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Conformational analysis of cyclolinopeptides A and B

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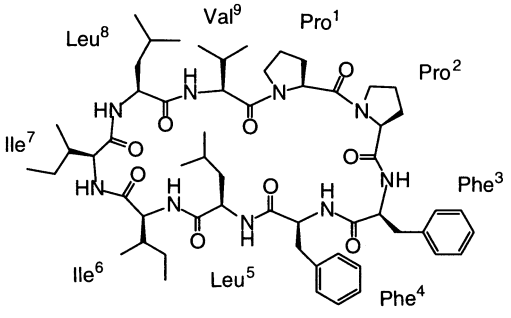
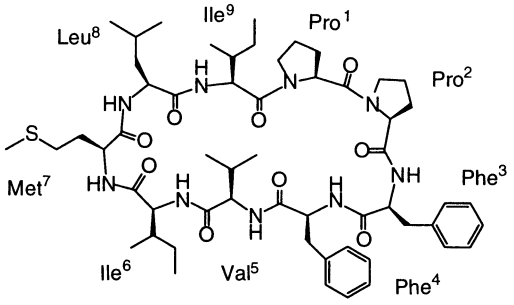
Abstract—Three dimensional structures of cyclolinopeptides A (CLA; cPPFFLIILV) and B (CLB; cPPFFVIMLI) isolated from the seeds of *Linum usitatissimum* were examined by X-ray analysis and distance geometry calculations using nuclear Overhauser effect constraints. Conformation in the solid state of CLA was similar to those in the solution state of CLA and CLB.

Unique cyclic nonapeptide, cyclolinopeptide A (**1**, CLA) isolated from the seeds of *Linum usitatissimum* (Linaceae),[1] have attracted interests from a biological point of view.[2] Recently, a kind of novel cyclic peptides, cyclolinopeptides A–I with an immunosuppressive activity on murine splenocytes have been isolated from seeds of *L. usitatissimum* (Linaceae).[3,4] Among them, cyclolinopeptide B (**2**, CLB),[3] consisting of different amino acids from those of CLA, showed potent immunosuppressive activity equivalent to that of CLA. The above evidence and our interest in cyclic peptide formation led to elucidate the three-dimensional stereostructures of CLA and CLB. In this paper, we describe the details of conformations of CLA and CLB in the solid and solution states by using X-ray and distance geometry (DG) calculations using nuclear Overhauser effect (NOE) constraints.



Cyclolinopeptide A (**1**)



Cyclolinopeptide B (**2**)

1. X-Ray structure of cyclolinopeptide A (1)

Cyclolinopeptide A (**1**) was crystallized from MeOH–isopropanol in orthorhombic crystals of space group $P2_12_12_1$ (Z=4), mp 243°C. A summary of the final crystallographic data was presented in Table 1. The ORTEP drawing was shown in Fig. 1. The torsion angles (ϕ, ψ, ω) were within the permissible ranges for peptides[5] and

Table 1. Crystal data of cyclolinopeptide A (**1**)

Empirical formula	$C_{57}H_{85}N_9O_{14}$
Color, habit	Colorless, prismatic
Dimensions	0.25×0.20×0.15 mm^3
System	Orthorhombic
D_{calc}	1.059 g cm^{-3}
Lattice parameters (Å)	a=9.866(1) Å
	b=21.690(2) Å
	c=33.054(3) Å
	V=7073.34(1) Å^3
Space group	$P2_12_12_1$
Z value	4
Final R value (R_w)	0.068 (0.086)

Keywords: cyclic peptide; cyclolinopeptide; *Linum usitatissimum*; conformation; NMR; distance geometry.

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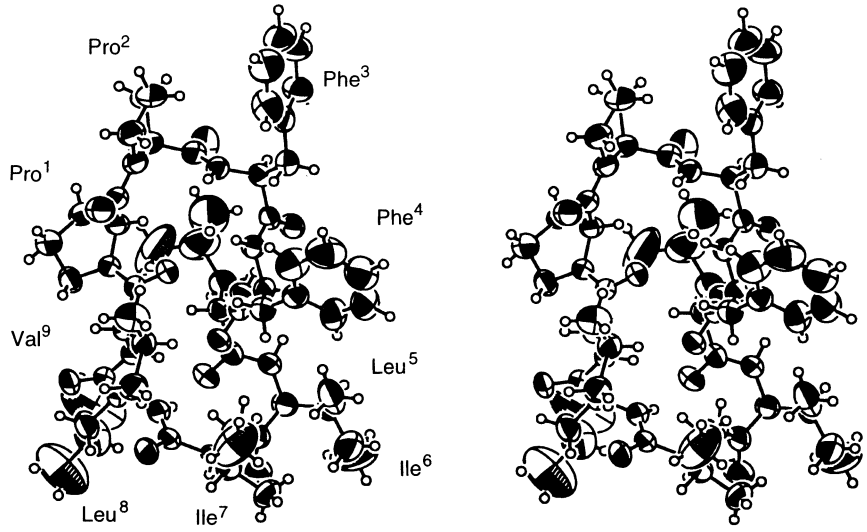

Figure 1. Stereoscopic view of ORTEP drawing of cyclolinopeptide A (**1**).

Table 2. Backbone torsion angles (°) for the crystal structure of CLA (**1**) and the mean structures **Aa** and **Ba** of CLA (**1**) and CLB (**2**), respectively

		Pro[1]	Pro[2]	Phe[3]	Phe[4]	Leu[5]	Ile[6]	Ile[7]	Leu[8]	Val[9]
CLA (**1**)	ϕ	−63.6	−90.5	−98.8	−85.7	−64.6	−51.6	−117.3	55.3	−124.6
	ψ	161.2	−16	−47.4	59.4	−28.4	−33.9	23.1	46.8	76.9
	ω	9.3	−176	−169.6	−178.5	177.1	179.9	175.6	169.6	176.8
	χ_1	25.6	30.8	−61	−70.5	−71.4	−174.0	60.3	−71	−56.2
	χ_2	−37.2	−37.2	−66	−84.6	−59.8	63.3	160.9	178.3	
	χ_3	33.9	27.7							
	χ_4	−18.4	−8.3							
	θ	−5.0	−13.8							
CLA (**Aa**)	ϕ	−73.5	−77.9	−77.3	−81.3	−58.9	−49.8	−104.5	59.8	−113.9
	ψ	160.6	−40.1	−53.3	49.6	−35.5	−39.9	24.3	30.2	90.6
	ω	0.0	−172.6	−167.3	179.2	177.3	172.3	179	180	180
		Pro[1]	Pro[2]	Phe[3]	Phe[4]	Val[5]	Ile[6]	Met[7]	Leu[8]	Ile[9]
CLB (**Ba**)	ϕ	−74.2	−77	−63.4	−84.5	−52.5	−54.1	−95.2	57.9	−123.2
	ψ	161.4	−42.3	−60.9	53.4	−46.7	−33.6	12	29.5	96.1
	ω	0.0	−174.2	−164.8	177	175.2	173.4	−176.6	178.4	−180

suggested that the molecule of this solid state conformation was not under any extra strain (Table 2).

The distances between O and N involved in the five intramolecular hydrogen bonds between Phe[4]-NH and Val[9]-CO, between Leu[5]-NH and Phe[3]-CO, between Ile[7]-NH and Phe[4]-CO, between Leu[8]-NH and Leu[5]-CO, and between Val[9]-NH and Phe[4]-CO were given in Table 3. In the crystal form, in addition to the five intramolecular hydrogen bonds, two β-turns (type III and type I) formed by the residues of Leu[5] and Ile[6], and of Ile[6] and Ile[7], one inverse γ-turn formed by the residue of Phe[4], and one α-turn formed by the residues of Pro[1], Pro[2], and Phe[3] restrained the cyclic nonapeptide backbone. The former was denoted as type III (Leu[5] ϕ, ψ (−64.6, −28.4); Ile[6] ϕ, ψ (−51.6, −33.9)) and the latter type I (Ile[6] ϕ, ψ (−51.6, −33.9); Ile[7] ϕ, ψ (−117.3, 23.1)). These torsion angles were well consistent with their ideal values (type III ϕ_{i+1}, ψ_{i+1} (−60.0, −30.0), ϕ_{i+2}, ψ_{i+2} (−60.0, −30.0); type I ϕ_{i+1}, ψ_{i+1} (−60.0, −30.0), ϕ_{i+2}, ψ_{i+2} (−90.0, 0.0); inverse γ-turn ϕ_{i+1}, ψ_{i+1} (−70 to −85, 60 to 70)). The amide bonds except Pro–Pro bond had *trans* geometry. The side chain of Phe[3], Phe[4], Leu[5], Ile[6], Ile[7], and Leu[8] took *gauche*$^-$, *gauche*$^-$, *gauche*$^-$, *trans*, *gauche*$^+$, and *gauche*$^-$ conformations, respectively. The conformation of pyrolidine rings of Pro[1] and Pro[2] were both C^2-Cγ-*endo*.[6] In the Leu[8] residue, ϕ and ψ angles (55.3° and 46.8°, respectively) indicated that it was in specific left handed α-helix region.

Table 3. Hydrogen bonds in cyclolinopeptide A (**1**)

From	To	Distance (Å)	Angle (°)
Phe[4]-N	Val[9]-O	3.34	122
Leu[5]-N	Phe[3]-O	2.87	145
Ile[7]-N	Phe[4]-O	3.29	144
Leu[8]-N	Leu[5]-O	2.78	167
Val[9]-N	Phe[4]-O	2.87	165

2. Three dimensional structures of cyclolinopeptides A (1) and B (2) in solution

The three-dimensional structures of CLA and CLB, which satisfy the NOE constraints, were constructed by DG calculations using distance constraints derived from the integrated volumes of NOE correlations in phase sensitive

Table 4. Summary of DG calculations for cyclolinopeptides A (**1**) and B (**2**)

Structural parameters	Cyclolinopeptide A (**1**)					Cyclolinopeptide B (**2**)				
No. of constraints										
Distance	48					48				
Torsion	9					9				
No. of calc. conformers[a]	284					331				
	Group									
	Aa	**Ab**	**Ac**	**Ad**	**Ae**	**Ba**	**Bb**	**Bc**	**Bd**	**Be**
No. of conv.[b]	17	23	12	10	12	59	22	14	11	10
Mean energy (kcal mol^{-1})	78.95	90.07	101.89	85.21	91.5	90.97	96.48	122.39	97.99	98.24
Mean RMS NOE	0.005	0.016	0.019	0.003	0.005	0.03	0.028	0.033	0.032	0.031
RMSD (Å)[c]	0.47	0.79	0.64	0.73	0.83	0.65	0.76	0.64	0.71	0.58

[a] Number of calculated conformers.
[b] Number of converged conformers.
[c] RMSD for backbone heavy atoms of mean structures.

ROESY spectra[7] and torsional constraints for amide bonds (eight *trans* amide bonds and one *cis* amide bond of CLA and CLB). No hydrogen bonding constraints were used. The initial structures satisfying the experimental constraints were generated by DG calculations with the program SYBYL.[8] Finally, the produced conformers were then subjected to constraint energy minimization with the AMBER all-atom force field.[9] The results of calculations were shown in Table 4.

The converged group was selected as those whose pairwise backbone (root mean square deviations) RMSDs were less

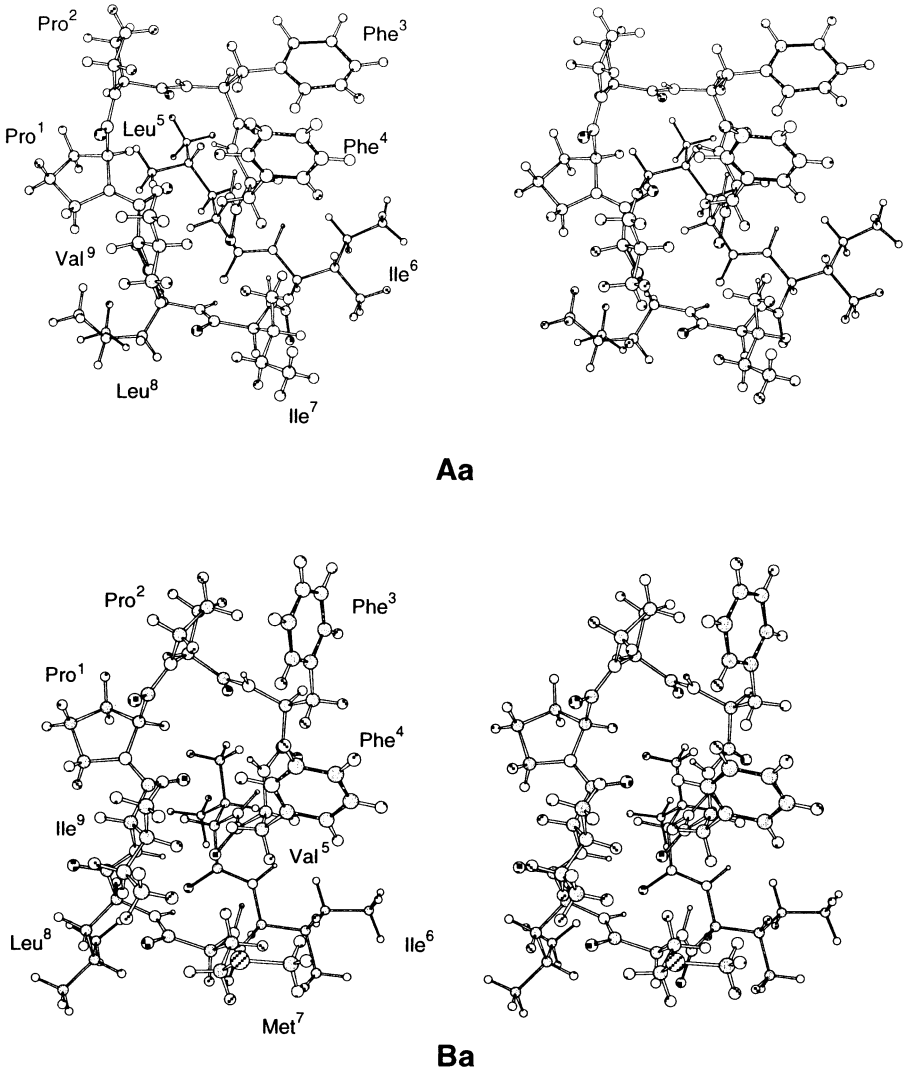

Figure 2. Stereoscopic views of mean structures **Aa** and **Ba** of cyclolinopeptides A (**1**) and B (**2**).

Table 5. Intramolecular hydrogen bonds and short contacts (Å) of the mean structure of group **Aa** of **1** and **Ba** of **2** obtained by DG calculations

Aa				**Ba**			
From	To	Distance (Å) O···HN	Angle (°) O···HN	From	To	Distance (Å) O···HN	Angle (°) O···HN
Phe^4-NH	Val^9-CO	2.43	110	Phe^4-NH	Ile^9-CO	2.43	110
Leu^5-NH	Phe^3-CO	1.88	153	Val^5-NH	Phe^3-CO	1.95	152
Ile^7-NH	Phe^4-CO	2.4	143	Met^7-NH	Phe^4-CO	2.11	137
Leu^8-NH	Leu^5-CO	1.85	153	Leu^8-NH	Val^5-CO	1.86	153
Val^9-NH	Phe^4-CO	1.89	156	Ile^9-NH	Phe^4-CO	1.92	167

than 1.00 Å and resulted in five structural families (groups **Aa**–**Ae** and **Ba**–**Be**). Seventeen structures among 284 structures generated by the DG method were defined as the converged group **Aa**, whose mean RMSD of the restraint violations was 0.005 Å. The mean structure generated, followed by energy minimization, was shown in Fig. 2. The overall atomic RMSDs between the individual structures and the mean coordinate positions were 0.47 Å for the backbone atoms. On the other hand, 59 structures among 331 structures for CLB were converged (group **Ba**: pairwise RMSDs for the backbone heavy atom is 0.65 Å). Fig. 2 showed their mean structures. Each of a total energy of the other groups (**b**–**e**) in CLA and CLB was higher than those of **Aa** and **Ba**, respectively, though mean RMS NOE value was similar to those of group a. In addition, the correlation with the NMR data concerning to hydrogen bonds, such as temperature dependence $\Delta\delta/\Delta T$ of the NH signals as described below, was not found in the groups **b**–**e**.

The mean structures adopted a type III β-turn at Leu^5–Ile^6 in CLA and Val^5–Ile^6 in CLB [CLA: Leu^5 ϕ, ψ (−58.9, −35.5); Ile^6 ϕ, ψ (−49.8, −39.9), CLB: Val^5 ϕ, ψ (−52.5, −46.7); Ile^6 ϕ, ψ (−54.1, −33.6)], a type I β-turn at Ile^6–Ile^7 in CLA and Ile^6–Met^7 in CLB [CLA: Ile^6 ϕ, ψ (−49.8, −39.9); Ile^7 ϕ, ψ (−104.5, 24.3), CLB: Ile^6 ϕ, ψ (−54.1, −33.6); Met^7 ϕ, ψ (−95.2, 12.0)] and a inverse γ-turn at Phe^4 in CLA and Phe^4 in CLB [CLA: Phe^4 ϕ, ψ (−81.3, 49.6), CLB: Phe^4 ϕ, ψ (−84.5, 53.4)], respectively (Table 2). The amide bonds except Pro–Pro bond had *trans* geometry. The distances involved in the five intramolecular hydrogen bonds between Leu^5-NH and Phe^3-CO, between Ile^7-NH and Phe^4-CO, between Leu^8-NH and Leu^5-CO, between Val^9-NH and Phe^4-CO, and between Phe^4-NH and Val^9-CO in CLA (**1**), and between Val^5-NH and Phe^3-CO, between Met^7-NH and Phe^4-CO, between Leu^8-NH and Val^5-CO, between Ile^9-NH and Phe^4-CO, and between Phe^4-NH and Ile^9-CO in CLB (**2**), respectively (Table 5). Temperature dependence studies,[10] which were recorded in 10°. intervals over the range 310–350 K in $CDCl_3$/DMSO=1:1 by NMR, suggested the presence of the above intramolecular hydrogen bonds. Low temperature coefficient values of Leu^5, Ile^7, and Val^9 in CLA (**1**) and Val^5, Met^7, and Ile^9 in CLB (**2**) indicated that these amide protons are involved in the formation of intramolecular hydrogen bonds (Table 6). These conformational characteristics of CLA and CLB implied by the NMR study were compatible with those observed in the crystal conformation of CLA by X-ray study (Table 3) and in the molecular models of **Aa** and **Ba** by DG calculations (Table 5).

Table 6. Temperature gradients $\Delta\delta/\Delta T$ (ppb/K) of the NH signals of cyclolinopeptides A (**1**) and B (**2**) in $CDCl_3$/DMSO-d_6=1:1

	Residue	$\Delta\delta/\Delta T$
Cyclolinopeptide A	Phe^3	6.6
	Phe^4	5.4
	Leu^5	−1.2
	Ile^6	6.8
	Ile^7	2.5
	Leu^8	4.2
	Val^9	1.8
Cyclolinopeptide B	Phe^3	7.4
	Phe^4	3.8
	Val^5	−2.0
	Ile^6	7.7
	Met^7	2.2
	Leu^8	4.8
	Ile^9	3.1

The assessment of a low energy group **Aa** and **Ba**, which considered ^{1}H NMR information (NOE effects, hydrogen bonds, and torsion angles calculated from vicinal coupling constants),[11] led to a proposal of the solution conformation for CLA and CLB, respectively. Superposition (RMSD 0.25 Å) of the backbone heavy atoms of the X-ray structure of **1**, the mean structures of **Aa** and **Ba** was shown in Fig. 3.

The partial conformations of CLA and CLB were similar to that of cycloleonurinin,[12] cyclo(-Gly-Pro-Thr-Gln-Tyr-Pro-Pro-Tyr-Tyr-Thr-Pro-Ala-), showing immunosuppressive

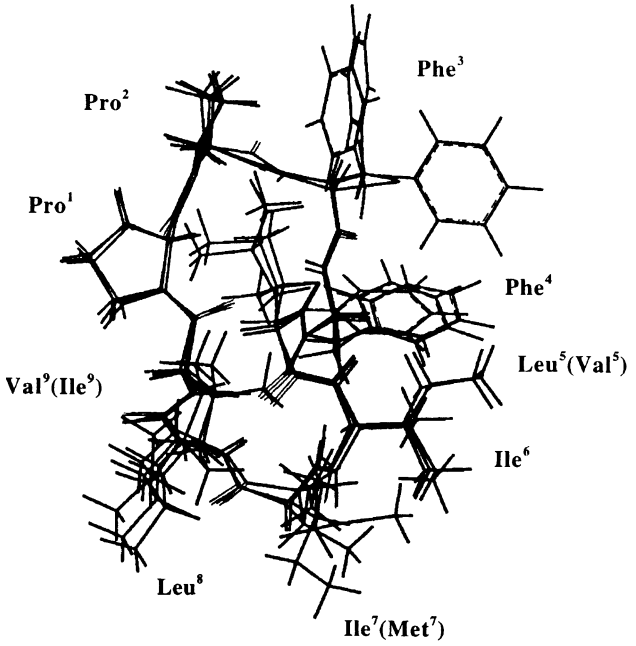

Figure 3. Superposition of the backbone of (a) X-ray structure of **1**, (b) mean structure **Aa** of **1**, and (c) mean structure **Ba** of **2**. RMSD, 0.25 Å.

activity and having amino acid sequences of a definite similarity, i.e. two adjacent prolines and two adjacent aromatic amino acid residues. In addition, geometry between two Pro amide bonds in cycloleonurinin, CLA, and CLB was of *cis* configuration and their conformations in the region of the above sequences are similar to each other. Common conformational feature of CLA and CLB may be important for the biological activity in addition of their sequential feature.

3. Experimental

3.1. General methods

^{1}H, ^{13}C, and 2D NMR spectra were recorded in $CDCl_3$/DMSO-d_6=1:1 (degassed) on a 400 MHz spectrometer at 330 K. Standard pulse sequences were employed for the 2D NMR experiments. A phase sensitive ROESY experiment was made with a mixing time of 200 ms. The temperature effect on NH chemical shifts was measured to assess the solvent accessibility to the amide protons at 10 intervals, over the range of 310–350 K, using linear regression analysis.

3.2. Material

Cyclolinopeptides A and B have been isolated from the seeds of *L. usitatissimum* according to the method described previously. A voucher specimen has been deposited in the herbarium of Tokyo University of Pharmacy and Life Science.

3.3. Computational methods

Computer modeling and all calculations were carried out using the molecular-modeling software package SYBYL ver. 6.22 (Tripos, Inc, St. Louis, MO) on an IRIS 4D computer. DG, SA and molecular mechanics calculations were performed with the AMBER all-atom force field[9] and the conditions were described in our previous paper.[12] The dielectric constant (ε) was assumed to be proportional to the interatomic distances (r) as $\varepsilon=r$. Solvent molecules were not included in the calculations. The NOE relationships (total 48 constraints in each **1** and **2**) were classified into three ranges, 1.9–2.5, 1.9–3.5, and 1.9–5.0 Å, corresponding to strong, medium, and weak NOEs, respectively, and were taken into account in the calculations of the constrained minimizations and dynamics, with an extra harmonic term of the form $E=(1/2)k(d-d^{\mathrm{low}})^2$ for $d<d^{\mathrm{low}}$, $(1/2)k(d^{\mathrm{high}}-d)^2$ for $d^{\mathrm{high}}<d$ and $E=0.0$ for $d^{\mathrm{low}}\leq d\leq d^{\mathrm{high}}$ added to the force field (k=200 kcal mol^{-1} Å^{-2}). Torsion constraints (the amide bond between Pro^6 and Pro^7 was only *cis* geometry), with an extra harmonic form of the form $E=(1/2)k(\omega-\omega^0)^2$ (k=0.01 kcal mol^{-1} °$^{-2}$) were also added to the force field. Each energy minimization was carried out until the derivatives became less than 0.01 kcal mol^{-2} Å^{-1} using the MAXMIN program.

3.4. X-Ray analysis of cyclolinopeptide A (1)

The crystal data are shown in Table 1. A colorless prismatic crystal of approximately 0.25×0.20×0.15 mm^3 in length was sealed in a thin walled glass capillary and was mounted on a Mac science DIP2020 diffractometer with graphite-monochromated Mo Kα radiation at 298 K. A total of 3919 reflections were observed. The structure was determined by the direct method using the SHELXS-86 program and the refinement was carried out by the block-diagonal-matrix least-squared method. The molecular structures determined by this methods are shown in Fig. 1. The refined fractional atomic coordinates, bond lengths, bond angles, hydrogen-atom coordinates and thermal parameters have been deposited at the Cambridge Crystallographic Data Centre (CCDC).

Acknowledgements

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11. The backbone dihedral angles (ϕ) calculated via Karplus-type equation proposed by Donzel from $^3J_{\mathrm{NH}}$-Cα coupling constants, corresponded closely to these conformers (**Aa** and **Ba**); CLA (**1**: ^3J, calc. angle, angle of the X-ray structure):

Phe[3] (8.8, −99°, −99°), Phe[4] (6.6, −83°, −86°), Leu[5] (6.8, −85°, −65°), Ile[6] (9.2, −102°, −52°), Ile[7] (7.9, −92°, −117°), Leu[8] (6.8, 29°, 55°), and Val[9] (9.3, −137°, −125°); CLB (**2**: ^3J, calc. angle): Phe[3] (8.8, −99°), Phe[4] (7.0, −86°), Val[5] (6.0, −80°), Ile[6] (8.8, −99°), Met[7] (8.3, −95°), Leu[8] (7.3, 33°), and Ile[9] (9.0, −140°).

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