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# Conformation of *cyclo*(L-Alanylglycyl-ϵ-aminocaproyl), a Cyclized Dipeptide Model for a β Bend. 2. Synthesis, Nuclear Magnetic Resonance, and Circular Dichroism Measurements[1]

**R. Deslauriers,[2a] D. J. Evans,[2b] S. J. Leach,[2b] Y. C. Meinwald,[2c] E. Minasian,[2b] G. Némethy,[2c] I. D. Rae,[2d] H. A. Scheraga,*[2c] R. L. Somorjai,[2e] E. R. Stimson,[2c] J. W. Van Nispen,[2c] and R. W. Woody[2f]**

*Division of Biological Sciences and Division of Chemistry, National Research Council of Canada, Ottawa, Canada K1A 0R6, Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia, Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853, Department of Chemistry, Monash University, Clayton, Victoria 3168, Australia, and Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523.* *Received August 7, 1980*

ABSTRACT: *cyclo*(L-Alanylglycyl-ϵ-aminocaproyl) has been synthesized by means of classical solution techniques. The Ala-Gly moiety of this molecule is constrained to form a β bend because of the closure of the ring by the five-carbon alkyl chain of the ϵ-aminocaproyl residue. $^{13}C$ NMR spectroscopic measurements indicate the absence of rapid internal segmental motion. Reorientation takes place by means of anisotropic rotational diffusion. $^1H$ NMR spectroscopic measurements show that the molecule has little flexibility and that its predominant backbone conformation is a type II β bend, with a strongly bent NH···O=C bond in the ϵ-aminocaproyl residue. Circular dichroism measurements confirm this conclusion. In addition, they indicate the presence of a minor component in a type I (and III) bend conformation. The distribution between these bend types depends on solvent and on temperature. The results are consistent with infrared and Raman spectroscopic data and with conformational energy calculations. Thus, the cyclized molecule can serve as a model for β bends, especially since the dihedral angles of the model compound fall in the range observed for β bends in proteins. The open-chain analogues Ac-L-Ala-Gly-NHMe and Boc-L-Ala-Gly-Aca-OMe exist as ensembles of conformations in solution, but with significant amounts of type II β-bend structures in the ensemble. This confirms earlier theoretical studies which predicted a high probability of the L-Ala-Gly dipeptide to form type II bends.

## I. Introduction

This paper is part of a series[3,4] which reports studies of the conformational properties of *cyclo*(L-alanylglycyl-ϵ-aminocaproyl), abbreviated *cyclo*(L-Ala-Gly-Aca). The Ala-Gly dipeptide, flanked by two peptide groups, must be in a β-bend conformation because of the steric constraint of the $(CH_2)_5$ chain in the Aca residue used to cyclize the molecule. See Figure 1 of paper 1[3] for nomenclature for the Aca methylene groups.

Experimental investigations of small open-chain and cyclic oligopeptides, using primarily various spectroscopic techniques, led to results that have been interpreted in

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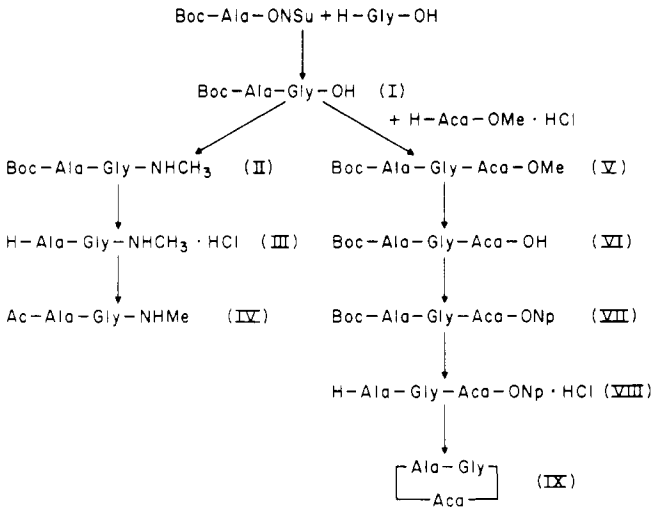


**Figure 1.** Schematic representation of the synthesis of *cyclo*(L-Ala-Gly-Aca) and of the open-chain analogue Ac-L-Ala-Gly-NHMe. The L form is implied for Ala throughout the scheme.

terms of the existence of $\beta$ bends.[5] Open-chain oligopeptides generally exist as an ensemble of many conformations,[6,7] however. This makes it difficult to calibrate spectroscopic experiments in terms of $\beta$ bends. While the ensemble of conformations of cyclic peptides is more limited, nevertheless it is sufficiently large to introduce similar difficulties in the interpretation of experimental results.

By contrast, *cyclo*(L-Ala-Gly-Aca) is well-suited to study the behavior of an oligopeptide chain in a bend conformation, because of steric constraints. Its properties can be used to obtain a unique interpretation of spectroscopic experiments in terms of bends. The rationale for choosing this molecule has been outlined in the first paper of elsewhere.[9] It was also shown there, by means of conformational energy calculations, that the molecule is highly constrained and that it can exist only in a few bend conformations. The experimental work reported here and in the following paper[4] confirms the prediction of the theoretical study by showing that the predominant form of the molecule is a type II bend, with a minor fraction of type I (and III) bends. Some open-chain analogues of *cyclo*(L-Ala-Gly-Aca) have also been investigated. This paper reports the synthesis and the results of NMR and CD studies. The third part of the series[4] describes the results of infrared and Raman studies. Preliminary data have been presented in a short communication.[8] Some of the results shown there have been superseded by this work. The spectroscopic work described earlier[8] was that of a peptide which was subsequently found to have been the DL compound. The spectroscopic data for *cyclo*(L-Ala-Gly-Aca) are presented here. The correlation of completed theoretical calculations with NMR, CD, and IR data has led to a slightly different conformational model here. A similar study of the two related compounds *cyclo*(L-Ala-L-Ala-Aca) and *cyclo*(L-Ala-D-Ala-Aca) will be reported elsewhere.[9]

## II. Syntheses

Classical solution techniques were used to synthesize the cyclic compound and the open-chain analogue (Ac-L-Ala-Gly-NHMe).[10] The path of synthesis is represented schematically in Figure 1, where the dipeptide Boc-L-Ala-Gly-OH (I) is the common intermediate for obtaining both the cyclic and open-chain compounds. The synthesis of intermediates is described in the supplementary material. Boc-L-Ala-Gly-OH and Boc-L-Ala-Gly-NHMe were prepared by using published methods.[11-14] Of several methods tried for cyclization, the best yield (50%) was obtained when the *p*-nitrophenyl ester of L-Ala-Gly-$\epsilon$-aminocaproic acid was cyclized according to the procedure of Sugihara et al.[15]

***cyclo*(Ala-Gly-Aca) (IX).** H-Ala-Gly-Aca-ONp·HCl (302 mg, 0.73 mmol) was dissolved in 5 mL of DMF. In the course of 3.5 h, this solution was added dropwise, with stirring, to 400 mL of pyridine, to which 0.09 mL of *N*-ethylmorpholine had been added, at ca. 50 °C. After an additional 1 h at 50 °C, the solution was kept overnight at room temperature and then evaporated. The residue was dissolved in MeOH and the solution was taken to dryness again. The yellow residue was dissolved in water and the solution applied to a weakly acidic cation exchange resin (Amberlite CG-50); water was used as the eluant. The fractions, containing a high-running (solvent system a; see supplementary material) UV-negative, ninhydrin-negative, and Cl-positive spot, were collected and lyophilized to give a white, fluffy material: yield 85 mg (48%); mp ca. 300 °C dec; $[\alpha]^{21}_D$ −7° (*c* 0.40, MeOH); TS, $R_f$ 0.54 (a), 0.43 (b), 0.60 (c) (Cl). Anal. Calcd for $C_{11}H_{19}N_3O_3$ (241.29): C, 54.76; H, 7.94; N, 17.41. Found: C, 54.9; H, 7.8; N, 17.4.

*cyclo*([1-$^{13}$C,10%]-L-Ala-Gly-Aca) and *cyclo*(L-Ala-[1,2-$^{13}$C,10%]Gly-Aca) were synthesized in a similar manner, using $^{13}$C-enriched alanine and glycine, respectively.

No racemization was detected with the use of the Manning–Moore test[16] and by a reversed-phase HPLC procedure.[17]

Mass spectra (both low and high resolution) of *cyclo*(L-Ala-Gly-Aca) were obtained with an AEI MS 902 spectrometer, with a CIS-2 source, from Scientific Research Instruments. The spectra demonstrated that the material was monomeric and cyclic.

## III. Methods

**$^1$H Nuclear Magnetic Resonance Measurements.** Solutions of Ac-L-Ala-Gly-NHMe (0.083 M), Boc-L-Ala-Gly-OMe (0.027 M), and *cyclo*(L-Ala-Gly-Aca) (0.069 M) were prepared in $Me_2SO$-$d_6$ of 100% isotopic purity (Aldrich) and degassed by bubbling with pure dry nitrogen for 10 min immediately before the spectra were recorded. Room-temperature $^1$H NMR spectra were recorded at 250 MHz, using the HF-250 spectrometer of the NMR Facility for Biomedical Research, Carnegie-Mellon University, Pittsburgh. Variable-temperature work and the NH exchange experiment were performed at 90 MHz, using Bruker HX-90 and WH90 spectrometers at Cornell University and Monash University, respectively. $Me_4Si$ was employed as an internal standard; the HF-250 was locked to this signal, but the deuterium lock was used with the HX-90 and WH90.

For the NOE experiment, fresh solutions of Ac-L-Ala-Gly-NHMe, Boc-L-Ala-Gly-Aca-OMe, and *cyclo*(L-Ala-Gly-Aca) in 100% $Me_2SO$-$d_6$ were flushed with dry nitrogen for 10 min immediately before the experiment. Measurements of peak areas were recorded electronically for spectra arising from 40–100 scans in a correlation mode and were standardized against the areas of resonances from hydrogens not involved in the irradiation. In the reference scan, the irradiating frequency was moved some 30–50 Hz from the peak of interest. Changes in area also were visualized as difference spectra and by digital integration to confirm the observations.

**$^{13}$C Nuclear Magnetic Resonance Measurements.** Proton-decoupled $^{13}$C NMR spectra were obtained at 20 MHz. Two different Varian CFT-20 spectrometers were used, operating at ambient temperatures of 32 and 38 °C, respectively. $Me_4Si$ was used as an external standard in $D_2O$ but as an internal standard in $Me_2SO$-$d_6$ and TFE. Chemical shifts are reported in parts per million (ppm) downfield from $Me_4Si$. Solutions of Aca (1 M) were prepared in $D_2O$, and the pH was adjusted with solutions of either HCl or NaOH dissolved in $D_2O$ (to pH meter readings of 1.0, 7.6, and 12.2). Solutions of *cyclo*(L-Ala-Gly-Aca) (0.07 M) were prepared in TFE, $Me_2SO$-$d_6$, and $D_2O$ (neutral pH, unadjusted). Also, solutions of Ac-L-Ala-Gly-NHMe (0.25 M) and Boc-L-Ala-Gly-Aca-OMe (0.05 M) were prepared in $D_2O$ (neutral pH, unadjusted).

**$^{13}$C Spin–Lattice Relaxation Time ($T_1$) Measurements.** Spin–lattice relaxation times ($T_1$) were measured by using the fast inversion–recovery Fourier transform (FIR FT) pulse sequence, $[180°-\tau-90°-T]_n$, where $\tau$ is a variable delay time, $T$ is a delay sufficient to allow the decay of the transverse magnetization before the next 180° pulse,[18] and $n$ is the number of scans. The value of $T$ was at least 1 s for the partially relaxed spectra

and 5 s for the fully relaxed spectra. The 90° pulse was 24 μs. At least ten values of $\tau$ and three values of the fully relaxed spectrum were obtained for each measurement of $T_1$. A total of 10 000 scans of the spectrum was acquired. Experiments were performed in triplicate. Reproducibility of measurements was ±10%. The values of $T_1$ were calculated from a least-squares fit to the best straight line on a semilogarithmic plot, using

$$M_\tau = M_\infty[1 - \alpha \exp(-\tau/T_1)] \quad (1)$$

$$\alpha = 2 - E_1(n - 1)/n \quad (2)$$

where $E_1 = \exp(-T/T_1)$, $n$ is the number of scans, $M_\tau$ is the value of the magnetization for a given value of $\tau$, and $M_\infty$ is the equilibrium value of the magnetization.[18] For $\tau = 0$, $M = -M_\infty$.[18]

**Calculation of the $^{13}C$ Spin–Lattice Relaxation Times.** Theoretical spin–lattice relaxation times ($T_1$) were calculated for the computed minimum-energy conformations[3] and fitted to the observed $T_1$ data by means of a nonlinear least-squares method. It was assumed that rigid anisotropic overall rotational diffusion describes adequately the molecular reorientation and provides the dominant mechanism for the dipolar relaxation of the $^{13}C$–H bonds.[19] The method of computation was described earlier.[20] It uses the moments of inertia $I_1$, $I_2$, and $I_3$, calculated from the computed coordinates,[3] and it fits the eight experimentally obtained values of $T_1$ with three effective correlation times $\tau_1$, $\tau_2$, and $\tau_3$, corresponding to rotations about the three main axes of inertia. The sum $F$ (defined in eq 6 of ref 20) of squared deviations between experimental and calculated $T_1$'s is minimized. The set of physically reasonable $\tau_i$'s with the lowest $F$ is retained; i.e., fittings are rejected in which the $\tau_i$'s differ by 2 or 3 orders of magnitude. The relaxation time of the alanyl $C^\beta H_3$ group was not included in the fitting because relaxation mechanisms other than dipolar are equally or more important for this group.[21]

**Circular Dichroism Spectra.** Circular dichroism (CD) spectra were recorded with a Jasco J20 circular dichroism spectrometer. The procedures used were those described by Nicola et al.[22]

Ellipticity readings were confined to the wavelength range in which absorbance values were ≤1.5 and signal-to-noise ratios were considered acceptable. The lower wavelength limit for these conditions varied between 190 and 197 nm. Linearity of response was checked for the cyclic peptide by measuring the amplitude of the 202/204- and 225-nm extrema as a function of concentration in the range 0.37–1.5 mg/mL in methanol solution. Peptide concentrations normally used were 1.5–1.8 mg/mL and spectra were recorded by using methanol, trifluoroethanol, and water in 0.1-mm jacketed cells at 5, 22, and 35 °C. All values of $[\theta]_M$ are quoted per mole of peptide, using the molecular weight of the acyclic or cyclic peptide, respectively. To convert these values to values per mole of amide groups, they would need to be divided by 3.

**Circular Dichroism Calculations.** The CD of *cyclo*(L-Ala-Gly-Aca) was calculated by methods described earlier.[23] CD curves were calculated for each of the ten low-energy computed conformations with trans peptide bonds, obtained by conformational energy calculations (Table III of ref 3). The calculation of the CD curve for each conformation follows methods described in detail elsewhere.[24,25] Only the $n\pi^*$ and $\pi\pi^*$ transitions of the peptide group were considered. The methyl side chain of the Ala residue and the methylene groups of the Aca residue were not included in the calculation. The optical parameters used assume a planar trans geometry ($\omega = 180°$) but, since the deviations from planarity in the ten computed conformations[3] used in this calculation are at most 13°, the use of these optical parameters with a nonplanar geometry should lead to negligible errors. A resultant theoretical CD curve of the cyclic peptide was than calculated at 0 and 25 °C as a Boltzmann-weighted average of the computed CD curves for the individual conformations.

## IV. Results

**$^1H$ Nuclear Magnetic Resonance Measurements.** The chemical shifts and coupling constants are given in Table I. Temperature coefficients of NH chemical shifts are given in Table II. The NH resonances of the Ala and Gly residues coincided in each compound except the Boc derivative, even at 250 MHz. Therefore, the combined resonances had to be considered for the first two compounds in Table I when examining temperature coefficients, exchange rates, and NOE behavior.

The $C^\alpha H$ resonances were assigned by consideration of their multiplicities and by means of extensive decoupling experiments. It was also established that the hydrogen giving rise to the signal at 0.96 ppm in the spectrum of *cyclo*(L-Ala-Gly-Aca) was not coupled appreciably to the $C^\alpha$ nor to the $C^\epsilon$ hydrogens of the Aca residue. Therefore, it is not a $C^\beta$ or a $C^\delta$ hydrogen and, hence, must be one of the $C^\gamma$ hydrogens. Attempts to confirm this assignment by single-frequency $^{13}C\{^1H\}$ decoupling experiments were inconclusive. This high-field signal is absent in the spectrum of Boc-L-Ala-Gly-Aca-OH, suggesting that the environment of one of the $C^\gamma$ hydrogens is unusual in the cyclic compound.

Some of the chemical shifts reported in Table I were assigned from analyses of the signals of the methylene protons, Gly $C^\alpha H$, Aca $C^\alpha H$, and Aca $C^\epsilon H$, as the AB parts of ABX, ABXY, and ABMNX multiplets, respectively.[26]

The results of the NOE experiments are reported in Table III. The enhancements were measured by simple techniques[27] because more sophisticated pulse sequences were not available to us.[28]

**$^{13}C$ Nuclear Magnetic Resonance Measurements. Assignment of $^{13}C$ NMR Spectra.** The $^{13}C$ NMR chemical shifts in $D_2O$ are given in Table IV. The $^{13}C$ NMR spectra were assigned by comparison with spectra of free amino acids corrected for incorporation into peptides.[29] The assignments for proton-bearing carbons were confirmed by observing the multiplet structures in off-resonance decoupled spectra. The assignment for glycine was confirmed by $^{13}C$ enrichment at the $\alpha$ carbon of this residue. Carbonyl carbons were assigned, as stated above, by using calculated chemical shifts (i.e., the shift for the free amino acid, corrected for incorporation into a peptide) and by observing the effects of pH titration on chemical shifts. The carbonyl assignments were also shown to be correct by selective $^{13}C$ enrichment of the Ala and Gly carbonyl carbons.

In the case of free Aca, the resonances were assigned by calculating the chemical shifts of Aca, starting with those of pentane[30–32] and adding to these the contributions from the amino and carboxyl substituents.[33] The assignment of the carboxyl carbon was based on that of hexanoic acid.[34] The assignments were confirmed by observing the effect of pH titration of the amino and carboxyl groups on the $^{13}C$ chemical shifts of the individual carbon resonances (see below). The shifts observed in the titration of free amino acids were used as a basis for these assignments.[29]

In Ac-L-Ala-Gly-NHMe, the assignments of the acetyl resonances were based on those of *N*-methylacetamide, determined here. The assignment of the methyl amide $CH_3$ group was based on that of ethyl methylcarbamate.[35]

The $C^\beta$ resonance of Ala in *cyclo*(L-Ala-Gly-Aca) is shifted upfield by 2.1–2.3 ppm compared to the calculated chemical shift and to those observed for the model compounds. In a similar comparison, the other resonances of Ala and Gly change little. In the Aca moiety, the resonance of $C^\alpha$ is downfield by 1.0 ppm compared to that of the acyclic peptide, whereas all other resonances in this residue show upfield shifts of 2–3 ppm (assuming no crossover of resonances, as in oxytocin[36]), the largest (3.0 ppm) being observed for the $C^\gamma$ resonance. Thus, it appears that cyclization produces the largest perturbation in the Aca residue—in particular at the $\gamma$ carbon.

Table I
$^1$H NMR Data for *cyclo*(L-Ala-Gly-Aca) and Its Open-Chain Analogues in $Me_2SO$-$d_6$

| residue | | Ac-L-Ala-Gly-NHMe | | *cyclo*(L-Ala-Gly-Aca) | | Boc-L-Ala-Gly-Aca-OMe | |
|---|---|---|---|---|---|---|---|
| | | δ [a] | *J*, Hz | δ [a] | *J*, Hz | δ [a] | *J*, Hz |
| Ac | $CH_3$ | 1.856 | | | | | |
| Boc | $CH_3$ | | | | | 1.385 | |
| Ala | NH | 8.157 m | | 8.605 | | 7.152 m | |
| | $C^\alpha H$ | 4.179 p | | 4.321 p | | 3.908 p | |
| | $C^\beta H_3$ | 1.199 d | | 1.195 | | 1.161 d | |
| | $J_{NH-C^\alpha H}$ | | ~7.0 | | ~7.1 | | 6.4 |
| | $J_{C^\alpha H-C^\beta H}$ | | 7.0 | | 7.2 | | 7.3 |
| Gly | NH | 8.157 m | | 8.605 m | | 8.127 m | |
| | $C^\alpha H_A$ | 3.593 dd | | 3.349 dd | | 3.592 dd | |
| | $C^\alpha H_B$ | 3.663 dd | | 3.802 dd | | 3.656 dd | |
| | $J_{NH-C^\alpha H_A}$ | | 5.4 | | 5.0 | | 6.4 |
| | $J_{NH-C^\alpha H_B}$ | | 6.2 | | 7.1 | | 6.4 |
| | $J_{C^\alpha H_A-C^\beta H_B}$ | | 16.5 | | 15.7 | | 16.8 |
| NHMe | NH | 7.641 q | | | | | |
| | $CH_3$ | 2.589 d | | | | | |
| | $J_{NH-C^{Me}H}$ | | 4.6 | | | | |
| Aca | NH | | | 6.962 t | | 7.630 m | |
| | $C^\epsilon H_A$ | | | 2.87 tt | | 2.993 dq | |
| | $C^\epsilon H_B$ | | | 3.3 m [b] | | 3.078 dq | |
| | $C^\beta H_2$, $C^\gamma H$, $C^\delta H_2$ | | | 1.2–1.7 | | 1.2–1.6 m [c] | |
| | $C^\gamma H$ | | | 0.96 m | | | |
| | $C^\alpha H_A$ | | | 2.045 dt | | 2.281 t | |
| | $C^\alpha H_B$ | | | 2.177 td | | | |
| | $J_{NH-C^\epsilon H_A}$ | | | | ~5 | | 6.4 |
| | $J_{NH-C^\epsilon H_B}$ | | | | | | 6.4 |
| | $J_{C^\epsilon H_A-C^\epsilon H_B}$ | | | | ~11 | | 13.2 |
| | $J_{C^\alpha H_A-C^\alpha H_B}$ | | | | ~13 | | |
| | $J_{C^\alpha H_A-C^\beta H_{A,B}}$ | | | | 4.0 | | 7.3 |
| | $J_{C^\alpha H_B-C^\beta H_A}$ | | | | ~13 | | ~7 |
| | $J_{C^\alpha H_B-C^\beta H_B}$ | | | | ~4 | | ~7 |
| OMe | $CH_3$ | | | | | 3.352 s | |

[a] In ppm downfield from internal $Me_4Si$ (at 250 or 270 MHz). Abbreviations used: d = doublet; t = triplet; q = quartet; p = pentet; m = multiplet; dd = doublet of doublets; tt = triplet of triplets; dt = doublet of triplets; td = triplet of doublets; dq = doublet of quartets. The larger coupling constant gives rise to the first-mentioned splitting in each case. [b] This peak overlapped the one for Gly $C^\alpha H_A$. Although its chemical shift could be determined, its multiplicity could not. [c] These multiplets include a quintet ($J$ = 7.5 Hz) with an area equivalent to two hydrogens, at δ = 1.509.

Table II
Temperature Coefficients of the NH Chemical Shifts in $Me_2SO$-$d_6$

| peptide | $-d\delta/dT$, ppb/deg | | | |
|---|---|---|---|---|
| | Ala NH | Gly NH | $NHCH_3$ | Aca NH |
| Ac-L-Ala-Gly-NHMe | 5.7 | 5.7 | 4.3 | |
| Boc-L-Ala-Gly-Aca-OMe | 9.0 | 6.3 | | 4.9 |
| *cyclo*(L-Ala-Gly-Aca) | 8.1 | 8.1 | | 3.3 |

The $^{13}C$ chemical shifts of *cyclo*(L-Ala-Gly-Aca) and Ac-Ala-Gly-NHMe in several solvents, and the differences (Δδ) between various solvent pairs, are shown in Tables V and VI, respectively.

**Experimental Spin–Lattice Relaxation Times.** Table VII gives the values of $NT_1$ determined for Boc-L-Ala-Gly-Aca-OMe and *cyclo*(L-Ala-Gly-Aca) in $D_2O$ at 32 °C, where $N$ is the number of hydrogens directly bonded to the carbon under investigation. The effective correlation times for rotational reorientation ($\tau_c$) were determined from the equation[37]

$$1/NT_1 = \langle r_{CH}^{-6} \rangle \hbar \gamma_C^2 \gamma_H^2 \tau_C \qquad (3)$$

where $\hbar$ is Planck's constant divided by $2\pi$, $\gamma_C$ and $\gamma_H$ are the gyromagnetic ratios of carbon and hydrogen, respectively, and $\langle r_{CH}^{-6} \rangle$ is the vibrationally averaged inverse sixth power of the C–H internuclear distance.

Table III
Results of NOE Experiments in $Me_2SO$-$d_6$

| peptide | irradiated | observed | increase in area, % 90 MHz | 250 MHz |
|---|---|---|---|---|
| Ac-L-Ala-Gly-NHMe | Ala NH + Gly NH | Ala $C^\alpha H$ | 22 | 21 |
| | Ala NH + Gly NH | Gly $C^\alpha H$ | 8 | 3 |
| | Ala $C^\alpha H$ | Ala NH + Gly NH | 5.5 | 9 |
| Boc-L-Ala-Gly-OMe | Ala $C^\alpha H$ | Gly NH | 10.8 [a] | |
| | Ala $C^\alpha H$ | Ala NH | 1.0 | |
| *cyclo*(L-Ala-Gly-Aca) | Ala NH + Gly NH | Ala $C^\alpha H$ | *b* | 9.4 |
| | Ala NH + Gly NH | Gly $C^\alpha H$ | *b* | 0 [c] |
| | Ala $C^\alpha H$ | Ala NH + Gly NH | *b* | 0 |

[a] The reverse experiment was not possible because of the proximity of the water peak to that of the Ala $C^\alpha H$. [b] Experiment not performed. [c] Result for low-field $C^\alpha H$; the high-field signal was obscured.

The small value of $NT_1$ of the α carbon of Gly (1.1 s) in Boc-L-Ala-Gly-Aca-OMe (Table VII) suggests that the motion of Gly is restricted (while anisotropic overall molecular motion would lead to reduced values of $NT_1$, such

Table IV
$^{13}C$ Chemical Shifts[a] of *cyclo*(L-Ala-Gly-Aca) and Its Open-Chain Analogues in $D_2O$ at 32 °C

| residue | | calcd[b] | Aca pH 1.0 | Aca pH 7.6 | Aca pH 12.2 | Ac-L-Ala-Gly-NHMe | Boc-L-Ala-Gly-Aca-OMe | *cyclo*(L-Ala-Gly-Aca) |
|---|---|---|---|---|---|---|---|---|
| Boc | $CH_3$ | | | | | | 28.80 | |
| | -C< | | | | | | 82.90 | |
| Ac | $CH_3$ | 22.8 | | | | 22.80 | | |
| | C=O | 175.6 | | | | 175.66 | | |
| Ala | α-CH | 50.8 | | | | 51.21 | 53.20 | 50.82 |
| | β-$CH_3$ | 17.7 | | | | 17.51 | 17.60 | 15.42 |
| | C=O | 175.8 | | | | 177.66 | 177.97 | 176.00 |
| Gly | α-$CH_2$ | 43.5 | | | | 43.67 | 43.70 | 44.57 |
| | C=O | 172.7 | | | | 172.97 | 172.20 | 172.78 |
| Aca[c] | α-$CH_2$ | 38.8 | 34.62 | 38.40 | 38.83 | | 34.70 | 35.66 |
| | β-$CH_2$ | 27.9 | 24.81 | 26.57 | 27.89 | | 25.03 | 22.65[d] |
| | γ-$CH_2$ | 26.6 | 26.18 | 26.58 | 26.91 | | 26.57 | 23.64[d] |
| | δ-$CH_2$ | 27.5 | 27.50 | 27.55 | 32.70 | | 29.04 | 26.97[d] |
| | ε-$CH_2$ | 40.5 | 40.52 | 40.45 | 41.72 | | 40.31 | 38.12 |
| | C=O | 179.7 | 179.69 | 184.50 | 185.14 | | 178.61 | 179.03 |
| OMe | $CH_3$ | | | | | | 52.3 | |
| NHMe | $CH_3$ | 27.4 | | | | 27.01 | | |

[a] In ppm downfield from external $Me_4Si$. Accuracy of chemical shifts is ±0.05 ppm. [b] Calculated as explained in text. [c] Spectrum calculated for zwitterionic species. α-Carbon is next to the carboxyl group. [d] The resonances were assigned according to the calculations described in the text. These calculations assumed that structural changes (due to the substitution of a different terminal group) are the major factors determining the $^{13}C$ chemical shift. Conformational changes are generally of secondary importance. Unambiguous assignment would require $^{13}C$ enrichment of specific carbons or, alternatively, specific proton decoupling of assigned $^1H$ resonances.

Table V
$^{13}C$ Chemical Shifts of *cyclo*(L-Ala-Gly-Aca) in Several Solvents at 38 °C[a]

| residue | | δ $D_2O$ | δ $Me_2SO$-$d_6$ | δ TFE | Δδ $D_2O$-$Me_2SO$-$d_6$ | Δδ TFE-$Me_2SO$-$d_6$ |
|---|---|---|---|---|---|---|
| Ala | α-CH | 51.13 | 48.88 | 51.30 | 2.25 | 2.42 |
| | β-$CH_3$ | 15.54 | 15.34 | 15.13 | 0.20 | −0.21 |
| | C=O | 176.13 | 172.73 | 176.08 | 3.40 | 3.35 |
| Gly | α-$CH_2$ | 44.79 | 43.37 | 45.03 | 1.42 | 1.66 |
| | C=O | 172.88 | 168.59 | 172.85 | 4.29 | 4.26 |
| Aca | α-$CH_2$ | 35.94 | 34.15 | 36.29 | 1.79 | 2.12 |
| | β-$CH_2$ | 22.95 | 21.59 | 22.26 | 1.36 | 0.67 |
| | γ-$CH_2$ | 23.79 | 22.08 | 23.69 | 1.71 | 1.61 |
| | δ-$CH_2$ | 26.90 | 26.17 | 27.25 | 0.73 | 1.08 |
| | ε-$CH_2$ | 38.32 | 35.75 | 38.38 | 2.57 | 2.63 |
| | C=O | 179.18 | 174.67 | 178.99 | 4.51 | 4.32 |

[a] Differences between these data and those of Table IV are believed to be due to differences in temperature. Also, since the error in δ is ~±0.05 in each value of δ, differences of ±0.1 are not significant. Values of δ are in ppm downfield from external $Me_4Si$ for $D_2O$ and from internal $Me_4Si$ for $Me_2SO$-$d_6$ and TFE.

Table VI
$^{13}C$ Chemical Shifts of Ac-L-Ala-Gly-NHMe in Several Solvents at 38 °C[a]

| residue | | δ $D_2O$ | δ $Me_2SO$-$d_6$ | Δδ $D_2O$-$Me_2SO$-$d_6$ |
|---|---|---|---|---|
| Ac | $CH_3$ | 22.84 | 22.46 | 0.38 |
| | C=O | 175.50 | 169.63 | 5.87 |
| Ala | αCH | 51.15 | 48.71 | 2.44 |
| | β$CH_3$ | 17.52 | 17.57 | −0.05 |
| | C=O | 177.03 | 172.68 | 4.35 |
| Gly | α$CH_2$ | 43.70 | 42.13 | 1.57 |
| | C=O | 172.95 | 169.13 | 3.82 |
| NHMe | $CH_3$ | 26.98 | 25.40 | 1.58 |

[a] Differences between these data and those of Table IV are believed to be due to differences in temperature. Also, since the error in δ is ~±0.05 in each value of δ, differences of ±0.1 are not significant. Values of δ are in ppm downfield from external $Me_4Si$ for $D_2O$ and from internal $Me_4Si$ for $Me_2SO$-$d_6$.

an effect would be smaller than the experimental errors in our measurements for molecules of the size studied here[38]). The rest of the Boc-L-Ala-Gly-Aca-OMe molecule (especially the Aca portion, with values of $NT_1$ ranging from 1.3 to 2.3 s, and the N and C termini, with values of 3.0 and 2.4 s, respectively) shows segmental motion in addition to overall molecular rotation, with the $CH_3$ groups undergoing internal motion with respect to the backbone. The correlation time for overall molecular motion in Boc-L-Ala-Gly-Aca-OMe is estimated from eq 3 to be $4.7 \times 10^{-11}$ s, based on a minimum value of $NT_1 = 1.1$ s for the Gly $C^\alpha$ resonance.

The $^{13}C$ spin–lattice relaxation times of *cyclo*(L-Ala-Gly-Aca) are of the same magnitude as those of Boc-L-Ala-Gly-Aca. In the backbone of the cyclic compound, the values of $NT_1$ of all the proton-bearing carbons range between 1.3 and 1.5 s, which corresponds to an effective correlation time of ca. $3.3 \times 10^{-11}$ s, assuming *isotropic* rigid-body reorientation. The $CH_3$ group of the alanyl residue (with a value of $NT_1 = 2.7$ s) shows evidence of faster internal motion. We find no conclusive evidence for greater internal motion of the Gly residue compared to other carbon atoms in the backbone, as has been observed in larger cyclic and acyclic peptides;[39] i.e., all backbone carbons appear to have a similar degree of flexibility or rigidity.

Table VII
$^{13}C$ Spin-Lattice Relaxation Times and Effective Rotational Correlation Times in Boc-L-Ala-Gly-Aca-OMe and *cyclo*(L-Ala-Gly-Aca) in $D_2O$ at 32 °C

| residue | | Boc-L-Ala-Gly-Aca-OMe | | *cyclo*(L-Ala-Gly-Aca) | |
|---|---|---|---|---|---|
| | | $NT_1$,[a] s | $\tau_c \times 10^{11}$, s | $NT_1$,[a] s | $\tau_c \times 10^{11}$, s |
| Boc | $CH_3$ | 3.0 | 1.6 | | |
| | -C< | n.o.[b] | | | |
| Ala | α-CH | 1.5 | 3.1 | 1.3 | 3.5 |
| | β-$CH_3$ | 2.1 | 2.2 | 2.7 | 1.7 |
| Gly | α-$CH_2$ | 1.1 | 4.7 | 1.5 | 3.1 |
| Aca | α-$CH_2$ | 2.3 | 2.0 | 1.4 | 3.3 |
| | β-$CH_2$ | 2.3 | 2.0 | 1.3 | 3.5 |
| | γ-$CH_2$ | 2.1 | 2.2 | 1.4 | 3.3 |
| | δ-$CH_2$ | 1.5 | 3.1 | 1.4 | 3.3 |
| | ε-$CH_2$ | 1.3 | 3.6 | 1.3 | 3.5 |
| OMe | $CH_3$ | 2.4 | 1.9 | | |

[a] Values of $NT_1$ are observed values of $T_1$ multiplied by $N$, the number of hydrogens directly bonded to the carbon under study. Reproducibility of measurements is ±10%. [b] Not observed.

Another estimate of an approximate value of the rotational correlation time of the *cyclo*(L-Ala-Gly-Aca) molecule can be made, using the Stokes–Einstein relation[40]

$$\tau_c = 4\pi R^3\eta/3kT = V_m\eta/kT \quad (4)$$

where $V_m$ is the molecular volume, $\eta$ is the viscosity of the solution, $T$ is the absolute temperature, and $k$ is the Boltzmann constant. The value of $V_m$ of *cyclo*(L-Ala-Gly-Aca), estimated by the method of atomic increments,[41] is 232 Å$^3$, and $T$ and $\eta$ were taken as 305 K and 0.7 cP, respectively, leading to a value of $\tau_c = 4 \times 10^{-11}$ s. This value is close to those given in the last column of Table VII. Thus, the molecule does not appear to aggregate to any significant extent. On the basis of the above calculations, the experimental results suggest that there is no sufficiently rapid internal flexibility which would influence the values of $T_1$.

Further support for the absence of rapid internal motion (relative to the rate of overall molecular reorientation) in *cyclo*(L-Ala-Gly-Aca) can be obtained by comparing the observed values of $NT_1$ with those observed for cycloalkanes which are thought to contain unhindered $CH_2$ groups.[42] Cycloalkanes dissolved in $CDCl_3$ show decreasing values of $T_1$ for $CH_2$ resonances as the size of the cyclic molecule increases and reach a limiting value of 1.2 s ($NT_1$ = 2.4 s), which is thought to be the relaxation time of an unhindered segment of a $CH_2$ chain dominated by segmental motion rather than overall tumbling. This limiting value is achieved when the cyclic molecule contains ca. 32 carbons.[42] Thus, in *cyclo*(L-Ala-Gly-Aca), segmental motion does not seem to be very rapid when compared with the rate of overall molecular motion.

**Fitting of the Spin–Lattice Relaxation Times to Specific Computed Conformations.** Fitted values of $NT_1$ are shown in Table VIII for various computed conformations[3] and for Boltzmann-weighted averages over various groups of related conformations, assuming a model of *anisotropic* rigid-body reorientation. The quality of the fitting of observed data is comparable for all seven of the lowest energy computed conformations,[3] independently of bend type. The magnitudes of the computed values of $\tau_i$ are physically reasonable (cf. the effective $\tau_i$, derived from observed $T_1$ values, in Table VII). The anisotropy of all seven conformations is small and similar in magnitude, as indicated by the computed moments of inertia: $I_1$ ranges from 860 to 940 amu Å$^2$, $I_2$ from 1072 to 1237 amu Å$^2$, and $I_3$ from 1646 to 1822 amu Å$^2$. Because of the low anisotropy and because the fitted $T_1$'s and $\tau_i$'s are so close to each other, it is reasonable to calculate Boltzmann averages of the $T_1$'s and $\tau_i$'s over the various conformations. These averages are shown in the last two columns for the two type II bends (conformations 1 and 3) and for all seven low-energy conformations, respectively. No large differences are seen for the two averages.

The similarity of the goodness of fitting (as indicated by similar $F$ values) by various individual or averaged conformations leads to the conclusion that the $^{13}C$ $T_1$ measurement is not a sensitive monitor of relatively small conformational differences, as they occur in this small cyclic molecule. The fitting parameters (i.e., the values of $\tau_i$) are physically reasonable. Thus, it is not possible, on the basis of the $T_1$ data alone, to select one of the

Table VIII
Comparison of Observed and Calculated $^{13}C$ Spin-Lattice Relaxation Times of *cyclo*(L-Ala-Gly-Aca)

| | | | | calcd $NT_1$ (s), fitted to the computed minimum-energy conformations, as indicated[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| residue | carbon | exptl[a] $NT_1$ | conformation[b] bend type[f] | 1[c] II | 2 I | 3 II | 4 III | 5 III | 6 III | 7 I | 1 + 3[d] | 1 to 7[e] |
| Ala | $C^\alpha H$ | 1.3 | | 1.34 | 1.37 | 1.33 | 1.36 | 1.37 | 1.35 | 1.31 | 1.34 | 1.35 |
| Gly | $C^\alpha H$ | 1.5 | | 1.37 | 1.40 | 1.36 | 1.39 | 1.40 | 1.38 | 1.39 | 1.37 | 1.38 |
| Aca | $C^\varepsilon H$ | 1.3 | | 1.35 | 1.34 | 1.35 | 1.36 | 1.32 | 1.35 | 1.28 | 1.35 | 1.35 |
| | $C^\delta H$ | 1.4 | | 1.37 | 1.33 | 1.36 | 1.37 | 1.34 | 1.34 | 1.39 | 1.37 | 1.36 |
| | $C^\gamma H$ | 1.4 | | 1.38 | 1.36 | 1.38 | 1.40 | 1.38 | 1.38 | 1.41 | 1.38 | 1.38 |
| | $C^\beta H$ | 1.3 | | 1.39 | 1.38 | 1.39 | 1.40 | 1.39 | 1.38 | 1.37 | 1.39 | 1.39 |
| | $C^\alpha H$ | 1.4 | | 1.36 | 1.38 | 1.37 | 1.38 | 1.37 | 1.37 | 1.43 | 1.36 | 1.37 |
| Ala | $C^\beta H_3$[g] | 2.7 | | (1.37) | (1.35) | (1.38) | (1.39) | (1.34) | (1.37) | (1.43) | (1.37) | (1.37) |
| | | | $\tau_1$[h] | 3.21 | 2.90 | 3.41 | 3.22 | 2.79 | 3.08 | 3.02 | | |
| | | | $\tau_2$ | 3.79 | 3.77 | 3.61 | 3.62 | 3.85 | 3.79 | 4.85 | | |
| | | | $\tau_3$ | 3.32 | 3.91 | 3.14 | 3.32 | 4.06 | 3.48 | 2.53 | | |
| | | | $10^4F$[i] | 2.92 | 2.75 | 2.88 | 3.03 | 2.63 | 2.93 | 1.65 | | |

[a] From column 5 of Table VII. [b] See Table III of ref 3 for these conformations. The numbering used here refers to that table. [c] The lowest energy computed conformation. [d] Boltzmann-weighted average at $T$ = 305 K over the two type II bend conformations. [e] Boltzmann-weighted average at $T$ = 305 K over all seven low-energy conformations (type I, II, and III bends). [f] As given in ref 3. [g] This group was not included in the fitting. See Methods. Hence the computed values of $NT_1$ are shown in parentheses. [h] Correlation times obtained from the least-squares fitting, in s × $10^{11}$. [i] Sum of squared deviations obtained for best fit (see Methods).

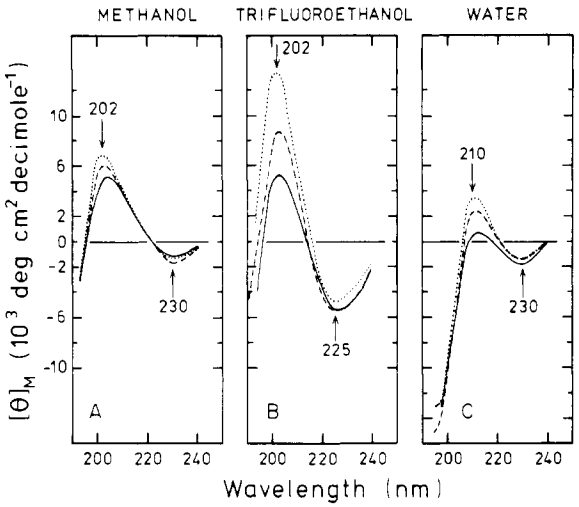


**Figure 2.** CD spectra of Ac-L-Ala-Gly-NHMe in (A) methanol at 1.45 mg/mL, (B) trifluoroethanol at 1.52 mg/mL, and (C) water at 1.51 mg/mL, at (···) 5, (- - -) 22, and (—) 35 °C.

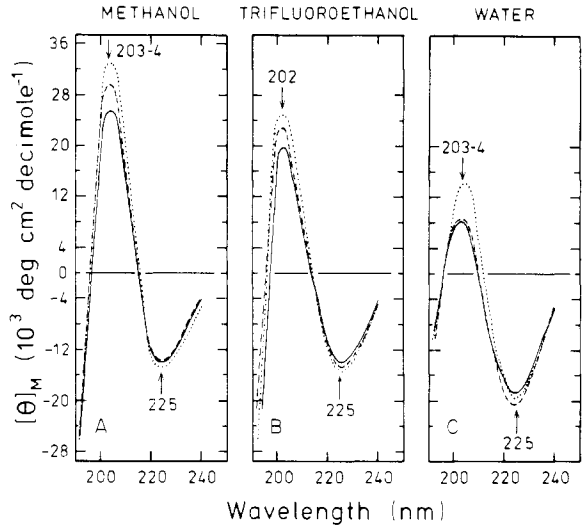


**Figure 3.** CD spectra of *cyclo*(L-Ala-Gly-Aca) in (A) methanol at 1.50 mg/mL, (B) trifluoroethanol at 1.52 mg/mL, and (C) water at 1.70 mg/mL, at (···) 5, (- - -) 22, and (—) 35 °C.

conformations or one set of them as more likely than the others. The $T_1$ measurements indicate that the molecules can be considered as an *effectively* rigid body, reorienting by means of anisotropic rotational diffusion, but with very little anisotropy.

**Circular Dichroism Spectra.** The CD spectra of Ac-L-Ala-Gly-NHMe in methanol, trifluoroethanol, and water are shown in Figure 2. In all three solvents, there is a negative extremum at 230 nm (methanol and water) or 225 nm (trifluoroethanol), the amplitude being largest in trifluoroethanol (−5500 deg cm$^2$ dmol$^{-1}$) and smallest in methanol (−1500 deg cm$^2$ dmol$^{-1}$). There is also a positive extremum which, at 5 °C, is most prominent in trifluoroethanol (+13 500 deg cm$^2$ dmol$^{-1}$, 202 nm) and the least prominent in water (+3500 deg cm$^2$ dmol$^{-1}$, 210 nm). This positive extremum increases in magnitude as the temperature is decreased in all three solvents, while the negative extremum is insensitive to temperature changes. Within the concentration range examined (0.37–1.5 mg/mL in methanol), the magnitude of the positive extremum increases linearly with the concentration; the negative extremum, however, deviates from linearity below 0.37 mg/mL. The blue shift from 230 to 225 nm induced by trifluoroethanol has been observed before.[23,43,44]

The spectra are, in general, similar to the class B types of CD spectra computed by Woody[25] for $\beta$ bends. Brahms et al.[45] observed CD spectra for poly(Ala$_2$-Gly$_2$) which had qualitatively similar features to those seen in Figure 2B for Ac-L-Ala-Gly-NHMe in trifluoroethanol, viz., an intense positive band at 207.5 nm, a weak negative extremum at 228 nm, and an intense negative extremum at 191 nm. However, the magnitudes of the CD bands for poly(Ala$_2$-Gly$_2$) are much larger than those for Ac-L-Ala-Gly-NHMe. CD spectra resembling more closely those observed for Ac-Ala-Gly-NHMe in trifluoroethanol were reported by Bush et al.[46] for cyclic hexapeptides of the type *cyclo*(X-L-Pro-Y)$_2$, where X may be either Gly or an L-amino acid and Y is Gly or a D-amino acid. The presence of two type II $\beta$ bends has been demonstrated in these peptides in the solid state by X-ray diffraction[47-49] and in solution by NMR.[50] Similar spectra were also observed by Brahmachari et al.[51] for Ac-L-Pro-Gly-L-Leu-OH in trifluoroethanol at temperatures ranging from −4 to −40 °C. The presence of a strong negative band around 180 nm and a large value for the ratio $[\theta]_{201}/[\theta]_{225}$ was taken as characteristic of the $\beta$ bend conformation, this ratio increasing in magnitude from 5.6 at −4 °C to 11.5 at −40 °C. Urry et al.[52] have also observed CD spectra of Woody's class B, very similar in shape and comparable in magnitude to those reported in our Figure 2B, for Boc-L-Val-L-Pro-Gly-Gly-OMe as well as cyclic and linear polypeptides with this repeating sequence. They have identified such spectra as indicative of type II $\beta$ bends. The CD spectra reported by Kawai and Fasman[53] for Cbz-Gly-L-Ser(O-*t*-Bu)-L-Ser-Gly-O-stearyl ester in cyclohexane show extrema at 198 and 221 nm. Infrared evidence has been reported[53] for a $\beta$ bend. If the peptide of Kawai and Fasman[53] forms a $\beta$ bend, its sequence would require it to be a type I bend, although the wavelengths reported are considerably shifted from those predicted for type I $\beta$ bends.[25]

Brahms et al.[45] and Zimmerman and Scheraga[54,55] have pointed out, on the basis of frequencies of bends in proteins, that the probability for the formation of type II bends is quite high for the sequence X-L-Ala-Gly-Y. In the light of these observations for proteins and the reported CD spectra for various peptides in solution, the CD spectra of Figure 2 for Ac-L-Ala-Gly-NHMe, especially in less polar solvents, may be taken as indicative of the presence of a significant fraction of type II $\beta$-bend conformations. This fraction is greatest in trifluoroethanol (Figure 2B) and least in water (Figure 2C), where the observed spectrum can be simulated by mixing the CD spectrum for a type II $\beta$-bend with that for an unordered peptide. (Numerous studies, e.g., ref 51 and 53, and unpublished work by Evans, Minasian, and Leach, have shown that the CD spectra of unordered peptides are characterized by large negative ellipticity extrema at 195–205 nm.)

The CD spectra of *cyclo*(L-Ala-Gly-Aca) in methanol, trifluoroethanol, and water are shown in Figure 3. The spectra in all three solvents are very similar, with a positive extremum at 202–204 nm and a negative extremum at 225 nm. The positions and signs of these bands agree with those predicted by Woody[25] for $\beta$ bends and with those observed for various other peptide models for $\beta$ bends.[45,46,51-53] The ratios of the ellipticities do not agree, however, presumably because of distortions from the standard $\beta$-bend conformations and/or averaging over several conformations.

The amplitude of the measured ellipticity at 202–204 nm is proportional to the peptide concentration between 0 and 1.5 mg/mL in methanol. This suggests that there is no aggregation or that its effects on the CD spectrum are negligible, at least in methanol.

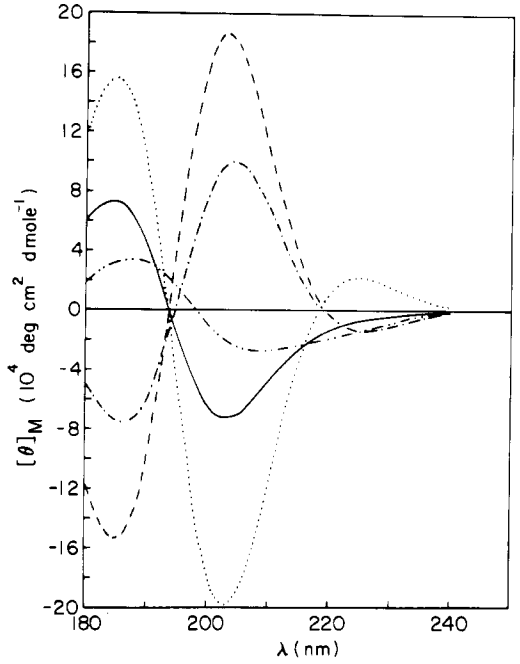


**Figure 4.** Theoretical CD spectra, calculated for computed low-energy conformations of *cyclo*(L-Ala-Gly-Aca) (Table III, ref 3). (—) Conformation 1, a type II bend; (-·-) conformation 2, a type I bend; (-··) conformation 3, a type II bend; (---) conformation 4, a type III bend; (···) conformation 8 a type III′ bend.

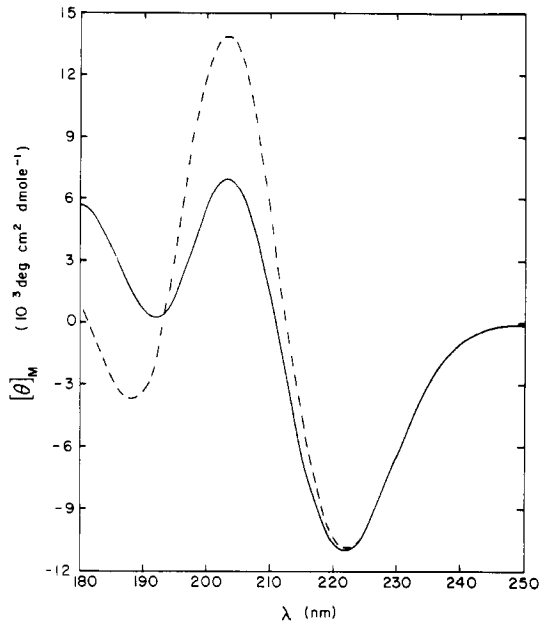


**Figure 5.** Theoretical CD spectra of *cyclo*(L-Ala-Gly-Aca), calculated as a Boltzmann-weighted average of the computed CD curves for the computed minimum-energy conformations, at (—) 0 and (---) 25 °C.

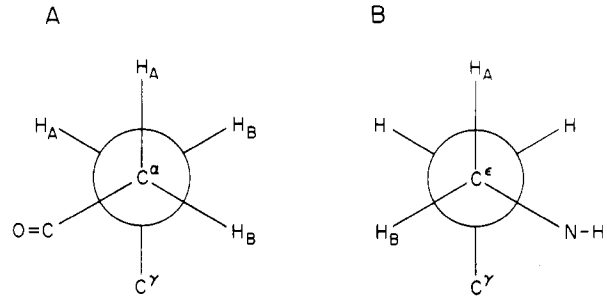


**Figure 6.** Newman projections along (A) the $C^\alpha$–$C^\beta$ bond and (B) along the $C^\epsilon$–$C^\delta$ bond of the Aca residue, in the computed[3] low-energy conformations 1–6, i.e., in all low-energy type II and type I (or III) bends. The conformation around the $C^\alpha$–$C^\beta$ bond is $g^-$ (i.e., $\theta_5 \approx -60°$); the conformation around the $C^\delta$–$C^\epsilon$ bond is $g^+$ (i.e., $\theta_2 \approx +60°$). Part A illustrates the relationships between the $C^\alpha$ and $C^\beta$ hydrogens, and part B those between the $C^\delta$ and $C^\epsilon$ hydrogens, used in the text to discuss the $^1$H NMR coupling constants.

The cyclic molecule appears to have some flexibility, as suggested by the solvent and temperature dependence of the ellipticities. The 202–204-nm positive band is considerably more sensitive to temperature than is the 225-nm band, as is the case with the linear blocked dipeptide. The amplitude of the 202–204-nm peak and its ratio to the amplitude of the trough at 225 nm are the greatest in methanol and at low temperatures (+33 000 deg cm$^2$ dmol$^{-1}$ and 6.6, respectively). Water has a much smaller effect on the spectrum of the cyclic peptide (Figure 3C) than on the linear peptide (Figure 2C). In the latter, it appears to reduce the bend content considerably.

**Calculated Circular Dichroism Spectra.** The calculated CD spectra for five computed minimum-energy conformations of *cyclo*(L-Ala-Gly-Aca) are shown in Figure 4. For the type II bend, both low-energy conformations (no. 1 and 3) are represented, while for each of the type I, type III, and inverted (type I′ and III′) bends only the CD curve of the lowest energy conformations are given. Figure 5 shows the calculated CD spectra of *cyclo*(L-Ala-Gly-Aca) at 0 and 25 °C, obtained by taking a Boltzmann-weighted average of the calculated CD spectra for all ten lowest energy computed conformations (Table III of ref 3).

## V. Discussion

***cyclo*(L-Ala-Gly-Aca).** The cyclic compound appears to have a well-defined conformation as the major component. The $^{13}$C spin–lattice relaxation times for the backbone $CH_2$ and CH groups do not show any gradient of motion within the peptide, indicating little overall flexibility. The analysis of the $^1$H NMR data, to be discussed next, also indicates the existence of one major conformation. First, the difference in chemical shifts of the geminal Gly $C^\alpha H_2$ hydrogens is very large (0.45 ppm) for a compound which contains no aromatic residues, suggesting that there is little conformational averaging in this part of the molecule. Second, the $C^\alpha H_2$ and $C^\epsilon H_2$ hydrogens of the Aca residue give discrete coupling patterns [(dt, td) and (tt), respectively; Table I] which are typical of a conformationally locked cyclohexane, rather than a mobile hydrocarbon chain.[56,57] The observed coupling constants are consistent with essentially rigid staggered arrangements about the $C^\alpha$–$C^\beta$ and $C^\delta$–$C^\epsilon$ bonds. $C^\alpha H_B$ has a large vicinal coupling (13 Hz) with one of the $C^\beta H$ hydrogens and a small coupling (4 Hz) with the other, indicating that it is approximately in the trans and gauche positions, respectively, relative to these two $C^\beta$ hydrogens. On the other hand, $C^\alpha H_A$ shows two small couplings (with an average value of 4 Hz) to its vicinal neighbors (the $C^\beta H$ hydrogens). This coupling scheme is consistent with the conformation about the $C^\alpha$–$C^\beta$ bond (Figure 6A), i.e., with a dihedral angle[58] $\theta_5$ near −60°. With the $C^\epsilon H_2$ hydrogens, the situation is complicated because of coupling to the NH hydrogen and the obscuring of one $C^\epsilon H$ signal by other multiplets, but we can identify the signal of the $C^\epsilon H_A$ as a triplet of triplets, which is reduced to a triplet of doublets when the NH is exchanged for deuterium. This indicates that the predominant form is the conformation about the $C^\delta$–$C^\epsilon$ bond shown in Figure 6B, i.e., with dihedral angle[58] $\theta_2$ near 60°.

Table IX
Comparison of Dihedral Angles in the Type II Bend Conformation of *cyclo*(L-Ala-Gly-Aca), Derived from Experiment and from Conformational Energy Computations

| | dihedral angles, deg | | | | | | |
|---|---|---|---|---|---|---|---|
| | ωAca | φAla | ψAla | ωAla | φGly | ψGly | ωGly |
| experimental model[a] | 180[b] | −80 | 70 | 180[b] | 120 | 20 | 180[b] |
| theoretical result[c] | 171 | −89 | 85 | −176 | 81 | 74 | −167 |

[a] Derived in the text. The dihedral angles are rounded to the nearest 10°. [b] The peptide bonds were assumed to be planar trans in the model. [c] Computed in ref 3.

The resonance of the Aca NH occurs at higher field than the corresponding resonance in Boc-L-Ala-Gly-Aca-OMe (6.96 and 7.63 ppm, respectively), and it has a lower temperature coefficient (Table II) than do the other NH's in this molecule. Thus, it is almost certainly located in the interior of the molecule.

All these observations are compatible with a β-bend conformation, possibily with a bent hydrogen bond beween the Aca NH and the Aca C=O. Most bends in proteins either do not have a corresponding hydrogen bond, or the hydrogen bond is strongly bent.

The NOE data (Table III) indicate that Ala $C^{\alpha}H$ is near either Ala NH or Gly NH. Conformations in which Ala $C^{\alpha}H$ is near the Ala NH (i.e., $\phi$ is in the range 0–120°) have high energy,[59] except for a small region near $\phi = 60°$, $\psi = 60°$, i.e., the left-handed α-helix structure. In this region, however, the coupling constant $^3J_{NH-C^{\alpha}H}$ would be >8 Hz,[60] whereas the observed value (see Table I) is 7.1 Hz. Hence, the NOE probably arises from the proximity of Ala $C^{\alpha}H$ and Gly NH. Subject to the caveats outlined in earlier papers,[61,62] the 9.4% enhancement of the Ala $C^{\alpha}H$ signal (Table III) indicates that the Ala $C^{\alpha}H$ and the Gly NH are separated by a short distance, i.e., 2.95 Å or less. (The observed enhancement may be smaller than the true NOE for various reasons.[61,62] If this is the case, 2.95 Å represents the maximal distance.) Such a short distance can occur only for positive values of $\psi_{Ala}$ and, hence, only in type II bends. In type I (or III) bends, with $-80° < \psi_{Ala} < 0°$, the distance mentioned would be ≈3.5Å. Hence, type I (or III) bends do not occur as major components. The presence of *small* amounts of these bends cannot be excluded by the NOE measurements.

The observed $^1H$ vicinal (HN–$C^{\alpha}H$) and geminal (H–C–H in Gly) coupling constants (Table I) correspond to limited ranges[60] of the dihedral angles $\phi_{Ala}$ and $\phi_{Gly}$. Some of these ranges can be eliminated because they occur in high-energy regions of the respective conformational energy maps.[59] The ranges of dihedral angles which are consistent with the coupling constants and with the NOE data occur only near the values $\phi_{Ala} \approx -160$ or −80°, $\psi_{Ala} > 60°$, $\phi_{Gly} \approx \pm 40$ or ±150°. Because of the constraints of ring closure using the Aca chain,[3] the cyclic molecule cannot have $\phi_{Ala}$ near −160°, nor $|\psi|_{Gly} \geq 80°$. Negative values of $\phi_{Gly}$ could occur only in combination with $\psi_{Ala} < 0°$. This leaves only the following possibilities: $(\phi,\psi)_{Ala} = (\approx -80, >60°)$, $\phi_{Gly} \approx 60$ or 130°, $\psi_{Gly} \approx \pm 40°$, with a tolerance of about ±20°.

A model which is consistent with the above experimental data and with steric and energetic constraints can be constructed with planar trans peptide bonds and the following dihedral angles[63] (rounded to the nearest 10°): $(\phi,\psi)_{Ala} = (-80, 70°)$, $(\phi,\psi)_{Gly} = (120, 20°)$, and nearly staggered arrangements around the $C^{\alpha}$–$C^{\beta}$ and $C^{\delta}$–$C^{\epsilon}$ bonds. This conformation is a type II β bend.[64–66] It is also close to the lowest energy conformation obtained in the theoretical computation[3] (Table IX). The differences in the $(\phi,\psi)$ dihedral angles are due in part to the constraint of $\omega = 180°$ in the model but not in the computation. One of the $C^{\gamma}$ hydrogens of the Aca residue projects into the interior of the molecule, where it is shielded by the peptide bonds.[67] This can account for the unusually high-field resonance (0.96 ppm) observed in the $^1H$ NMR spectrum and possibly for some of the shielding of the $C^{\gamma}$ carbon resonance in the $^{13}C$ NMR spectrum.

The presence of small amounts of type I (+III) bends cannot be excluded on the basis of the $^1H$ NMR data. Most of the dihedral angles, viz., all θ's, $\phi_1$ and $\psi_2$ are similar in the type II and I bend conformations (lines 1 and 2 in Table III of ref 3). Thus, the conformation of much of the molecule and the corresponding $^1H$ NMR observations would be similar for the two bends. Interconversion between the two bends does not require a major conformational change of the Aca residue and only small transient changes in the orientation of the Aca-Ala and Gly-Aca peptide groups. This is consistent with the $^{13}C$ spin–lattice relaxation data.

Solvent perturbation of the $^{13}C$ chemical shifts provides evidence that is not inconsistent with the proposed type II β-bend structure. The underlying premise in this technique[68] is that, in going from a non-proton-donating solvent (like $Me_2SO$) to a proton-donating one (like $D_2O$ or TFE), an "exposed" carbonyl carbon exhibits a larger downfield chemical shift than one that is intramolecularly hydrogen bonded, *provided that the change of solvent does not induce a change of conformation.* This behavior presumably arises because a hydrogen-bonded carbonyl is already shifted partly downfield by the intramolecularly bonded NH, and consequently the effect of forming another hydrogen bond with the solvent is not as large. In a type II bend, the C=O of both Ala and Aca should be less exposed than that of the Gly. The exposure of the Ala C=O is only slightly decreased, because of the vicinity of the Ala $CH_3$ group, while the Aca C=O is in the interior of the bend. In a type I bend, both the Ala and the Gly C=O groups are exposed (the former being distant from the Ala $CH_3$ group), while the C=O of Aca is less exposed. Since the data of Table V show that Δδ is 4.4, 4.3, and 3.4 for the Aca, Gly, and Ala carbonyls, respectively, for two solvent pairs, it appears that the Aca C=O and Gly C=O are the most exposed and the Ala C=O the least exposed. The latter suggests that the major conformation is a type II bend. Because of the closeness of the values of Δδ, it is difficult to use them for identifying the conformation.

Studies of *cyclo*(L-Ala-L-Ala-Aca) and *cyclo*(L-Ala-D-Ala-Aca), which will be described in detail in a subsequent paper,[9] help to clarify the interpretation of the CD of *cyclo*(L-Ala-Gly-Aca). In agreement with qualitative considerations of the $(\phi,\psi)$ conformational space for L-Ala and D-Ala residues,[59] conformational energy calculations indicate that *cyclo*(L-Ala-L-Ala-Aca) can assume only a type I (+III) β-bend conformation, while *cyclo*(L-Ala-D-Ala-Aca) can form only a type II bend. Moreover, the low-energy conformations of *cyclo*(L-Ala-L-Ala-Aca) and *cyclo*(L-Ala-D-Ala-Aca) are predicted to resemble closely (within 17° or less in all dihedral angles) the corresponding conformations of *cyclo*(L-Ala-Gly-Aca). The CD of *cyclo*(L-Ala-

L-Ala-Aca) shows a double minimum above 200 nm, remarkably resembling the CD of an $\alpha$ helix (Woody's[25] class C spectrum). We may thus assume that this type of spectrum is characteristic of the type I (+III) bends in *cyclo*(L-Ala-L-Ala-Aca) and in *cyclo*(L-Ala-Gly-Aca). The CD of *cyclo*(L-Ala-D-Ala-Aca) shows a negative band at 230 nm and a strong positive band at 203 nm, corresponding to the class B spectrum of Woody.[25] The class B spectrum can therefore be associated with type II bends in *cyclo*-(L-Ala-D-Ala-Aca) and in *cyclo*(L-Ala-Gly-Aca). This spectrum has been suggested earlier for various $\beta$-bend models.[45,46,51,52]

Using the results on these uniquely defined $\beta$-bend conformations, we can interpret the CD of *cyclo*(L-Ala-Gly-Aca). Type I (+III) and II $\beta$ bends both have negative CD bands near 230 nm. The two types of bends, however, have CD of opposite sign near 203 nm. The observed positive CD band near 203 nm for *cyclo*(L-Ala-Gly-Aca) reflects a preponderance of type II $\beta$ bends, consistent with conformational energy calculations[3] and with the other experimental results reported in this and the following[4] paper. The observed temperature dependence of the 203-nm band is consistent with the presence of type I (+III) $\beta$ bends at somewhat higher energies than the type II bend.

An estimate of the relative amounts of the two types of bends can be made from the CD of *cyclo*(L-Ala-Gly-Aca), if we assume that the observed CD spectra of *cyclo*(L-Ala-L-Ala-Aca) and *cyclo*(L-Ala-D-Ala-Aca) are representative of type I (+III) and II bends, respectively. An approximate analysis of this type indicates that *cyclo*(L-Ala-Gly-Aca) contains about 85% type II bend conformations in trifluoroethanol, about 80% in methanol, and about 65% in water at 22 °C. A detailed analysis will be presented subsequently.[9]

The shapes of the theoretical CD curves for the type I (+III) and II bends (Figure 4) are opposite to those inferred from the experimental data[9] on *cyclo*(L-Ala-L-Ala-Aca) and *cyclo*(L-Ala-D-Ala-Aca). Previous calculations[25] showed that the calculated CD is very sensitive to variations in conformation near the "Venkatachalam $\beta$ bend"[66,69] conformations. When $\phi$ and $\psi$ were varied by ±10° around the Venkatachalam values for the type II bend, most of these bend conformations were predicted[25] to exhibit CD spectra qualitatively resembling those inferred for the type II bends in *cyclo*(L-Ala-Gly-Aca). A significant number of such variants, however, exhibited quite different CD spectra. Calculations in which $(\phi,\psi)$ dihedral angles are varied by ±10° around the minimum-energy conformations for *cyclo*(L-Ala-Gly-Aca) (Table III of ref 3) show that the CD is correspondingly sensitive in these regions of conformational space, especially for type II bends. Thus, small errors in either the CD calculations or the conformational energy computations could account for the discrepancies observed. For example, failure to include the effects of solvent in either the conformational energy or the CD calculations might be responsible because interactions with the solvent could easily cause small changes in the dihedral angles.

It should be noted in Figure 5 that, although the Boltzmann-weighted theoretical CD spectrum qualitatively resembles the spectrum observed (Figure 3) for *cyclo*(L-Ala-Gly-Aca), the temperature dependence of the 203-nm band is opposite to that observed. This discrepancy results from the failure to predict correctly the spectra of type I (+III) and II $\beta$ bends, as discussed above.

**Ac-L-Ala-Gly-NHCH$_3$ and Boc-L-Ala-Gly-Aca-OMe.** The results suggest that these compounds, too, have a preferred conformation in solution. While the chemical shift differences of the two Gly C$^\alpha$H$_2$ hydrogens (0.070 and 0.064 ppm, respectively) are common in X-Gly dipeptides,[70] the view has been expressed[57,71,72] recently that nonequivalence of even this small extent indicates some restriction of the motion of Gly.

The temperature coefficient of $\delta$ for the NHCH$_3$ amide hydrogen in Ac-L-Ala-Gly-NHMe and for the Aca NH in Boc-L-Ala-Gly-Aca-OMe is lower than that of the other NH's (Table II), suggesting the existence of some structure in which this hydrogen is somewhat sheltered from the solvent. The restrictions on internal motion are not severe, because the $T_1$ measurements indicate segmental motion.

In Ac-L-Ala-Gly-NHMe, the $^1$H NOE is stronger than in the other two compounds (Table III). Following the same argument as used above for the cyclic compound, the observed increase in area of 9% for the combined NH signals may be ascribed to an 18% increase in the area of the Gly NH signal. The 21% enhancement of the Ala C$^\alpha$H signal, upon irradiation of the combined (Ala NH + Gly NH) signal is consistent with this. Subject to the caveats[61,62] expressed earlier, we conclude that the Ala C$^\alpha$H and Gly NH are separated by at most 2.6 Å. This restricts the possible values of $\psi_{Ala}$ and suggests that the preferred form is a type II bend.

In the same way as for the cyclic molecule, we have interpreted the observed coupling constants and NOE data for Ac-L-Ala-Gly-NHMe in terms of probable $(\phi,\psi)$ dihedral angles and discarded those that correspond to high-energy structures. The remaining values are ($\phi_{Ala} \approx -160$ or $-80°$), ($60° < \psi_{Ala} < 175°$), ($\phi_{Gly} \approx \pm 60$ or $\pm 130°$). These data are compatible with a type II bend as the most frequently occurring species. Conformational energy calculations[73] for the terminally blocked dipeptide Ac-L-Ala-Gly-NHMe indicate a high probability for the existence of a type II bend in this molecule. The CD measurements are also consistent with this conclusion.

This structure is not supported by data on solvent perturbation of $^{13}$C chemical shifts (Table VI); $\Delta\delta$ is 5.87, 4.35, and 3.82 for the acetyl, Ala, and Gly carbonyls, respectively. Hence, it appears that the acetyl C=O is the most exposed and the Gly C=O the least exposed. In a type II bend, the Gly C=O is the most exposed and the other two C=O groups less so; in the type I bend, both the Gly and Ala C=O are exposed, and the acetyl C=O less so. The method of solvent perturbation may not be applicable here if the conformation is altered by a change of solvent. The analysis of CD, infrared, and Raman spectra suggests that changes of solvent may displace the conformational equilibria.[4]

Generally, the properties of Boc-L-Ala-Gly-Aca-OMe are similar to those observed for Ac-L-Ala-Gly-NHMe, indicating a high probability for the existence of a type II bend in this molecule as well. The probability may be somewhat lower for the Boc derivative, though, as suggested by the changes in the coupling constants and the $d\delta/dT$ values (Tables I and II).

## VI. Conclusion

The experimental data reported here and in the accompanying paper[4] suggest that the predominant conformation of *cyclo*(L-Ala-Gly-Aca) in solution is a type II bend, with limited flexibility. As a minor component, a type I (or III) bend is present as well, in amounts which depend on the solvent and the temperature. It may constitute as much as 35% in water at 22 °C, as indicated by the CD measurements. Its presence is not detected by the NMR, Raman, and IR measurements, but it is consistent with those measurements. Its percentage in Me$_2$SO solution

might be less than in water. The conformation of most parts of the molecule is essentially the same for the type II and I bends. Only the orientation of the Ala-Gly peptide group changes.[3,66] Thus, the portion of the molecule extending from $C^{\alpha}_{Gly}$ through the Aca residue to $C^{\alpha}_{Ala}$ has the same conformation in the two bends, and it would give rise to similar $^1H$ NMR signals. The environment of the Gly $C^{\alpha}H_2$ group undergoes some change between the two conformations (because of the changed orientation of the neighboring Ala-Gly peptide group), but there is no change of orientation of the Gly-Aca peptide group. Hence, the relative orientations of the Gly $C^{\alpha}H_2$ group and the Gly C=O bond remain the same, giving rise to the same large difference of the chemical shift of the two geminal hydrogens. The results of the Raman and IR spectroscopic measurements in solution[4] also are consistent with the presence of one or more closely related type II bends as the dominant conformations.

This conclusion agrees with the result of the theoretical conformational energy calculation,[3] and thus it confirms the theoretical predictions. The computed lowest energy conformation is a type II $\beta$ bend. In this bend, the orientation of all three peptide groups is the same as in the model proposed in section V. A type I bend was computed to have relative energy 0.74 kcal/mol above that of the type II bend global minimum. Another type II bend and four type I and III bends have relative energies of 0.93–1.59 kcal/mol. Therefore, the computation predicts[3] that the predominant species in a Boltzmann distribution would be the type II bend, with a fractional population of 0.63 at 25 °C.

The conformational energy computations did not include interactions with the solvent. Such interactions may contribute as much as 1–2 kcal/mol to the energy difference between the various types of bends. A small lowering of the energy of the type II bend conformations, due to solvation, could easily render these conformations the predominant ones. Thus, the experimental and theoretical results are fully compatible. The energy barriers between the two type II conformations and between the type I and III conformations, respectively, are low (estimated[3] to be 3–4 kcal/mol). The differences in energies between the various conformations also are small. Differences in the interactions with the solvent may alter the relative energies of the various conformations and shift the equilibrium. The barrier between the type II conformations on the one hand, and the type I + III conformations on the other, is high for fixed bond geometry and for planar peptide groups. Thus, the interconversion between these bend types may involve strain of the geometry.

The approximate dihedral angles of the model for the predominant component, constructed on the basis of the NMR measurements, and those of the computed lowest energy conformation are close to each other (Table IX). Even though both sets of dihedral angles differ from those of the idealized reference conformations of Venkatachalam,[64] both do fall in the range observed for $\beta$ bends in proteins.[66] Part of the differences between the two sets of dihedral angles can be accounted for by the constraint of planarity of the peptide groups in the construction of the model, while this constraint was absent in the computations. The differences may reflect an actual difference in details of the conformation, however, caused by interactions with the solvent. The possibility of small conformational adjustments in solution also is suggested by the CD spectra, by IR and Raman spectra,[4] and by the differences of the theoretical and experimental conclusions about intramolecular hydrogen bonds.[3] The NMR results show that the Aca C=O group is closer to the Aca NH group than to the Gly NH group, while the opposite is true for the computed conformation.

In terms of the overall shape of the molecule and the orientation of the peptide groups, all results reported here and in the accompanying papers[3,4] concur. All of them indicate that *cyclo*(L-Ala-Gly-Aca) exists predominantly as a type II bend.

**Supplementary Material Available:** Details of the synthetic scheme shown in Figure 1 (9 pages). Ordering information is given on any current masthead page.

## References and Notes


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(2) (a) Division of Biological Sciences, National Research Council of Canada. (b) Russell Grimwade School of Biochemistry, University of Melbourne. (c) Baker Laboratory of Chemistry, Cornell Univeristy. (d) Department of Chemistry, Monash University. (e) Division of Chemistry, National Research Council of Canada. (f) Department of Biochemistry, Colorado State University.

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