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Compatibility of the Thioamide Functional Group with β-Sheet Secondary Structure: Incorporation of a Thioamide Linkage into a β-Hairpin Peptide

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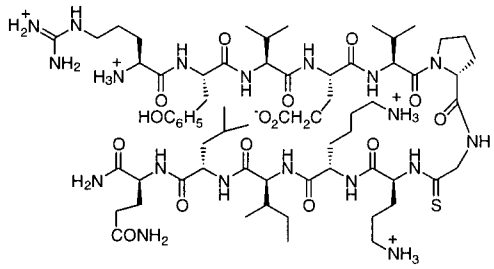
ABSTRACT



We report the incorporation of a thioamide linkage between the *i* + 2 and *i* + 3 residues of the type II′ β-turn of a peptide known to fold into a β-hairpin conformation. Two-dimensional NMR spectroscopy and circular dichroism spectroscopy indicate that the thioxo peptide adopts a hairpin conformation similar to that of the oxo peptide and that the hairpin conformation persists at elevated temperatures. The results show that a thioamide linkage is compatible with β-sheet secondary structure.

In efforts to understand and control peptide and protein conformations and interactions, "unnatural" amino acids (amino acids other than the 20 standard coded amino acids) are useful tools. Unnatural amino acids with restricted conformational space can introduce novel folds or help lock peptides into desired conformations.[1] Unnatural amino acids can also confer resistance to enzymatic degradation.[2] The modification of amino acids by thioxylation, in which the carbonyl oxygen is replaced by a sulfur atom, has received attention recently for several reasons. Thioxylated analogues of biologically active peptides, in which a thioamide linkage replaces a backbone amide linkage, have shown increased enzymatic stability[3] and increased potency and selectivity.[4] The conformational restrictions introduced by the thioamide, together with its ability to confer increased enzymatic stability, should make thioxylated amino acids useful in drug design. The thioamide introduces special spectroscopic properties into the peptide, making it a potential probe of local conformation.[5]

The thioamide is a nearly isosteric replacement for the amide.[6] Although the larger size of the sulfur atom and the longer carbon−sulfur bond length restrict the allowed ϕ and ψ angles of the amino acids flanking the thioamide group,

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conformations compatible with the three major types of regular secondary structure (α-helix, β-sheet, and β-turn) are accessible to thioxylated amino acids.[7] Nevertheless, thioamide linkages have not been incorporated into peptides that adopt defined, regular secondary structure; experimental studies of thioxo peptides have been limited to very short, flexible peptides. An understanding of the thioamide's influence on the surrounding secondary structure is necessary for its use as a tool in protein design.

We sought to incorporate a thioamide linkage into a β-hairpin, which comprises two antiparallel strands joined by a β-turn and is the minimal model of an antiparallel β-sheet. Several short peptides have been reported that display partial β-hairpin formation in aqueous solution.[8] We chose the β-hairpin designed by Stanger and Gellman[9] because it adopts a highly populated hairpin conformation at room temperature in aqueous solution and comprises α-amino acids in both the strand and turn regions of the hairpin.[10]

We chose to insert the thioamide linkage between the Gly-7 and Orn-8 residues (the $i + 2$ and $i + 3$ residues of the turn) of the hairpin peptide. Calculated ϕ,ψ maps for thioxo peptides indicate that a thioxylated amino acid can adopt a conformation compatible with the $i + 2$ position of a type II′ β-turn and that an *N*-thioacyl amino acid can adopt the extended conformation required for an antiparallel β-sheet.[7] In this position, the sulfur atom points toward the exterior of the hairpin, while the thioamide NH participates in the hydrogen bonding network of the hairpin. Thus, the larger size of the sulfur atom should not interfere with interstrand interactions in the hairpin. The thioamide NH is a stronger hydrogen bond donor than the amide NH,[11] and this interstrand hydrogen bond may provide additional stability to the hairpin conformation. In the oxo peptide, the turn sequence was found to be essential for inducing the hairpin conformation, so this location provides a test of the thioamide's ability to conform to this secondary structural element.

The thioxo peptide H_2N-Arg-Tyr-Val-Glu-Val-dPro-Gly-ψ[CS-NH]Orn-Lys-Ile-Leu-Gln-NH_2 (**1**) was synthesized using standard solid-phase synthesis methodology with Fmoc/*tert*-butyl protection on the Rink amide resin. The thioamide linkage was introduced using Fmoc-Gly-thioxo-6-nitrobenzotriazolide for thioacylation,[12] and the resin-bound thioxo peptide was elongated by solid-phase peptide synthesis. The thioxo peptide was cleaved from the resin with TFA:H_2O:triisopropylsilane (95:2.5:2.5) and purified by RP-HPLC. The purified peptide was characterized by mass spectrometry (electrospray ms calcd 1430, found 1430 $(M + H)^+$, 715 $(M + 2H)^{2+}$) and NMR spectroscopy.

Figure 1 summarizes ROESY data obtained for **1** in aqueous solution. The long-range NH−NH NOE between

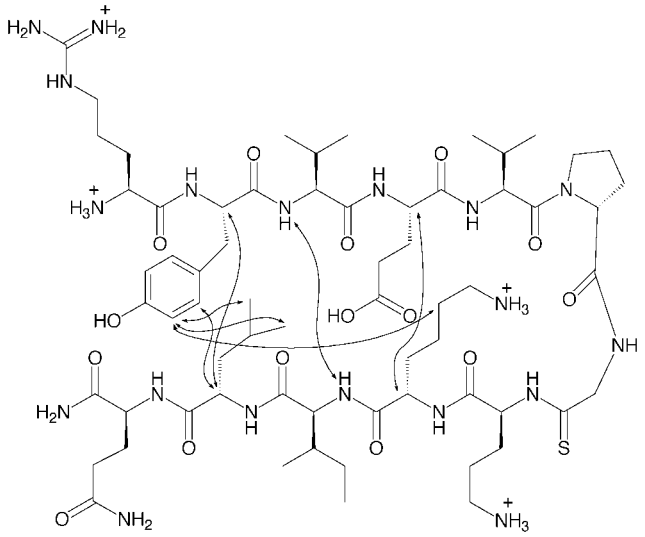

Figure 1. Long-range NOEs observed in ROESY spectra of 3.7 mM **1** in 100 mM aqueous sodium deuterioacetate buffer, pH 3.9 (uncorrected), 25 °C. All NOEs were observed in both D_2O and 9:1 H_2O:D_2O except the NH−NH NOE between Val-3 and Leu-10 (observed only in 9:1 H_2O:D_2O) and the H_α−H_α NOE between Glu-4 and Lys-9 (observed only in D_2O). One additional NOE was observed between a C-terminal amide NH and the Val-3 H_α (see text). Resonance assignments were obtained from COSY and TOCSY spectra and confirmed by sequential H_α−NH NOEs in the ROESY spectrum.

Val-3 and Ile-10 and the C_αH−C_αH NOEs between Tyr-2 and Leu-11, and between Glu-4 and Lys-9, indicate that the expected β-hairpin conformation is highly populated. Side chain−side chain NOEs were also observed between Tyr-2 and Leu-11 and between Tyr-2 and Lys-9. Similar NOEs were reported by Stanger and Gellman for the corresponding oxo peptide.[9] The hairpin conformation of thioxo peptide **1** appears to be quite stable, as all cross-strand NOEs were observed at 50 °C as well as at 25 °C. An NOE between a C-terminal amide NH and the Val-3 C_αH (not shown) indicates some disorder at the C-terminus of the peptide.

The chemical shifts of the C_α protons provide another means for the qualitative determination of peptide secondary structure.[13] A comparison of the C_αH chemical shifts of **1** with the expected chemical shifts for a peptide in a random coil conformation provides further evidence that **1** adopts a β-hairpin conformation.[14] Figure 2 summarizes these data and compares them to reported C_αH chemical shifts for the oxo peptide.[9] Downfield shifts of the C_αH resonances ($\Delta\delta_{\alpha H} \geq 0.1$) indicate β-strand structure in the segments Tyr-2 to Val-5 and at Ile-10. The magnitudes of the shift offsets match

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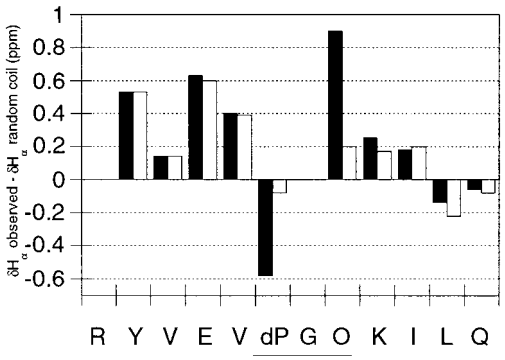

Figure 2. $C_\alpha H$ chemical shift deviations from random coil chemical shift: ■ = thioxo peptide **1**, □ = oxo peptide (data from reference 9). Underlined residues (dP−O) are shifted due to proximity to the thioamide. Data for Arg are omitted because its amino group is not acylated, and data for Gly are omitted because there are two $C_\alpha H$ resonances ($\Delta\delta H_\alpha$ = 0.21, 0.47). ^{1}H NMR spectrum of 3.5 mM **1** in 100 mM aqueous sodium deuterioacetate buffer, pH 3.9 (uncorrected), 25 °C was externally referenced to sodium [3-trimethylsilyl-2,2,3,3-^{2}H] propionate (TSP).

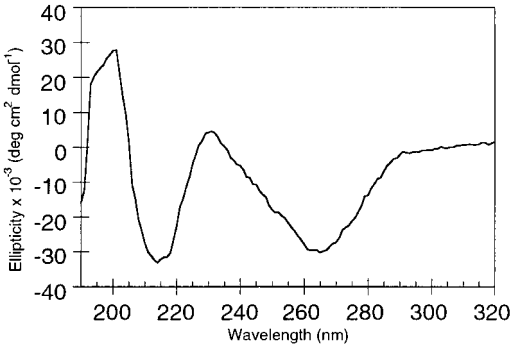

Figure 3. Circular dichroism data for 0.2 mM **1** in 100 mM aqueous sodium acetate buffer, pH 3.9, 25 °C. Data obtained on an Aviv 62DS spectrometer.

those for the oxo peptide and indicate that the two peptides have similar conformations. Residues near the thioamide group exhibit large changes in $\delta_{\alpha H}$ and cannot be used for analysis. Again, the results suggest some disorder at the C-terminus of the peptide, as the $\delta_{\alpha H}$ values for Leu-11 and Gln-12 are shifted upfield relative to random coil peptides.

Circular dichroism spectroscopy can be used to analyze the conformation of the amino acids immediately surrounding the thioamide linkage as well as the conformation of the thioxo peptide as a whole. The $\pi\pi^*$ band of the thioamide appears in the range 280−250 nm, well separated from the $\pi\pi^*$ band of the amides, while the nπ^* band of the thioamide appears above 300 nm.[15] The CD spectrum of **1** (Figure 3) shows the characteristics of a β-sheet in the amide regions of the spectrum: a minimum at 216 nm and a maximum at 200 nm. The thioamide has a strong negative $\pi\pi^*$ band at 268 nm, while the weak positive signal above 310 nm indicates a positive nπ^* band.

Hollósi et al. have analyzed the CD spectra of a series of thioxylated *N*-acyl amino acid and *N*-acyl dipeptide *N*′-methylamide models.[15] The dominant influences on the signs of the nπ^* and $\pi\pi^*$ absorbances were the orientations of the substituted α-carbons on either side of the thioamide. Therefore, one should be able to correlate the thioamide CD signal with the ψ angle of the thioxylated residue and the ϕ angle of the residue following it in the sequence. In our case, the thioxylated residue, glycine, has no C_α substituent, so the ϕ angle of the ornithine residue should be the major determinant of the thioamide CD signals. The strong negative thioamide $\pi\pi^*$ band of **1** is similar to that reported for Z-Gly[ΨCSNH]Ala-OEt in polar solvents. Hollósi et al. attribute this negative $\pi\pi^*$ band to a ϕ angle of approximately −70° in Z-Gly[ΨCSNH]Ala-OEt.[15] The expected ϕ value for Orn in the hairpin conformation is approximately −140°, suggesting that there is a large range of ϕ angles that give rise to a negative $\pi\pi^*$ band for the thioamide. The CD spectrum of **1** is unchanged at temperatures up to 70 °C, indicating that the hairpin conformation persists even at high temperatures.

The NMR and CD data demonstrate that thioxo peptide **1** adopts a hairpin conformation quite similar to that of the corresponding oxo peptide and that the conformation is stable at high temperatures. The results provide the first experimental demonstration of the ability of a thioxo peptide to adopt β-sheet secondary structure.

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