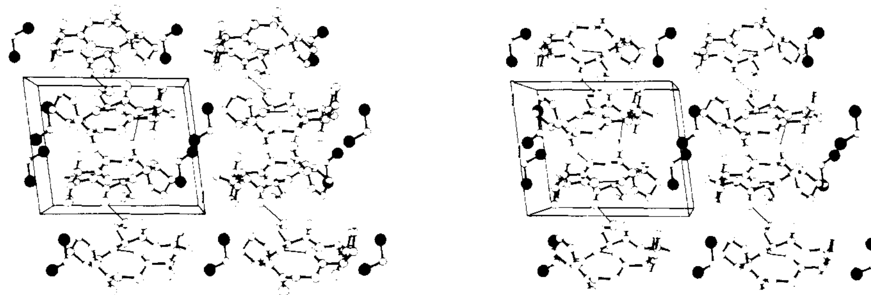

**Figure 5.** A stereodiagram of the packing. Layers of solvent molecules $CH_2Cl_2$, where the Cl atoms are represented by ●, intersperse layers of peptide molecules. Hydrogen bonds between peptide molecules are indicated by thin lines. The lipophilic side groups of the Pro and Ala residues are directed toward the lipophilic solvent layers.

between the peptide and the $CH_2Cl_2$ molecules are $O_2 \cdots C_{CH_2Cl_2}$ and $O_4 \cdots C_{CH_2Cl_2}$ at 3.03 and 3.13 Å respectively.

The central portion of the peptide stacks contains the polar regions of the peptide molecules, $N_1H$, $N_3H$, $N_4H$, and $O_3{}^{\gamma}H$, that participate in hydrogen bond formation with $O_1$, $O_3$, and $O_5$. The intermolecular hydrogen bonds are located around the screw axes at ($x = 1/2$, $z = 0$) and ($x = 1/2$, $z = 1/2$). The hydrogen bonds connect the stacked peptide molecules into infinite layers parallel to the solvent layers. Thus in proceeding parallel to the *a* axis, one encounters the lipophilic solvent layer, a lipophilic region of the peptide, the polar region of the peptide, a lipophilic region of the peptide, and the next lipophilic solvent layer.

A comparison of the conformation of cyclic Gly-Pro-Ser-D-Ala-Pro in the crystalline state with the conformation in solution deduced from NMR data will be presented in the accompanying paper.[1]

**Supplementary Material Available:** Listings of observed and calculated structure factors as well as tables of anisotropic thermal parameters for the nonhydrogen atoms and coordinates for hydrogen atoms (10 pages). Ordering information is given on any current masthead page.

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# Solution Conformation of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro). Hydrogen-Bonded Reverse Turns in Cyclic Pentapeptides


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**Abstract:** The synthesis and conformational analysis in solution of *cyclo*-(glycyl-L-prolyl-L-seryl-D-alanyl-L-prolyl) [*cyclo*-(Gly-Pro-Ser-D-Ala-Pro)] are reported. $^1H$ and $^{13}C$ NMR results indicate that one conformer (all-trans) predominates in $CD_2Cl_2$ and $Me_2SO$-$d_6$, and that intramolecular hydrogen bonding involving the Gly N–H is a feature of the preferred conformer. $^1H$ and $^{13}C$ NMR results suggest a likelihood of a 1←3 hydrogen bond, though not analogous to that observed in the related molecule *cyclo*-(Gly-Pro-Gly-D-Ala-Pro). A structure is proposed which contains a D-Ala-Pro type II′ $\beta$ turn, with the Gly N–H involved in the 1←4 hydrogen bond. The Ser N–H is suggested to participate in the 1←3 interaction though to remain accessible to solvent or other peptide molecules. Comparisons are discussed between the conformer proposed and the crystal structure reported in an accompanying paper. In addition, these structures are compared with other proline-containing cyclic pentapeptides which have been studied.


Recently recognized to be important and widespread conformational features in proteins are so-called "turns", wherein the polypeptide chain reverses direction, often with concomitant formation of a hydrogen bond.[2-5] Indeed, it has been suggested that the formation of turns may nucleate polypeptide chain folding.[6] Cyclic peptides serve as ideal models for studies of the details of conformation in turns, and offer the attractive possibility of examining various amino acid sequences to establish conformational variables for different types of turns.

In previous studies,[7,8] a cyclic pentapeptide, *cyclo*-(Gly(1)-Pro-Gly(2)-D-Ala-Pro),[9] was observed to take up an all-trans $\beta,\gamma$-turn conformation both in the crystal and in solution in a variety of solvents. The observed conformation contained transannular hydrogen bonds from the N–H of

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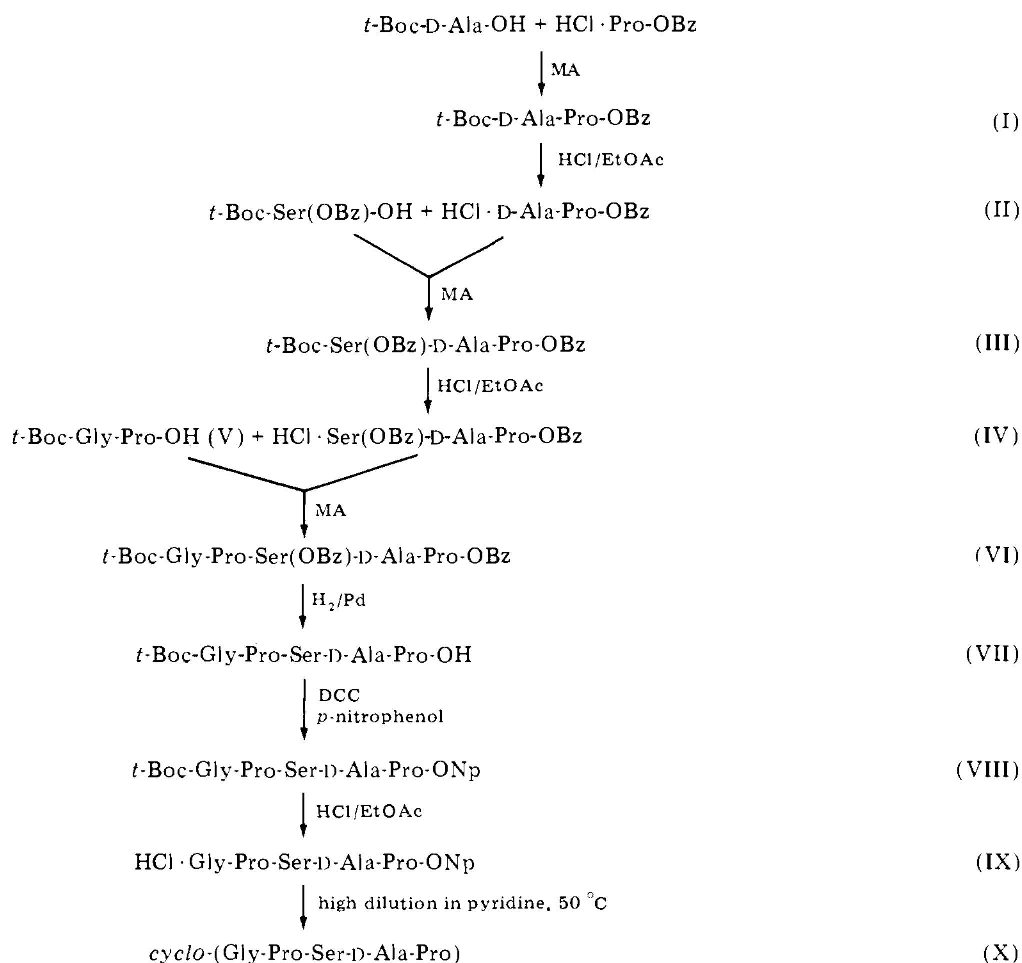
Scheme I. Scheme of Synthesis of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) (X)




D-Ala to the Gly(1) C=O (1←4) and from the N–H of Gly(1) to the D-Ala C=O (1←3). The 1←4 hydrogen bond was part of a type II $\beta$ turn.

In the present report, we describe results of an investigation of an analogue of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro), in which one of the Gly's has been replaced by an L-serine, viz., *cyclo*-(Gly-Pro-Ser-D-Ala-Pro). Theoretical predictions[3,10] and results from studies on other cyclic peptides[11] suggested that the same overall $\beta,\gamma$-turn conformation might obtain as in the previous peptide, but with a type I (Pro-Ser) $\beta$ turn. However, it was anticipated that the introduction not only of a new side chain, but also of a new potential hydrogen-bonding group (OH), might alter markedly the conformational energetics of the pentapeptide. The results of an NMR[12] study of the solution conformation (this paper), and those of a crystal structure determination (reported in the accompanying paper[13]) reveal two all-trans intramolecularly hydrogen-bonded conformations, with that observed in solution distinct from the one observed in the crystal structure, *and* each of these different from the conformation favored by *cyclo*-(Gly-Pro-Gly-D-Ala-Pro). Both contain type II′ (X-Pro) $\beta$ turns.[3]

## Experimental Section

**Synthesis.**[12] *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) was synthesized according to the scheme outlined (Scheme I). The identity and purity of all intermediate compounds were checked by thin layer chromatography (TLC), NMR, and amino acid analysis.

***t*-Boc-D-Ala-Pro-OBz (I).** *t*-Boc-D-Ala-OH (3.0 g, 16 mmol) was dissolved in chloroform (~50 mL) and cooled to −20 °C in dry ice–$CCl_4$. Then *N*-methylmorpholine (1.76 mL, 1 equiv) and isobutyl chloroformate (2.21 mL, 1.05 equiv) were added successively. After 20 min, HCl·Pro-OBz (3.87 g, 1 equiv) with 1 equiv of *N*-methylmorpholine dissolved in chloroform (~25 mL) was added dropwise. The reaction mixture was allowed to warm slowly to 0 °C, and stirring was continued overnight at 4 °C. After evaporation of the solvent, the residue was dissolved in ethyl acetate (~300 mL) and successively extracted with 20-30-mL portions of 0.05 N $H_2SO_4$, 8% $NaHCO_3$, $H_2O$, and saturated NaCl (two times each). After drying with $Na_2SO_4$, the solvent was evaporated. The crude product was crystallized from ether-hexane to give I in 85% yield (6.1 g), mp 89–91 °C.

**HCl·D-Ala-Pro-OBz (II).** A solution of *t*-Boc-D-Ala-Pro-OBz (3.9 g, 10.4 mmol) in ethyl acetate (~50 mL) was cooled to 0 °C and treated with dry HCl gas for 20 min. Stirring was continued for an additional 20 min at room temperature. The solvent was evaporated, and the product was triturated with ether and dried overnight in vacuo, yield 3.24 g (100%).

***t*-Boc-Ser(OBz)-D-Ala-Pro-OBz (III).** *t*-Boc-Ser(OBz)-OH (3.0 g, 10.2 mmol) was dissolved in chloroform (~50 mL) at −20 °C, and its mixed anhydride prepared with *N*-methylmorpholine (1.1 mL) and isobutyl chloroformate (1.40 mL) as described above. After 20 min, a chloroform solution of II (3.24 g, in ~25 mL) with 1 equiv of *N*-methylmorpholine was added dropwise. After the solution was stirred overnight in the cold (4 °C), workup as usual afforded the desired tripeptide in quantitative yield (5.35 g, 95%) as a solid foam which could not be crystallized.

**HCl·Ser(OBz)-D-Ala-Pro-OBz (IV).** A solution of III (5.35 g) in ethyl acetate (~75 mL) was cooled to 0 °C and treated with dry HCl gas for 20 min. Evaporation of solvent left the tripeptide as a foam, which was triturated with ether and dried in vacuo, yield 4.74 g (100%).

***t*-Boc-Gly-Pro-OH (V).** Preparation was as previously reported.[14]

***t*-Boc-Gly-Pro-Ser(OBz)-D-Ala-Pro-OBz (VI).** *t*-Boc-Gly-Pro-OH (2.63 g, 9.7 mmol) was dissolved in chloroform (~50 mL), cooled to −20 °C, and treated in the usual manner with *N*-methylmorpholine (1.07 mL) and isobutyl chloroformate (1.33 mL). After 20 min of

Table I. N-H Data for *cyclo*-(Gly-Pro-Ser-D-Ala-Pro)[a]

| residue | δ, ppm | | $J_{N\alpha}$ | | $\Delta\delta/\Delta T$[b] | | $\Delta\delta_{soln}$[c] |
|---|---|---|---|---|---|---|---|
| | $CD_2Cl_2$ | $Me_2SO$-$d_6$ | $CD_2Cl_2$ | $Me_2SO$-$d_6$ | $CD_2Cl_2$ | $Me_2SO$-$d_6$ | |
| Gly | 7.77 | 7.53 | 8.3, ~2 | 6.5, ~4 | 0 | 0.5 | +0.24 |
| Ser | 6.57 | 7.59 | 8.9 | 8.8 | 11 | 4.2 | −1.02 |
| D-Ala | 7.45 | 7.73 | 8.4 | 8.1 | 15 | 3.2 | −0.28 |

[a] Data given are for 22 °C, 5 mg/mL, unless otherwise noted. [b] $\times 10^3$ ppm/deg; temperature range studied, −15 to 20 °C in $CD_2Cl_2$, 30 to 100 °C in $Me_2SO$-$d_6$. [c] $\delta_{CD_2Cl_2} - \delta_{Me_2SO\text{-}d_6}$.

Table II. $^{13}C$ Chemical Shifts of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro)[a]

| carbon | $CD_2Cl_2$ | $Me_2SO$-$d_6$ |
|---|---|---|
| Gly α | 151.32 | 151.27 |
| Pro α | 130.59 | 130.28 |
| α | 133.86 | 134.18 |
| Ala α | 145.64 | 146.48 |
| Ser α | 138.31 | 138.27 |
| Pro β | 163.15 | 163.60 |
| β | 166.84[b] | 167.49[b] |
| Ser β | 129.82 | 131.68 |
| Ala β | 176.80 | 176.39 |
| Pro γ | 167.61[b] | 168.09[b] |
| γ | 168.02[b] | 168.79[b] |
| Pro δ | 145.54 | 146.40 |
| δ | 146.64 | 147.80 |

[a] Peptide concentration 30 mg/mL. Temperature 30 ° C. Chemical shifts given in parts per million upfield from external $CS_2$. On this scale, the resonance of $Me_4Si$ occurs at 193.7 ppm. [b] Assignment speculative; cannot distinguish between Pro $C^\beta$ and $C^\gamma$ resonances.

stirring at −20 °C, the chloroform solution of IV (4.74 g) with 1 equiv of *N*-methylmorpholine was added dropwise. Stirring was continued overnight in the cold, and workup as above yielded the desired pentapeptide as a solid foam which could not be crystallized, yield 6.98 g (98%).

**t-Boc-Gly-Pro-Ser-D-Ala-Pro-OH (VII).** A catalytic amount of 10% palladium/charcoal was added to the pentapeptide VI (6.98 g) in 50 mL of 90:10 (v/v) *tert*-butyl alcohol/ethanol, and the solution was hydrogenated for 3 days at ca. 35 psi. The catalyst was removed by filtration through Celite, and solvent was evaporated. Thorough pumping in vacuo yielded VII as a solid foam (4.94 g, 95%).

**t-Boc-Gly-Pro-Ser-D-Ala-Pro-ONp (VIII).** A solution of 2.0 g (3.8 mmol) of the pentapeptide acid VII and 0.63 g (1.2 equiv) of *p*-nitrophenol in chloroform (50 mL) was cooled to 4 °C and treated with 0.78 g (1 equiv) of dicyclohexylcarbodiimide. Stirring was continued overnight at 4 °C, and a few drops of acetic acid were added. The solution was filtered to remove dicyclohexylurea (DCU). To ensure complete removal of DCU, chloroform was evaporated and the reaction mixture was dissolved in ethyl acetate. Two crops of DCU were collected from ethyl acetate solution. Evaporation of solvent left a pale yellow foam, which was triturated with ether and dried in vacuo, yield 2.2 g (90%).

**HCl·Gly-Pro-Ser-D-Ala-Pro-ONp (IX).** The pentapeptide active ester VIII (2.2 g, 3.4 mmol) dissolved in ethyl acetate (30 mL) was cooled to 0 °C and treated with dry HCl gas for 20 min. Evaporation of solvent left the pentapeptide hydrochloride as a solid foam. The product was triturated with ether and dried in vacuo, yield 1.85 g (93%).

***cyclo*-(Gly-Pro-Ser-D-Ala-Pro) (X).** A 50-mL solution of 1.85 g (3.2 mmol) of IX in dimethylformamide (DMF) (distilled over BaO) was added dropwise over a 4-h period to 1.5 L of pyridine at 50 °C. The reaction was allowed to proceed at 50 °C for 3 days. The pyridine and DMF were evaporated completely, using a vacuum pump and 40 °C water bath. The crude product was dissolved in 100 mL of 50:50 ethanol/water and treated with 50 g of Rexyn 1-300 ion-exchange resin for 1 h. The resin was removed by filtration, and the solvents were evaporated to yield glassy product. The crude product was crystallized from ethanol/ether to give 0.5 g of X (38% yield). High-resolution mass spectrometry gave *m/e* 409.196 13 (calcd for $C_{18}H_{27}N_5O_6$, 409.196 23).

**NMR Spectroscopy.** $^1H$ NMR spectra were recorded on either a Brucker HX-270 or a Varian HR-220 instrument, using 5-mm sample tubes. The peptide concentrations in deuterated solvents ranged from 0.01 to 0.02 M. All spectra were recorded in the Fourier transform mode. $^{13}C$ NMR data were obtained on a Brucker 270 (NIH modified) with 10-mm sample tubes, using solutions of concentration 0.08 M. Typical spectral conditions for $^1H$ NMR were 32 scans, acquisition time 1.6 s, 2-s pulse delay, and 90° pulse; for $^{13}C$ NMR, 8000 scans, 0.54-s acquisition time, 1-s pulse delay, and 60° pulse. Proton spectra are referenced to internal $Me_4Si$ (δ scale) and carbon spectra to external $CS_2$.

## Results and Discussion

**$^1H$ NMR.** Spectra were assigned by the usual spin decoupling methods, yielding the results shown in Figure 1 for solutions of the pentapeptide in $CD_2Cl_2/CDCl_3$ (1:3)[15] and in $Me_2SO$-$d_6$. Salient points which can be inferred from proton spectra such as this are that one conformation predominates in each solvent, that there are no unusual chemical shifts by comparison with other model peptides, and that no large shifts occur in non-N-H or O-H resonances between the two solvents. In $Me_2SO$-$d_6$, a small population of a conformer interconverting slowly with the predominant species gives rise to an extra set of signals (notably the N-H's and the Ala $CH_3$'s).

The N-H region was analyzed in detail as solvent composition and temperature were varied. A summary of the results is given in Table I. The $J_{N\alpha}$ data reveal only small changes for the coupling-constant values between the two solvents. This finding together with the fact that protons not bound to N or O show only small shifts between the two solvents (cf. Figures 1a and 1b), leads to the conclusion that no major conformational changes occur upon changing from $CD_2Cl_2$ to $Me_2SO$-$d_6$ solution.

The normal criteria of accessibility of N-H's to solvent (or intermolecular interactions) indicate strongly that the Gly N-H does not participate in intermolecular hydrogen bonding, and hence is *not* exposed. Note that its N-H resonance is essentially independent of temperature in both solvents, and moves *upfield* by a small amount on changing from $CD_2Cl_2$ solution to the more strongly hydrogen-bonding solvent $Me_2SO$-$d_6$.

By contrast, the Ser N-H is clearly exposed to intermolecular interactions, as evidenced by temperature dependence of its resonance in both solvents and the large chemical shift downfield between $CD_2Cl_2$ and $Me_2SO$-$d_6$ solutions. The Ala N-H also appears to be exposed, though its behavior is not so readily characterized. Its resonance shows strong dependence on temperature in $Me_2SO$-$d_6$, and the highest of the three in $CD_2Cl_2$. Yet, upon changing from $CD_2Cl_2$ to $Me_2SO$-$d_6$ solutions, the Ala N-H resonance undergoes only a 0.28-ppm shift downfield, as compared to 1.02 ppm for the Ser N-H peak.[16]

**$^{13}C$ NMR.** Data for $^{13}C$ NMR spectra of solutions of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) in $CD_2Cl_2$ and $Me_2SO$-$d_6$ are presented in Table II, and the spectra from which they were taken are shown in Figure 2. Assignments indicated are based upon comparisons with other model peptides and with synthetic precursors. In some cases, undecoupled spectra were examined to remove ambiguity. Of particular note is the clear observation of one predominant conformation with similar chemical shifts for most resonances in both solvents. All chemical shift vari-

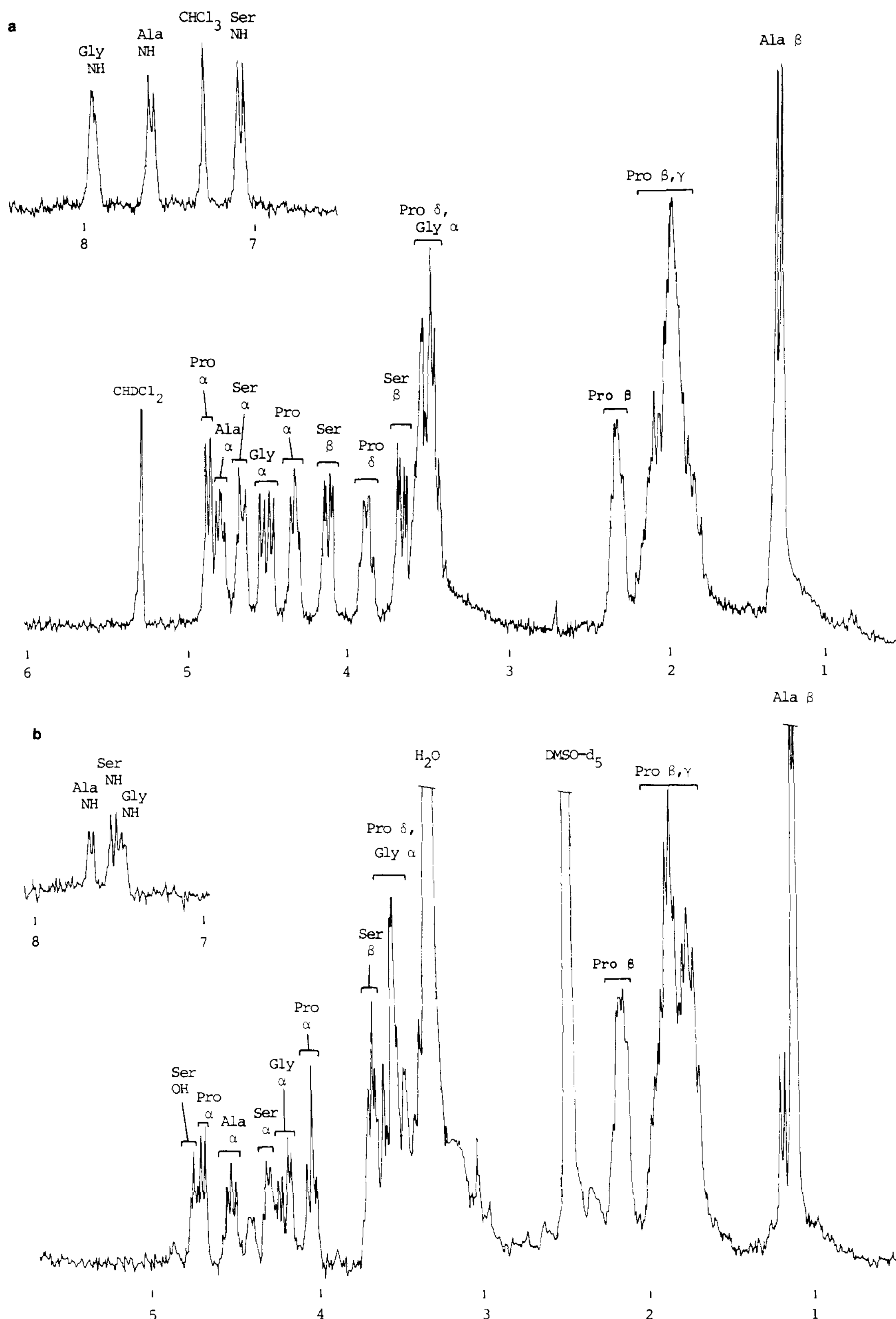



**Figure 1.** $^1H$ NMR spectra of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) at 270 MHz: (a) in $CDCl_3/CD_2Cl_2$, 3:1 (v/v), concn 6.2 mg/mL, temp 25 °C; (b) in $Me_2SO$-$d_6$, 6.2 mg/mL, temp 25 °C. The Ser OH resonance did not appear in 3:1 $CDCl_3/CD_2Cl_2$. Upon addition of a small quantity of $Me_2SO$-$d_6$, it became visible at 4.6 ppm. Precise assignments of peaks near 3.5 ppm is not possible in (a) or (b) owing to multiple overlaps.

ations in the peptide as the solvent is changed are <1 ppm, except for the Ser $C^{\beta}$, which is 1.9 ppm upfield in $Me_2SO$-$d_6$ relative to $CD_2Cl_2$. In $Me_2SO$-$d_6$, a small population (ca. 6% of the total peptide) of a minor conformer, separated by a high energy barrier from the major species, is evident, as was the case in $^1H$ spectra. Its resonances in the Pro $C^{\beta}$ and $C^{\gamma}$ region identify it as containing at least one cis X-Pro bond, in contrast to the all-trans major conformer.

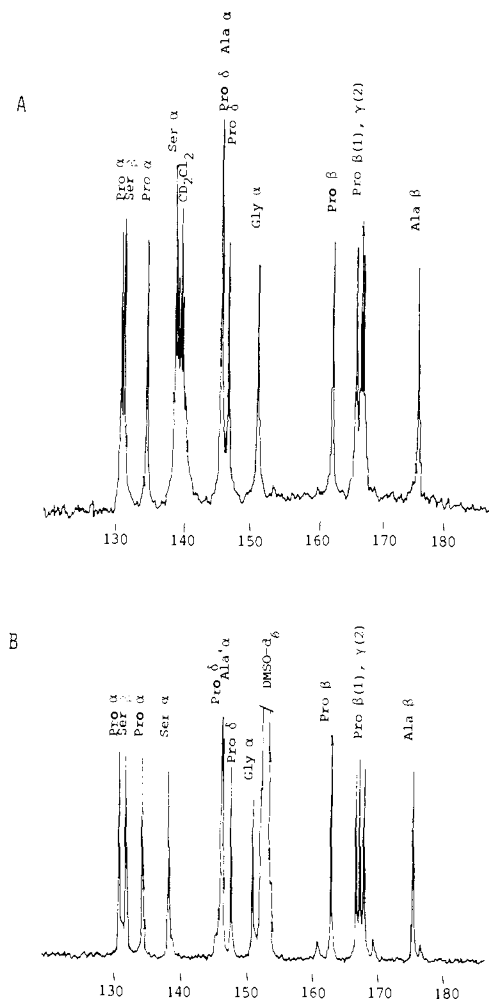
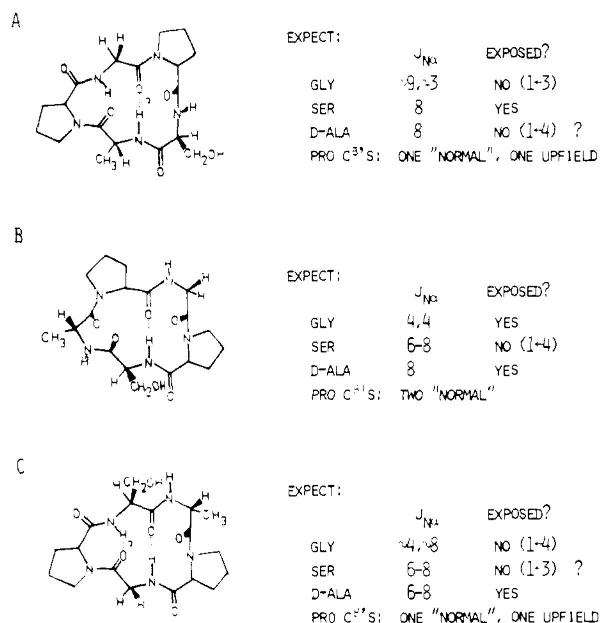



**Figure 2.** $^{13}C$ NMR spectra of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) at 67.5 MHz: (A) in $CD_2Cl_2$; (B) in $Me_2SO$-$d_6$; both at 25 °C and concn 33 mg/mL.

Scheme II




Approximate Dihedral Angles (Low trans' Pro ψ Angles Underlined):

| | | Gly | Pro | Ser | D-Ala | Pro |
|---|---|---|---|---|---|---|
| A | φ | 120 | -60 | -90 | 160 | -80 |
| | ψ | 180 | -60 | 0 | -120 | <u>70</u> |
| B | φ | 50 | -75 | -170 | 90 | -70 |
| | ψ | -130 | -20 | 110 | -120 | 170 |
| C | φ | -150 | -80 | 60 | -60 | -60 |
| | ψ | -90 | <u>90</u> | -150 | -80 | 0 |

In both $CD_2Cl_2$ and $Me_2SO$-$d_6$ solutions, the $^{13}C$ results show that the two prolines are in very different environments. Differences in chemical shifts between the two Pro $C^{\alpha}$ and the two Pro $C^{\beta}$ resonances corresponding to the different prolines in the sequence are particularly marked (ca. 3 ppm). Precedents have now been established from results with *cyclo*-(Pro-Gly)$_3$,[17] *cyclo*-(Gly-Pro-Gly-D-Ala-Pro),[7] cyclic dipeptides,[18] and others,[19] for the correlation of an unusually high-field $C^{\beta}$ resonance (which seems to occur concurrently with a high-field $C^{\alpha}$ resonance) with geometries of the proline residue in which there is eclipsing of the $C^{\beta}$ and the Pro C=O, i.e., a low trans' $\psi$ angle. In the present case, the observed shifts are not so large as those observed for the analogue lacking the serine side chain, but are still greater than those reported for *cyclo*-(Pro-Gly)$_3$ in nonpolar solvents.[17]

**Conformational Interpretation.** Principal findings which must be taken into account in proposing a solution conformation for *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) are solvent shielding of the Gly N-H, coupling constants, and a high-field Pro $C^{\beta}$ signal. It is possible to infer from the clear-cut behavior of the Gly N-H, from the large (ca. 1.0 ppm) difference between the two Gly $H^{\alpha}$ resonance positions, and from the distinction between the $^{13}C$ shifts of the two prolines that the conformation is relatively rigid. In addition, the evidence suggests strongly that the peptide does not undergo major changes in conformation between $CD_2Cl_2$ and $Me_2SO$-$d_6$ solutions.

Furthermore, comparison of $^1H$ spectra of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) with those of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro)[7] reveal striking similarities in the upfield resonance positions. Indeed, the chemical shifts of protons of like residues in the two compounds in $Me_2SO$-$d_6$ differ by less than 0.1 ppm. Also, the $J_{N\alpha}$ coupling constant values are similar for analogous residues of the two peptides: Gly(1), 9.5 and 2.5 Hz for *cyclo*-(Gly(1)-Pro-Gly(2)-D-Ala-Pro) (independent of solvent) compared to 8.3 and ca. 2 Hz ($CD_2Cl_2$) or 6.5 and ca. 4 Hz ($Me_2SO$-$d_6$) for Gly in the title compound, and 8.5 Hz for the D-Ala in *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) compared to 8.4 ($CD_2Cl_2$) or 8.1 ($Me_2SO$-$d_6$) Hz for the title compound. The observed proline $H^{\alpha}$ resonance positions and coupling patterns for these two pentapeptides are virtually the same, although it is *not* possible to associate the signals with the specific prolines in the sequence.

Interestingly, the two proline $H^{\alpha}$ resonances within each pentapeptide are distinctly different from one another, one being a doublet (splitting ~8 Hz), with a chemical shift near 4.7 ppm, and the other a triplet ($\Sigma J_{\alpha\beta} \approx 15$ Hz), with chemical shift near 4.1 ppm (exact values vary slightly depending on the peptide or solvent). Examination of data from a number of proline-containing peptides[11] suggests that the usual chemical shift range for Pro $H^{\alpha}$ resonances may be considered to be 4.1–4.3 ppm. Therefore, 4.7 ppm (the chemical shift of the doublet) is a *low-field* position for the $H^{\alpha}$ signal. Also, proline $H^{\alpha}$ resonances are most commonly triplets, indicating $J_{\alpha\beta}$ values which are moderate in magnitude, and not very different from one another. The occurrence of a doublet (arising from one large and one small $J_{\alpha\beta}$) has been correlated with a cis X-Pro-bond conformer.[20,21] We suggest that the doublet pattern may also arise in prolines which are in trans X-Pro bonds when the ring geometry is strongly influenced by other steric factors, notably a low trans' $\psi$ angle and consequent crowding between the carbonyl oxygen and the $C^{\beta}$ methylene.[22]

More specifically, model building suggests that an unusually large (negative) Pro $\phi$ angle may occur in prolines which are

in γ turns, both as a result of the crowding and in order to optimize the 1←3 hydrogen bonding. The X-ray structure analysis of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro)[8] demonstrates that this situation obtains in the crystal conformation of this peptide: the proline in the β turn has a "normal" trans′ ψ angle (126°) and a typical φ angle of −52°, while the proline in the γ turn has a low trans′ ψ angle (70°) and a high (negative) φ angle, −86°.

Based on the above comparisons, one might initially consider for *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) in solution a β,γ conformation analogous to that seen for *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) both in solution and in crystals. This structure is shown for the title peptide as A in the diagram (Scheme II), and a set of approximate φ, ψ angles is given.[23,24] Listed on the diagram are expected $J_{N\alpha}$ values,[25,26] solvent accessibility of N–H's, and whether a proline with a high-field $C^{\beta}$ resonance is anticipated. B and C illustrate alternative intramolecularly hydrogen-bonded conformers which are sterically feasible from model-building studies (and indeed are consistent with general predictions for the given amino acid sequence).[3,10] Both of these contain type II′ β turns. They may be considered as frame shifts of the original β,γ-turn conformer proposed for *cyclo*-(Gly-Pro-Gly-D-Ala-Pro).

It is possible to exclude conformer B immediately on the basis of N–H accessibility. The results indicate that the Gly N–H is solvent shielded in the present peptide. Interestingly, B is the type of conformer which is observed in crystals of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro). In the solid, as described in the accompanying paper,[8] no evidence of any 1←3 hydrogen bonding is seen. The structure observed contains a type II′ Gly-Pro β turn, and the resulting position of the second proline precludes the possibility of an additional intramolecular hydrogen bond.

The distinction between conformers A and C as the predominant solution conformer is more difficult. Note that, despite the small size and relative rigidity of the cyclic pentapeptide, expected coupling constant values for these two quite different conformations are very close. The prediction of degrees of solvent exposure of the N–H's requires careful consideration. Comparisons with the analogous cyclic pentapeptide *cyclo*-(Gly(1)-Pro-Gly(2)-D-Ala-Pro) suggest that involvement in either 1←4 or 1←3 intramolecular hydrogen bonding may give rise to inaccessibility to solvent.[7] However, *previous reports with other model peptides indicate that in* some cases, N–H's in 1←3 hydrogen bonds may participate in intermolecular hydrogen bonding, as signaled by the dependence of their NMR chemical shifts on temperature and solvent composition. Indeed, Bystrov et al.[27] found infrared evidence in a series of linear dipeptides for 1←3 intramolecular hydrogen bonding, but no indication from NMR results of inaccessibility to solvent or to other peptide molecules. In contrast, many examples are now available supporting the premise that 1←4 hydrogen bonding of an N–H leads to a reduced involvement in intermolecular interactions.[11,28-30]

In the present case, the data on solvent exposure of N–H's (Gly sequestered, D-Ala and Ser exposed) would be consistent with either conformer A with the D-Ala N–H accessible (little or no 1←4 hydrogen bonding) and the γ-turn N–H sequestered, or conformer C with the γ-turn N–H accessible and the β-turn N–H sequestered. The generalizations discussed above based on other systems favor the latter, an interpretation which is supported by examining models of conformation C for the present peptide. First, the presence of the Ser in the 3 position of the γ turn causes some steric crowding with the Pro carbonyl oxygen. This can be relieved by opening the γ turn (raising the Pro ψ value) somewhat, which makes the Ser N–H more accessible to solvent. Second, it appears that the cyclic peptide ring is puckered as a result of the geometry of the type II′ β turn such as to direct the Ser N–H (in the γ turn) toward solvent.

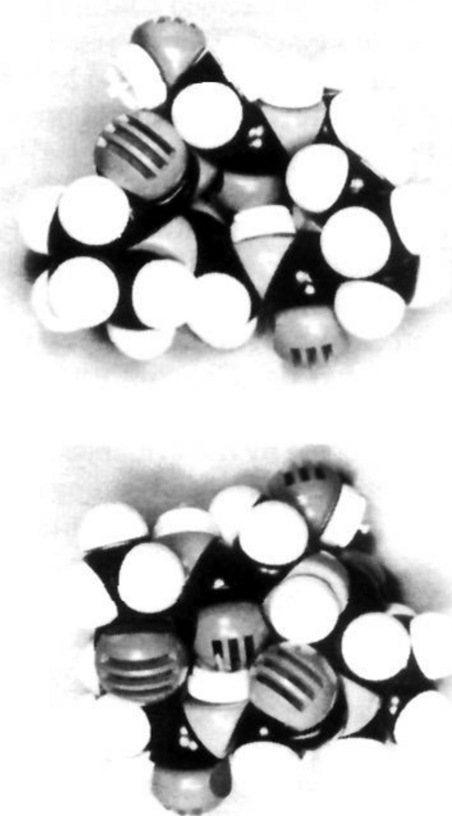


**Figure 3.** Photographs of a CPK model (two views) of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) in the proposed solution conformation (C).

This occurs with the retention of a still rather low trans′ Pro ψ angle in the γ turn, as is indicated by the $^{13}C$ NMR data.

*The fact that the* $^{1}H$ *NMR data seem to indicate strong* similarity of the environments of like residues in *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) and *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) does not argue strongly for either conformer A or C. First, the proline $H^{\alpha}$ shifts and coupling patterns are consistent with *either* conformer A or C, both of which contain one proline expected to be "normal" (in one case cis′, ψ = −60°; in the other, trans′, ψ = 0°; both unperturbed by any steric crowding), and one proline with a low trans′ ψ angle. (Examination of samples of each of the peptides synthesized with one perdeuterioproline would make possible the unequivocal assignment of particular prolines in the sequence.) Second, proton chemical shifts are sensitive to a number of factors, including sequence and conformation. It is not surprising that, for example, the $H^{\alpha}$ resonances of corresponding glycines in the two pentapeptides resemble one another, even if *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) is in the C conformation. Inspection of molecular models suggests that the environments around the comparable Gly $H^{\alpha}$'s in conformer A and C are quite similar (with respect to proximity and orientation of electron-rich anisotropic groups, viz., carbonyls). The use of specifically deuterated glycine residues, as exemplified by the work of Ballardin et al. on [Pro[3],Gly[4]] oxytocin,[31] could aid this analysis considerably.

Conformer C appears, given the available data, to be the best representation of the solution structure of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) in $CD_2Cl_2$ or $Me_2SO$-$d_6$, although alternatives cannot be rigorously excluded. In Figure 3 are shown photographs of a CPK model of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) in conformation C. The φ, ψ angles consistent with NMR data and model building follow.[24]

| | Gly | Pro | Ser | D-Ala | Pro |
|---|---|---|---|---|---|
| φ | −150 | −80 | 60 | −60 | −60 |
| ψ | −90 | 90 | −150 | −80 | 0 |

It is impossible to account in detail for the preference of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) for the proposed conformer (C). However, several points can be mentioned with respect to the relationship between amino acid sequence and preferred conformation in this compound. These will be stated with reference to the peptide *cyclo*-(Gly-Pro-Gly-D-Ala-Pro),

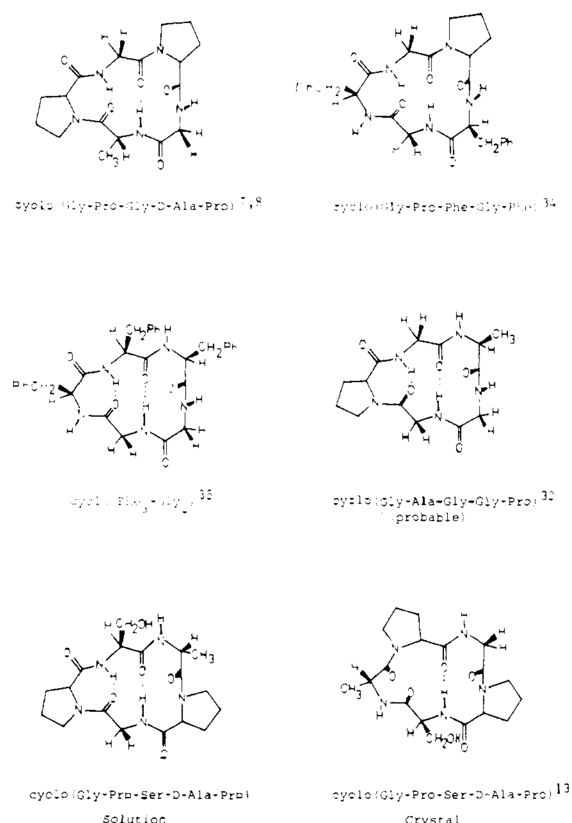
which adopts one rigid, well-defined conformation in a variety of solvents and in the crystal. The presence of an L residue (Ser) following one of the Pro's appears to have disfavored the occurrence of a Pro-X $\beta$ turn. Theoretical predictions[3,10] and results with (X-Pro-Y)$_2$ cyclic hexapeptides[11] are consistent with this general idea, i.e., that a Pro-Gly $\beta$ turn is lower in energy than a Pro-L-X $\beta$ turn, although many examples of Pro-L-X turns are known. It can then be surmised that other possible intramolecularly hydrogen-bonded structures are energetically competitive with the *cyclo*-(Gly-Pro-Gly-D-Ala-Pro)-type conformation. The conformer proposed in solution (C) has two intramolecular hydrogen bonds, though one may be weak (as suggested by the evidence that its N-H participates in intermolecular hydrogen bonding). In contrast, the structure observed in the solid state[13] contains only *one* intramolecular hydrogen bond (from the Ser N-H to the C=O of one of the Pro's), and the molecules are packed so that all other potential hydrogen-bond donors or acceptors are involved in intermolecular hydrogen bonding. Thus, a rationale for the preference for conformer C emerges. It seems to be the conformer which maximizes the number of intramolecular hydrogen bonds, in the most preferable turn geometries, given the sequence and configuration of its amino acids. A deduction is implicit in this statement, namely, that in the framework of this molecule a D-Ala-Pro $\beta$ turn is preferred over a Pro-L-Ser $\beta$ turn (particularly since the $\gamma$-turn part of the molecule would be essentially equivalent for the two possibilities). The Ser OH undoubtedly plays a role in the conformational energetics of this cyclic pentapeptide, but it is not possible to assess its influence without examining an analogue lacking this function. It appears from model building that the Ser OH can be hydrogen bonded to an adjacent group in most of the likely local conformations of this residue. Perhaps the introduction of Ser into the sequence alters the preferred conformation relative to the compound with Gly in the corresponding position primarily because of its steric influence. Confirmation of this hypothesis awaits studies of other analogues.

To date the conformations of four proline-containing cyclic pentapeptides have been studied in detail in solution, and two in crystals. Evidence of intramolecularly hydrogen-bonded structures is found in all cases. The peptide *cyclo*-(Gly-Ala-Gly-Gly-Pro), which may be considered an analogue to *cyclo*-(Gly-Pro-Gly-D-Ala-Pro), was studied by Meraldi et al.[32] Although the three glycines were not distinguished, the N-H's of two appeared to be intramolecularly hydrogen bonded. This result would be consistent with a $\beta,\gamma$-turn conformer of type A, as was proposed for *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) in solution and crystals, or with one of type C, provided that the $\gamma$-turn N-H were sequestered. Deuteration studies would allow discrimination of these two possibilities. The reported $^{13}$C NMR[33] of this compound suggests strongly that the proline is occurring in a $\gamma$-turn conformation with a low trans' $\psi$ angle, as the $C^{\beta}$ chemical shift for the all-trans major conformer is high (167.00 ppm). This datum favors the occurrence of a conformer of type A.

*cyclo*-(Pro-Phe-Gly-Phe-Gly) was synthesized and its conformation studied in solution by Demel and Kessler.[34] It was proposed to adopt a $\beta,\gamma$-turn conformer, to account for two clearly sequestered N-H's. The proposed conformer is stabilized by hydrogen bonding of both of the Gly N-H's. The result is that the Pro-Phe sequence occurs in a type I $\beta$ turn, and the other Phe as residue 2 of the $\gamma$ turn. *cyclo*-(Phe$_3$-Gly$_2$) has been recently reported[35] by the same group to occur also in a $\beta,\gamma$-turn conformer in solution, in this case with a Phe-Gly type II $\beta$ turn.

In general it appears that the preference for intramolecularly hydrogen-bonded structures is strong in cyclic pentapeptides. Depending on the amino acid sequence, $\beta$-turn-containing conformers of various types may occur; examples of types I, II, and II' have been described. In Scheme III is shown a comparison of the structures proposed for the various cyclic pentapeptides. Those peptides with type I or II $\beta$ turns display sequestering of two N-H's, one of which has been inferred to participate in a $\gamma$ turn. The peptide discussed here is the only example thus far proposed to adopt a type II' $\beta$ turn in solution, and does *not* show sequestering, by the usual criteria, of two N-H's. However, evidence has been presented ($^{13}$C chemical shifts and proline $^1$H NMR data) which suggests strongly that a $\gamma$ turn is present. Hence, while only a small number of proline-containing cyclic pentapeptides is available for comparison, it can be stated that this class of model peptide, which is simultaneously restricted in conformational freedom, and yet may adopt several quite different hydrogen-bonded conformations in response to variations in sequence, has yielded insight into expected experimental parameters for $\beta$ and $\gamma$ turns in peptides.

Scheme III




## Conclusions

The conformation of *cyclo*-(Gly-Pro-Ser-D-Ala-Pro) has been studied in $CD_2Cl_2$ and $Me_2SO$-$d_6$ solutions by $^1$H and $^{13}$C NMR. It is proposed to adopt an all-trans conformation containing one 1←4 intramolecular hydrogen bond in a type II' D-Ala-Pro $\beta$ turn, which is consistent with the observation that the Gly N-H does not participate in intermolecular interactions. In addition, it appears that a 1←3 hydrogen bond ($\gamma$ turn) is present in the solution conformer, although it is not signaled by a sequestering of the N-H (in this case the Ser N-H). Evidence offered in support of the existence of the $\gamma$ turn includes a high-field Pro $C^{\beta}$ resonance and a Pro $H^{\alpha}$ resonance which appears as a doublet at relatively low field (ca. 4.7 ppm).

Comparisons with the crystal structure of the title compound (see preceding paper) and with solution and crystal structures of other proline-containing cyclic pentapeptides reveal a general preference for intramolecular hydrogen bonding, although

several types of overall conformation are possible depending on sequence.

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(16) Note that the interpretation of the relative magnitudes of solvent shifts of N–H resonances is complicated by the fact that the peak positions are also dependent upon temperature in each solvent. Hence, the size of the observed shift is not simply related to accessibility. The finding of large dependences on temperature and a significant downfield shift between $CD_2Cl_2$ and $Me_2SO$-$d_6$ solutions for the D-Ala N–H resonance indicates that this N–H is exposed to solvent.
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(22) Note that *cyclo*-(Pro-Gly)$_3$, which has a low-field (4.75 ppm) doublet Pro $H^\alpha$ resonance in $CD_2Cl_2$, was originally proposed to occur in a three-cis Gly-Pro-bond conformer in this solvent.[21] This peptide is now considered[17] to adopt an all-trans conformation in nonpolar solvents like $CD_2Cl_2$ with three 1←3 ($\gamma$-turn) intramolecular hydrogen bonds and low trans' Pro $\psi$ angles.
(23) Models may be constructed with either a type I or a type II Pro-Ser $\beta$ turn, though the former appears to be more likely. Theoretical predictions[3] and findings with *cyclo*-(X-Pro-Y)$_2$ cyclic hexapeptides[11] also suggest that type I would be favored for the Pro-Ser sequence.
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# Proton, Carbon-13, and Nitrogen-15 Nuclear Magnetic Resonance and CNDO/2 Studies on the Tautomerism and Conformation of Amiloride, a Novel Acylguanidine

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**Abstract:** The favored ground-state structures were determined for the novel acylguanidine diuretic, amiloride (**2a**·HCl), and its free base form (**2a**) using natural-abundance $^1$H, $^{13}$C, and $^{15}$N NMR techniques and CNDO/2 theoretical calculations. Amiloride was found to exist primarily in the acylamino tautomer form as planar conformer F1, whereas free base **2a** was shown to prefer the acylimino tautomer form as planar conformer A1 (and/or A4). The conformational preference (i.e., as conformer A1 or as conformer A4) of **2a** was not established. The dynamic mechanism(s) for the experimentally observed rapid equilibration of the terminal amino groups in **2a** and **2a**·HCl and, when N-substituted, their substituents were explored by the CNDO/2 method. Of the six possible pathways considered for effecting N-10–N-11 interconversion in **2a**, a novel mechanism involving a synchronous rotation around $\phi_2$ and $\phi_3$ (path F) was calculated to have the lowest barrier to interconversion. Experimental verification of this novel mechanism was attempted, but not found, by preparation of an appropriate model, **11**, and subsequent determination of the $\Delta G^{\ddagger}$ values (14.7–14.8 kcal/mol) for **11** and pyrazine analogues **4e** and **4e**·HCl using the dynamic $^{13}$C NMR technique in $Me_2SO$-$d_6$-$CD_3OD$. Based on the results of these studies, it is concluded that free base **2a** is likely to undergo N-10–N-11 interconversion via simple $\phi_3$ rotation and/or $\phi_2$ rotation plus inversion. Accordingly, amiloride (**2a**·HCl) must equilibrate by a $\phi_3$ rotation mechanism.

The noteworthy discovery[2] that certain acylguanidines such as compound **1** display saluretic–diuretic activity, i.e., promote loss of sodium chloride and water, while repressing potassium excretion in experimental animals provided impetus

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