Observation of 3 → 1 Intramolecular Hydrogen Bonds (γ Turns) in the Cyclic Tetrapeptides, [Ala⁴]-Desdimethylchlamydocin and *cyclo*-(D-Phe-Pro-D-Phe-Pro), by NMR Spectrometry. Effect of Solvent on Solution Conformation[1]

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Abstract: The solution conformations of *cyclo*-(Gly-Phe-D-Pro-Ala) (**5**) and *cyclo*-(D-Phe-Pro-D-Phe-Pro) (**6**) have been studied by ^1H and ^{13}C NMR in $CDCl_3/Me_2SO$-d_6 mixtures, and by circular dichroism spectroscopy in chloroform, methanol, acetonitrile, and water. The data from these studies indicate that the conformations of **5** and **6** are solvent dependent. The conformation of **5** in $CDCl_3$ is characterized by four transoid amide bonds, at least one 3 → 1 intramolecular hydrogen bond, and a peptide ring backbone with the following torsion angles (±20°): ϕ_1 72°, ψ_1 −60°, ω_1 168°; ϕ_2 −110°, ψ_2 95°, ω_2 −156°; ϕ_3 72°, ψ_3 −60°, ω_3 168°; ϕ_4 −110°, ψ_4 95°, ω_4 −154°. The conformation of **6** in $CDCl_3$ has four transoid amide bonds, two inverse γ turns, and a peptide ring backbone conformation with the following torsion angles: ϕ(D-Phe) 120°, ψ(D-Phe) −115° ω(D-Phe) 160°; ϕ(Pro) −60°, ψ(Pro) 60°, ω(Pro) −160°. Solvent titration, NMR variable-temperature, and ^{13}C NMR studies indicate the presence of a γ turn between the Ala NH and Phe carbonyl group and possibly between the Phe NH and Ala carbonyl group in **5** and between the Phe NH and Pro CO in **6**. Evidence is presented that these γ turns stabilize the all-transoid ring conformations of **5** and **6** in chloroform. As the mole fraction of Me_2SO-d_6 in $CDCl_3$ increases, cis X-Pro amide bond conformations are found. In neat Me_2SO-d_6, **5** exists in equilibrium between 30% all-transoid and 70% cis X-Pro amide bond containing conformers, while **6** has only cis D-Phe-Pro amide bonds.

Conformational studies of cyclic tetrapeptides have provided interesting examples of unusual peptide bond geometries[2,3] and conformational interconversions.[4] Variations in biological activities of analogues of naturally occurring cyclic tetrapeptides,[5] e.g., tentoxin,[6,7] and the related *cyclo*-tetradepsipeptides, e.g., AM-toxins,[8] also have been rationalized in conformational terms. Extension of these studies to other systems is dependent upon the complete characterization of the possible solution conformations available to a cyclic tetrapeptide of a given sequence.

Theoretical calculations indicate that cyclic tetrapeptides can exist in multiple ring conformations[9-12] and that one with four transoid amide bonds **1** (where transoid designates a trans amide bond deviating from planarity by ω twist angles of ±14–25°) may be more stable than conformations with alternating planar cis,trans,cis,trans amide bonds **2** (Figure 1).[10,11] Although several examples of the latter conformation have been studied,[4,13] the all-transoid conformation has been observed unambiguously only in the crystal structure of dihydrochlamydocin **3a**,[3] an analogue of the cytostatic cyclic tetrapeptide chlamydocin **3b** (Figure 1).[14] *cyclo*-Tetraglycyl **4** has been reported to exist in all-transoid conformation like **1** in Me_2SO or TFA,[15] but this conclusion has been disputed, and conformation **2** has been proposed for *cyclo*-tetraglycyl.[16]

While investigating the solution conformation of several cyclic tetrapeptides related to chlamydocin,[17] we found that the conformation of [Ala⁴]-desdimethylchlamydocin, *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) (**5**),[18] was extremely dependent on the nature of the solvent. Solvent dependency of cyclic peptide conformations is known for other peptide systems,[19] but the solvent dependency of cyclic tetrapeptide **5** was unusual because it appeared to be the first example of conformational solvent dependency in a cyclic tetrapeptide ring system. To characterize fully the conformation of **5**, it was necessary also to determine the conformation of the model cyclic tetrapeptide, *cyclo*-(D-Phe-Pro-D-Phe-Pro) (**6**).[20]

We report here the results of an investigation to determine the solution conformations of the cyclic tetrapeptides, *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) (**5**) and *cyclo*-(D-Phe-L-Pro-D-Phe-L-Pro) (**6**). Our results show that the solution conformations of each peptide are unusually solvent dependent. In chloroform both peptides have four transoid amide bonds, and this is the first observation of these conformations in solution. As the solvent polarity is increased, other conformations with cis amide bonds predominate. Our data suggest that the transoid conformation for a cyclic tetrapeptide in solution is stabilized by 3 → 1 intramolecular hydrogen bonds (γ turns).[21a,b]

Experimental Section

The syntheses of [Ala⁴]-desdimethylchlamydocin (**5**)[18] and *cyclo*-(D-Phe-L-Pro-D-Phe-L-Pro) (**6**)[20] have been described. The optical purity of each chiral amino acid was established by hydrolyzing the peptide and reacting the free amino acids with L-amino acid oxidase as previously described.[18] A mass spectrum of each peptide gave the correct molecular ion for a cyclic tetrapeptide. Proton NMR spectra were recorded on a Bruker HX-90E (90 MHz) or WH-270 (270 MHz) spectrometer operated in the FT mode at nominal solute concentrations of 10–100 mM. Carbon NMR spectra were obtained on a Bruker HX-90E (22.63 MHz) or a JEOL FX-90Q (22.5 MHz) spectrometer operated in the FT mode at nominal solute concentrations of 0.03–0.3 M. The circular dichroism spectra were measured on a Jasco J-40-A spectropolarimeter in 0.5-mm cells and reported as mean residue ellipticities. Infrared spectra of cyclic peptide **5** were obtained in chloroform, which was dried and freed of ethanol by filtration through alumina, using a Nicolet 7199 FT infrared spectrophotometer. 4-Amino-2,2,6,6-tetramethylpiperidinooxy, free radical, was obtained from Aldrich.

Results

***cyclo*-(D-Phe-Pro-D-Phe-Pro) (6). ^1H NMR.** The chemical shift and coupling constant data for peptide **6** in 95% chloroform-d/Me_2SO-d_6 are given in Table I and Figure 2. This solvent system was used because of the low solubility of **6** in pure chloroform. The proton spectrum obtained is consistent with a single C_2 symmetrical conformation of **6** in this solvent. Minor peaks attributable to alternate conformations are not observable, and the large coupling constant for the Phe NH-C$^\alpha$H does not vary from 20 to 50 °C, indicating that conformational averaging of the peptide ring system does not occur. The Phe-NH resonance is relatively independent of

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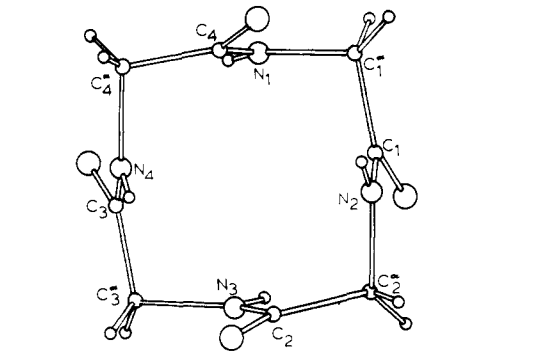

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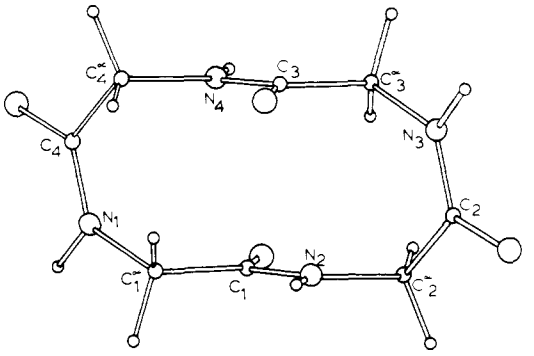

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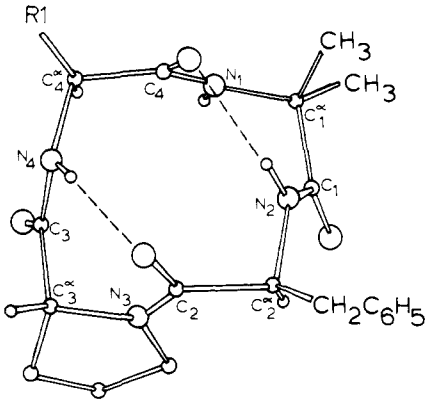


3a $R_1 = (CH_2)_5CHOH-CH-CH_2$ (O)

b $R_1 = (CH_2)_5CO-CH-CH_2$ (O)

Figure 1. Diagrammatic representations of proposed conformations for cyclic tetrapeptides. **1**, all-transoid amide bond sequence, S_4 symmetry; **2**, cis,trans,cis,trans amide bond sequence, i_1 symmetry; **3**, all-transoid amide bond sequence, bis-γ-turn conformation.

temperature ($\Delta\delta/\Delta T$, -1.4×10^{-3} ppm/°C) and peptide concentration (Figure 3), consistent with shielding of the Phe-NH from solvent.

When the concentration of Me_2SO-d_6 in chloroform-*d* is increased to 20%, additional resonances appear in the ^1H NMR spectra (Figure 2b). Above 50% Me_2SO-d_6 in chloroform-*d*, only the new resonances are visible and at 100% Me_2SO-d_6 there is evidence that an additional set of resonances now predominates (Figure 2e). These changes are consistent with the partitioning of *cyclo*-(D-Phe-Pro-D-Phe-Pro) into one or more new conformations.

^{13}C NMR. Schematic drawings of the ^{13}C NMR spectra of *cyclo*-(D-Phe-Pro-D-Phe-Pro) in 20% Me_2SO-d_6 in chloroform-*d* and 100% Me_2SO-d_6 are shown in Figure 4. We were unable to obtain ^{13}C NMR spectra at lower Me_2SO-d_6 concentrations because of the low solubility of **6** in chloroform. In 20% Me_2SO-d_6 in chloroform-*d*, the carbon spectrum of **6**

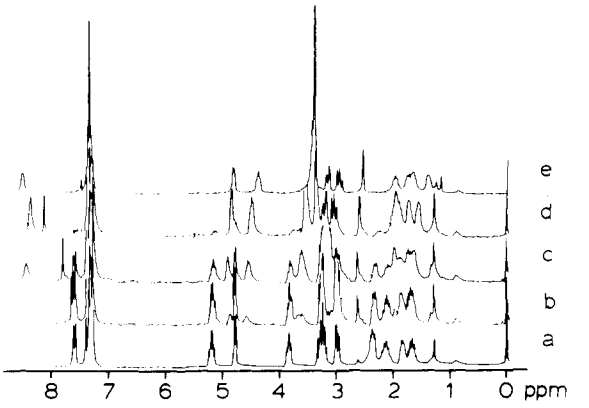


Figure 2. 270-MHz ^1H NMR spectra of *cyclo*-(D-Phe-Pro-D-Phe-Pro) as a function of Me_2SO-d_6 concentration in chloroform-*d*: (a) 5%; (b) 20%; (c) 30%; (d) 50%; (e) 100%. Peptide concentration was 15 mg/mL, 20 °C.

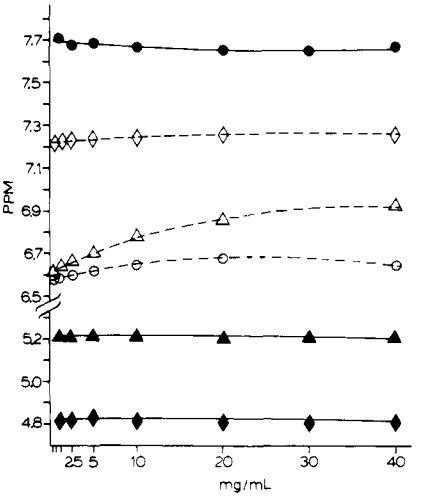


Figure 3. Chemical shift vs. concentration of peptide at 20 °C: *cyclo*-(D-Phe-Pro-D-Phe-Pro) (**6**) (—), Phe NH (●), Phe αH (▲), Pro αH (♦); *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) (**5**) (- - -), Gly NH (△), Phe NH (○), Ala NH (◇).

Table I. 270-MHz ^1H NMR Data for *cyclo*-(Gly-L-Phe-D-Pro-L-Ala)[a] and *cyclo*-(D-Phe-Pro-D-Phe-Pro)[b]

residue	δ, ppm	$J_{N\alpha}$, Hz	$\Delta\delta/\Delta T$[c]
	cyclo-(D-Phe-Pro-D-Phe-Pro)		
Phe, NH	7.65 (d)	11.0	−1.4
α-H	5.21 (ddd)	11.0, 10.2, 6.8	~0
Pro, α-H	4.81 (dd)	8.0, 1.5	~0
	cyclo-(Gly-L-Phe-D-Pro-L-Ala)		
Gly, NH	6.86 (dd)	9.2, 4.3	−5.6
α-H_i	4.58 (dd)	9.2, 12.5	
α-H_o	3.22 (dd)	4.3, 12.5	
Phe, NH	6.68 (d)	10.1	−3.4
α-H	5.20 (ddd)	10.1, 9.7, 5.9	
D-Pro, α-H	4.74 (dd)	8.6, 2.0	
Ala, NH	7.27 (d)	10.0	−0.8
α-H	4.64 (m)	m	

[a] Concentration 20 mg/mL, 20 °C (δ from Me_4Si), in $CDCl_3$. [b] Concentration 20 mg/mL, 20 °C (δ from Me_4Si), in 95% $CDCl_3$, 5% Me_2SO-d_6. [c] $\times 10^3$ ppm/deg; temperature range studied, 20 to 50 °C for *cyclo*-(D-Phe-Pro-D-Phe-Pro) and −40 to 10 °C for *cyclo*-(Gly-L-Phe-D-Pro-L-Ala).

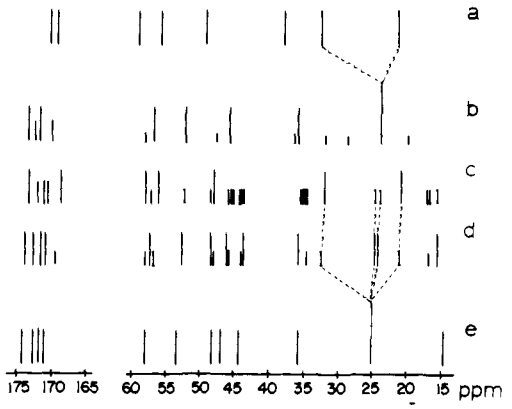


Figure 4. Schematic of ^{13}C NMR at 30 °C. *cyclo*-(D-Phe-Pro-D-Phe-Pro) (**6**), 28 mg/mL: (a) 100% Me_2SO-d_6; (b) 20% Me_2SO-d_6 in $CDCl_3$. *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) (**5**), 70 mg/mL; (c) 100% Me_2SO-d_6; (d) 50% Me_2SO-d_6 in $CDCl_3$; (e) 100% $CDCl_3$.

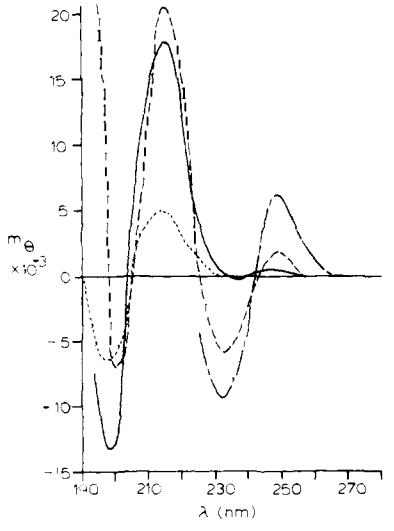


Figure 5. Circular dichroism spectra of *cyclo*-(D-Phe-Pro-D-Phe-Pro) (**6**) in various solvents at 0.43 mM: $CHCl_3$ (— · —); CH_3CN (- - -); MeOH (—); H_2O (· · ·). The H_2O contained 1% MeOH to increase solubility of the peptide.

shows a resonance at 24.1 ppm for both the Pro C^β and Pro C^γ carbons (Figure 4b). Thus, in this solvent system, the X-Pro bond is predominantly trans.[22] The unusually high field resonance for the Pro C^β carbon is consistent with a Pro ψ torsion angle near 60°[23] and suggests the existence of an inverse γ turn.[21b,24,25]

In 100% Me_2SO-d_6, the carbon resonances for the Pro C^β and C^γ carbons are separated by 11.0 ppm, indicating that the X-Pro bond is cis (Figure 4a).[22] The carbon spectrum has only one line for each Pro and Phe carbon, and therefore the conformation of **6** is C_2 symmetrical in Me_2SO-d_6. A comparison between spectra in Figures 4a and 4b shows that the cis X-Pro conformation of **6** is a minor component of **6** in 20% Me_2SO-d_6 in chloroform-*d*. This conformer probably gives rise to the minor resonances in the ^1H NMR spectrum of **6** in 20% Me_2SO-d_6 in chloroform-*d* (Figure 2b). However, when the Me_2SO-d_6 concentration is only 5% (Figure 2a), the proton resonances attributable to the cis X-Pro bond conformers are not seen. Thus, we conclude that in chloroform solutions containing 5% or less Me_2SO-d_6 the conformation of **6** contains only trans X-Pro bonds.

Circular Dichroism. The CD spectra of **6** in different solvents are shown in Figure 5. The most important feature of these is the strong, negative ellipticity at 232 nm in chloroform. This

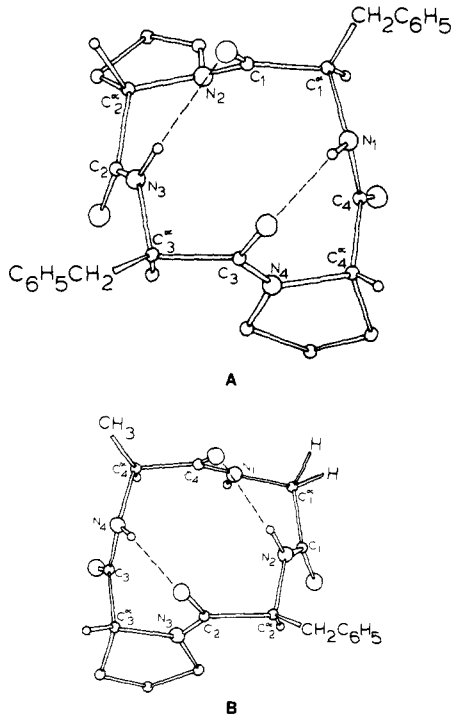


Figure 6. Diagrammatic representations of proposed solution conformation of cyclic tetrapeptides in chloroform: (A) *cyclo*-(D-Phe-Pro-D-Phe-Pro) (**6**); (B) *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) (**5**).

is close to the value calculated for proline inverse γ turns[26] and observed in cyclic hexapeptides[27] and cyclic pentapeptides[24] shown to contain inverse γ turns. As the polarity of the solvent is increased, the amplitude of the bands at 233 and 250 nm diminishes. In methanol or water no CD band is apparent in this region. It is probable that this reflects the partitioning of **6** into additional, non-γ-turn conformations, but additional data are needed to establish this.

Proposed Conformation of 6. The data presented for **6** are consistent only with a conformation with four trans amide bonds. Alternate conformations with single cis amide bonds can be eliminated from consideration because of the obvious conformational symmetry for **6** apparent in the ^1H and ^{13}C NMR spectra. A cis,trans,cis,trans conformation can be eliminated because it does not account for the shielding of the Phe-NH from solvent or for the high-field Pro C^β resonance in the ^{13}C NMR.

The data presented for *cyclo*-(D-Phe-Pro-D-Phe-Pro) are consistent with the bis inverse γ-turn conformation in chloroform shown in Figure 6A. The conformation is C_2 symmetrical with both X-Pro bonds trans. The hydrogen bonding studies indicate the Phe-NH groups are either shielded from solvent or in intramolecular hydrogen bonds. The CD spectrum in chloroform is consistent with an inverse γ turn, and this is supported by the high-field Pro C^β resonance in the ^{13}C NMR, which is comparable to those found in inverse γ turns. The Phe NH-C^αH coupling constant also is compatible with this conformation. The torsion angles for the chloroform conformation of **6** (Table II) are derived from molecular models and are consistent with the NMR data.[28]

We have assumed that the chloroform conformation of **6** (Figure 6A) contains four transoid instead of four planar trans amide bonds. This assumption is based on theoretical calculations that show that closure of a linear tetrapeptide to a cyclic form with four trans amide bonds is not possible unless ω deviates ±15-25° from 180°.[29,30] Our data show that the amide bonds cannot be cis and that Pro is in an inverse γ turn, but we do not have direct evidence that all amide bonds are not planar.

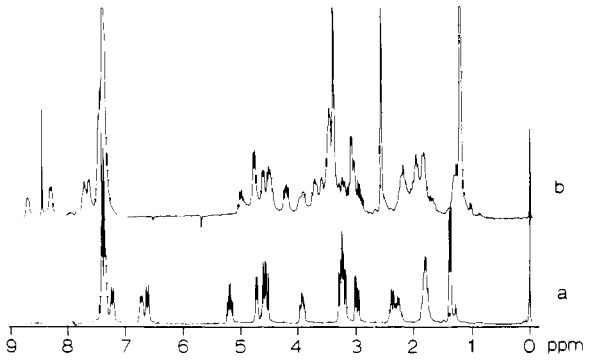


Figure 7. 270-MHz ^{1}H NMR spectra of *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) (**5**). Peptide concentration was 14.7 mg/mL at 20 °C: (a) 100% $CDCl_3$; (b) 100% Me_2SO-d_6.

Table II. Torsion Angles[a] for Proposed Solution Conformations of *cyclo*-(D-Phe-Pro-D-Phe-Pro) and *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) in Chloroform

	cyclo-(D-Phe-Pro-D-Phe-Pro)			
	D-Phe	Pro	D-Phe	Pro
$\phi^{b,d}$	120°	−60° ω^c	160°	−160°
ψ^d	−115°	60°		

	cyclo-(Gly-L-Phe-D-Pro-L-Ala)[e]			
	Gly- (A^{t}bu)	L-Phe- (L-Phe)	D-Pro- (D-Pro)	L-Ala- (L-Aoe)
$\phi^{b,d}$	72 (71.8)	−110 (−105.5)	72 (71.8)	−110 (−105.5)
ψ^d	−60 (−63.7)	95 (94.4)	−60 (−63.7)	95 (104.7)
ω^c	168 (162)	−156 (−165.7)	168 (162)	−154 (−163.7)

[a] For a definition of torsion angles, see ref 28. [b] All values are ±20°. [c] The ω twist angles are assumed to be approximately the same as those found in dihydrochlamydocin.[3] [d] In dihydrochlamydocin, τ angles are 105°.[3] Torsion angles for **5** and **6** were derived using Dreiding models in which τ = 109°. [e] Values in parentheses are derived from X-ray crystal structure of dihydrochlamydocin.[3]

However, when one builds molecular models incorporating the observed conformational features, it becomes necessary to incorporate nonplanar amide bonds into the conformation. To do this we assumed the ω twist angles in **6** are similar to those found in dihydrochlamydocin **3a**.[3] Large $^3J_{\alpha,NH}$ values have been observed in other polypeptide systems,[31,32] and DeMarco et al. have suggested that $^3J_{\alpha,NH} \geq 10$ Hz represent $\theta_{\alpha,NH}$ values $\simeq 180°$ for transoid peptide bonds.[33] Thus, the large $^3J_{\alpha,NH}$ found for **6** (Table I) is consistent with the nonplanar Phe-Pro amide bond proposed for **6** in chloroform.

***cyclo*-(Gly-L-Phe-D-Pro-L-Ala). ^{1}H NMR.** The chemical shift, coupling constant data, and temperature dependencies of the amide protons are listed in Table I and representative spectra shown in Figure 7. Assignments were made by the usual decoupling methods. The absence of minor peaks, the large $J_{N\alpha}$ values, and the large differences in chemical shift between glycyl geminal protons indicate that one ring conformation predominates in chloroform solution. No evidence for conformational heterogeneity was observed as the temperature was raised from −50 to 20 °C.

The N-H region was analyzed in detail, as the solute concentration (Figure 3), temperature (Table I), and solvent composition (Figure 8) were varied. In addition, we looked for differences in the broadening of the peptide proton (NH) resonances that are produced by small additions of a stable free radical, 4-amino-2,2,6,6-tetramethylpiperidinooxy (Figure 9). The results clearly indicate that the Ala-NH is shielded

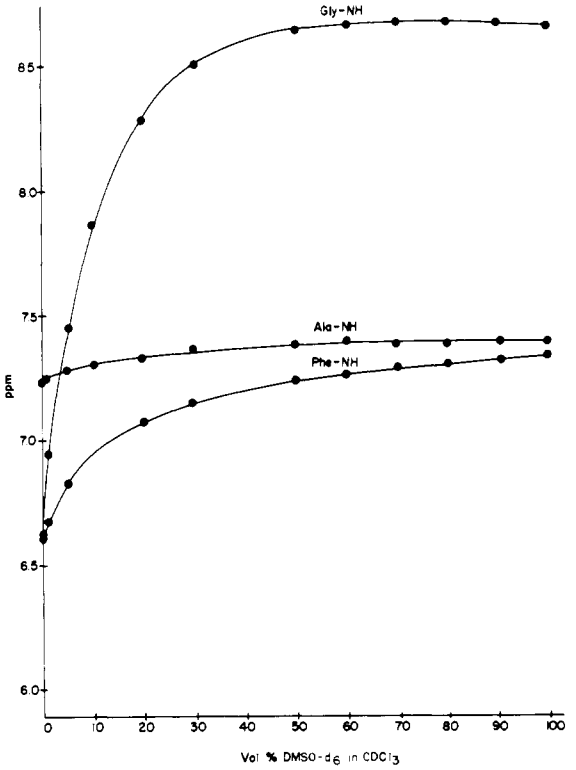


Figure 8. Amide proton chemical shifts of *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) (**5**) at varying volume percent Me_2SO-d_6 in $CDCl_3$ (peptide concentration 14.7 mg/mL, 20 °C).

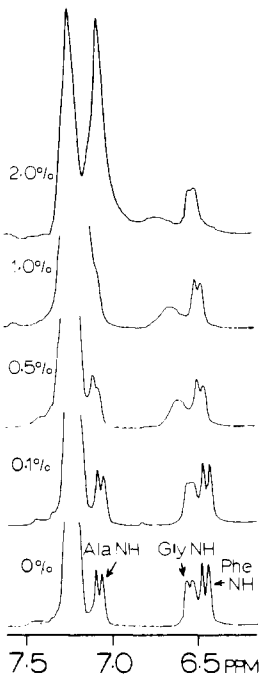


Figure 9. Effect of varying amounts (w/v %) of the stable free radical 4-amino-2,2,6,6-tetramethylpiperidinooxy on the amide resonances of *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) (**5**) at 270 MHz. Peptide concentration was 8.9 mg/mL in $CDCl_3$ at 20 °C.

from the solvent or from intermolecular interactions.[34] In contrast, all data are consistent with exposure of the Gly-NH to solvent. The Phe-NH appears to be shielded from solvent on the basis of the stable nitroxide experiment in which equal broadening of the Ala-NH and Phe-NH resonances is observed. The remaining criteria indicate the Phe-NH may be

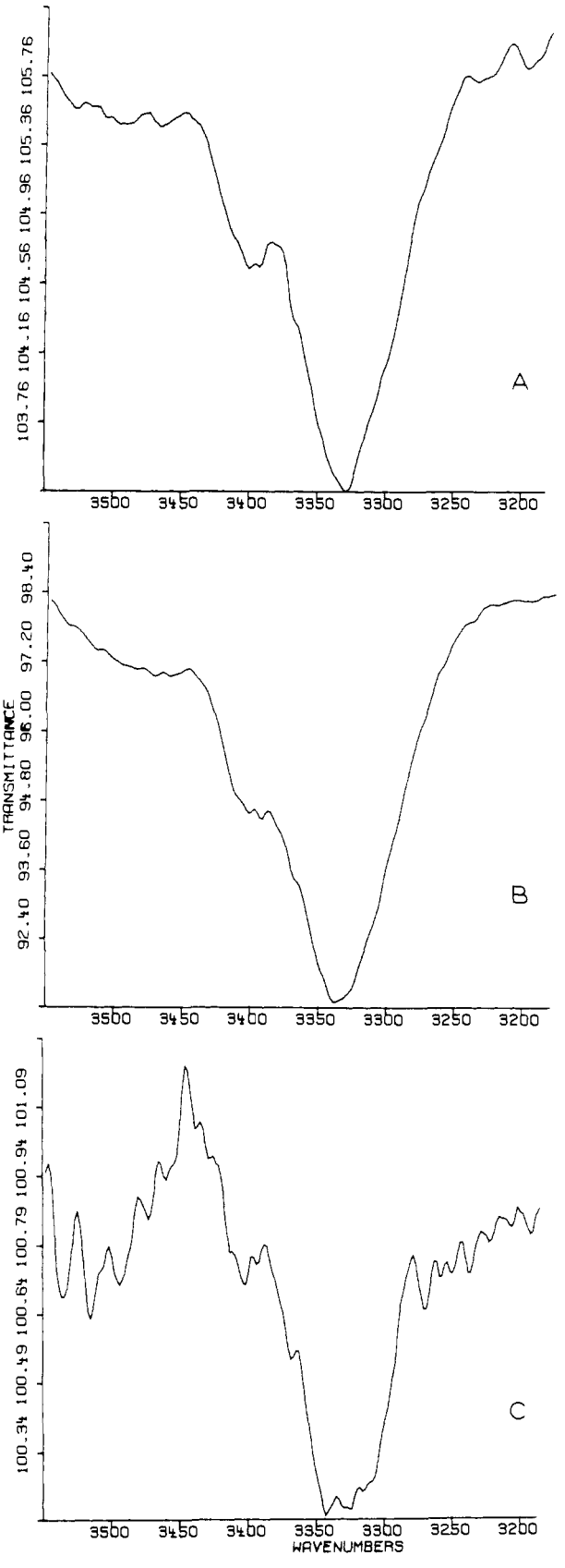


Figure 10. Infrared N–H stretching absorptions of *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) (**5**) as a function of peptide concentration in $CHCl_3$: (A) 10^{-2} M; (B) 10^{-3} M; (C) 10^{-4} M.

more accessible to solvent than the Ala-NH but less accessible to solvent than the Gly-NH. The solvent shielding of the Ala-NH (and Phe-NH) could be caused by a stable aggregate (e.g., a dimer) of peptide **5**, or by an intramolecular hydrogen bond.

The variable concentration study (Figure 3) shows a small change in chemical shift with peptide concentration at concentrations below 10 mg/mL, which suggests that the peptide is still aggregated. However, FT-IR studies of **5** at 10^{-2}, 10^{-3}, and 10^{-4} M (Figure 10) show the presence of both a hydrogen-bonded NH (3330 cm^{-1}) and a non-hydrogen-bonded NH (3400 cm^{-1}). The ratio of bonded to nonbonded NH absorption is about 2:1, and this ratio does not appear to change as the sample concentration is diluted. This type of data has been interpreted as indicating the presence of intramolecular hydrogen bonds in the conformations of peptides.[34]

Analysis of the chemical-shift and coupling constant data for glycine established that the Ala-Gly amide bond is trans. As has been observed in other cyclic tetrapeptide systems,[6,35] the glycyl geminal protons are nonequivalent. The proton resonating at 4.58 ppm is unusually downfield and must be eclipsed by the deshielding region of the alanyl carbonyl group. The downfield shift of protons eclipsed by an amide carbonyl has been observed in several conformationally defined cyclic tetrapeptide systems.[6,35] The large vicinal coupling constant (9.2 Hz) between the deshielded glycyl proton and the Gly-NH establishes that the vicinal bond angle is about 155°.[33,40] This angle and the deshielding of the α proton can be obtained only when the Ala-Gly amide bond is approximately trans.

^{13}C NMR. Schematic representations of the carbon nuclear magnetic resonance spectra obtained for **5** are given in Figure 4. A striking feature of the ^{13}C NMR spectrum of **5** is that both the β- and γ-prolyl carbons resonate at 25 ppm in chloroform (Figure 4e) and at 25.5 and 25 ppm in chloroform–methanol mixtures, and, therefore, the acyl-Pro amide bond must be trans.[22] The upfield resonance of the prolyl β carbon provides additional conformational information, and indicates that the D-Pro ψ torsion angle is near −60°.[23] The high-field Pro C^{β} resonance also is found in cyclic pentapeptides[24,25] and in cyclic hexapeptides[27] in which an L-Pro is in an inverse γ turn with ψ(L-Pro) = 60°.

As the solvent composition is changed from pure chloroform to Me_2SO-d_6, additional resonances appear in the ^{13}C NMR spectra (Figures 4c and d). Of particular interest is the appearance of resonances at 32 and 21 ppm, which signal the presence of cis acyl-Pro amide bonds.[22] Additional resonances can be attributed to the doubling of signals due to the presence of two or more conformations which interconvert slowly on the NMR time scale. These data are consistent with ring conformations with either cis,trans,cis,trans or cis,trans,trans,trans amide bond sequences in Me_2SO. Additional data, which will be reported separately, support the former possibility.

Circular Dichroism. The circular dichroism spectra of peptide **5** as a function of solvent are shown in Figure 11. In chloroform peptide **5** shows a strong negative ellipticity at 238 nm. This spectrum is more complicated than it appears because the n→π* band is red shifted from what is anticipated for a γ turn,[26,27] and the ellipticity is negative instead of positive. The NMR data obtained for **5** strongly suggest that the D-Pro residue in **5** is part of a γ turn. This γ turn should produce a positive n→π* transition because it is the mirror image of the inverse γ turn in **6** which has a negative n→π* transition. The unsymmetrical shape of the CD spectrum of **5** between 230 and 245 nm is not caused by transitions involving the aromatic ring of **5** because the CD spectrum of *cyclo*-(Gly-L-β-cyclohexylalanyl-D-Pro-L-Ala) (**7**), synthesized by hydrogenation of **5** over PtO_2,[36] is essentially identical with that of **5** between 200 and 245 nm in acetonitrile and between 230 and 250 nm in chloroform.

We are unable to rationalize completely the CD spectrum of **5** with the NMR data. One possible explanation is that the band between 230 and 245 nm is a composite of two transitions,

one positive at ~232 nm and one negative at ~235 nm.[37] Assuming the CD band for the D-prolyl γ turn occurs at the same wavelength as the L-prolyl inverse γ turn in **6** but is positive, it is possible to calculate the negative CD band needed to produce the observed CD (Figure 11).

The origin of such a negative band is not clear. It could arise from an n→π* transition for an inverse γ turn involving the Ala-Gly-Phe sequence (ψ(Gly) = 60°). However, $J_{N\alpha}$ for Phe in **5** (Table I) is too large for the vicinal bond angle (θ) near 15°, which would be present in this inverse γ turn.[33,40] Furthermore, the Phe α-H resonates farthest downfield of all α protons in **5**, and this is consistent with its being eclipsed by the glycyl carbonyl group.[4,35] These data indicate to us that the Ala-Gly-Phe sequence is part of a γ turn (ψ(Gly) = −60°), and that an inverse γ turn cannot explain the anomalous CD spectrum of **5**.

Alternatively, the hypothetical negative 235-nm band could be due to some other non-γ-turn, n→π* transition of the type observed in *cyclo*-(L-azetidine-2-carboxyl)$_3$,[38] for which the observed ellipticity (−13 100) is comparable to that calculated for **5** (−9000). As the solvent polarity is increased, the observed CD band for **5** at ~238 nm shifts to lower wavelength, and decreases in intensity. Both results are consistent with the known effects of solvent on CD spectra of n→π* transitions. However, these effects could also arise from conformational interconversions which NMR data indicate occur in the more polar solvent systems.

Proposed Conformation of 5. The spectroscopic data presented establish that the conformation of *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) (**5**) in chloroform must be based on a ring system with four trans amide bonds. This conclusion is based on the following analysis. Since the X-Pro and X-Gly amide bonds are trans, there are only four possible amide bond sequences for the cyclic system: A, cis,trans,cis,trans; B, trans, trans,trans,cis (Pro-Ala); C, trans,trans,trans,cis (Gly-Phe); D, all trans. Conformer A can be eliminated since intramolecular hydrogen bonding is not possible in this ring system and because it does not account for the high-field Pro C$^\beta$ resonance in the ^{13}C NMR. Conformer B can be eliminated for the same reasons as A plus the fact that the Phe NH-C$^\alpha$H vicinal bond angle would be too small (<130°) for the large Phe $J_{N\alpha}$ observed.[33,40] Conformer C is more difficult to exclude since it permits the intramolecularly hydrogen bonded γ turn involving Ala-D-Pro. However, this conformation does not account for the shielding of the Phe-NH from solvent, and it also places the β carbon of Phe within 2.5 Å of the δ carbon of Pro. This is a severely hindered interaction[39] and one that has been shown to be disfavored energetically in other cyclic tetrapeptide systems.[4,6,7]

We therefore conclude that the conformation of *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) (**5**) must have four trans amide bonds. The hydrogen-bonding studies suggest that one intramolecular bond involving the Ala-NH is especially likely and that a second intramolecular hydrogen bond to the Phe-NH is probable. The ^{13}C NMR chemical shift for the Pro-C$^\beta$ resonance is consistent with a γ turn involving the Pro residue. The conformational constraints imposed by the γ turn, the trans X-Pro and Ala-Gly amide bonds, and the observed ϕ angles[33,40] can be accommodated in a cyclic tetrapeptide conformation with four transoid amide bonds as shown in Figure 6B. Values for the ϕ, ψ, and ω torsion angles in **5** are given in Table II. The nonplanar transoid amide bonds were assumed to have ω twist angles close to those found in dihydrochlamydocin **3a** for the reasons cited previously.[29,30] A hydrogen bond between the Phe-NH and the Ala-CO is proposed on the basis of the hydrogen-bonding data.

Discussion

The conformation of [Ala^4]-desdimethylchlamydocin (**5**) in chloroform-*d* and the conformation of *cyclo*-(D-Phe-Pro-D-Phe-Pro) (**6**) in 5% Me_2SO-d_6/chloroform-*d* represent the first time cyclic tetrapeptides with four transoid amide bonds have been observed unambiguously in solution. In the case of *cyclo*-(D-Phe-Pro-D-Phe-Pro) (**6**), ^{13}C NMR data establish that the D-Phe-Pro bonds must be trans. The Pro-D-Phe bond normally would be expected to be trans, since this is a secondary amide bond and this expectation is borne out by the hydrogen bonding and NMR data.

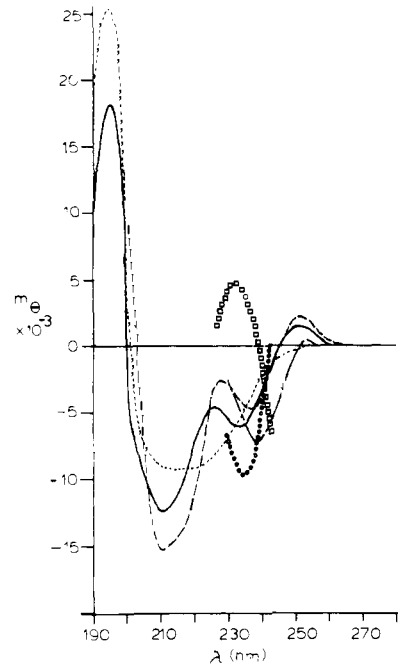


Figure 11. Circular dichroism spectra of *cyclo*-(Gly-L-Phe-D-Pro-L-Ala) (**5**) in various solvents: $CHCl_3$ (— · —) (6.56 × 10^{-4} M); CH_3CN (- - -) (6.56 × 10^{-4} M); CH_3OH (—) (2.21 × 10^{-4} M); H_2O (· · ·) (3.52 × 10^{-4} M; the water contained 1% methanol). Predicted band of n→π* transition for D-Pro γ turn (□); calculated negative n→π* band (○).

Cyclic peptide **5** contains only one proline residue, which was assigned a trans configuration from the ^{13}C NMR data, and it was necessary to find other methods for assigning the geometry of the remaining amides. To do this we relied upon the unusual downfield shift of one glycine α proton when it is eclipsed by a neighboring carbonyl group in a synperiplanar fashion. This same argument previously was used to assign the s-cis geometry to the dehydrophenylalanyl residue to tentoxin,[4] and also was used here (vide supra) to exclude the possibility that the conformation of **5** is based on a γ turn, inverse γ turn conformation. The NMR spectroscopic evidence is consistent with the conformation of **5** depicted in Figure 6B. Thus, the interpretation that the downfield glycine inner proton can be used to establish ω(Ala) appears to be justified and may prove useful in determining the conformations of other conformationally constrained peptide systems.

In addition to finding that the conformations of **5** and **6** contained four transoid amide bonds, we found that intramolecular hydrogen bonds are an important conformational feature of these ring systems in chloroform. The ^{1}H and ^{13}C NMR data strongly support the interpretation that the hydrogen bonds in each molecule participate in γ (or inverse γ) turns. Peptide **6** clearly has two inverse γ turns, while peptide **5** has one γ turn at proline and probably a second γ turn at the glycyl residue. Thus, both conformers **5** and **6** (Figure 6) are closely related in their ring conformations to the conformation of dihydrochlamydocin **3a** (Figure 1) as observed in the crystalline state. All structures have four transoid amide bonds and at least one (and probably two) γ turns. It is interesting that

these conformations are related to the one originally proposed by Schwyzer et al. for *cyclo*-(Gly)$_4$.[42]

In assigning γ turns (or inverse γ turns) to the proline residues in **5** and **6**, we relied upon the previous interpretation that an unusually high field ^{13}C NMR resonance for Pro-C$^{\beta}$ signaled a ψ(L-Pro) $\simeq$ 60°.[24,25,27] All lines of evidence reported here support conformations in which ψ(Pro) = 60° (for **6**) and −60° (for **5**), and Pro-C$^{\beta}$ resonated at 24 and 25 ppm for both **5** and **6** in the ^{13}C NMR in chloroform. Therefore, this correlation has been extended to the cyclic tetrapeptide ring systems. The correlation of ψ(L-Pro) = 60° with the high-field Pro-C$^{\beta}$ resonance appears to be a general phenomenon and one that may be diagnostic for a γ turn (inverse γ turn in the case of L-Pro) in other peptide systems.

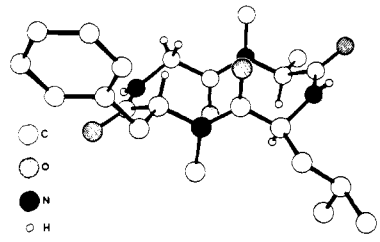
The origin of the negative CD band between 230 and 245 nm in **5** is ambiguous and does not appear to be rationalizable in terms of the proposed conformation (Figure 6B). Nevertheless, the NMR and IR data are consistent with a cyclic tetrapeptide ring system with four transoid amide bonds and at least one γ turn. A theoretical calculation may be required to explain the CD spectra of this strained cyclic tetrapeptide system.

The availability of the first cyclic tetrapeptide established to have four transoid amide bonds in chloroform enabled us to study the factors stabilizing this conformation. Solvent titration studies (Figures 4 and 8) show that the conformation of **5** has four transoid amide bonds in chloroform containing low Me$_2$SO-d_6 concentrations, but at higher Me$_2$SO-d_6 concentrations, **5** has cis Phe-D-Pro amide bonds. In neat Me$_2$SO-d_6, [Ala^4]-desdimethylchlamydocin exists in equilibrium between the transoid conformation (Figure 6B) and two or more additional conformations in which 70% of the X-Pro bonds are cis. Molecular models as well as studies of related cyclic tetrapeptides[4,35] indicate that [Ala^4]-desdimethylchlamydocin can adopt cis,trans,cis,trans conformations related to **2** (Figure 1). These conformations in Me$_2$SO will be reported separately. Similar results were observed for the effect of solvent on the conformation of *cyclo*-(D-Phe-Pro-D-Phe-Pro). Our studies show that this peptide exists in a transoid conformation (Figure 6A) in 5% Me$_2$SO in chloroform, but when the Me$_2$SO concentration exceeds 50%, *cyclo*-(D-Phe-Pro-D-Phe-Pro) adopts conformations with only cis X-Pro bonds. Thus, the conformation of *cyclo*-(D-Phe-Pro-D-Phe-Pro) is as sensitive to solvent as **5** and this shows that the solvent dependency may be a general property of cyclic tetrapeptides capable of forming γ turns or inverse γ turns and not a property of the chlamydocin sequence alone.

The stability of the all-transoid conformations of peptides **5** and **6** in chloroform appears to result from the intramolecular hydrogen bonds in the γ turns or inverse γ turns. Addition of hydrogen bond acceptors like Me$_2$SO to chloroform solutions of **5** or **6** causes the peptides to adopt cis,trans,cis,trans conformations (Figures 4 and 8). This change appears to be caused by hydrogen bonding of solvent to the amide group rather than by the increased polarity of solvent because both peptides remain predominantly in transoid conformations in acetonitrile. Other hydrogen-bonding solvents, e.g., methanol or water, promote cis amide bond conformations for these cyclic tetrapeptides but not as effectively as Me$_2$SO.

Although the conformations of **5** and **6** shown in Figure 6 are related to calculated cyclic tetrapeptide conformations with S_4 ring symmetry,[10,12] intramolecular hydrogen bonds are not a stabilizing feature in the theoretical conformations. Our data indicate that the conformations of peptides **5** and **6** are stabilized over either pure S_4 or cis,trans,cis,trans conformers (Figure 1) in chloroform by the hydrogen bond in the γ turn. As this intramolecular hydrogen bond is broken by addition of dimethyl sulfoxide or other hydrogen-bonding solvents to the chloroform solution, the equilibrium between transoid and cis X-Pro conformers shifts toward cis conformers. Thus, in contrast to the energy calculations,[10-12] the equilibrium between all-transoid and alternating cis,trans conformations, in the absence of intramolecular hydrogen-bonding or unfavorable transannular steric interactions, appears to lie in favor of species with cis amide bonds, although the energy differences between conformations cannot be great. However, while hydrogen bonding of Me$_2$SO to amide NH groups would remove a stabilizing hydrogen bond in the γ turn, the hydrogen-bonded solvent molecule may also destabilize the transoid conformations through steric interactions with transannular ring atoms as does a *N*-methyl group.[9] The apparent preference for cis amide bond conformers in Me$_2$SO therefore may not accurately reflect the relative stability of cyclic tetrapeptide conformations in the absence of solvent effects.

In the crystal structures of these compounds, intermolecular hydrogen bonds or other crystal packing forces could shift the conformation of a cyclic tetrapeptide from an all-transoid to a cis,trans,cis,trans conformation. This may account for the crystal structure of dihydrotentoxin **8**, which has a cis,



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trans,cis,trans amide bond conformation (with the secondary amide bonds cis[2]), even though molecular models suggest this molecule can adopt a bis γ turn conformation without obvious strain or steric interactions developing.

The results we have obtained for cyclic tetrapeptides **5** and **6** may relate to the uncertainty about the solution conformation of *cyclo*-(Gly)$_4$. Wuthrich et al.[15] reported that *cyclo*-(Gly)$_4$ in Me$_2$SO or TFA adopted an all-transoid or S_4 conformation as shown in Figure 1. Their analysis was based on the unusual ^{13}C chemical shift of the carbonyl groups vs. model compounds. They assigned ^{13}C resonances near 172–175 ppm to transoid amide bond carbonyl carbons and resonances between 168 and 172 ppm to planar trans carbonyl carbons. As shown in Figure 4, we observe downfield carbonyl resonances (172, 174 ppm). However, these can be attributed to either transoid amide bonds or to hydrogen-bonded carbonyls.[41]

Dale and Titlestad reported a cis,trans,cis,trans conformation **2** for *cyclo*-(Gly)$_4$ in Me$_2$SO or TFA and suggested that Wuthrich et al. were studying *cyclo*-(Gly)$_8$.[16] The results which we obtained for peptides **5** and **6** show that the all-transoid conformations predominate in chloroform but that conformations with cis X-Pro bonds predominate in pure Me$_2$SO or other hydrogen-bonding solvents. Thus, our results suggest that the conformation of *cyclo*-(Gly)$_4$ in Me$_2$SO would be related to the cis,trans,cis,trans conformation **2** (Figure 1) and not to the S_4 conformation **1**. It is possible that we cannot extrapolate the solvent dependency of **5** and **6** to *cyclo*-(Gly)$_4$ because both **5** and **6** contain proline residues and therefore may assume the cis amide bond more readily than a Gly-Gly sequence. However, the differences in the amount of cis X-Pro bonds in Me$_2$SO between peptide **5** (1 Pro) and peptide **6** (2 Pro) are not sufficient to ensure the absence of cis amide bonds in *cyclo*-(Gly)$_4$ in Me$_2$SO.

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