3-5] However, most of these couplings supply information about the ϕ or side chain angles (i.e., χ_1, χ_2); there are no ^3J couplings about the ψ angle which are easily obtained. This led us to explore the use of one-bond couplings as a source of conformational information, namely, the $^1J_{C^{\alpha}\text{-}H^{\alpha}}$ coupling, which depends on both the ϕ and ψ torsions and is easy to measure (HMQC[6] without proton decoupling). Here we describe the implementation of a penalty function in MD simulations based on the $^1J_{C^{\alpha}\text{-}H^{\alpha}}$ coupling.

The penalty function is similar to that commonly used for NOE restraints and recently utilized for three-bond couplings:[1,2]

$$E_J = \tfrac{1}{2}K_J(^1J - {}^1J_{exp})^2 \qquad (1)$$

where E_J is the potential energy of the penalty function, K_J is the force constant, ^1J is the coupling constant calculated from the dihedral angles, and J_{exp} is the experimental value. The coupling constant is calculated from the ϕ and ψ dihedral angles using the equation developed for L-amino acids by Egli and von Philipsborn:[7]

$$^1J = A + B\cos^2(\phi + 30°) + C\cos^2(\psi - 30°) \qquad (2)$$

with A, B, and C coefficients of 136.2, 14.0, and −4.9. From an extensive study of a series of peptides and proteins, we have obtained a better fit for alanine using slightly different values: 138.4, 13.7, and −5.2, for A, B, and C, respectively.[8]

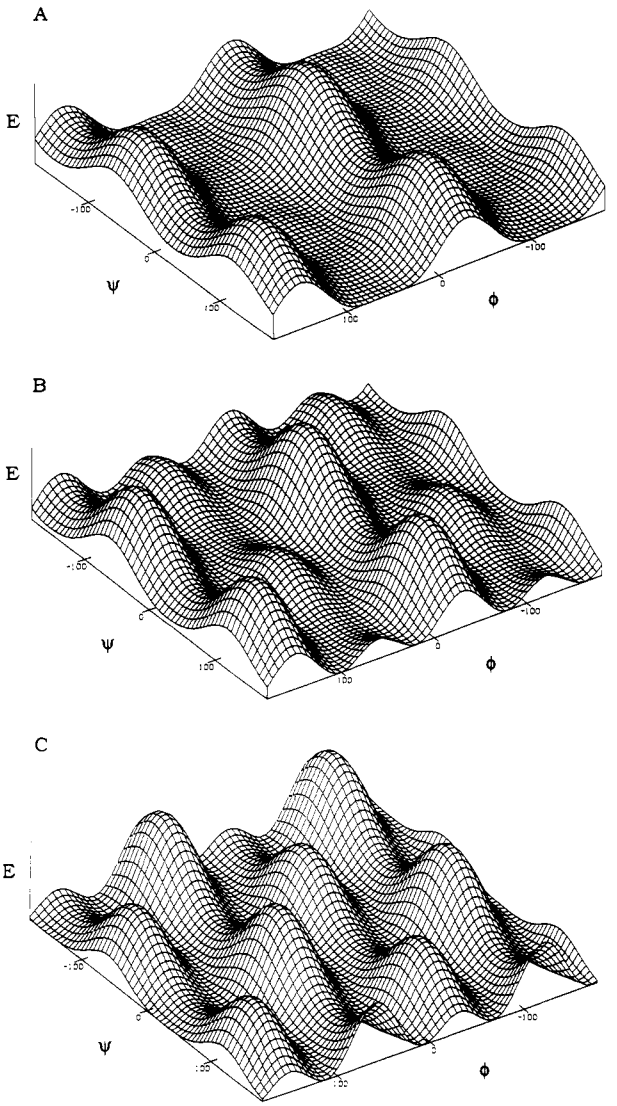

Figure 1. Plot of potential energy as a function of the ϕ and ψ dihedral angles. The energy was calculated using eqs 1 and 2 with K_J = 1.0 kJ mol^{-1} Hz^{-2} for (A) Ala^3 ($^1J_{C^{\alpha}\text{-}H^{\alpha}}$ = 135.8 Hz), (B) Ala^4 ($^1J_{C^{\alpha}\text{-}H^{\alpha}}$ = 140.4 Hz), and (C) Ala^5 ($^1J_{C^{\alpha}\text{-}H^{\alpha}}$ = 143.2 Hz).

The coupling constant restraint function was tested on a model peptide, cyclo[Pro^1-Pro^2-Ala^3-Ala^4-Ala^5], which has been extensively examined in DMSO by NMR.[9] The ^1J couplings (Table

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