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Macrocyclic Design Strategies for Small, Stable Parallel β-Sheet Scaffolds

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Cyclization is a powerful strategy for inducing antiparallel β-sheet secondary structure in relatively short peptide segments. Biological examples feature cyclization via the backbone,[1] as seen in gramicidin S and θ-defensin, and cyclization via side chains (disulfide formation),[2] as seen in tachyplesins and protegrins. These natural prototypes have inspired the use of cyclization in β-sheet design efforts aimed at both structural and functional goals. For example, both backbone and side chain cyclization have been used to generate peptides that serve as spectroscopic references for the β-sheet conformations adopted by flexible, linear peptides.[3] Cyclic β-sheet scaffolds have provided a fruitful basis for development of peptides with a variety of biological activities, including antibiotics, vaccine epitopes, RNA ligands, and, perhaps most intriguingly, helix-mimetic inhibitors of protein−protein recognition.[4]

The demonstrated utility of cyclically enforced antiparallel β-sheet scaffolds raises the prospect that analogous parallel β-sheet scaffolds would be comparably useful. Biology does not offer a clear basis for achieving this structural goal in relatively small molecules. Interstrand disulfides seem to be incompatible with a parallel β-sheet secondary structure, given the rarity of such cross-links in proteins.[5] β-Strand-forming segments must be linked N-terminus-to-C-terminus within peptides and proteins; therefore, covalent connection of β-strands in parallel orientation requires a peptidic linker that is at least as long as the β-strands themselves. This topological limitation has inspired many efforts to devise small nonpeptide units that can be used to connect peptide segments in a C-to-C or N-to-N fashion.[6−8] Such units should ideally have a strong turn-forming propensity that can encourage β-sheet interactions between attached peptide segments. We have developed both preorganized C-to-C linkers and preorganized N-to-N linkers that promote (but do not enforce) parallel β-sheet formation between peptide segments in aqueous solution (Figure 1a).[9] Here we evaluate these linkers in the context of backbone cyclization. Unexpectedly, we find that only one of the two turn units needs to be preorganized to enforce a high level of parallel β-sheet folding.

We previously showed that a linker containing D-proline and 1,2-diamino-1,1-dimethylethane (D-Pro-DADME) promotes parallel β-sheet formation between peptide segments attached via their C-termini[9a,b] and that linkers containing *cis*-1,2-cyclohexanedicarboxylic acid and glycine (*cis*-CHDA-Gly) promote parallel β-sheet formation between peptide segments attached via their N-termini.[9c] Substituting L-Pro for D-Pro in the C-to-C linker abolished folding, for strands containing exclusively L-residues, but the two configurations of the *cis*-CHDA unit in the N-to-N linker (that is, the *cis*-CHDA-Gly unit shown in Figure 1a and its enantiomer) displayed comparable promotion of a parallel β-sheet. Macrocycles **1**−**4** contain varying combinations of these two types of preorganized linkers and flexible analogues, along with an invariant pair of hexapeptide strands. In **1**, both linkers are preorganized to promote folding. In **2**, however, only the N-to-N linker is preorganized, because the C-to-C linker contains Gly in place of D-Pro. A linear analogue containing the two strand segments connected via only

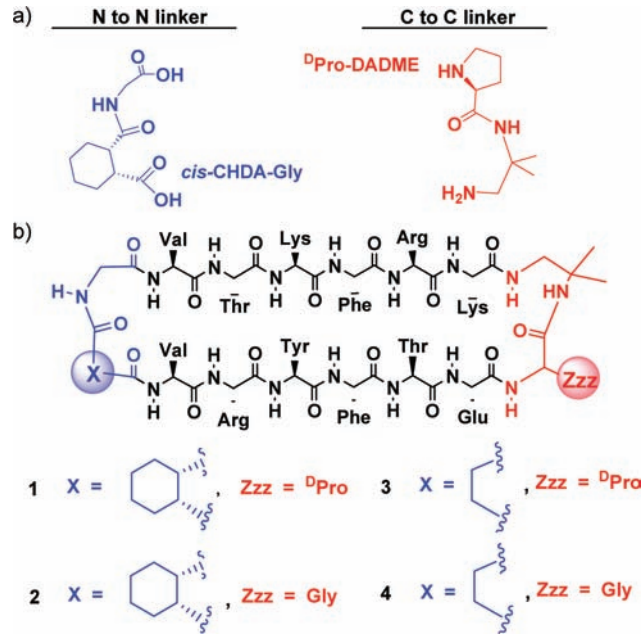

Figure 1. (a) Linkers used to promote parallel β-sheet secondary structure between attached peptide strands. (b) Macrocycles **1**−**4**.

the flexible Gly-DADME unit showed no evidence for folding,[10] which confirms the importance of linker preorganization in the absence of a macrocyclic constraint. Both **1** and **2** contain one particular configuration of the *cis*-CHDA unit; the diastereomers with the other *cis*-CHDA configuration displayed very similar behaviors.[10] In **3**, only the C-to-C linker is preorganized, because the N-to-N linker contains a succinyl unit in place of *cis*-CHDA. In **4**, neither linker is preorganized.

Chemical shifts observed for protons attached to amino acid residue α-carbons have proven to be useful site-specific indicators of secondary structure formation.[11] A β-sheet secondary structure is suggested by sets of three or more sequential $\delta C_\alpha H$ values that are downfield by ≥0.1 ppm relative to $\delta C_\alpha H$ expected for the random coil state. Measurement of $\Delta\delta C_\alpha H = \delta C_\alpha H(\text{observed}) - \delta C_\alpha H(\text{random coil})$, which is referred to as the "chemical shift deviation" (CSD), requires a source of "random coil" data. We used noncyclic molecule **5** to provide these data (Figure 2a), because the flexible succinyl-Gly linker does not induce parallel β-sheet interactions between the attached strands. Indeed, $\delta C_\alpha H$ values for **5** are very similar to $\delta C_\alpha H$ values that have been used to represent sequence-independent random coil references.[12] Figure 2b shows $\Delta\delta C_\alpha H$ data for the 12 strand residues common to **1**−**4**. These NMR data were acquired in 9:1 H_2O/D_2O containing 100 mM sodium acetate, pH = 3.8, with 2.5 mM peptide samples.[13] DOSY measurements[14] showed that peptide diffusion coefficients are invariant in 0.3 and 5 mM solutions, which suggests that there is little or no peptide aggregation under these conditions.

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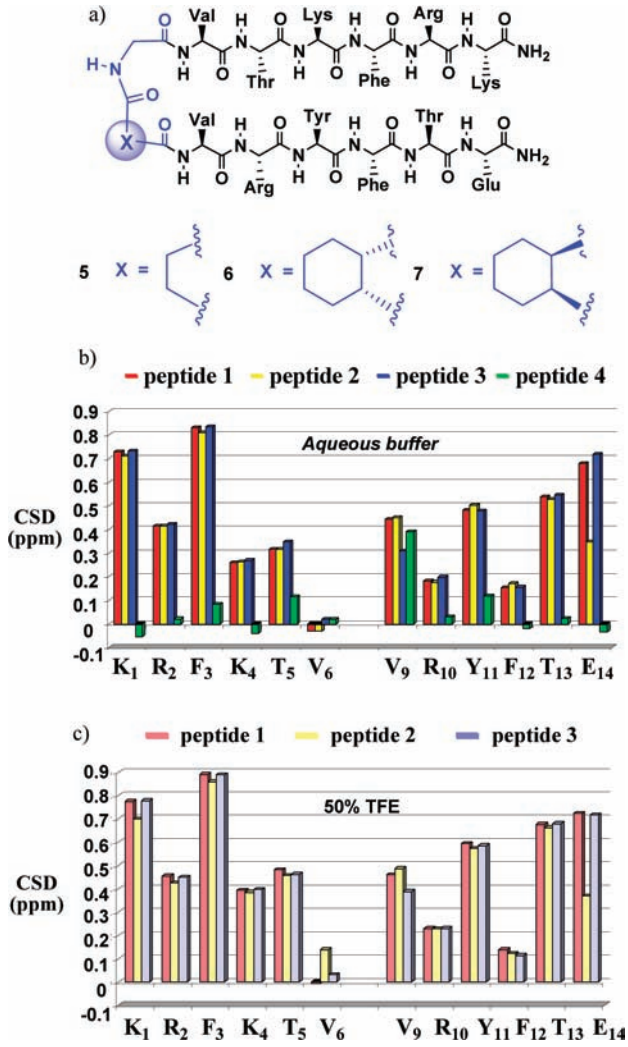

Figure 2. (a) N-linked peptides **5**−**7**. (b) $\Delta\delta C_\alpha H$ = $\delta C_\alpha H$(observed) − $\delta C_\alpha H$(random coil), or CSD, for α-amino acid residues of macrocycles **1**−**4** dissolved in aqueous buffer. (c) CSD for α-amino acid residues of macrocycles **1**−**3** dissolved in a 1:1 vol/vol solution of aqueous buffer and 2,2,2-trifluoroethanol (TFE). For (b) and (c), $\delta C_\alpha H$(random coil) values were obtained from peptide **5** in the appropriate solvent.

The $\Delta\delta C_\alpha H$ data indicate extensive parallel β-sheet formation for **1**−**3**, but not for **4**, in aqueous buffer (Figure 2b). Among **1**−**3**, 11 of 12 strand residues show $\Delta\delta C_\alpha H > 0.1$ ppm, and the absolute values for each residue are very similar in these three cyclic peptides. The $\Delta\delta C_\alpha H$ values change only slightly in the presence of 50 vol % TFE (Figure 2c), which suggests that the antiparallel β-sheet populations for **1**−**3** are very high in pure aqueous buffer. In contrast, the $\Delta\delta C_\alpha H$ data for **4** suggest that this molecule forms little or no β-sheet secondary structure in aqueous solution. The behavior of **4** shows that merely placing the two strand segments in a macrocyclic context by using flexible linkers is not sufficient to induce parallel β-sheet folding; conformational preorganization of linking segments plays a vital role. However, the lack of significant distinction among **1**−**3** reveals a conclusion that we did not anticipate: only one of the two linkers must be preorganized to achieve maximum β-sheet promotion. The data suggest that the D-Pro-DADME (C-to-C) and *cis*-CHDA-Gly (N-to-N) linkers have comparable sheet-promoting propensities.

To examine the conformations adopted by **1**−**3** in greater detail, we used NOE-restrained dynamics to determine the structures in aqueous buffer.[15] Superimposition of the 10 most favorable conformations identified by this approach for each macrocycle led to very good structural overlap (rmsd among backbone atoms = 0.035 ± 0.017 Å for **1**, 0.284 ± 0.179 Å for **2**, and 0.300 ± 0.186 Å for **3**). Figure 3 shows an overlay of the most favorable

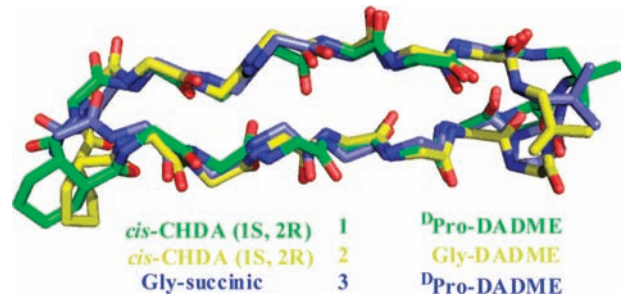

Figure 3. Overlay of NMR-derived conformations for macrocycles **1**−**3** in aqueous buffer. Rmsd for backbone atoms in the strand segments is 0.659 Å for **1** vs **3** and 0.691 for **2** vs **3**.

conformation for each of **1**−**3** according to the NOE-restrained dynamics analysis. Each molecule forms a two-stranded parallel β-sheet, as intended. The β-strand segments overlap quite well, and the major deviations are seen in only the linkers. In contrast to the many NOEs between protons from sequentially nonadjacent residues observed for **1**−**3**, no medium- or long-range NOEs were detected for **4**, in which both linkers are flexible.

The two six-residue strand segments common to **1**−**4** were designed to be prone to parallel β-sheet formation, based on interstrand neighbor preferences deduced by Fooks et al. from the protein structure database.[16] Previously we showed that N-to-N linkage of these two strands via a *cis*-CHDA-Gly unit, as in **6** or **7**, leads to significant population of the parallel β-sheet secondary structure.[9c] The lack of detectable folding in analogue **5**, in which *cis*-CHDA has been replaced by succinyl, shows the importance of linker preorganization for parallel β-sheet formation. However, our earlier study revealed that if the strand positions are swapped on a *cis*-CHDA-Gly linker, to generate **8**, then no parallel β-sheet forms.[9c] The dramatic difference between strand-swapped isomers **6** and **8** shows that a *cis*-CHDA-Gly cannot enforce parallel β-sheet interactions between strands that have a low intrinsic propensity to pair in this way.

For design purposes, it would be very valuable to identify a strategy that induces a parallel β-sheet secondary structure even when the strand segments have a low intrinsic propensity for parallel sheet interactions. We therefore examined macrocycle **9**, which has the same strand juxtaposition as in **8** and features a preorganized D-Pro-DADME linker for C-to-C linkage and a flexible N-to-N linker. Macrocycle **9** displays numerous NOEs between protons on residues that are not adjacent in sequence; these data are consistent with parallel β-sheet secondary structure in the strand segments. NOE-restrained dynamics[15] (Figure 4b) suggest a backbone conformation very similar to that of **3**, which features the same pair of linkers. $\Delta\delta C_\alpha H$ data for **9** are consistent with high population of the parallel β-sheet secondary structure in aqueous solution (Figure 4c). Thus, using a macrocyclic backbone to link two peptide strands in parallel orientation, with at least one linker appropriately preorganized, appears to be a robust strategy for inducing a parallel β-sheet secondary structure.

The results reported here provide new and possibly general guidelines for creating peptidic scaffolds that display substantial conformational stability in aqueous solution. There are well-established strategies for creating relatively short peptides that display high population of an α-helix[17] or antiparallel β-sheet[18]

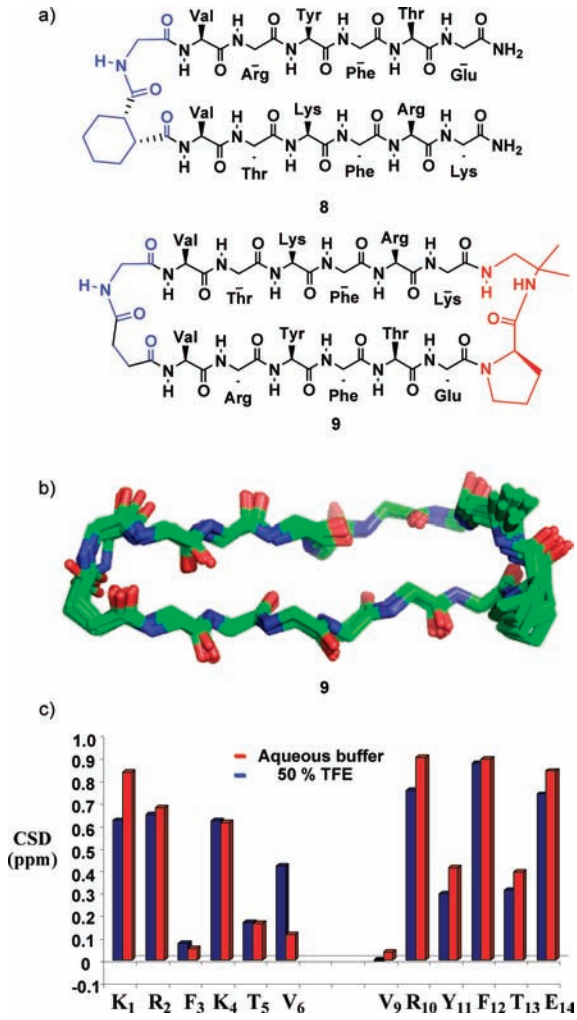

Figure 4. (a) Peptides **8** and **9**. (b) Overlay of the 10 best structures for **9** in aqueous buffer obtained via NOE-restrained dynamics (see text for details). Rmsd for the backbone atoms in the strand segments is 0.320 ± 0.117 Å. (b) $\Delta\delta C_\alpha H = \delta C_\alpha H(\text{observed}) - \delta C_\alpha H(\text{random coil})$, or CSD, for α-amino acid residues of macrocycle **9** dissolved in aqueous buffer or in a 1:1 vol/vol solution of aqueous buffer and 2,2,2-trifluoroethanol (TFE). For (c) the $\delta C_\alpha H$(random coil) values were obtained from peptide **8** in the appropriate solvent.

secondary structure in aqueous solution; the approach documented above complements these strategies by providing a *parallel* β-sheet secondary structure. Two nonpeptide linkers are necessary to generate macrocycles that promote parallel strand interactions, and the finding that only one of these linkers needs to be preorganized in cyclic systems is useful because the chiral element in the D-Pro-DADME linker is commercially available, while the chiral element in a *cis*-CHDA-Gly linker must be generated via asymmetric synthesis.

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Supporting Information Available: Experimental details, compound characterizations, and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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