BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **219,** 572–579 (1996)
ARTICLE NO. 0275

Conformational Study of Two Linear Hexapeptides by Two-Dimensional NMR and Computer-Simulated Modeling: Implication for Peptide Cyclization in Solution

Aih-Jing Chiou,* Geok-Toh Ong,† Kung-Tsung Wang,† Shyh-Horng Chiou,*,† and Shih-Hsiung Wu*,†,[1]

*Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan; and †Institute of Biological Chemistry, P.O. Box 23-106, Academia Sinica, Taipei, Taiwan

Received January 3, 1996

Two linear peptides, D-leucyl-L-prolyl-L-isoleucyl-L-valyl-L-alanyl-β-alanine (**I**) and D-leucyl-L-prolyl-L-isoleucyl-L-valyl-N-methyl-L-alanyl-β-alanine (**II**), whose sequences were designed from protodestruxin and desmethyldestruxin B by replacing D-leucic acid with D-leucine, two cyclic hexadepsipeptides with insecticidal and immunodepressant activities, have been found to be cyclized in unusually high yields (>85%). In order to gain insight into the conformation and the relative flexibility of different constituent residues in these linear peptides, we have applied various techniques of 2D-NMR spectroscopy coupled with dynamic simulated annealing by computer modeling to establish the solution conformations of these two linear peptides. Based on the derived structures, it is found that the distances between N- and C-terminal residues of both peptides are short enough to facilitate the cyclization, thus collaborating the observation of favorable cyclization yields for both linear peptides.

Cyclic peptides with ring structure from natural products are well known to play important roles in their biological activities. A lot of microbial peptides such as peptide antibiotics possess structure-constrained ring structure [1]. Since the synthesis of gramicidin S was accomplished in 1950s, many cyclization methods including both the cyclization and cleavage of peptides on the solid-phase resins have been developed [2,3]. For a successful chemical synthesis of biologically-active cyclic peptides, the key step is usually the cyclization reaction. In most cyclization reactions of synthetic peptides, lower yields are obtained [4]. The yield of cyclization for linear peptides depends on several factors such as sequence and length of linear peptides, concentration of linear peptides, reagents and conditions of cyclization [2,5], etc.

Destruxins are cyclic hexadepsipeptides first isolated from the culture filtrate of *Metarrhizium anisopliae*, an insect-pathogenic fungus [6]. They are composed of 5 amino acids: L-Pro, L-Ile, N-methyl-L-Val, N-methyl-L-Ala and β-alanine plus a D-α-hydroxy acid which differs in five congeners, destruxins A, B, C and D. and desmethyldestruxin B [6–8]. Destruxin B and desmethyldestruxin B are proposed to be biosynthesized from protodestruxin by N-methylation at two sites and subsequent demethylation at one site on the cyclic ring [9].

Our recent interests in these conformation-constrained cyclodepsipeptides lie in their potential application as useful immunodepressants similar to cyclosporins [10] plus their suppressive effect on hepatitis B virus surface antigen (HBsAg) production in human hepatoma cells [11]. In an effort

Abbreviations: NMR, nuclear magnetic resonance; BOP, benzotriazolyloxycarbonyl *N*-oxytridimethylaminophosphonium hexafluorophosphate; DQF-COSY, double-quantum filtered correlation spectroscopy; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; XH-COSY, heteronuclear shift-correlated spectroscopy; NOESY, nuclear Overhauser and exchange spectroscopy; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; NOE, nuclear Overhauser effect; RMSD, root mean square deviation; DMF, dimethylformamide; DMSO, dimethylsulfoxide; HMP resin, *p*-hydroxymethylphenoxymethylpolystyrene resin; Fmoc-amino acid, N-fluorenylmethoxycarbonyl-amino acid.

[1] Corresponding address: Institute of Biological Chemistry, Academia, P.O. Box 23-106, Taipei, Taiwan. Fax: (886)-2-3635038. E-mail: shwu@gate.sinica.edu.TW.

0006-291X/96 $18.00

to prepare useful analogues of these biologically active cyclic peptides, we have synthesized two linear peptides, D-leucyl-L-prolyl-L-isoleucyl-L-valyl-L-alanyl-β-alanine (**I**) and D-leucyl-L-prolyl-L-isoleucyl-L-valyl-N-methyl-L-alanyl-β-alanine (**II**), whose sequences were designed from protodestruxin and desmethyldestruxin B by replacing D-leucic acid with D-leucine. We have analyzed these flexible short polypeptides using 2D-NMR spectroscopy coupled with dynamic simulated annealing to derive the solution conformations for these two linear peptides. Based on the derived structures, it is found that the separation between N- and C-terminal residues of both linear peptides are in a close distance favorable for the cyclization, in agreement with a high yield obtained in the final cyclization reaction for these synthetic analogues of destruxins.

MATERIALS AND METHODS

Preparation of linear peptides. The peptides used for NMR determination in this work was synthesized by solid-phase peptide synthesis in HMP resin using an ABI automatic peptide synthesizer (Model 431A) essentially according to the previous report [12]. The Fmoc-amino acids were introduced using the manufacturer's prepacked cartridges (1 mmol each) with a stepwise *FastMoc* protocol. The synthesized peptide was further purified by HPLC on a Vydac C_{18} semi-preparation column. The purified peptides were characterized by mass spectroscopy (JEOL JMS-HX 110 Mass Spectrometer) and amino acid analysis (Beckman 6300 Amino Acid Analyzer).

Cyclization of linear peptides. To a solution of the linear peptide (50 mg; 0.085 mmol) in 120 ml of DMF at 0°C was added BOP (442 mg; 0.1 mmol) and solid sodium bicarbonate (30 mg; 0.357 mmol) [13,14]. The mixture was stirred at 0°C for 1 h and then at room temperature for further reaction. The progress of reaction was monitored by HPLC. The reaction was stopped by quick freezing and lyophilization and the cyclic peptide was isolated by HPLC. The purified cyclic peptide was also characterized by mass spectroscopy and amino acid analysis.

NMR spectroscopy. The NMR samples were made of ca. 15 mg in 0.5 ml DMSO-d_6. All NMR experiments were performed on either Bruker AM-400, or AXL-400 or ARX-500 MHz spectrometer. Chemical shifts are referred to the solvent signal at 2.49 ppm. The assignments of the proton spectra of the two hexapeptides were made via the use of 1D proton and carbon spectra, DQF-COSY [15,16], HOHAHA [17], XH-COSY [18], NOESY [19] and ROESY [20] 2-D spectra. Spectra were recorded in the phase-sensitive mode with the time-proportion phase increments (TPPI) [19] method except XH-COSY, which was recorded in the magnitude mode. Typical two-dimensional spectra were recorded at 306 K with 512 t_1 increments, 2048 complex points (256 t_1, 1024 for ROESY and NOESY, 80 t_1, 2048 for XH-COSY). For the HOHAHA experiments, mixing time 80–200 ms was used. A mixing time of 150–500 ms was used for the NOESY and ROESY experiments. The ROESY experiment was performed by using a small flip angle (17°) in the pulsed spin-locked experiment to suppress chemical exchange [20]. After zero filling to a 1K × 1K or 2K × 2K matrix, $\pi/2$-shifted squared sine-bell functions were applied in both dimensions prior to Fourier transformation. The ROESY and NOESY spectra were processed by Felix program (version 2.05, Hare Research, Inc.). The temperature coefficients were determined over a temperature range of 27 to 67 °C at 5°C interval.

Computer-simulated modeling. The interproton distance information was derived by integrating the volume of the ROESY spectra's cross peak similar to that described in the previous report [12]. The ROESY cross peak intensities of linear peptides **I** and **II** were integrated and used for the computation of the tertiary structure. The dihedral angles of Ile^3 and Val^4 residues in both peptides were constrained within the limits [−180°, −80°] based on the coupling constants $^3J_{HN\alpha}$. All calculations were carried out on a Silicon Graphics, Iris 4D/35 and Iris Indigo Elan 4000 workstation. Structures were examined by the macromodel computer-modeling system "Insight II" from Biosym (version 2.2). Structures were calculated in two steps using NMRchitech program: DGII (distance geometry) and SA (simulated annealing) methodology. Both were executed with ROE constraints and dihedral angles. DGII includes smoothing, embedding and optimizing steps. Twenty structures obtained from DGII calculation were then used as starting points for the second-stage modeling based on the simulated annealing. From 20 random structures, 10 structures were selected based on the maximum distance violation of less than 0.5 Å, low total energy and the smallest root-mean-square deviation (RMSD) value.

RESULTS AND DISCUSSION

The characterization of conformational and dynamic properties of linear peptides in solution is a challenging and important focus in current structural biology. NMR spectroscopy remains the method of choice in this realm of research. During the process of the synthesis of destruxin analogues, it was found that in contrast to most synthetic peptides a higher than 85% yield of cyclization was obtained in this series of cyclodepsipeptides. The high yield of the ring closure was probably due to a favorable conformation of the linear chain precursor. Therefore a systematic analysis of two linear peptides, D-leucyl-L-prolyl-L-isoleucyl-L-valyl-L-alanyl-β-alanine (**I**) and

D-leucyl-L-prolyl-L-isoleucyl-L-valyl-N-methyl-L-alanyl-β-alanine (**II**), was carried out by 2D-NMR coupled with computer modeling to derive their solution conformation.

Preparation and Cyclization of Linear Peptides

The two linear peptides (**I**) and (**II**) were synthesized by automatic solid-phase peptide synthesizer. The linear peptides were purified by HPLC and characterized by mass spectroscopy and amino acid analysis. The cyclization of the two linear peptides was carried out in DMF solution with low concentration to avoid dimerization or polymerization. The final reaction solution analyzed by HPLC showed almost only one product in the cyclization reaction; **I** → **III,** cyclo-(D-leucyl-L-prolyl-L-isoleucyl-N-methyl-L-valyl-N-methyl-L-alanyl-β-alanyl) and **II** → **IV,** cyclo-(D-leucyl-L-prolyl-L-isoleucyl-L-valyl-N-methyl-L-alanyl-β-alanyl). Both of these two cyclic peptides showing longer retention times than their corresponding linear peptides in reverse-phase HPLC were isolated by semi-preparative HPLC with over 85% yield, and their structures were confirmed by mass spectroscopy and amino acid analysis. It is noteworthy that Lee and Izumiya [21] failed to cyclize D-leucic acid-L-prolyl-L-isoleucyl-L-valyl-L-alanyl-β-alanine for the synthesis of protodestruxin. Therefore the cyclization of linear peptides by amide-bond formation should be easier than by ester-bond formation.

NMR Chemical-Shift Assignments for Linear Peptides

DMSO instead of DMF was used as solvent in NMR study for two reasons: (1) the solvent peaks of DMF had more interference for the assignment of NMR spectra. (2) DMSO which possesses similar polar property to DMF seems more suitable as a solvent for peptides.

The ^{1}H-NMR spectra of **I** and **II** were shown in **Fig. 1.** The ^{1}H chemical shift assignments for **I** and **II** are relatively straightforward from HOHAHA, COSY and the conventional procedures of sequence assignment. Judging from the spectra, there are two forms existed in these linear peptides due to the presence of proline residue. The ratio of major and minor forms is about 3:1 on the basis of integrated peak areas. From the ROESY spectra, it was found that strong correlation was observed between D-Leu^{1} αH and L-Pro^{2} δH in the major form, indicating clearly the major form as the *trans* Leu^{1}-Pro^{2} conformer. In contrast, cross-peak could be observed between D-Leu^{1} αH and L-Pro^{2} αH in the minor form which should be the *cis* Leu^{1}-Pro^{2} conformer (**Fig. 2**). [22]. The two conformers in the two linear peptides were also demonstrated in the two-dimensional XH-COSY experiment. All ^{1}H and ^{13}C chemical shifts for two forms of both linear peptides are listed in **Table 1** and **Table 2.** There are two sets of ^{13}C signals of Pro^{2}α, Pro^{2}β, Pro^{2}γ, Pro^{2}δ and Leu^{1}α for **I** and **II.**

The ^{1}H-NMR spectra of cyclic peptides **III** and **IV** (data not shown) revealed only a single form in these cyclic peptides. Strong correlation between D-Leu^{1} αH and L-Pro^{2} δH indicated that the cyclic peptides were the *trans* Leu^{1}-Pro^{2} conformers.

Structure Calculation by Distance Geometry and Refinement by Simulated Annealing

The final structures of **I** and **II** were calculated from distance geometry and simulated annealing based on ROESY distance constraints and dihedral angles. The minor forms of these linear peptides had too few ROE distance constraints to be calculated. The superpositions of 10 structures of **I** and **II** major forms are shown in **Fig. 3.** The conformational ensembles of **I** and **II** major forms show good convergence and indeed provide qualitative evidence for folding. The root mean square deviation (RMSD) values between all atom pairs among the ten structures of **I** and **II** major forms are as follows: the average pairwise RMSD for all atoms is 1.661 ± 0.552 and 1.558 ± 0.645 Å for **I** and **II** respectively whereas that for backbone atoms is 0.448 ± 0.368 and 0.296 ± 0.300 Å for **I** and **II** respectively. The distances between N- and C-terminal residues in the *trans* forms of **I** and

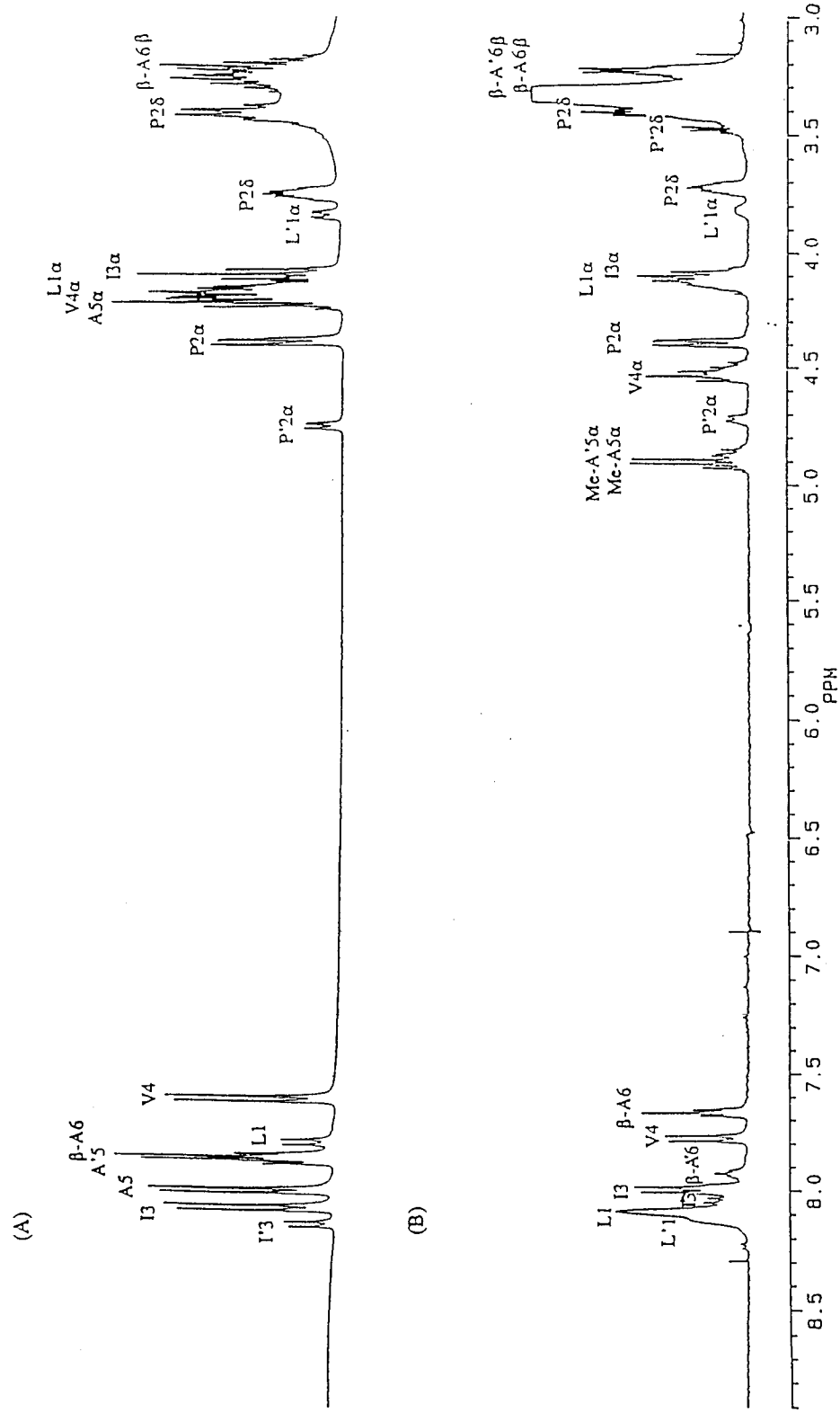

FIG. 1. H-NMR spectra of linear hexapeptides (A) peptide **I** and (B) peptide **II**. The NMR peaks corresponding to the minor forms of two peptides are labeled with prime (') marks.

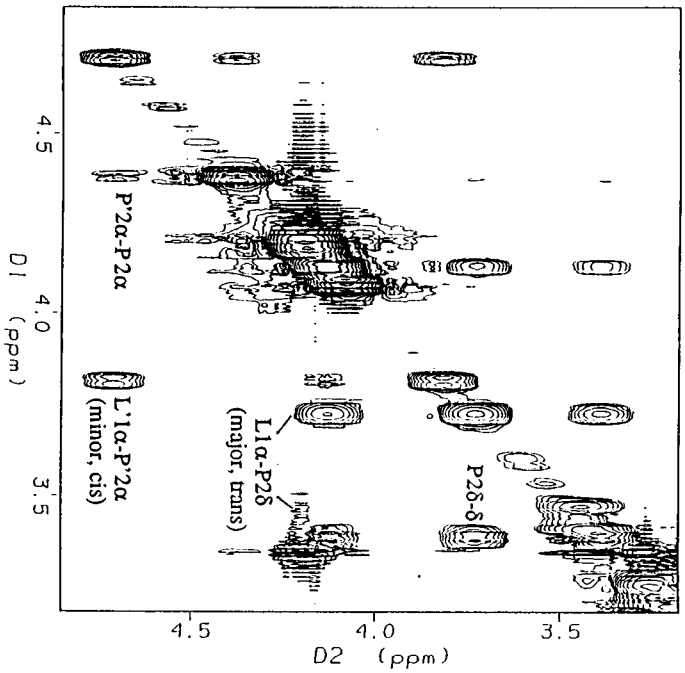

FIG. 2. Part of ROESY spectra (τ_m = 300 ms) for Peptide **I** which shows major (*trans*) and minor (*cis*) forms.

II are 5.26 and 4.30 Å, respectively, which are short enough to facilitate the cyclization of **I** and **II** in high yields.

Conformation of the Linear Hexapeptides in Relation to Cyclization

Small linear peptides are usually found to exist in random-coil structure and they exhibit a high degree of conformational flexibility. However the high yields of cyclization obtained for the linear peptides studied here would imply that the conformation of these linear peptides in organic solvent does not exist in completely random-coil like states and possesses somewhat stable folded structures in order to facilitate the cyclization reaction. Based on the solution structures generated from NMR data and computer-modeling, the major form of linear peptides with *trans* Leu^1-Pro^2 conformer possesses a distinct β-turn in DMSO solution. However, the structure of minor species could not be calculated owing to weaker NMR signals with overlapping peaks from those of major species. Major species of linear peptides and cyclic peptides exist as conformers of *trans* Leu^1-Pro^2. It is believed that cyclic peptides are produced directly from the cyclization of major *trans* forms of linear peptides and the yield of cyclization increases by shifting gradually *cis* isomer (minor) to *trans* (major) isomer with the equilibrium and subsequent conversion of both forms of linear peptides to a cyclic peptide of *trans* form.

CONCLUSION

In most cyclization reactions of linear peptides, lower yields were observed [4]. In the synthesis of two destruxin analogues reported here we have obtained high yields of cyclization for both linear peptides **I** and **II.** The high yields of the ring closure in these two linear peptides probably arise from a favorable conformation of the linear chain precursor [23]. By 2-D NMR coupled with computer simulated annealing we have demonstrated qualitative evidence for the existence of well-defined and folded conformation in the solution structures of these two supposedly flexible and random-coil like short hexapeptides. The distances between N- and C-terminal residues in the

TABLE 1

The Chemical Shifts (ppm), $^3J_{NH\text{-}CH}$(Hz) and the Temperature Coefficients of δ(NH)(10^{-3}ppm/K) of the Major Form and Minor Form[£] of Peptide **I** in DMSO-d_6 at 306K

^{1}H chemical shifts

Residue	NH	α-CH	β-CH	γ-CH	δ-CH	Others	$^3J_{NH\text{-}CH}$	Δδ(NH)/ΔT
D-Leu[1]	7.78(−)	4.13(3.82)	1.55(1.41)	1.75(1.40)	0.88(0.79)		8.77	
					0.92(0.91)			
Pro[2]	–	4.37(4.74)	2.02(2.09)	1.89(1.67)	3.73			
			1.81		3.38(3.42)			
Ile[3]	8.06(8.13)	4.08(4.10)	1.73(−)	1.08(−)	0.80(−)	γ-$CH_3$0.81(0.81)	8.68(8.09)	4.53
				1.48(−)				
Val[4]	7.60(−)	4.17(−)	1.95(1.95)	0.88(0.88)			8.86	4.14
Ala[5]	7.99(7.87)	4.20(−)	1.15(1.14)				7.36	4.94
β-Ala[6]	7.85(−)	2.34(−)	3.23(−)					

^{13}C chemical shifts

Residue	α-C	β-C	γ-C	δ-C	Others
D-Leu[1]	49.71(49.36)	38.88(38.75)	23.40(−)	21.10, 23.02(21.05,23.02)	
Pro[2]	59.69(59.44)	29.42(29.30)	23.95(23.10)	46.59(47.05)	
Ile[3]	57.62(57.59)	36.05(−)	24.58(−)	11.00(−)	γ-$CH_3$15.45(15.32)
Val[4]	57.21(−)	30.65(−)	17.84,19.11(17.93,−)		
Ala[5]	48.13(−)	18.22(18.27)			
β-Ala[6]	33.73(−)	34.72(−)			

[£] chemical shifts of minor form in parentheses. Some assignment of the minor species are not listed owing to weak signal or overlap.

TABLE 2

The Chemical Shifts (ppm), $^3J_{NH\text{-}CH}$(Hz) of Major Form and Minor Form[£] of Peptide **II** in DMSO-d_6 at 306K

^{1}H chemical shifts

Residue	NH	α-CH	β-CH	γ-CH	δ-CH	Others	$^3J_{NH\text{-}CH}$
D-Leu[1]	8.12(8.20)	4.15(3.81)	1.49(1.58)	1.75(1.73)	0.89(0.89)		
					0.92(0.92)		
Pro[2]	–	4.38(4.74)	1.81	1.89(1.69)	3.40(3.42)		
			2.05(2.10)		3.73(−)		
Ile[3]	8.00(8.07)	4.10(−)	1.71(1.72)	1.08(−)	0.82(0.82)	γ-$CH_3$0.79(0.79)	8.70
				1.54(−)			
Val[4]	7.78(8.06)	4.52(4.54)	1.98(1.98)	0.84(0.85)			8.74
				0.87(−)			
Me-Ala[5]		4.91(4.88)	1.16(1.23)			$NCH_3$2.63(2.62)	
β-Ala[6]	7.67(7.87)	2.35(2.39)	3.22(3.32)				

^{13}C chemical shifts

Residue	α-C	β-C	γ-C	δ-C	Others
D-Leu[1]	49.69(49.31)	38.88(38.75)	23.39(23.08)	21.08, 23.00(21.02, 23.10)	
Pro[2]	59.67(59.35)	29.41(29.35)	23.95(23.95)	46.59(46.67)	
Ile[3]	57.30(−)	36.31(36.10)	24.52(−)	11.02(11.10)	γ-CH_3 15.34(15.21)
Val[4]	53.56(53.78)	30.11(29.98)	17.97(18.21), 19.15(−)		
Me-Ala[5]	51.77(51.64)	14.21(15.11)			NCH_3 27.11(26.85)
β-Ala[6]	33.72(33.64)	34.86(35.07)			

[£] chemical shifts of minor form in parentheses. Some assignment of the minor species are not listed owing to weak signal or overlap.

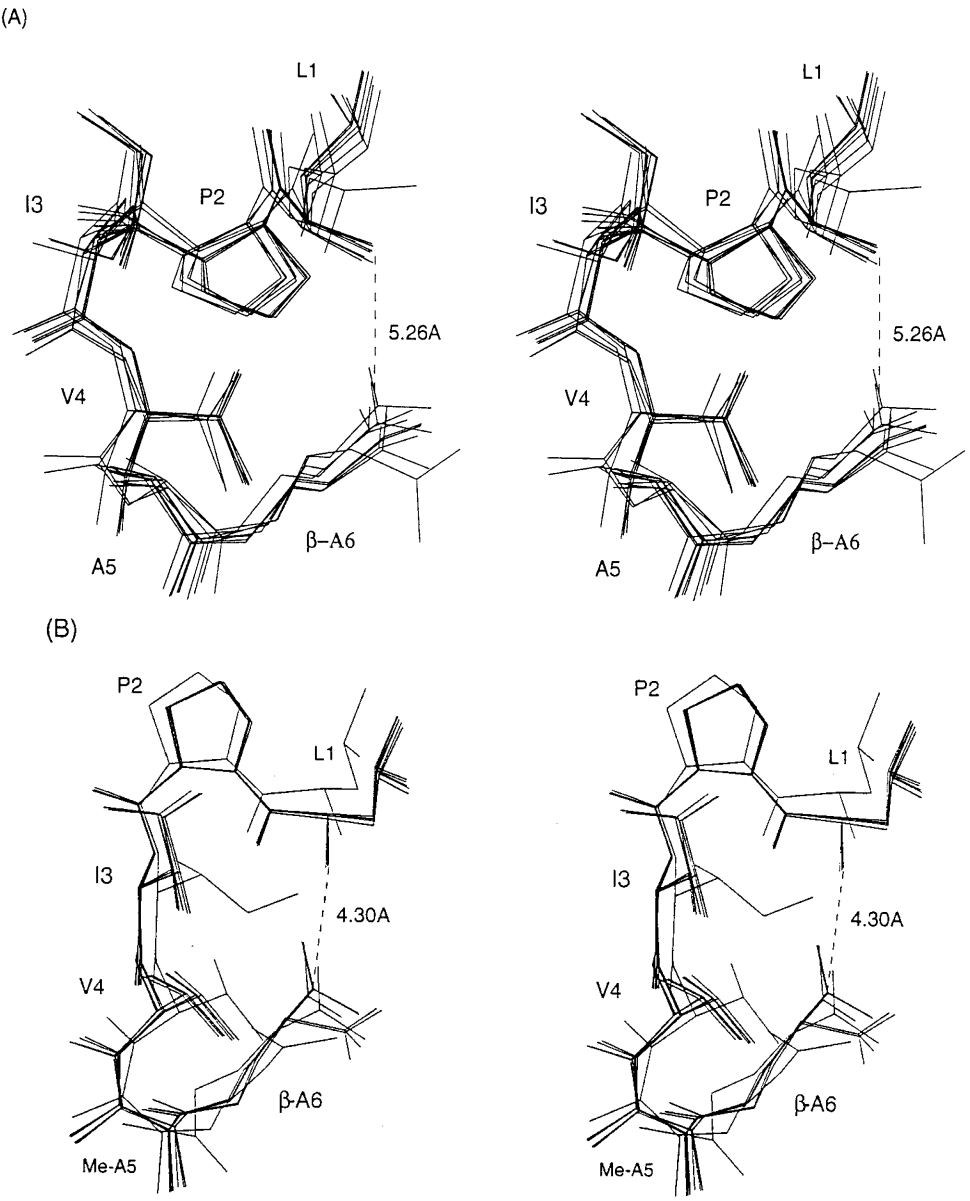

FIG. 3. Superposition of ten energy-minimized structures for the major (*trans*) forms of (A) peptide **I** and (B) peptide **II.** The dashed lines with numerical values indicate the distances between the N- and C-terminal residues of two linear peptides.

trans forms of **I** and **II** are found to be 5.26 and 4.30 Å, respectively, which are indeed close enough to facilitate the cyclization of **I** and **II** in high yields.

ACKNOWLEDGMENTS

This work was supported by Academia Sinica and the National Science Council, Taipei, Taiwan. We also thank Mr. Fong-Ku Shi in M & Vactek Corporation, Taiwan for measuring 2-D ROESY spectra.

REFERENCES

1. Kessler, H. (1982) *Angew. Chem. Int. Ed. Engl.* **21,** 512–523.
2. Kopple, K. D. (1972) *J. Pharmaceutical Sci.* **61,** 1345–1356.

3. Nishino, N., Xu, M., Ueno, Y., Kumagai, H., Mihara, H., and Fujimoto, T. (1992) *in* Peptide Chemistry 1991 (A. Suzuki, Ed.), pp. 135–140, Protein Research Foundation, Osaka, Japan.
4. Bodanszky, M., and Bodanszky, A. (1984) *in* The Practice of Peptide Synthesis, pp. 207–210, Springer-Verlag, Berlin.
5. Bodanszky, M. (1984) *in* Principles of Peptide Synthesis, pp. 217–225, Springer-Verlag, Berlin.
6. Kodaira, Y. (1961) *Agr. Biol. Chem.* **25,** 261–262.
7. Tamura, S., Kiyama, S., Kodaira, Y., and Higashikawa, S. (1964) *Agr. Biol. Chem.* **28,** 137–138.
8. Suzuki, A., Kawakami, K., and Tamura, S. (1971) *Agr. Biol. Chem.* **35,** 1641–1643.
9. Naganawa, H., Takita, T., Suzuki, A., Tamura, S., Lee, S., and Izumiya, N. (1976) *Agr. Biol. Chem.* **40,** 2223–2229.
10. Vey, A., Quiot, J. -M., Vago, C., and Fargues, J. (1985) *C.R. Acad. Sci. Paris* **300,** series III, 647–651.
11. Chen, H. C., Yeh, S. F., Ong, G. -T., Wu, S. -H., Sun, C. -M., and Chou, C. -K. (1995) *J. Nat. Prod.* **58,** 527–531.
12. Liao, S. -Y., Ong, G. -T., Wang, K. -T., and Wu, S. -H. (1995) *Biochim. Biophys. Acta* **1252,** 312–320.
13. Spatola, A. F., Anwer, M. K., and Rao, M. N. (1992) *Int. J. Peptide Protein Res.* **40,** 322–332.
14. Fournier, A., Wang, C. -T., and Felix, A. M. (1988) *Int. J. Peptide Protein Res.* **31,** 86–97.
15. Marion, D., and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.* **113,** 967–974.
16. Rance, M., Sørensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.* **117,** 479–485.
17. Bax, A., and Davies, D. G. (1985) *J. Magn. Reson.* **65,** 355–360.
18. Bax, A., and Morris, G. A. (1981) *J. Magn. Reson.* **42,** 501–505.
19. Bodenhausen, G., Kogler, H., and Ernst, R. R. (1984) *J. Magn. Reson.* **58,** 370–388.
20. Kessler, H., Griesinger, C., Kerssebaum, R., Wagner, K., and Ernst, R. R. (1987) *J. Am. Chem. Soc.* **109,** 607–609.
21. Lee, S., and Izumiya, N. (1977) *Int. J. Peptide Protein Res.* **10,** 206–218.
22. Wüthrich, K. (1986) *in* NMR of Protein and Nucleic Acids, pp. 123–125, John Wiley & Sons, New York.
23. Bodanszky, M., and Henes, J. B. (1975) *Bioorg. Chem.* **4,** 212–218.