Pergamon

Tetrahedron Vol. 50, No. 22, pp. 6537-6548, 1994

0040-4020/94 $7.00+0.00

0040-4020(94)E0274-W

Conformational Analysis of a New Cyclic Depsipeptide Calcium Blocker, Leualacin, by NMR Spectroscopy

Keiko Yoda, Hideyuki Haruyama*, Harumitu Kuwano, Kiyosi Hamano [a], Kazuhiko Tanzawa [b]

Analytical and Metabolic Research Laboratories,
[a] Biomedical Research Laboratories,
[b] Biological Research Laboratories, Sankyo Co., Ltd.,
1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan

Key words: Leualacin, conformational analysis, NMR spectroscopy, Ca^{2+} Blocker, Depsipeptide

Abstract: The three-dimensional structure of leualacin, a novel calcium channel blocker from *Hapsidospora irregularis* was determined in $CDCl_3$. Based upon the dihedral angle constraints from the analysis of $^3J_{H,H}$ and $^3J_{C,H}$, and the distance constraints deduced from ^1H-{^1H} NOEs and ^{13}C-{^1H} NOEs, conformers completely satisfying the NMR data were obtained by the conformational grid search program SYBYL followed by the energy minimization program XPLOR. Leualacin's structure is characterized by γ- and β-turn like moieties analogous to cyclic peptides, which are fixed by trans-annular hydrogen bonds formed between L-leucine and β-alanine. L-N-methylphenylalanine and S-leucic acid were found to be connected by *cis* peptide bond. The RMSDs (Root Mean Square Differences) calculated for the backbone atoms among four structures are 0.6 Å. The ring current effect caused by the phenylalanine moiety was reproduced by a calculation based on the resulting structures according to Bovey and Johnson's formula.

INTRODUCTION

Leualacin is a new calcium blocker isolated from *Hapsidospora irregularis* during our search for novel calcium blockers from microbial products [1]. Its structure has been established as a cyclic pentadepsipeptide consisting of L-leucine (**L**), L-N-methylphenylalanine (**F**), β-alanine(**A**) and S-and R-leucic acids (**V**and**V1**), by MS and NMR spectra [2]. Accordingly with the fact that ^3H-nitrendipine binding to porcine heart membranes was competitively inhibited *in vitro*, leualacin was categorized as an L-type specific Ca^{2+} channel blocker [1]. Besides clinically used Ca^{2+} blockers, which are mostly derivatives of dihydropyridine and/or benzothiazepine, there exist some proteineous Ca^{2+} blockers, which

consist of ca. 30-80 amino acids [3), 4), 5)]. Due to the significant structural differences among these two classes of Ca^{2+} blockers, it is fairly difficult to identify the structural motif responsible for the Ca^{2+} channel antagonist activities of these proteins. The identification of the structural motif would be a clue to generating Ca^{2+} blockers with novel molecular skeletons. In this context, we were interested in the three dimensional structure of leualacin, a small cyclic depsipeptide with reduced molecular flexibility. In this report, we describe the three dimensional structure of leualacin in $CDCl_3$, which was derived from distance constraints from ^1H-^1H and ^1H-^{13}C NOEs and dihedral angle constraints calculated from $^3J_{H,H}$ and $^3J_{C,H}$, supplemented with the ring current shift.

RESULTS

Signal assignments

The assignments for ^1H and ^{13}C signals, which relied mainly on the ^{13}C detected ^{13}C-^1H long-range couplings, have been reported previously [2)]. In the present report, **V1**γ-H is assigned to the signal at 1.65 ppm based on its ^1H-^{13}C long-range coupling to **V1**β-C at 37.3 ppm in the HMBC spectrum, leading to the assignment of the other unidentified signal at ca. 1.75 ppm to **L**γ-H. Thus the corresponding carbon-signals of **V1**γ and **L**γ were identified at 24.2 and 25.2 ppm, respectively. The high field shift of **V**β(b)-H resonating at –0.4 ppm, which should be ascribed to the ring current effect of the **F** residue, suggests that leualacin should have a rigid conformation in $CDCl_3$.

Hydrogen bonding

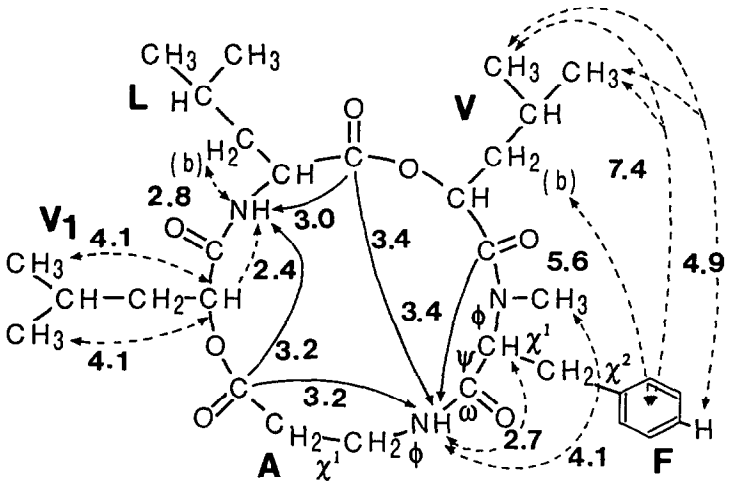
Another line of evidence for the stable conformation of leualacin in solution came from the slow exchange of two involved amide protons. These amide protons did not exchange during several days at 300 K after adding a trace amount of D_2O. The coefficients of the temperature dependence of their chemical shifts in $CDCl_3$ were 0.2×10^{-3} ppm/K for **A**β-NH and 0.6×10^{-3} ppm/K for **L**α-NH. These significantly smaller coefficients, compared with $(2.4 \pm 0.5) \times 10^{-3}$ ppm/K, that was reported for the solvent-exposed amide protons in $CDCl_3$[6)], indicate the presence of hydrogen bonds, in which these amide protons form a part. The proton acceptors of the two hydrogen bonds were identified during determination of the three-dimensional structures.

Table 1. The Dihedral Angle Constraints with NMR Parameters

Angles	Constraints (deg)	Assignments [a]		$^3J_{m,n}$(Hz)	NOE [b] (m,n)
A ϕ	120 (-120) [c]	NH	Hβ(a)	9.8	
		NH	Hβ(b)	2.4	S
A χ^1	60 (-60) [c]	Aα-C=O	Hβ(a)	9.7	
		Aα-C=O	Hβ(b)	4.9	
		Aα-C=O	NH		S
F ϕ	-160 or -80	C(NMe)	Hα	5.8	
		H(NMe)	Hβ(b)		S [d]
F χ^1	-60	Hα	Hβ(a)	3.4	S
		Hα	Hβ(b)	11.5	W
		H(NMe)	Hβ(b)		S [d]
		Cγ	Hα	2.8	
V1 ϕ	75 or 165 or -115 or -5	Aα-C=O	Hα	3.6	
V χ^1	-60	Hα	Hβ(a)	11.4	W
		Hα	Hβ(b)	2.4	S
		Hβ(b)	H(Fo)		W
		Hβ(b)	H(Fm)		S
V χ^2	180	Hγ	Hβ(b)	9.0	
		Hδ(0.57ppm)	Hα		S
		Hδ(0.57ppm)	Hβ(b)		S
		Hδ(0.68ppm)	Hα		W
		Hδ(0.68ppm)	Hβ(b)		S
L ϕ	-120	Hα	NH	10.1	W
		NH	Hβ(b)		S
L χ^1	-60 (180) [c]	Hα	Hβ(a)	3.9	S
		Hα	Hβ(b)	10.7	
		NH	Hβ(a) or Hγ		S
		NH	Hβ(b)		S
		Cγ	Hα	3.4	
L χ^2	180 (60) [c]	Hγ	Hβ(b)	1.5	
		Hδ(0.95ppm)	Hβ(b)		S
		Hδ(0.91ppm)	Hα		S

[a] Two atoms in this column correspond to m and n in the succeeding columns on the same line.
[b] S:strong, W:weak
[c] The combinations of the dihedral angles, Aϕ, Aχ^1, Lχ^1 and Lχ^2 were restricted to the listed values or those in the parentheses.
[d] These intensities were measured from ^{1}H-^{1}H NOE difference spectra.

Fig.1. The distance constraints deduced from ^{1}H-^{1}H NOEs (◂------▸) and ^{13}C-^{1}H NOEs(——▸). The arrows point from ^{13}C to ^{1}H. The NOEs including aromatic and/or methyl protons were constrained to the pseudoatoms placed at the center of the aromatic ring and/or methyl carbons by adding 2.4 Å and 1.0 Å, respectively, to the NOE derived distances.



Conformational analysis

Distance constraints were collected from ^{1}H-{^{1}H} and ^{1}H-{^{13}C} NOEs observed in the NOESY spectrum and the ^{1}H-{^{13}C} NOE difference spectra with selective irradiation of amide protons at 7.48 and 6.18 ppm. The upper bounds of the distance constraints, which were calculated according to the procedure described in the experimental section, are summarized in Fig.1. The dihedral angle constraints from $^{3}J_{H,H}$, $^{3}J_{C,H}$ and NOEs are listed in Table 1. As for the prochiral methylene protons of **V** and **F** residues, stereospecific assignments [7] were made by combined analysis of the intra and sequential NOEs and ^{3}J-coupling constants.

The three dimensional structure of leualacin was calculated as follows. In the first stage, a grid search was applied to the partial structure consisting of **F** and **V** residues (**FV** part) to obtain conformers satisfying the 9 distance constraints (Fig.1) and dihedral angle constraints (Table 1) assigned to this part. In the second stage, fixing the range of 6 dihedral angles involved in the **FV** part to the values obtained in the previous calculation, a grid search of the pseudo cyclic molecular model corresponding to the complete leualacin

(where the ester linkage between **V** and **L** residues was open to take care of the ring closure), was carried out applying 13 distance constraints (Fig.1) and 13 dihedral angle constraints (Table 1). For technical reasons, the dihedral angles of the isopropyl side chain of the **L** residue were fixed to $L\chi^1 = -60°$, $L\chi^2 = 180°$, one of the rotamers shown in Table 1, and the χ^1 and χ^2 angles of the **V1** residue were manually adjusted to the values so as to satisfy the distance constraints between **V1**α and **V1**δ shown in Fig.1. By this two-step grid search, 839 conformers were obtained. However, the dihedral angle constraints in the β-alanine residue, **A**φ and $A\chi^1$, were not evaluated at this stage.

The peptide bond between **F** and **V** residues was assigned to be a *cis* amide because a NOE was observed on **F**β(b)-H at 2.95 ppm and not on **V**α-H at 4.68 ppm or **F**α-H at 4.67 ppm on irradiating **F**α-NMe at 2.88 ppm. To confirm the *cis* peptide bond, an independent calculation assuming a *trans* peptide bond between the **F** and **V** residues was carried out, and this gave no structures satisfying the geometrical constraints [8].

In the third stage of the structure calculations, reproducibility of the ring current effect on the **V**β(b)-H at –0.41 ppm was evaluated according to Bovey and Johnson's formula [9]. The magnitude of the high field shift of **V**β(b) was expressed as the chemical shift difference between **V**β(b) and the average of the corresponding protons in the **V1** and **L** residues, where the ring current effects from the phenyl ring of the **F** residue could be considered to be negligible. Thus, 143 conformers, in which the calculated high field shift was in the

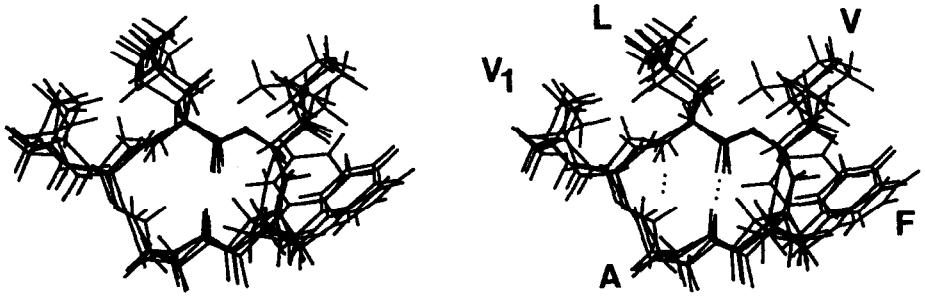


Fig.2 Stereoplots of leualacin in $CDCl_3$. (RMSD=0.59 Å)
-------: Hydrogen bonds.

range from −1.9 to −2.2 ppm in comparison with the observed high field shift of −2.06 ppm, were selected. Among them, 41 conformers were found to satisfy the dihedral angle constraints on ($A\phi$, $A\chi^1$)= (120°, 60°) or (−120° , −60°) , which were not evaluated in the previous stage.

In the final stage, energy minimization was applied to these 41 conformers, where the distance constraints between carbonyl carbon and amide proton or nitrogen, 2.3 or 3.3 Å [10] respectively, were added to hold two hydrogen bonds between the **L** and **A** residues. All conformers except one converged to the four conformer types represented by conformers 7, 11, 19 and 3. The backbone RMSDs were 10^{-2}–10^{-4} Å within each conformer type, while the backbone RMSDs among the four types were 0.59 Å. The statistics of the calculations are summarized in Table 2. The ring current effects for these four conformers calculated as mentioned above are shown in Table 3. Fig.2 is a superposition of the four conformers.

Table 2. Structural Statistics of leualacin

RMS differences from experimental restraints (Å)	**0.59**
E total (kcal/mol)	**27.5 ± 1.8**
E noe (kcal/mol)	**0.1 ± 0.1**
E dihe (kcal/mol)	**0.0 ± 0.0**
Deviations from idealized geometry	
bond (Å)	**0.01 ± 0.00**
angles (deg)	**2.23 ± 0.03**
impropers (deg)	**1.30 ± 0.25**

Table 3. Ring current effects of leualacin

Assignments	**Calculated values** [a] **(ppm)**	**Measured values (ppm)**
Vα	−1.08 ± 0.31	−0.49
Vβ(a)	−0.48 ± 0.06	−0.37
Vβ(b)	−1.64 ± 0.35	−2.06
Vγ	−0.30 ± 0.01	−0.24
Vδ	−0.28 ± 0.03	−0.30

[a] The ring current effects were calculated for all protons using Bovey and Johnson's formula [9].

DISCUSSION

As can be easily seen in Fig.2, the solution conformation of leualacin is characterized by the type VI β-turn [11), 12)] -like moiety, consisting of the **F** and **V** residues, and the γ-turn [12), 13)] -like part, consisting of the **A, V1** and **L** residues. In the β-turn mimetic part, the **V** and **F** residues correspond to residue i+1 and i+2 of a normal β-turn, and the hydrogen bond between **L**-C=O and **A**-NH assumes the role of hydrogen bonding between the carbonyl oxygen of residue i and the NH of residue i+3 which fixes the type VI β-turn geometry. In the γ-turn mimetic part, the **A** and **L** residues correspond to residue i and i+2 of a normal γ-turn, in which hydrogen bonding is formed between **A**-C=O and **L**-NH.

In Table 4, the geometries of the hydrogen bonds and averages of the φ and ψ angles of the four conformers are shown. To compare the geometrical similarities between these turn mimetics and normal turn structures, the φ and ψ angles were plotted on pseudo Ramachandran plots as shown in Fig.3 and Fig.4. The φ and ψ angles of leualacin equivalent to the φ and ψ angles of the peptide bond were defined as C=O(i−1) - N or O(i) - Cα(i) - C=O(i) and N or O(i) - Cα(i) - C=O(i) - N or O(i+1), respectively (Fig.1). Thus the "pseudo type VI β- and γ-turn " in analogy with the peptide conformation, could be quantitatively rationalized.

Table 4. The geometries of hydrogen bonds and backbone dihedral angles of leualacin

Distance between H and O of hydrogen bonds (Å)	
type VI β-turn like part	1.91 ± 0.03
γ-turn like part	2.03 ± 0.06
H-N-O angle of hydrogen bonds (deg)	
type VI β-turn like part	13.0 ± 5.0
γ-turn like part	26.5 ± 3.5
The backbone dihedral angles (deg)	
V1 φ	80.4 ± 3.2
ψ	-78.0 ± 5.2
L φ	-110.8 ± 11.4
ψ	-170.4 ± 43.8
V φ	-55.7 ± 28.1
ψ	102.9 ± 8.5
F φ	-133.9 ± 1.6
ψ	71.9 ± 3.3

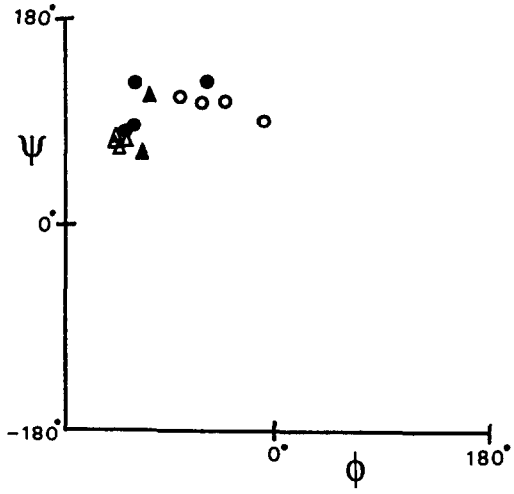


Fig.3 Ramachandran plot, ϕ and ψ angles.

o : V residue of leualacin. ● : The i+1 residue of peptides forming VI β-turn [11), 12)].

Δ : F residue of leualacin. ▲ : The i+2 residue of peptides forming VI β-turn [11), 12)].

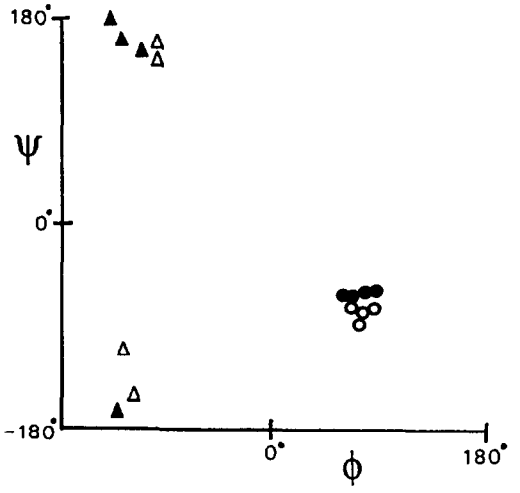


Fig.4 Ramachandran plot, ϕ and ψ angles.

o : V1 residue of leualacin. ● : The i+1 residue of peptides forming γ-turn [13)].

Δ : L residue of leualacin. ▲ : The i+2 residue of peptides forming γ-turn [13)].

The result that leualacin has a stable conformation characterized by well defined γ- and β-turn like motifs containing two trans-annular hydrogen bonds in $CDCl_3$, is interesting considering the following facts. Firstly, cyclic pentapeptides consisting of γ- and β-turn structures have been frequently seen in physiologically active peptides [12], and the strategy of replacing these turn structures with non-peptide conformational mimetics is an important research area in medicinal chemistry. The three dimensional structure of leualacin determined in this study presents an example of successfully replacing amide bonds with esters, and maintaining the biologically important turns.

According to Ripka et al.[14], benzodiazepine structures can be fitted to β-turns with RMSDs less than 1 Å, while benzodiazepines such as diazepam are known to inhibit ^3H nitrendipine binding competitively in smooth muscle [15]. Thus, in order to compare the three dimensional structures of leualacin and diazepam, leualacin's structure was superimposed on the x-ray crystal structures of clinically used L-type Ca^{2+} channel blockers including diazepam (data not shown). Based on the preliminary results, it may be speculated that the functionally essential motifs of leualacin are the carbonyl and alkyl groups of the **V** residue and the phenyl ring of the **F** residue. We are now performing structure determination of calciseptine [3], a small protein which exhibits L-type Ca^{2+} channel specific antagonism in aqueous solution, and this may give another clue to understanding the structural motifs essential for the function of proteineous Ca^{2+} blockers.

EXPERIMENTAL

Nuclear Magnetic Resonance

All NMR spectra were recorded at 303K on JEOL JNM-GSX500 and -GX400 spectrometers. All the spectra were processed using NMR2 software [16]. Leualacin was dissolved in 0.5 ml of $CDCl_3$, to give final concentrations of 35, 157, 350, 776 mM.

One-dimensional ^1H-NMR spectra were recorded with 16k data points at several temperatures ranging from 253-313K in order to observe the temperature dependence of the chemical shifts of amide protons. NOESY spectra of the 35mM leualacin were recorded with mixing times of 150, 300, 500, 800 and 1200 ms, and NOESY spectra of the 350 mM sample were measured with a mixing time 300 ms, to check the concentration dependence of the spectra. All NOESY spectra consisted of 256 experiments of 2048 data points with 96 scans. For evaluating the cross peak intensities of the NOESY spectra, a NOESY

spectrum of 35 mM leualacin was run twice with the mixing time 300 ms. For the stereospecific assignments of β-methylene protons, separation of the zero quantum coherence was applied [17].

^{13}C-{^{1}H}NOE difference spectra of the 776 mM leualacin irradiating amide protons at 7.48 and 6.18 ppm, were recorded with 5s presaturation and 3s relaxation delay, and 9408 scans (ca.45 hours) were accumulated using 32k data points, covering a spectral width of 30,000Hz. Two experiments with different irradiation powers for one irradiation position were performed, and the distances were calculated from the average of the signal enhancements of the two experiments.

^{1}H-^{13}C long-range *J*-resolved spectra of 350 mM leualacin were recorded with selectively irradiating amide or α-protons. The spectra consisted of 32 experiments of 2048 data points with 208 scans (ca. 7.5 hr). Spectral widths of 22,000 Hz in f2 and 40 Hz in f1 were used.

Distance constraints from NOE

The NOE peak volumes were calculated using the surface fitting function in NMR2. The H-H distances were estimated using the ratio of peak volume of a cross peak to that of the relevant reference peak. The average of two NOEs observed between the geminal methylene protons of the **A** and **V** residues was employed as reference, assuming the distance between them to be 1.81 Å. Practically, the peak volume of a specific NOE cross peak was evaluated as the average of the volumes of two sets of paired cross peaks observed at symmetrical positions in two separately recorded spectra. To set upper distance limits, 0.2 Å, the maximum standard deviation of the calculated distances, was added to the obtained distances. In the case of methyl or phenyl protons, the pseudo atom corrections were added as stated in the caption for Fig.1. C-H distance constraints were calculated in the same way, assuming that the distance between peptide amide proton and carbonyl carbon is 2.08 Å.

Dihedral angle constraints from coupling constants

$^{3}J_{H,H}$ coupling constants were obtained from the fine coupling pattern in the ^{1}H-NMR spectrum, while ^{13}C-^{1}H long-range *J*-resolved spectra were analysed to obtain $^{3}J_{C,H}$. The upper and lower bounds of the dihedral angle constraints were set by adding ±20° to the values calculated by applying Karplus type relationships [18), 19), 20)] to the observed $^{3}J_{H,H}$ and $^{3}J_{C,H}$ values. The **F**ϕ angle was set in the range from −180° to −60°, considering the effect

of electronegativity of the oxygen atom on the observed J values, as exhibited in the case of β-methylcellobioside [20].

Computer simulations

A three-dimensional structure of leualacin which completely satisfies the NMR data was obtained by the grid search algorithm program SYBYL [21], and energy minimization using the program XPLOR [22]. In the grid search, the rotatable bonds were rotated by 10° grid, and the size of van der Waals radii were reduced to 83%. To perform ring closure, the permitted deviation of the distances and angles involving the atoms forming the macrocyclic ring were set at 0.2 Å and 5°. Energy minimization was carried out using 3000-step Powell minimization until the derivatives became less than 0.01 kcal·mol^{-1}·Å^{-1}.

REFERENCES AND NOTES

1. Hamano, K.; Kinoshita, M.; Furuya, K.; Miyamoto, M.; Takamatsu, Y.; Hemmi, A.; Tanzawa, K., *J. Antibiotics*, 1992, *45*, 899-905.
2. Hamano, K.; Kinoshita, M.; Tanzawa, K.; Yoda, K.; Ohki, Y.; Nakamura, T.; Kinoshita, T., *J. Antibiotics*, 1992, *45*, 906-913.
   It should be noted that the one-letter code uesd in this paper do not coincide with the code commonly used for normal amino acids.
3. De Weille, J.R.; Schweitz, H.; Maes, P.; Tartar, A.; Lazdunski, M., *Proc. Natl. Acad. Sci. USA*, 1991, *88*, 2437-2440.
4. Hayakawa, N.; Morita, T; Yamaguchi, T.; Mitsui, H.; Mori, K. J.; Saisu, H.; Abe, T., *Biochem. Biophys. Res. Commum*, 1990, *173*, 483-490.
5. Mintz, I. M.; Venema, V. J.; Adams, M. E.; Bean, B. P., *Proc. Natl. Acad. Sci. USA*, 1991, *88*, 6628-6631.
6. Stevens, E. S.; Sugawara, N.; Bonora, G. M.; Toniolo, C., *J. Am. Chem. Soc.*, 1980, *102*, 7048-50.
7. Wagner, G.; Braun, W.; Havel, T. F.; Schaumann, T.; Gō, N.; Wüthrich, K., *J. Mol. Biol.*, 1987, *196*, 611-639.
8. A weak NOE was observed between **V**α-H and **F**α-NMe indicating the presence of a minor conformer, where the amide bond between **V** and **F** is *trans*, however the molar ratio of such a minor conformer was small and we could not get enough structural data to determine its three dimensional structure.
9. Johnson, C. E.; Bovey, F. A., *J. Chem. Phys*, 1958, *29*, 1012-1014.
10. Driscoll, P. C.; Gronenborn, A. M.; Beress, L.; Clore, G. M., *Biochemistry*, 1989, *28*, 2188-2198.
11. Morita, H.; Kondo, K.; Hitotsuyanagi, Y.; Takeya, K.; Itokawa, H; Tomioka, N.; Itai, A.; Iitaka, Y., *Tetrahedron*, 1991, *47*, 2757-2772.
12. Rose, G. D.; Gierasch, L. M.; Smith, J. A., *Adv. Protein Chem.*, 1985, *37*, 1-109.
13. Khaled, M. A.; Urry, D. W.; Okamoto, K., *Biochem. Biophys. Res. Commun.*, 1976, *72*, 162-169.
14. Ripka, W. C.; De Lucca, G. V.; Bach II, A. C.; Pottorf, R. S.; Blaney, J. M., *Tetrahedron*, 1993, *49*, 3593-3608.
15. Rampe, D.; Triggle, D. J., *Trends Pharmacol. Sci.*, 1986, *7*, 461-464.
16. Levy, G. C.; Delaglio, F.; Macur, A.; Begemann J.; *Computer enhanced spectroscopy*, 1986, *3*, 1-12.

17. Macura, S.; Huang, Y.; Suter, D.; Ernst, R. R., *J. Magn. Reson.*, 1981, *43*, 259-281.
18. Ramachandran, G. N.; Chandrasekaran, R.; Kopple, K. D., *Biopolymers*, 1971, *10*, 2113.
19. Kopple, K. D.; Wiley, G. R.; Tauke, R., *Biopolymers*, 1973, *12*, 627.
20. Marshall, J. L., *Carbon-Carbon and Carbon-Proton NMR Couplings*, Deerfield Beach, Florida, Verlag Chemie International, 1983; pp.22-26.
21. Dammkoehler, R. A.; Karasek, S. F.; Shands, E. F. B.; Marshall, G. R., *J. Comput.-Aided Mol. Design;* 1989, *3*, 3-21.
22. Powell, M. J. D., *Mathematical Programming*, 1977, *12*, 241-254.

(*Received in Japan* 15 *February* 1994; *accepted* 25 *March* 1994)