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β-Sheet Hydrogen Bonding Patterns in Cystine Peptides

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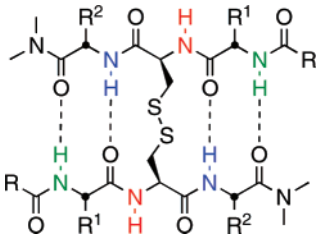
ABSTRACT



Cystine peptides have been shown to adopt conformations in organic solvents that mimic small β-sheets. Relative hydrogen bond strengths, β-strand aggregation, and the identity of individual hydrogen bond donors and acceptors have been identified through hydrogen/deuterium exchange.

β-Sheets are one of the fundamental secondary structures found in proteins. Much has been deciphered about the protein sequences that lead to β-sheets and the propensities for various amino acids to promote their formation.[1] Despite these advances, the ability to produce small-molecule mimics of β-sheet structure is hindered by their inherent thermodynamic instability.[2] Successful approaches have relied on either creating peptides that are large enough to maintain a stable fold or by incorporating non-natural elements that lend structural stability.[3] These small β-sheet structures provide insight into the fundamental forces that control protein folding as well as creating scaffolds capable of replicating β-sheet interactions with other substrates.

Our efforts to create minimal β-sheet mimics began with the principle of attaching two peptides at their α-carbons with an appropriate tether that would promote the hydrogen bonding patterns that replicate a β-sheet (Figure 1). This approach would permit propagation of the β-sheet in both directions from the central tether and could be tailored to closely resemble a natural β-sheet.

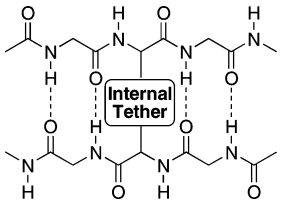


Figure 1. A strategy for β-sheet peptidomimetics using an internal tether between two peptide α-carbons.

The most synthetically accessible derivative that could serve as an internal tether is a disulfide-linked cystine that has been modified to contain additional hydrogen bonding groups, as in **1** and **2**.[4] These structures position two amino acid backbones separated by a four-atom tether, such that

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(2) Smith, C. K.; Regan, L. *Acc. Chem. Res.* **1997**, *30*, 153.

(3) (a) Blanco, F.; Rivas, G.; Serrano, L. *Nat. Struct. Biol.* **1994**, *1*, 584. (b) Simpson, E. R.; Meldrum, J. K.; Bofill, R.; Crespo, M. D.; Holmes, E.; Searle, M. S. *Angew. Chem., Int. Ed.* **2005**, *44*, 4939. (c) Nowick, J. S. *Org. Biomol. Chem.* **2006**, *4*, 3869. (d) Hughes, R. M.; Waters, M. L. *Curr. Opin. Struct. Biol.* **2006**, *16*, 514. (e) Tsang, K. Y.; Diaz, H.; Graciani, N.; Kelly, J. W. *J. Am. Chem. Soc.* **1994**, *116*, 3988. (f) Smith, A. B., III; Guzman, M. C.; Sprengeler, P. A.; Keenan, T. P.; Holcomb, R. C.; Wood, J. L.; Carroll, P. J.; Hirshman, R. *J. Am. Chem. Soc.* **1994**, *116*, 9947. (g) Kemp, D. S.; Bowen, B. R.; Muendel, C. C. *J. Org. Chem.* **1990**, *55*, 4650.

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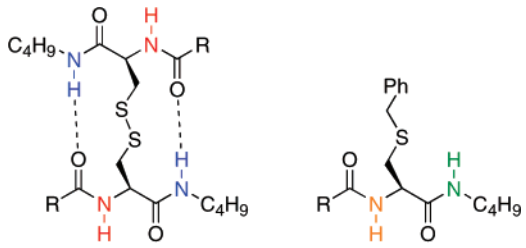
the C-terminal amide can serve as a hydrogen bond donor, and the N-terminal carbonyl can serve as a hydrogen bond acceptor, forming the first hydrogen bonds necessary to propagate a β-sheet outward from a central tether. The presence and orientation of hydrogen bonding was established through a variety of techniques. In each case, the hydrogen bonding patterns in β-sheet mimics **1** and **2** were compared with similar monomeric cysteine derivatives **3** and **4** to verify the role of the adjacent peptide strand.



A complete picture of the hydrogen bonding in these β-sheet mimics can only be determined by compiling the results from different techniques and includes both intramolecular hydrogen bonding as well as aggregation between molecules. Changes in NMR chemical shift that occur at different concentrations are indicative of intermolecular hydrogen bonding,[5] and the concentration dependence for **1**−**4** is shown in Figure 2. Both monomeric cysteine derivatives **3** and **4** show appreciable changes in chemical shift of both N−H protons (▲, ◆, △, ◇) above 50 mM, suggesting that greater aggregation occurs as concentration increases, more so for the diamide **4**. The disulfide dimer **1** is not soluble above 250 mM but shows little evidence of aggregation at these concentrations (●, ■). The acetamide-functionalized disulfide **2** is not soluble above 50 mM, but the changes in chemical shift suggest that it is engaging in intermolecular hydrogen bonds well below this concentration. The acetamide N−H protons (□) show changes at all concentrations above 1 mM, with larger shifts (0.3 ppm) between 25 and 50 mM. The butylamide N−H signal (○) is largely insensitive to concentration over the given range and only shows a small effect at 50 mM, suggesting that aggregation is mainly occurring through the acetamide N−H. The greater sensitivity of the acetamide N−H (□) to concentration as compared to controls suggests that **2** is aggregating to a greater extent at lower concentrations. Additionally, these curves point out the importance of performing subsequent experiments at concentrations beneath 10 mM to minimize intermolecular hydrogen bonding.

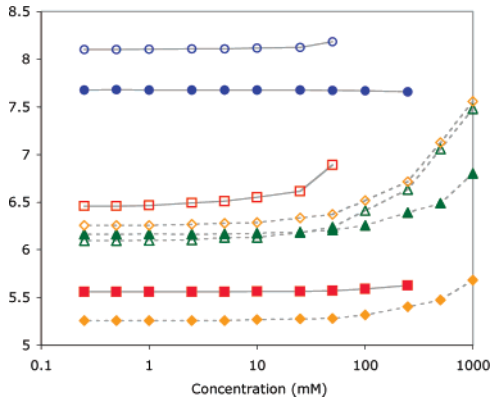


Figure 2. Dependence of NMR chemical shifts on concentration; including cystine dimer **1** (Boc-NH = ■; NHBu = ●), cystine dimer **2** (Ac−NH = □; NHBu = ○), cysteine monomer **3** (Boc-NH = ◆; NHBu = ▲), and cysteine monomer **4** (Ac−NH = ◇, NHBu = △).

Changes in chemical shift with the addition of a hydrogen bonding solvent also indicate the presence of hydrogen bonds.[6] In this case, the chemical shifts of **1**−**4** were determined in 100% C_6D_6 and with increasing percentages of DMSO-d_6.[7] Significant downfield shifts (>1 ppm) with increasing DMSO were observed for both N−H protons of cysteine monomers **3** and **4**, as well as the carbamate of **1** and the acetamide of **2**. The butyl amide of both **1** and **2**, on the other hand, showed a very small shift (<0.3 ppm) and ultimately an upfield shift at higher concentration. These results suggest that the butylamides of **1** and **2** are already participating in significant hydrogen bonds and are less sensitive to the competitive hydrogen bonding contribution of the added DMSO.

While the data above are consistent with the formation of interstrand hydrogen bonds, an even more detailed picture is possible using hydrogen/deuterium (H/D) exchange. This technique can be used to correlate a slower rate of H/D exchange with a stronger hydrogen bond donor and the increased rate of H/D exchange with a hydrogen bond acceptor.[8] The H/D exchange kinetics in 10% $CD_3OD/CDCl_3$ for **1** can be seen in Figure 3, along with comparisons with the analogous cysteine monomer **3** and controls **5** and **6**, which cannot engage in intramolecular hydrogen bonding. Exponential curve fits are included for all H/D exchange figures to indicate the correlation with pseudo-first-order kinetics.

The butyl amide of cysteine **3** (▲) exchanged more slowly than control **5** (+), indicating it was acting as a hydrogen bond donor. The carbamate of **3** (◆) exchanged more quickly than control **6** (×), suggesting it is functioning as a hydrogen bond acceptor. Since these kinetics were performed at a concentration below which significant aggregation was observed (Figure 2), this indicated that the observed in-

(4) Raj, P. A.; Soni, S. D.; Ramasubbu, N.; Bhandary, K. K.; Levine, M. J. *Biopolymers* **1990**, *30*, 73.

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(7) Complete data can be found in the Supporting Information.

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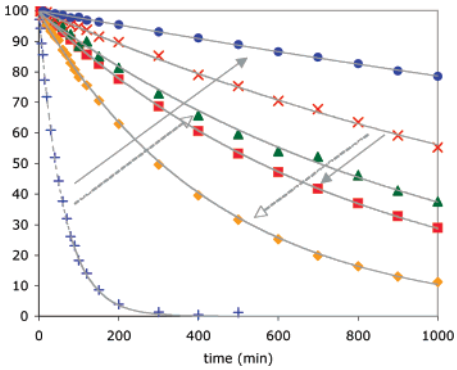
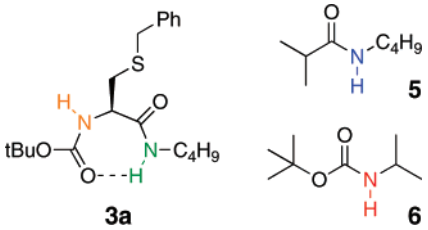


Figure 3. H/D exchange kinetics for cystine dimer **1** (Boc-NH = ■; NHBu = ●), cysteine monomer **3** (Boc-NH = ▲; NHBu = ◆), and non-intramolecular hydrogen bonding controls **5** (+) and **6** (×). Arrows indicate changes from controls.

tramolecular hydrogen bond was the cysteine folding into a seven-membered ring γ-turn as shown in **3a**.[8]



Cystine dimer **1** showed slightly different kinetics of H/D exchange. The butyl amide (●) was even more protected from H/D exchange than the analogous cysteine monomer **3**. This would suggest that it is not merely adopting a γ-turn but is forming a stronger hydrogen bond. The carbamate of **1** (■) also exchanged more quickly than control **6** (×), suggesting it is also functioning as a hydrogen bond acceptor. Interestingly, the carbamate of **1** does not exchange as quickly as the carbamate in monomer **3**. While this may indicate a lessened role as a hydrogen bond acceptor effect, it seems likely to be reflecting the aggregation observed earlier (Figure 2). If this is the case, this proton is experiencing both a rate acceleration as an intramolecular hydrogen bond acceptor and a slight rate deceleration from serving as an intermolecular hydrogen bond donor.

The acetamide-functionalized cystine **2** showed even more evidence of aggregation during H/D exchange (Figure 4). While the H/D exchange of cystine **1** was not sensitive to changes in concentration, the acetamide analogue showed varied kinetics over a limited concentration range. The most significant effect was observed in the acetamide exchange, where at low concentrations the exchange was comparable to the amide control **5**, but slowed appreciably as concentration increased. This is most consistent with hydrogen bonded aggregation using the outward-pointing acetamide N−H. The H/D exchange of the interior-pointing butylamide also slowed with increased concentrations, but to a lesser extent. The

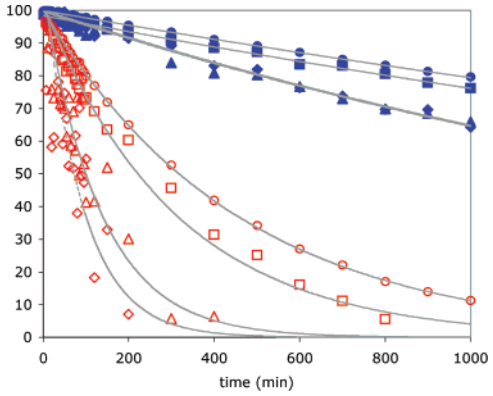


Figure 4. H/D exchange kinetics for acetyl−cystine dimer **2** at different concentrations. Closed symbols refer to butyl amide **1**, while open symbols refer to the acetamide. Concentrations include: 10 mM (●, ○), 5 mM (■, □), 2.5 mM (▲, Δ), and 1 mM (◆, ◇). Curve fits for $A_t = A_o \times \exp(-kt)$ are shown.

pseudo-first-order conditions used and insensitivity of **1** do not support a stoichiometry-dependent change in the exchange rate but rather a cooperative strengthening of the intramolecular hydrogen bond with the increasing intermolecular aggregation.

Following the thorough investigation of the potential for interstrand hydrogen bonding, the next step was to extend the structure along the peptide strand to start to build a small β-sheet. A cystine tripeptide dimer was created with the intention of forming four hydrogen bonds between two peptide strands.[9] This was created through solution-phase synthesis of linear tripeptide **7** (octanoyl-Leu-Cys(Trt)-Ala-NMe_2) followed by disulfide formation with iodine in methanol[10] to form a dimeric tripeptide **8** (Figure 5). An

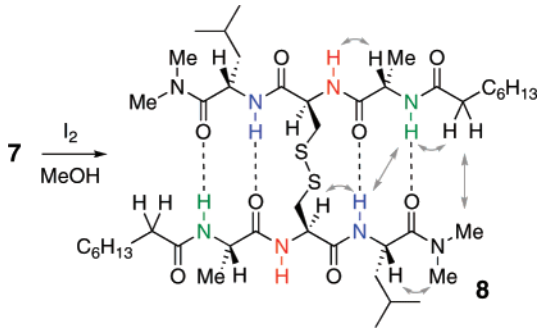


Figure 5. Synthesis of tripeptide cystine dimer **8**, and significant NOESY cross-peaks (arrows).

N-terminal octanoylamide was incorporated to enhance solubility, and a C-terminal dimethylamide was included to limit aggregation.[11]

(9) Analogous cyclic cystine peptides have previosuly been shown to form an antiparallel β-sheet. Kishore, R.; Kumar, A.; Balaram, P. *J. Am. Chem. Soc.* **1985**, *107*, 8019.

Dimeric tripeptide **8** extends the hydrogen bonding observed in **1** and **2**. Significant downfield shifts indicative of hydrogen bonding are observed when comparing the N−H protons of **7** and **8** (see Supporting Information). Changes in the chemical shift of the α-protons have also been used to support β-sheet formation,[12] with the α-protons for both leucine and alanine shifting slightly downfield and cysteine shifting significantly. All N−H protons of monomer tripeptide **7** show sensitivity to concentration and solvent,[7] although the leucine N−H shows the smallest change of the three. This insensitivity in combination with the significant change in the cysteine α-proton chemical shift suggests that the monomer tripeptide is adopting a γ-turn intramolecular hydrogen bond involving the leucine N−H and therefore does not make an ideal control for **8**.

The alanine and leucine amides of cystine dimer **8** are largely immune to changes in concentration,[7] while the cystine NH shows changes with concentration that are even larger than the monomer **7**, suggesting enhanced aggregation. The NMR shifts due to changes in C_6D_6/DMSO-d_6 ratio are also most pronounced for the cystine N−H, while the alanine N−H shifts slightly, and the leucine N−H shifts remarkably upfield.[7] β-Sheet formation is also supported by NOESY cross-peaks (Figure 5). Sequential α-amide connectivity is present, while interstrand connectivity is observed between the leucine and alanine amides, as well as between the terminal dimethylamide and octanoyl chain.

Hydrogen/deuterium exchange (Figure 6) supports the β-sheet folding pattern of **8**. The leucine-NH (●) exchanges more slowly than the analogous cross-strand hydrogen bond found in **2** (○), suggesting that the strength of the hydrogen bond has been increased with the additional peptide length. The alanine-NH (▲) exchanges at a faster rate than the leucine but still much slower than similar controls. This is consistent with β-sheet formation for the alanine and leucine amides, with the difference between these two exchange rates indicating the reduced stability of the hydrogen bond near the fraying end of the β-sheet. For the central cystine (■), the reduced exchange in comparison with **2** (□) is likely caused by aggregation, despite the incorporation of the dimethylamide. Enhanced aggregation of **8** in comparison with **2** suggests that the longer peptide chain produces a structure that is more preorganized and therefore more prone to aggregate, a common problem with both natural β-sheet proteins and artificial β-sheet peptidomimetics.[11]

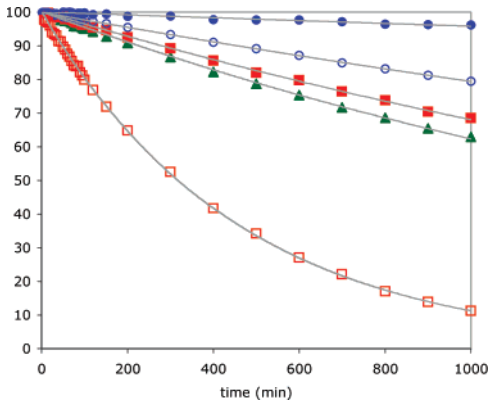


Figure 6. H/D exchange kinetics for **8** and **2**. Closed symbols refer to tripeptide dimer **8** (Ala-NH = ▲; Cys-NH = ■; Leu-NH = ●). Open symbols refer to cystine dimer **2** (Boc-NH = □; NHBu = ○). Curve fits for $A_t = A_o \times \exp(-kt)$ are shown.

These studies demonstrate the ability to create small molecules that replicate the hydrogen bonding patterns of a β-sheet, as well as our ability to assess the strength of these interactions and the role of individual hydrogen bond donors and acceptors. Simple tethers between α-carbons serve to position amide functional groups such that they can form a controlled array of hydrogen bonds. This effort continues with larger structures capable of forming a robust β-sheet in competitive solvents and in the creation of non-natural tethers that may have improved distance or structural characteristics. Additionally, the ability to use H/D exchange to assess relative hydrogen bond strength provides a useful method for evaluating the factors that stabilize a β-sheet fold, as well as the aggregation of these small β-sheet peptidomimetics into quaternary structures.

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Supporting Information Available: Synthetic procedures, spectral data, kinetic protocols, data for solvent dependence. This material is available free of charge via the Internet at http://pubs.acs.org.

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