the folded structure that predominates in solution. This is reflected in the reduced rate constants.

**Phosphate–Adenine–Isoalloxazine Complex (Steps 3–4 and 5–4 in Scheme II).** Structure 4 in Scheme II involves simultaneous interactions with the phosphate moiety and adenine and isoalloxazine ring systems. This complex is of course unique to the NiFAD system and corresponds to $\tau_4$ in Table III. Presumably, the complex is folded back upon itself, in a manner opposite to that in the free coenzyme. Space-filling models demonstrate the flexibility of the FAD molecule and clearly show the possibility of the 3-fold interaction.

In principle, there are two routes by which the complex $C_4$ can be formed: 3–4 and 5–4. Experimentally, we found the relaxation time $\tau_4$ for Scheme II to be relatively insensitive to step 3–4. A rate constant $k_{34} = 30\ s^{-1}$ was the best-fit value from the computer analysis, but values of 60 or even 0 $s^{-1}$ did not give markedly inferior fits. The rate constants for this step are essentially indeterminate.

On the other hand, the mechanism was quite sensitive to the value of the rate constant $k_{54}$. Since this step does not exist in the other systems, the rate constants cannot be compared with other values. It is not surprising, however, that step 5–4 is the slowest of the four that have been characterized for NiFAD.

## Conclusions and Significance

In conclusion, we have shown that Ni(II) interacts with FMN in a manner analogous to AMP5′. That is, two concentration-dependent relaxation times in the $10^{-4}$–$10^{-3}$-s time region were observed for each system. Rate constants were found to be comparable in the two systems for the phosphate and base interactions. For the NiFAD system, four separate relaxation times were characterized. A multistep mechanism involving a phosphate interaction followed by interactions with the individual components of the coenzyme was found to be consistent with the data. To the best of our knowledge, no other binary system has been observed with four relaxation times.

There could well be some biological importance in the fact that metal ions form several different bound structures with FAD. A redox metal ion, for example, may be tightly bound to the phosphate moiety and simultaneously interact weakly with the isoalloxazine ring, keeping the metal in the proper spatial orientation and thus facilitating electron transfer. Finally, it is evident that simple model experiments designed to demonstrate metal–flavin interactions in biological systems must take into account the fact that the strongest interaction is with the phosphate moiety.

**Acknowledgment.** This work was supported by the NIH in the form of a research grant to J.E.S. (GM-13,116). J. B. gratefully acknowledges support from the B. F. Goodrich Corp. in the form of a graduate fellowship. We thank Dr. Steven Feltch for his assistance with the computer analyses.

**Registry No.** FAD, 146-14-5; FMN, 146-17-8; Ni, 7440-02-0.

**Supplementary Material Available:** Kinetic data for the interaction of Ni(II) with FMN (Table II) (2 pages). Ordering information is given on any current masthead page.

# Compatibility of β- and γ-Turn Features with a Peptide Backbone Modification: Synthesis and Conformational Analysis of a Model Cyclic Pseudopentapeptide

**Arno F. Spatola,*† Mohmed K. Anwer,† Arlene L. Rockwell,‡ and Lila M. Gierasch*‡**

*Contribution from the Department of Chemistry, University of Louisville, Louisville, Kentucky 40292, and the Department of Chemistry, University of Delaware, Newark, Delaware 19711.* *Received March 11, 1985*

**Abstract:** A backbone-modified cyclic peptide has been synthesized and characterized by carbon-13 and proton NMR spectroscopies, and the results have contrasted with well-defined parent all-amide model cyclic pentapeptide. The pseudopeptide was prepared by solid-phase methods using two different linear sequences and then cyclized to yield a common structure. When a mixture of diphenylphosphoryl azide, hydroxybenzotriazole, and (dimethylamino)pyridine was used, the yield of cyclization was 85%. The pseudopeptide, cyclo[Gly-Pro$\psi$[$CH_2S$]Gly-D-Phe-Pro], containing a single thiomethylene group as an amide bond surrogate was nevertheless able to adopt both β- and γ-intramolecular hydrogen bonds in deuteriochloroform, as assessed by diagnostic chemical shift, temperature dependence, and solvent dependence data. However, in contrast to its all amide counterpart, the cyclic pseudopeptide showed evidence of *cis/trans*-proline peptide bond isomerism upon addition of dimethyl sulfoxide.

Variation of backbone elements in peptides can be expected to have profound consequences on conformation, secondary structure, and solubility as well as on biologically important factors such as receptor selectivity, altered transport properties, and different patterns of enzymatic degradation.[1] Reported peptide backbone changes have included not only configurational (*R,S*) interchanges, but also $N^2$ and C alkylations,[3,4] dehydroamino acids,[5] α-carbon substitutions ("aza" analogues),[6] and an increasing variety of amide bond replacements (surrogates). In the latter category are such amide substitutions as $\psi$[COO] (depsipeptides),[7] $\psi$[NHCO] (retroamides),[8] $\psi$[CSNH],[9] $\psi$[$CH_2$NH],[10] $\psi$[$COCH_2$],[11] $\psi$[NHCONH],[12] $\psi$[CH=CH],[13] and $\psi$[$CH_2S$][14] functionalities.

†University of Louisville
‡University of Delaware

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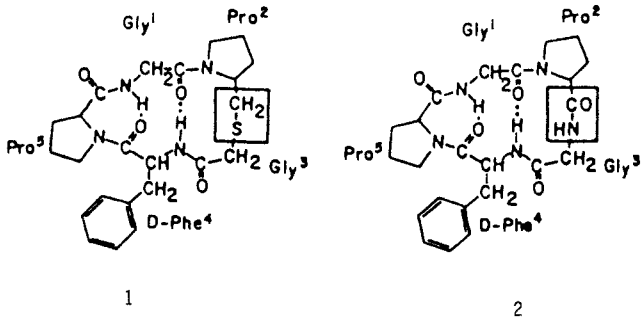


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**Figure 1.** Diagrams of cyclic peptide (right) and pseudopeptide (left), showing the amide bond replacement (indicated by a box). Note the $\beta$-turn formed by residues Gly¹-Pro²-Gly³-D-Phe⁴ with a hydrogen bond from the D-Phe⁴ NH to the Gly¹ C=O and the $\gamma$-turn formed by residues D-Phe⁴-Pro⁵-Gly¹ with a hydrogen bond from the Gly¹ NH to the D-Phe⁴ C=O.

The $\psi[CH_2S]$ moiety, first described in 1977,[15] is an amide bond replacement characterized by its facile synthetic accessibility,[16] reasonable isosterism, and resistance to proteolytic enzymes.[17] It has recently shown utility as a stabilizing factor when incorporated as part of an enkephalin antagonist,[18] and several enkephalin agonists have also been prepared containing this substitution.[1,19] The thiomethylene ether has been incorporated within a large number of LH-RH (luteinizing hormone-releasing hormone) analogues, both agonists and antagonists, and these analogues displayed a wide variation in biological activities, with some possessing activity essentially equipotent to that of the parents, while others showed a large decrease in potency.[14]

One important difference between the $CH_2S$ group and the normally planar amide linkage is increased flexibility. In support of this is the low potency observed for a [Gly$\psi$[$CH_2S$]Leu$^{6-7}$]-LH-RH analogue in which the 6-7 residues are believed to represent the corner positions of a $\beta$-turn feature comprising residues 5-8.[14b] The tentative explanation offered for this loss in potency was the introduction of increased flexibility at a position of the molecule believed to be important for the conservation of the parent's secondary structure; this is also consistent with the high LH-releasing activity reported for a "conformationally constrained" lactam-bridged LH-RH agonist[22] in which the cited $\beta$-turn has been preserved. Nevertheless, reasonable potency (5-10%) was obtained with a [Tyr$\psi$[$CH_2S$]Gly$^{5-6}$]LH-RH analogue for which a formal hydrogen bond-stabilized $\beta$-turn structure (though not necessarily the turn feature itself) is impossible.[14]

Molecular models suggest that most common peptide and protein secondary structural features such as $\beta$-turn and $\alpha$-helices are compatible with the $\psi[CH_2S]$ substitution. Recent X-ray evidence indicates that while minor changes in bond angles and bond lengths are observed with the thiomethylene ether-containing structures leading to a somewhat shorter (3.4 Å) $C_{\alpha i}$-$C_{\alpha i+1}$ distance,[23] other structural changes are minimal. Nevertheless, it might be anticipated that substitution of a "flexible" amide surrogate such as $\psi[CH_2S]$ would be more successful if built into cyclic peptide structures in which considerably greater conformational constraints exist vis-á-vis linear analogues.

The present study was designed to investigate the conformatinal consequences of incorporating the $\psi[CH_2S]$ within a well-studied model cyclic peptide. The parent structure chosen for this study was cyclo[Gly-Pro-Gly-D-Phe-Pro]. This compound belongs to a class of structures that have been extensively investigated both in solution[24] and in the solid state.[25] Most members of this class (Xxx-Pro-Yyy-D-Zzz-Pro) contain two intramolecular hydrogen bonds through $\beta$- and $\gamma$-turn features that involve four of the five amide bonds in the structure. Thus, it was of interest to incorporate the $\psi[CH_2S]$ in the only linkage (Figure 1) *not* participating as either a donor or acceptor in the hydrogen bonding scheme and to assess whether these structural features could still coexist with this type of peptide backbone modification. We find that the $\psi[CH_2S]$ *is* compatible with the preferred backbone conformation of the cyclic peptide: both $\beta$- and $\gamma$-turn features are conserved in the cyclopseudopeptide.

**Table I.** Methodology for the Solid-Phase Synthesis of cyclo[D-Phe-Pro-Gly-Pro$\psi$[$CH_2S$]Gly]

| step | reagent | no. of cycles | time, min |
|---|---|---|---|
| 1 | dichloromethane | 3 | 1 |
| 2 | 40% trifluoroacetic acid in dichloromethane containing 10% anisole and 10% mercaptoethanol | 2 | 15 |
| 3 | dichloromethane | 3 | 1 |
| 4 | 7% diisopropylethylamine in dichloromethane | 2 | 5 |
| 5 | dichloromethane | 3 | 1 |
| 6 | dimethylformamide | 2 | 1 |
| 7 | Boc-amino acid, DCC, HOBt in DMF[a] | 1 | 120 |
| 8 | dimethylformamide | 2 | 1 |
| 9 | dichloromethane | 3 | 1 |
| 10 | ethyl alcohol | 3 | 1 |
| 11 | dichloromethane | 3 | 1 |

[a] Ratios = 2.5:2.5:1.0 equiv.

## Results and Discussion

**Synthesis.** The synthesis of the cyclo[Gly-Pro$\psi$[$CH_2S$]Gly-D-Phe-Pro] was carried out by the solid-phase method of Merrifield.[26]

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**Table II.** $^1$H NMR Data for Cyclic Peptide and Pseudopeptide[a]

| | Gly$^1$ | | | | Pro$^2$ | X$^3$ | | | | D-Phe$^4$ | | | | Pro$^5$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\delta_{NH}$ | $J_{N\alpha}$ | $\Delta\delta/\Delta T_{NH}$[b] | $\delta_{H^\alpha}$ | $\delta_{H^\alpha}$ | $\delta_{NH}$ | $J_{N\alpha}$ | $\Delta\delta/\Delta T_{NH}$ | $\delta_{H^\alpha}$ | $\delta_{NH}$ | $J_{N\alpha}$ | $\Delta\delta/\Delta T_{NH}$ | $\delta_{H^\alpha}$ | $\delta_{H^\alpha}$ |
| | | | | | | X = Gly | | | | | | | | |
| $CDCl_3$[c] | 7.99 | 9.4 | 1.8 | 4.55 | 4.28 (t) | 6.61 | 7.5 | 5.8 | 4.17 | 7.79 | 7.5 | 3.9 | 4.88 | 4.78 (d) |
| | | 2.0 | | 3.37 | | | 5.7 | | 3.76 | | | | | |
| $Me_2SO$[d] | 7.55 | 8.4 | ≅0 | 4.30 | 4.14 (t) | 8.51 | 7.1 | 3.8 | 3.77 | 7.78 | 8.6 | 1.0 | 4.75 | 4.69 (d) |
| | | 2.8 | | 3.47 | | | e | | ≅3.6 | | | | | |
| | | | | | | X = $\psi$[CH$_2$S]Gly | | | | | | | | |
| $CDCl_3$[f] | 8.03 | 9.2 | 1.4 | 4.43 | 4.26 (m) | | | | | 7.92 | 8.1 | 3.2 | 4.92 | 4.76 (d) |
| | | <2 | | 3.31 | | | | | | | | | | |
| $Me_2SO$[g] | | | | | | | | | | | | | | |
| tr[h] | 7.69 | | 1.8 | | | | | | | 8.56 | 7.3 | 4.5 | ≅4.38 | |
| cis | 7.95 | | 3.0 | | | | | | | 9.14 | 3.9 | 4.2 | ≅4.49 | |

[a] Chemical shifts given in parts per million (ppm) downfield from internal tetramethylsilane (TMS); coupling constants ($J_{N\alpha} = J_{NH-H\alpha}$) are given in hertz; temperature, 23 °C. [b] Parts per billion/degree. [c] Concentration 19 mM. [d] Concentration 17 mM. [e] Not observable due to overlap. [f] Concentration 31 mM. [g] Concentration 31 mM; m and M refer to minor (all-trans) and major (one-cis) species, respectively. No entry indicates that the signal could not be assigned due to overlaps. [h] "tr" refers to the all-trans peptide bond-containing conformer, cis to the conformer proposed to contain one cis peptide bond.

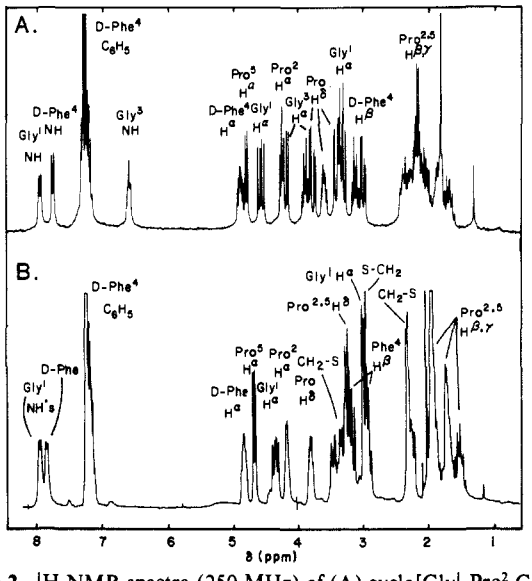


**Figure 2.** $^1$H NMR spectra (250 MHz) of (A) cyclo[Gly$^1$-Pro$^2$-Gly$^3$-D-Phe$^4$-Pro$^5$] in $CDCl_3$, concentration 19 mM, and (B) cyclo[Gly$^1$-Pro$^2\psi$-[CH$_2$S]Gly$^3$-D-Phe$^4$-Pro$^5$] in $CDCl_3$, concentration 31 mM. (singlet at 2.1 ppm likely due to acetate).

Two syntheses were performed, to provide two different linear sequences of the pseudopentapeptide, which after cyclization yielded the same cyclic product. In the first synthesis, Boc-Gly ((*tert*-butyloxycarbonyl)-glycine) was esterified to a chloromethylated polystyrene resin using the Marglin procedure,[27] and the chain was extended by successive couplings using Boc-Pro, Boc-D-Phe, and Boc-Pro$\psi$[CH$_2$S]Gly (Table I). The N-terminal Boc group was removed by treatment with trifluoroacetic acid, and the peptide was cleaved from the resin using anhydrous hydrogen fluoride.[28] Cyclization of the linear peptide [Pro$\psi$-[CH$_2$S]Gly-D-Phe-Pro-Gly] was carried out by using diphenylphosphoryl azide (DPPA) according to Brady et al.[29] The overall yield from this attempt was less than 10%, but the product was confirmed as the desired cyclic analogue by a variety of physical and analytical criteria.

The synthesis of the compound **1** was repeated by using the solid-phase method but with the initial resin-linked residue being Boc-Pro$\psi$[CH$_2$S]Gly incorporated via the Gisin method.[30] In this case, the chain was extended successively with Boc-Gly, Boc-Pro, and Boc-D-Phe, and H-D-Phe-Pro-Gly-Pro$\psi$[CH$_2$S]-Gly–resin thus obtained was cleaved by anhydrous hydrogen fluoride containing anisole and ethyl methyl sulfide as scavengers to give the crude linear pseudopentapeptide in 80% yield. This compound was cyclized by a modified version of the Brady procedure employing DPPA in the presence of (dimethylamino)-pyridine (DMAP) and 1-hydroxybenzotriazole (HOBt). This method gave a homogeneous product in 85% yield.

The final product, cyclo[Gly-Pro$\psi$[CH$_2$S]Gly-D-Phe-Pro], was assessed for purity by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) and was shown to be at least 98% pure in an acetonitrile/triethylammonium phosphate system

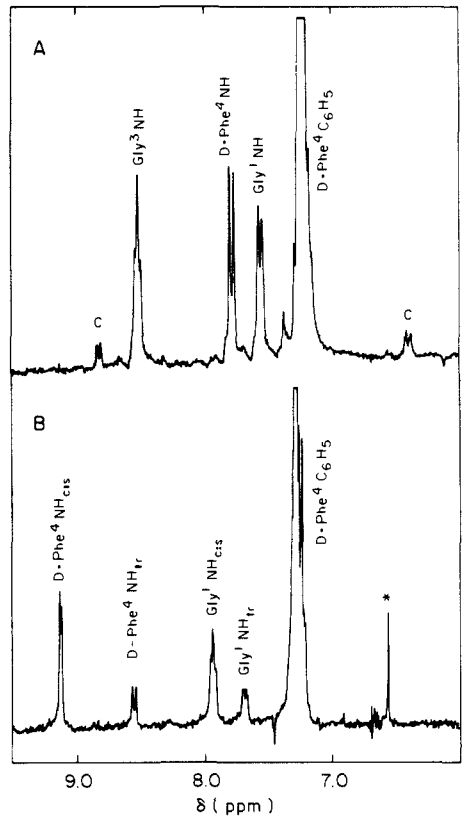


**Figure 3.** NH regions of $^1$H NMR spectra (250 MHz) of (A) cyclo-(Gly$^1$-Pro$^2$-Gly$^3$-D-Phe$^4$-Pro$^5$) in $Me_2SO$-$d_6$, concentration 17 mM (peaks marked "c" are from the minor [one-cis] conformer), and (B) cyclo-(Gly$^1$-Pro$^2\psi$[CH$_2$S]Gly$^3$-D-Phe$^4$-Pro$^5$) in $Me_2SO$-$d_6$, concentration 31 mM. (tr designates the all-trans conformer; cis the one-cis X-pro bond conformer; * designates an impurity.)

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**Table III.** $^{13}C$ NMR Chemical Shifts of Cyclic Peptide and Pseudopeptide[a]

| | Gly[1] | Pro[2] | | | | Gly[3] | D-Phe[4] | | Pro[5] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $C^\alpha$ | $C^\alpha$ | $C^\beta$ | $C^\gamma$ | $C^\delta$ | $C^\alpha$ | $C^\alpha$ | $C^\beta$ | $C^\alpha$ | $C^\beta$ | $C^\gamma$ | $C^\delta$ |
| peptide[b] | 41.56 | 61.44 | 28.57 | 24.57[c] | 47.26 | 43.32 | 53.40 | 37.62 | 58.41 | 25.38[c] | 24.66[c] | 45.92 |
| | Gly[1] | "Pro[2]" | | | | $\psi[CH_2S]Gly^3$- | D-Phe[4] | | Pro[5] | | | |
| | $C^\alpha$ | $C^\alpha$ | $C^\beta$ | $C^\gamma$ | $C^\delta$ | $CH_2$-S-$CH_2$ | $C^\alpha$ | $C^\beta$ | $C^\alpha$ | $C^\beta$ | $C^\gamma$ | $C^\delta$ |
| pseudopeptide[d] | 41.98 | 57.10 | 29.49 | 23.51[c] | 46.45[c] | 35.45[c] | 34.67[c] | 53.87 | 38.03 | 58.25 | 25.06[c] | 24.76[c] | 46.20[c] |

[a] Chemical shifts are given as parts per million (ppm) downfield from TMS; temperature 23 °C; solvent $CDCl_3$. [b] Concentration 21 mM. [c] Assignments not unambiguous; based on comparisons between the cyclic peptide and pseudopeptide and on observations in other model peptides. [d] Concentration 31 mM.

(see Experimental Section). It showed identical chromatographic behavior with a sample of the same cyclic structure prepared from the different (Pro$\psi$[$CH_2S$]Gly-D-Phe-Pro-Gly) linear precursor. Its molecular weight of 458 by mass spectrometry confirmed its monomeric nature (precise mass 458.199 ± 0.003, expected for $C_{23}H_{30}N_4O_4S$), as well as the absence of any oxidized ((*R*)- or (*S*)-sulfoxide) contaminants.

**Proton NMR Data for Cyclic Peptide and Pseudopeptide 1 in Chloroform and Dimethyl Sulfoxide.** Table II summarizes spectral parameters of the cyclic peptide and pseudopeptide in chloroform and in dimethyl sulfoxide. Spectra representative of those from which these data were obtained are shown in Figures 2 and 3. In chloroform, the two compounds show very similar spectral parameters: chemical shifts, coupling constants, and temperature dependences of NH resonances, for all analogous protons. The substitution of the amide linkage between $Pro^2$ and $Gly^3$ (see diagram of structures for the numbering scheme) by a $CH_2S$ leads to the disappearance of the $Gly^3$ NH and $H^\alpha$'s and to the appearance of new peaks for the $CH_2$ groups adjacent to the sulfur. Two-dimensional *J*-correlated (COSY) spectra of the dipeptide, Boc-Pro-Gly-OH, and the pseudodipeptide, Boc-Pro$\psi$[$CH_2S$]-Gly-OH, enabled us to assign a resonance centered at 2.4 ppm to the $CH_2$ preceding the sulfur and one at 3.2 ppm to the $CH_2$ that is essentially replacing the Gly-$CH_2$. These assignments are indicated for the cyclic pseudopeptide in Figure 2.

For purposes of conformational comparison (Table II and see below), note in particular that the proline $H^\alpha$ resonances are not only at nearly identical chemical shifts in the two compounds but also have the same form, indicating similar coupling interactions with the $H^\beta$'s. Also, the two NH's in common in the two molecules display small temperature coefficients, with the Gly NH having the smaller $\Delta\delta/\Delta T$ in both cases. In both the cyclic peptide and the cyclic pseudopeptide, the $H^\alpha$'s of the $Gly^1$ are strongly AB, having chemical shifts of 4.5 and 3.4 ppm in the peptide and 4.4 and 3.4 ppm in the pseudopeptide. Their $J_{N^\alpha}$'s are the same within experimental error in the two molecules.

By contrast, the spectra of these two molecules in $Me_2SO$ show several distinct differences (NH regions shown in Figure 3). First, it is apparent that there are two slowly interconverting conformers of the cyclic pseudopeptide **1** present from the appearance of two sets of resonances, while one predominant set of signals is seen in the spectrum of the peptide. (A small proportion (10%) of another conformer is seen in the cyclic peptide **2** spectrum.) In order to correlate the conformers of the pseudopeptide present in $Me_2SO$ with that observed in $CDCl_3$, a titration was carried out through differing proportions of the two solvents. Figure 4A illustrates the shifting of the pseudopeptide NH resonances as a function of solvent composition. No signals were observed in pure $CDCl_3$ for the conformer that eventually becomes the major one (ca. 65%) in $Me_2SO$ (filled symbols in the figure). A similar titration of the cyclic peptide revealed that the conformer that exists in $CDCl_3$ is the predominant conformer in $Me_2SO$ (Figure 4B). $^{13}C$ NMR results (presented below) allow the assignment of the predominant conformer of the pseudopeptide in $CDCl_3$ and of the peptide **2** in both solvents to an all-trans species. The major conformer of the pseudopeptide in $Me_2SO$ is inferred to be a one-cis X-Pro bond conformer, although the possibility of a species containing two cis X-Pro bonds cannot be ruled out. Hence, conformational comparisons of the minor species of the pseudopeptide in $Me_2SO$ with the major species of the peptide in $Me_2SO$ are appropriate. Comparing the temperature dependence of the corresponding NH resonances in $Me_2SO$ reveals that the Gly NH of the pseudopeptide shifts more than that of the peptide, although both have temperature coefficients smaller than those of the Phe NH's. The $^1H$ NMR spectral parameters for the cyclic peptide do not vary markedly between $CDCl_3$ and $Me_2SO$ solution (Table II). The complexity and overlaps in the spectrum of the pseudopeptide in $Me_2SO$ precluded analysis of most of the upfield signals even in two-dimensional NMR spectra.

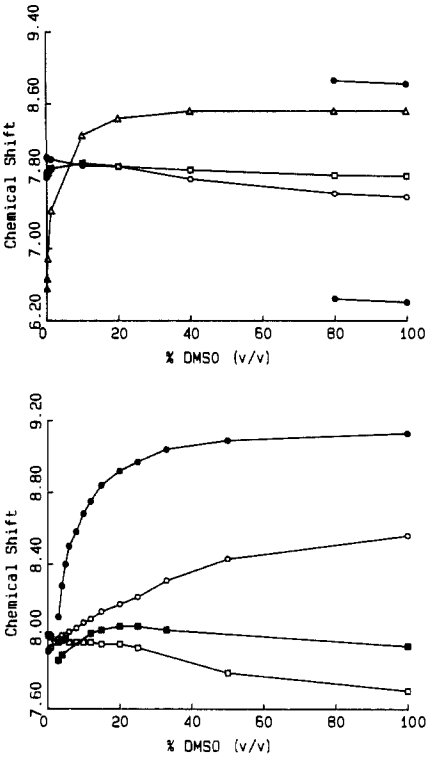


**Figure 4.** (A, top) $CDCl_3/Me_2SO$-$d_6$ titration of cyclo[$Gly^1$-$Pro^2$-$Gly^3$-D-$Phe^4$-$Pro^5$]; concentration 11 mM. Note that in this peptide, a one-cis X-Pro bond conformer does not appear until the solvent is 80% $Me_2SO$-$d_6$ and at 100% $Me_2SO$-$d_6$ constitutes only 10% of the total. (○) $Gly^1$NH, all-trans conformer; (□) $Phe^4$ NH, all-trans conformer; (Δ)$Gly^3$ NH, all-trans conformer; (●) small peaks due to apperance of one-cis conformer, residues not identified. (B bottom) similar titration of cyclo[$Gly^1$-$Pro^2\psi$[$CH_2S$]$Gly^3$-D-$Phe^4$-$Pro^5$], concentration 3 mM, showing the change in chemical shift of the NH's and the appearance of a one-cis X-Pro bond conformer as the sovent composition is changed. Note that this conformer appears at a solvent composition of 3% $Me_2SO$-$d_6$; at 100% $Me_2SO$-$d_6$, the cis conformer is the predominant one (65:35). (○) $Phe^4$ NH, all-trans conformer; (●) $Phe^4$ NH, one-cis conformer; (□) $Gly^1$ NH, all-trans conformer; (■) $Gly^1$ NH, one-cis conformer.

**$^{13}C$ NMR Spectra in Chloroform and Dimethyl Sulfoxide.** $^{13}C$ NMR data for the cyclic peptide and pseudopeptide in $CDCl_3$ are given in Table III, with Figure 5 illustrating the spectra from which these data were obtained. The assignments were made based on comparisons with analogous cyclic peptides (e.g., cyclo[Gly-Pro-Gly-D-Ala-Pro][24]) and by inspection of spectral differences between the dipeptide, Boc-Pro-Gly-OH, and the pseudodipeptide, Boc-Pro$\psi$[$CH_2S$]Gly-OH. In these latter two

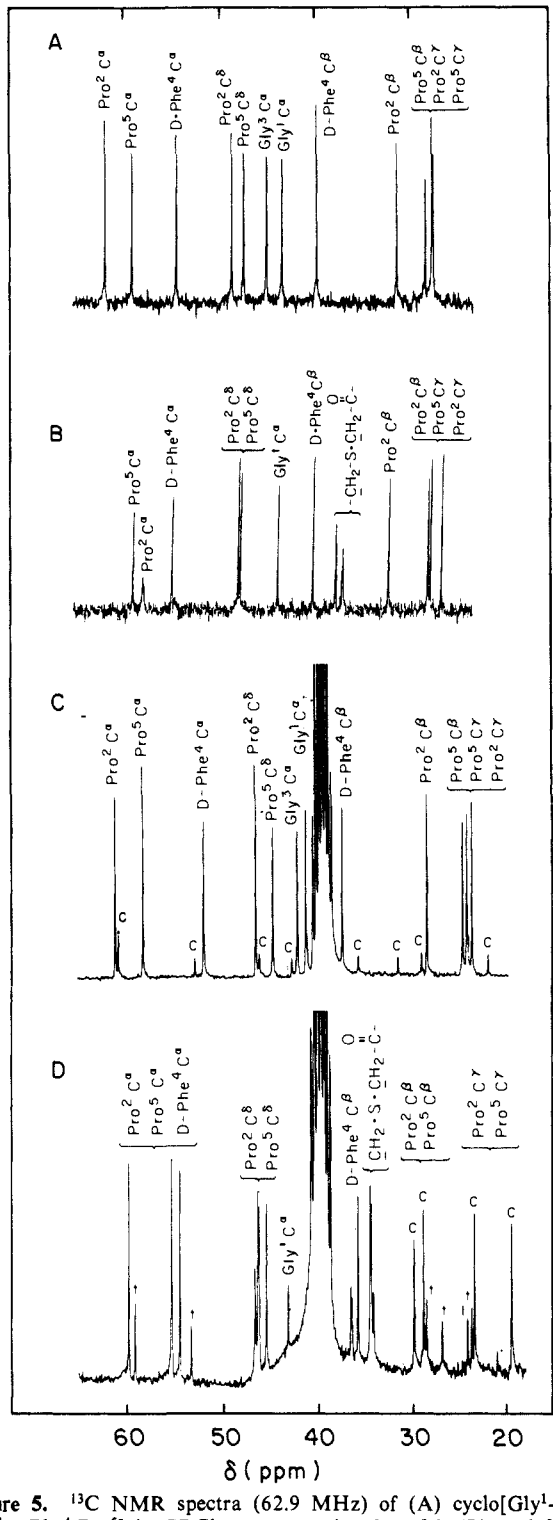


**Figure 5.** $^{13}C$ NMR spectra (62.9 MHz) of (A) cyclo[Gly$^1$-Pro$^2$-Gly$^3$-D-Phe$^4$-Pro$^5$] in $CDCl_3$, concentration 21 mM, (B) cyclo[Gly$^1$-Pro$^2\psi$[$CH_2$S]Gly$^3$-D-Phe$^4$-Pro$^5$] in $CDCl_3$, concentration 31 mM, (C) cyclo(Gly$^1$-Pro$^2$-Gly$^3$-D-Phe$^4$-Pro$^5$) in $Me_2SO$-$d_6$, concentration 21 mM, and (D) cyclo[Gly$^1$-Pro$^2\psi$[$CH_2$SyGly$^3$-D-Phe$^4$-Pro$^5$] in $Me_2SO$-$d_6$, concentration 23 mM. In (C), note the small (10%) amount of a one-cis X-Pro bond conformer (peaks denoted by "C"). In (D), t designates the all-trans conformer; C the one-cis X-pro bond conformer. (Large peaks at 40 ppm are due to $Me_2SO$.) Assignments are not unambiguous but are based on comparisons of spectra of the two cyclic and other model peptides.

compounds, it was observed that the presence of the amide replacement gave rise to a shifting of the Pro "$\alpha$" carbon signal upfield by ca. 4 ppm. The two carbons in the pseudopeptide that do not correspond directly to carbons in the peptide, i.e., that preceding the sulfur and that following the sulfur, appeared at ca. 35 ppm.

For purposes of conformational comparison of the two cyclic molecules, it is particularly noteworthy that the carbon signals from Pro$^5$ are nearly the same and are unusual for Pro residues. The upfield position of the Pro C$\beta$ in the two molecules is characteristic of a Pro in the $i + 1$ position of a $\gamma$-turn.[21] While the signals of the Pro$^2$ are somewhat shifted in the pseudopeptide due to the replacement of the amide bond by the thiomethylene moiety, there is still a close correspondence between its signals in the peptide and the pseudopeptide. Both Pro residues in the two molecules in $CDCl_3$ display resonance positions typical of trans X-Pro bonds.

The $^{13}C$ NMR spectrum of the pseudopeptide in $Me_2SO$, shown in Figure 5D, reveals (as did the $^1H$ spectrum) the presence of two competing conformers in similar proportions. In contrast, the $^{13}C$ NMR parameters of the cyclic peptide **2** in $Me_2SO$ indicate clearly a predominant all-trans species, with retention of the $\gamma$-turn (Figure 5C). A small proportion of a one-cis species can be observed, especially in the Pro C$\beta$ and C$\gamma$ region.

**Conformational Interpretation.** Both $^1H$ and $^{13}C$ NMR spectral data for the cyclic pentapeptide in $CDCl_3$ are consistent with a preferred conformation containing all-trans peptide bonds and stabilized by a $\beta$-turn involving Gly$^1$-Pro$^2$-Gly$^3$-D-Phe$^4$ with a hydrogen bond from the D-Phe NH to the Gly$^1$ C=O, and by a $\gamma$-turn involving D-Phe$^4$-Pro$^5$-Gly$^1$ with a hydrogen bond from the Gly$^1$ NH to the D-Phe C=O. The low-temperature coefficients of the resonances of the two NH's involved in intramolecular hydrogen bonding, the fit of the $J_{N^\alpha}$ values to those expected for this $\beta,\gamma$-turn conformation, and the diagnostic shifts of the prolines, especially Pro$^5$, are all supportive of this interpretation. Furthermore, the observed spectral parameters are extremely close to those previously reported for the cyclic pentapeptide, cyclo(Gly-Pro-Gly-D-Ala-Pro), that differs from the one reported here only in the presence of a D-Ala instead of a D-Phe.[24]

In $Me_2SO$, the cyclic pentapeptide maintains the same preferred conformation. The low-temperature coefficients of the NH resonance of Gly$^1$ and D-Phe relative to that of Gly$^3$, the proline $^{13}C$ NMR shifts, and the similar $J_{N^\alpha}$s values to those in $CDCl_3$ all indicate little conformational change between the two solvents. Also, a titration through varying proportions of $CDCl_3$ and $Me_2SO$ led to spectral changes expected for a retention of conformation but a change in solvation. The resonance position of the exposed NH (that of Gly$^3$) was dramatically shifted to low field by the increasing amounts of $Me_2SO$, while the shifts of the intramolecularly hydrogen-bonded NH's (Gly$^1$ and D-Phe) were only slightly changed (Figure 4A). No major changes in the positions of the upfield $^1H$ resonances were noted. The observation of gradual chemical shift changes, rather than the appearance of new signals, confirmed that the peptide did not undergo a peptide bond isomerization during $CDCl_3$ and $Me_2SO$ solvent interchanges.

The pseudopeptide displays spectral parameters that are remarkably similar to those of the peptide in $CDCl_3$ solution. The comparison of both $^1H$ and $^{13}C$ NMR data leads to the conclusion that the pseudopeptide also adopts a preferred all-trans conformation in $CDCl_3$, with both a $\beta$- and $\gamma$-turn. Hence, the two remaining NH's in this molecule are both involved in intramolecular hydrogen bonds. But unlike the peptide, the pseudopeptide clearly undergoes a conformational change, with a peptide bond isomerization upon addition of $Me_2SO$ (Figure 4B). The major form of this molecule in $Me_2SO$ appears to have one cis X-Pro bond. By comparison with other cyclic pentapeptides studied previously[21] and through inspection of molecular models, it is inferred that the Gly$^1$-Pro$^2$ bond undergoes isomerization. This model is also consistent with the greater solvent accessibility of the D-Phe NH than that of the Gly$^1$ NH (Figure 4B), since the reorientation of the Gly$^1$ C=O upon isomerization necessarily breaks its hydrogen bond with the D-Phe NH. $\Delta\delta/\Delta T$ data for the major (cis) form of the pseudopeptide in $Me_2SO$ indicate partial solvation of both NH's, with the Gly$^1$ NH less exposed. The smaller $J_{NH-H}$ for the D-Phe in the cis form is also consistent with its pointing out toward the solvent. The minor species present in solution of the pseudopeptide in $Me_2SO$ is all-trans, which is

instead the preferred form in $CDCl_3$. Although a thorough analysis was not carried out of the $^1H$ NMR spectrum of the pseudopeptide in $Me_2SO$ because of the severely overlapped spectrum (even in two-dimensional experiments), the temperature dependence data argue that at least the $\gamma$-turn involving the $Gly^1$ NH is retained in the minor form in $Me_2SO$. The D-Phe NH of the pseudopeptide, by contrast, appears from its midrange $\Delta\delta/\Delta T$ and moderate shift upon addition of $Me_2SO$ to be accessible to intermolecular interactions (not strongly intramolecularly hydrogen-bonded).

These results can be of utility in the design of peptide analogues containing amide bond surrogates such as the $CH_2$–S linkage. Here, the fact that the peptide and its pseudopeptide analogue both adopt the same conformation, including two intramolecularly hydrogen-bonded reverse turns, in $CDCl_3$, a solvent characterized by weak interactions toward peptides indicates that the driving forces within the molecules toward this $\beta,\gamma$-conformation and the steric restrictions on the backbone are retained upon substitution of the $CH_2$–S for the $Pro^2$-$Gly^3$ peptide bond. Note that this is the one peptide bond not involved in any intramolecular hydrogen bonding. In terms of analogue design, the present result points to like loci for amide bond replacements in biologically active peptides.

The different responses of these two molecules to solvation by $Me_2SO$ suggest that (1) intermolecular effects, such as solvation, will lead to perturbations resulting from the amide bond replacement, since the substituted groups are fully exposed, and (2) the region of the pseudopeptide near the $CH_2$–S bond may be conformationally more mobile than the comparable region of the peptide.

It is also of interest to relate these results to cases in which amide bonds that are believed to participate directly in hydrogen bond formation are replaced by a $\psi[CH_2S]$ linkage. The increased flexibility that accompanies the replacement of an amide linkage by a methylenethio group is somewhat counter to the growing trend toward conformational constraints, especially in linear peptides. Yet the residual biological activity observed for an LH–RH analogue containing a $\psi[CH_2S]$ amide replacement at the 6–7 position (a $\beta$-turn has been postulated at residues 5–8) suggests that only a modest contribution can be attributed to intramolecular 4 → 1 hydrogen bonding in linear peptides.

Even more significant is the effect of a $\psi[CH_2S]$ replacement in a biologically active cyclic peptide analogue. We have recently prepared a cyclo[Pro$\psi$[$CH_2$S]Phe-D-Trp-Lys-Thr-Phe] somatostatin analogue[20] based on the all-amide Merck minisomatostatin parent.[31] The activity of the latter exceeds that of the somatostatin tetradecapeptide analogue. In the case of the pseudopeptide derivative, the $\psi[CH_2S]$ replacement is at a site that would prevent formation of at least one of the two postulated intramolecular hydrogen bonds in the Merck compound. While the $\psi[CH_2S]$ analogue did possess somewhat diminished potency, the relatively small magnitude of the drop (25% of the activity of somatostatin and 6% of the hexapeptide parent in terms of in vivo GH release) argues against an absolute requirement for hydrogen bonding as an important feature for receptor interaction and confirms the utility of backbone modifications as structure-compatible choices in analogue design.[20] Further extrapolations are undoubtedly premature in the absence of additional, detailed physical data such as those obtained in the present study.

## Experimental Section

L-Prolinol and Merrifield resin were obtained from Sigma Chemical Co. Boc-D-Phe, Boc-Pro, and Boc-Gly were products of Vega Biochemicals. Di-*tert*-butyl dicarbonate, diphenylphosphoryl azide, (dimethylamino)pyridine, and the deuterated solvents were purchased from Aldrich Chemical Co. 1-Hydroxybenzotriazole was prepared according to the procedure of König and Geiger.[32] TLC was performed on silica gel (Merck 254 plates) in the following systems (v/v): (A) chloroform/methanol/acetic acid, 85:10:5; (B) ethanol/water, 7:3, (C) *n*-butanol/acetic acid/water, 4:1:1, (D) *n*-butanol/acetic acid/water, 4:1:5 (upper phase). HPLC was performed on a Du Pont 850 liquid chromatograph system. A Vega Model 50 was used for solid-phase synthesis. Optical rotations were determined on a Perkin-Elmer Model 241 at the sodium D line. Proton and carbon-13 NMR spectra were recorded on a Bruker WH-250 Fourier Transform NMR spectrometer.

**Preparation of $N^{\alpha}$-Boc-L-2-Pyrrolidinemethanol (Boc-Prolinol).** Di-*tert*-butyldicarbonate (9.82 g, 45 mmol) was added to a solution of L-2-pyrollidinemethanol (prolinol) (5.06 g, 50 mmol) in ethyl acetate (35 mL) at room temperature. After 2 h of mixing, the reaction mixture was transferred to a 250-mL separatory funnel with the aid of ethyl acetate (15 mL). The organic layer was washed in succession with 1 N HCl (2 × 20 mL) and saturated NaCl (2 × 30 mL) and dried over anhydrous $Na_2SO_4$. Evaporation of solvent under vacuum, followed by further drying of the residual clear oil under high vacuum yield Boc-prolinol (8.59 g, 95%) as white granular crystals: mp 57–58 °C (sharp); $R_f$ (A) 0.68.

**Preparation of $N^{\alpha}$-Boc-L-2-(Pyrrolidinemethyl)toluene Sulfonate (Boc-Pro-OTs).** To a solution of Boc-prolinol (4.02 g, 20 mmol) in DMF (20 mL) maintained at 0 °C, *p*-toluenesulfonyl chloride (4.77 g, 25 mmol) and DMAP (0.25 g, 2 mmol) was added. The reaction mixture was stirred at 0 °C for 0.5 h and then at room temperature for 6 h. TLC analysis of the reaction mixture indicated that the reaction was complete in 4 h. The reaction mixture was then poured into a separatory funnel containing saturated NaCl (50 mL) and ethyl acetate (30 mL) and fractionated. The aqueous layer was further extracted with ethyl acetate (2 × 30 mL). The pooled organic extracts were then washed with 1 N HCl (2 × 30 mL), saturated NaCl (2 × 30 mL), and 10% $K_2CO_3$ (2 × 30 mL) and dried over anhydrous $Na_2SO_4$. Evaporation of solvent in vacuo yielded Boc-Pro-OTs as an oil (5.89 g, 83%): $R_f$ (A) 0.78.

**Preparation of Boc-Pro$\psi$[$CH_2$S]Gly-OH.** To an aqueous solution of potassium hydroxide (1.684 g in 2 mL of water), mercaptoacetic acid (0.7 mL, 14 mmol) was added under an atmosphere of nitrogen. This was followed by the addition of Boc-Pro-OTs (5.86 g, 16.5 mmol) in DMF (10 mL), and the reaction flask was sealed with a rubber septum. The reaction mixture was stirred for 30 h, and as the reaction progressed, a white precipitate formed. The reaction mixture was diluted with 10% aqueous $K_2CO_3$ (50 mL) and partitioned with ethyl acetate (2 × 30 mL). The aqueous layer was brought to pH 3 by addition of 2 N HCl and extracted with ethyl acetate (2 × 30 mL). The combined organic extracts were dried over anhydrous $Na_2SO_4$ and evaporated under reduced pressure to an oil. Addition of ether (2 mL) followed by *n*-hexane (200 mL) and standing overnight at 4 °C yielded Boc-Pro$\psi$[$CH_2$S]Gly-OH (1.470 g, 38%) which was identical in all respects including TLC with an authentic sample: $R_f$ (A) 0.55.[14]

**Boc-Pro$\psi$[$CH_2$S]Gly-OCH$_2$-$C_6H_4$-Polymer.** Cesium carbonate (0.33 g, 1 mmol) in water (1 mL) was introduced into a methanolic solution of Boc-Pro$\psi$[$CH_2$S]Gly-OH (0.54 g, 1.95 mmol) and gently mixed for 15 min. Azeotropic distillation with a mixture of benzene and methanol (1:1 v/v) yielded the cesium salt in an anhydrous form. This was stirred with a suspension of Merrifield polymer (3.0 g, 1.00 mequiv of Cl/g) in DMF (25 mL) at 50 °C for 24 h. Boc-Pro$\psi$[$CH_2$S]Gly-OCH$_2$-$C_6H_4$-polymer thus obtained was treated in the same manner as described by Gisin. Incorporation level based on weight increase was assessed to be approximately 0.26 mmol/g of resin.

**Solid-Phase Synthesis of D-Phe-Pro-Gly-Pro$\psi$[$CH_2$S]Gly-OCH$_2$-$C_6H_4$-Polymer.** The synthesis was initiated with 3.0 g of Boc-Pro$\psi$-[$CH_2$S]Gly-OCH$_2$-$C_6H_4$-polymer, and the title pentapeptide was assembled by sequential addition of Boc-Gly, Boc-Pro, and Boc-D-Phe following the cycle of operations illustrated in Table I. The completion of coupling in each case was monitored by the ninhydrin test,[33] and the synthesis proceeded uneventfully to yield the Boc-protected pseudopeptide. After removal of the Boc group via steps 1–5, the resin was washed with $CH_2Cl_2$ (200 mL), EtOH (200 mL), and $CH_2Cl_2$ (200 mL) and dried in vacuum (3.15 g, total peptide content was 0.78 mmol as determined by both weight gain and quantitative amino acid analysis of a resin sample).

**Removal of Peptide from Resin: AcOH·H-D-Phe-Pro-Gly-Pro$\psi$-[$CH_2$S]Gly-OH.** Anhydrous hydrogen fluoride (20 mL, distilled over $CoF_3$) was added to a suspension of D-Phe-Pro-Gly-Pro$\psi$[$CH_2$S]Gly-OCH$_2$-$C_6H_4$-polymer (2.0 g) in anisole (2 mL) and ethyl methyl sulfide (2 mL) at 0 °C. After 30 min, the solvents were removed under vacuum and the resin was extracted with ether (2 × 30 mL), and the ether washes were discarded. The residual solid was then extracted with 30% aqueous acetic acid (3 × 30 mL) and filtered. Subsequent lyophilization of the filtrate and gel permeation chromatography (Sephadex G-15)

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yielded the linear pentapeptide as the acetate salt (214 mg, 80% of theory). The compound moved as a single component on TLC: $R_f$ (B) 0.52; $R_f$ (C) 0.36.

**Cyclo[D-Phe-Pro-Gly-Proψ[$CH_2$S]Gly].** To a solution of AcOH·D-Phe-Pro-Gly-Proψ[$CH_2$S]Gly (175 mg, 0.33 mmol) in dry degassed DMF (50 mL) maintained at -30 °C, DPPA (0.40 mmol), HOBt (62 mg, 0.4 mmol), DMAP (49 mg, 0.4 mmol), and triethylamine (0.8 mmol) were added. The reaction mixture was then placed in a freezer at -15 °C for 18 h. TLC analysis showed complete reaction. Water (6 mL) and Dowex MR-3 mixed-bed resin (6 mL) were introduced into the reaction mixture and stirred for 6 h. The resin was separated by filtration, and the solvents were completely removed in vacuum. Repeated lyophilization from 30% aqueous acetic acid solution yielded chromatographically pure cyclic pentapeptide (127 mg, 85% of theory): $R_f$ (C) 0.64; mp 110-112 °C; $[\alpha]^{25}_D$ -103.2° (*c* 1.1, 50% HOAc).

Analytical HPLC on a C-18 reversed-phase column at 50 °C (Spherisorb, 4.6 mm i.d. × 250 mm) indicated the compound to be at least 99% pure, $t_R$ = 10.5 min, $k'$ = 8.39. The mobile phase consisted of a linear gradient of $CH_3CN$ (20%-30% over 20 min) against 0.25 M triethylammonium phosphate buffer (pH 2.5). Mass spectral analysis indicated the precise mass to be 458.199 ± 0.003 (Calcd for $C_{23}H_{30}N_4O_4S$: 458.19876). Anal. Calcd for $C_{23}H_{30}N_4O_4S \cdot 1.5H_2O$: C, 56.88; H, 6.85; N, 11.33; S, 6.60. Found: C, 56.65; H, 6.14; N, 10.65; S, 6.96. Amino acid analysis: Phe, 1.00; Pro, 1.07; Gly, 0.99; Proψ[$CH_2$S]Gly, could not be detected due to its very weak color response with ninhydrin.

**Registry No.** 1, 99781-72-3; 2, 86044-88-4; Boc-Pro-OTs, 86661-32-7; Boc-Proψ[$CH_2$S]Gly-OH, 77489-32-8; Boc-Proψ[$CH_2$S]Gly-OH·Cs, 99726-40-6; Boc-Gly, 4530-20-5; Boc-Pro, 15761-39-4; Boc-D-Phe, 18942-49-9; H-D-Phe-Pro-Gly-Proψ[$CH_2$S]Gly-OH, 99781-71-2; L-prolinol, 23356-96-9; di-*tert*-butyl dicarbonate, 24424-99-5; Boc-prolinol, 69610-40-8; *p*-toluenesulfonyl chloride, 98-59-9; mercaptoacetic acid, 68-11-1.

# *Communications to the Editor*

## Generation of a *cis*-[$Mo^VO(OH)$] Center: $^1H$- and $^{17}O$-Superhyperfine Parameters Relevant to Molybdoenzymes

Frank Farchione,[1a] Graeme R. Hanson,[1a,d] Clyde G. Rodrigues,[1a] Trevor D. Bailey,[1a] Raman N. Bagchi,[1b] Alan M. Bond,[1b] John R. Pilbrow,[1c] and Anthony G. Wedd*[1a]

*Department of Chemistry, La Trobe University Bundoora, Victoria 3083, Australia*

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Analysis of molybdenum EXAFS data[2,3] indicates the presence of [$Mo^{VI}O_2(SR)_n$] (*n* = 2, 3) units at the oxidized molybdenum sites of chicken liver sulfite oxidase (SO), *Chlorella* nitrate reductase (NR), and the desulfo form of chicken liver xanthine dehydrogenase. [$Mo^{VI}OS(SR)_2$] units are detected[2,4] in active xanthine dehydrogenase and bovine milk xanthine oxidase (XO). A single oxo group only is observed in the respective reduced $Mo^{IV}$ forms and protonation of the other oxo or sulfido group to form an OH or SH ligand is suggested.[2,3] Indeed, the presence of $^1H$-superhyperfine coupling[5] in the $Mo^V$ ESR signals of SO (low pH form),[6,7] of E. coli and spinach NR (low pH forms),[8,9] and of XO (desulfo and active (rapid type 1) forms)[5,10] has been interpreted in the same way.[11] Further information is available from the extensive and inventive work of Bray:[12-15] substitution of $^{16}OH_2$ by $^{17}OH_2$ in solutions of oxidized SO and XO followed by interaction with reducing substrates permits observation of $^{17}O$-superhyperfine coupling in the various ESR-active forms of the enzymes. Ligand oxo, OH, $OH_2$, and OR (where R is derived from substrate) are possible sources of the coupling.

*cis*-[$Mo^{VI}O_2L$] ($LH_2$ = ($HSCH_2CH_2N(Me)CH_2-)_2$) (I) is unusual for such compounds in that it exhibits[16] a one-electron redox process ($E°$ = -1.3 V vs. SCE) which is chemically reversible on the timescale of cyclic voltammetry. Electrolysis[17] of I in THF containing 0.1 M $^1H_2O$ at -42 °C in the cavity of an X-band ESR spectrometer leads to a resonance typical of $Mo^V$, except that each feature is present as a doublet (Figure 1a). In the presence of 0.1 M $^2H_2O$, the structure collapses (Figure 1b), demonstrating the presence of superhyperfine coupling to a single hydrogen atom. Comparison of the derived parameters (*g*, 1.961; *a*(Mo), 39.3 × $10^{-4}$ $cm^{-1}$) with those[18,19] of *cis*-[MoOClL] (1.966; 37.8) and *cis*-[MoOClL'$_2$] (L'H = 8-mercaptoquinoline) (1.968; 37.6) is consistent with the expected spectrum of *cis*-[MoO(OH)L] (II). The $^1H$-superhyperfine coupling constant of 13.6 × $10^{-4}$ $cm^{-1}$ is listed with those for the "strongly coupled" protons of the

(1) (a) Department of Chemistry, La Trobe University, Bundoora, Victoria 3083, Australia. (b) Division of Chemical and Physical Sciences, Deakin University, Waurn Ponds, Victoria 3217, Australia. (c) Department of Physics, Monash University, Clayton, Victoria 3168, Australia. (d) Present address: Centre for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, MA 02115.

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(11) Note that the $^1H$ coupling under discussion is due to the so-called "strongly coupled" protons which exhibit $a$ = (9-15) × $10^{-4}$ $cm^{-1}$ and are exchangeable with solvent $H_2O$.[5] In active XO, the source of this proton is the C-8 proton of xanthine.[16] Also observed[5] in XO are "weakly coupled" protons with $a$ = (0.5-3.0) × $10^{-4}$ $cm^{-1}$ which are also exchangeable and, in certain "inhibited" signals, protons with coupling constants of intermediate magnitude and that are not exchangeable.

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