

# Templates That Induce $\alpha$ -Helical, $\beta$ -Sheet, and Loop Conformations

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## I. Introduction

Polypeptides composed of 30 amino acid residues or less typically do not adopt well-defined conformations in aqueous solution; rather they adopt an ensemble of energetically similar conformations. The small free energy difference (3–15 kcal/mol) that stabilizes the folded state of a native protein over the unfolded state is difficult to achieve in a small polypeptide as the difference in free energy between the folded and unfolded state is derived from the difference between two large numbers representing entropic and enthalpic contributions from both the solvent and the polypeptide chain.<sup>1</sup> Since the magnitude of the electrostatic interactions (hydrogen bonding, salt bridges, etc.) and the hydrophobic interactions are context dependent, it is very difficult to design a polypeptide whose thermodynamic stability can be predicted empirically. Although there are a number of elegant examples of *de novo* protein design, it is fair to characterize this undertaking as quite challenging and risky, especially for folds other than predominantly  $\alpha$ -helical folds. The work reviewed within demonstrates that it is possible to prepare relatively small peptides that are biased toward adopting the desired conformation owing to the incorporation of a single unnatural amino acid residue or template.

This review surveys what is currently state of the art in the use of peptidomimetic templates to propagate common protein structural motifs in predominantly  $\alpha$ -amino acid sequences. The use of templates to nucleate or propagate a certain conformation from an ordered region (template) through a disordered region ( $\alpha$ -amino acid-based portion) to form  $\alpha$ -helices



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and  $\beta$ -sheet structures has proven to be a viable method for achieving folding in the absence of a detailed understanding of how to design a linear sequence that will spontaneously fold. The chain entropy penalty associated with the folding of a linear peptide chain can be decreased by lowering the conformational entropy of the unfolded state through the incorporation of conformationally restricted tem-

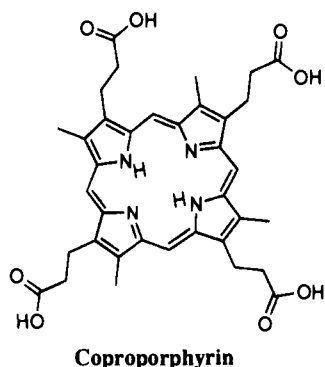
plates. The templates described within typically transmit their conformational preferences to the peptide chain by hydrogen bonding or by a combination of hydrogen bonding and hydrophobic interactions to nucleate the desired structure. The concept of introducing a conformationally rigid molecule into a peptide to stabilize a single bioactive conformation was first demonstrated by Hirschmann and his colleagues at Merck.<sup>2,3</sup> This review will focus on examples of templates that nucleate the formation of  $\alpha$ -helical,  $\beta$ -sheet, and  $\Omega$ -loop structures. We will not include work on  $\beta$ -turn mimics which are designed to position peptide-like side chains in the proper spatial orientation for recognition by proteins or receptors, as this work has been amply reviewed.<sup>4-9</sup>

## II. Templates That Induce $\alpha$ -Helical Folding

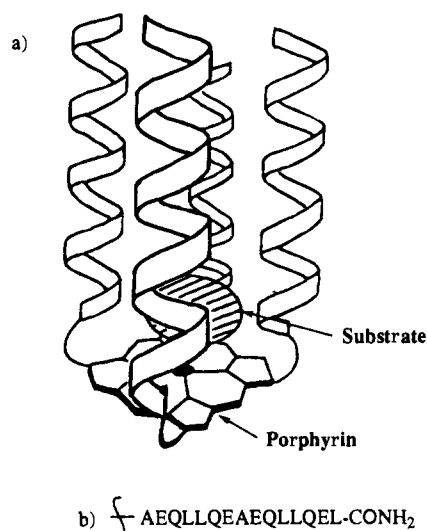
### A. Porphyrin Templates

Startling progress has been made in the *de novo* design of predominantly  $\alpha$ -helical proteins composed exclusively of  $\alpha$ -amino acid sequences over the past 10 years in the laboratories of Baldwin, DeGrado, Hamilton, Kaiser, Kallenbach, Kemp, Kim, Rose, Schultz, and Taylor among others.<sup>10-28</sup> However, it is still nontrivial to design a predominantly  $\alpha$ -helical protein from first principles using the full complement of natural amino acids. Hampered by the difficulties associated with predicting the folded structure of a polypeptide from its primary sequence, several laboratories have incorporated templates into  $\alpha$ -amino acid sequences in an effort to achieve a desired tertiary fold.

Kaiser and Sasaki utilized the tetrasubstituted coproporphyrin shown below as a template to direct the folding of attached amphiphilic peptides, affording a four helix bundle they named "helichrome".<sup>29</sup>



The four propanoic acid linkers on the porphyrin were coupled to the terminal amino groups of identical amphiphilic peptides employing a segment condensation reaction which afforded the templated helical bundle (Figure 1).<sup>30</sup> The synthetic protein "helichrome" was designed to mimic the hydroxylase activity of cytochrome P-450, whose porphyrin-based active site is surrounded by several  $\alpha$ -helices which serve as a hydrophobic substrate binding pocket. The sequence of the peptides in the helichrome was chosen such that the folded structure has a hydrophobic pocket or cavity near the base of the porphyrin as depicted in Figure 1. This pocket which is

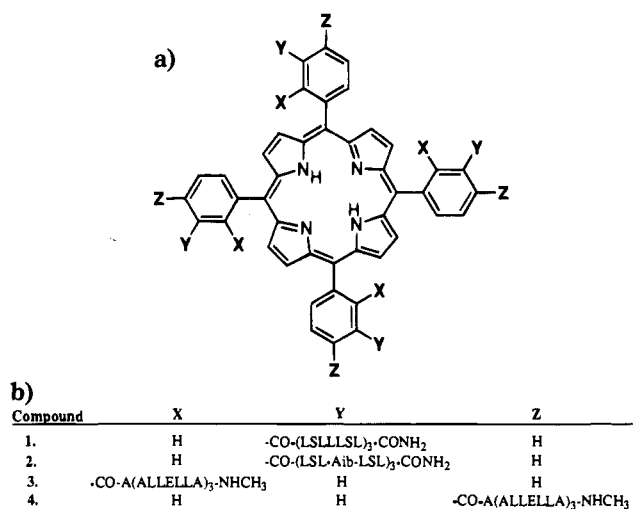


**Figure 1.** (a) A synthetic enzyme or "helichrome" consisting of an Fe(III)-coproporphyrin template which directs the folding of attached peptide strands resulting in a four-helix bundle and (b) the amphiphilic sequence of the attached peptides allows for the formation of a hydrophobic pocket responsible for the binding of substrate.

proximal to the porphyrin serves as the binding site for substrates of the hydroxylase reaction.

Circular dichroism (CD) studies reveal that the helichrome contains a predominantly  $\alpha$ -helical structure at pH 7.5 in aqueous phosphate buffer, whereas the isolated peptide segments adopt an ensemble of random conformations under identical conditions, strongly suggesting that the coproporphyrin is responsible for the formation and stabilization of the four-helix bundle. Gel filtration and analytical equilibrium ultracentrifugation experiments under identical conditions indicate that the helichrome exists as a monomeric helix bundle in aqueous solution. The  $\alpha$ -helical bundle structure observed by CD results from intramolecular folding of the attached peptide strands facilitated by the coproporphyrin which presumably reduces the chain entropy penalty of folding. The stability of helichrome has been accessed by monitoring the guanidine hydrochloride-initiated unfolding transition of the four-helix bundle by CD spectroscopy. The resulting denaturation curve yielded a free energy of stabilization of the folded state over the unfolded state, i.e.  $\Delta G_{\text{H}_2\text{O}}$  of  $-4.4$  kcal/mol and a sensitivity of the free energy difference to urea concentration, i.e.  $m$  value of  $0.84$  kcal  $\text{mol}^{-1} \text{M}^{-1}$ , which are consistent with parameters from other previously reported  $\alpha$ -amino acid-based four-helix bundles.<sup>31</sup>

The ability of the Fe(III)-helichrome complex to function as an effective aniline hydroxylase was tested by monitoring the formation of *p*-aminophenol as a function of the concentration of the aniline substrate. The assay, performed in the presence of fixed 7-acetylflavin and NADPH concentrations at pH 7.0 yielded a  $k_{\text{cat}}$  of  $0.02 \text{ min}^{-1}$  and a  $K_m$  of  $5.0 \text{ mM}$ , values which are similar to those of natural heme-proteins such as hemoglobin which possess this activity.<sup>32,33</sup> An Fe(III) complex of the isolated coproporphyrin showed no catalytic activity under identical conditions, suggesting that the rigid hydrophobic pocket formed by the peptide portion of the



**Figure 2.** (a) Structure of the tetraphenylporphyrin template used to direct the folding of attached peptide strands into a four-helix bundle and (b) sequence of peptides appended to the ortho, meta, and para positions of the four phenyl functionalities affording tetraphilins 1–4.

helichrome is essential for catalytic activity. The chemical mechanism of the helichrome's catalytic activity has not been studied in detail.

Tetraphenylporphyrins have also been employed to direct the folding of amphiphilic peptides into four-helix bundles, which were designed to insert into membranes and serve as ion channels. DeGrado and co-workers have functionalized the *meta* position of the phenyl groups in the tetraphenylporphyrin shown in Figure 2 with amphiphilic peptides resulting in tetraphilins 1 and 2.<sup>34</sup> Meta substitution provides optimal interhelical spacing for ion transport. The sequence of the  $\alpha$ -amino acid portion of tetraphilin (1) is based on model peptides developed by DeGrado and co-workers which self-assemble into proton-selective ion channels in membranes. However, tetraphilin (1) exhibited low solubility in 1 M HCl and was not suitable for evaluation as an ion channel. Replacement of three leucine residues in the peptide portion of tetraphilin (1) with less hydrophobic aminoisobutyric acid residues affords soluble tetraphilin 2 which forms proton-selective ion channels in diphytanoylphosphatidylcholine bilayers. Tetraphilin 2 displays a primary conductance state of 470 pS and secondary states of 320 and 100 pS. It is interesting that the lifetime of the conductance state of porphyrin-constrained tetraphilin 2 (5 ms) is considerably longer than that of previously studied unconstrained peptides (<0.2 ms) composed of similar sequences.<sup>35</sup> The authors suggest that the porphyrin template stabilizes the conducting state of the attached peptides, i.e. the four-helix bundle state in the membrane.

Nishino, Mihara, and co-workers also employed a tetraphenylporphyrin template in the synthesis of a four-helix bundle designed to penetrate lipid bilayers, Figure 2.<sup>36</sup> Amphiphilic peptide strands were attached to the ortho and para positions of a tetraphenyl-substituted porphyrin ring resulting in tetraphilins 3 and 4, respectively. Tetraphilin 3 displays a concentration-independent (CD) spectra in methanol indicative of a monomeric  $\alpha$ -helical structure, sug-

gesting that the porphyrin efficiently directs the intramolecular folding of the attached peptide strands resulting in the desired four-helix bundle. Tetraphilin 4 also forms a helical structure as discerned by CD; however, the helical signal is concentration dependent suggesting that the amphiphilic helical segments are associating intermolecularly. Apparently, attachment of the strands at the *para* positions of the tetraphenylporphyrin template results in a tetraphilin that is unable to exclusively fold intramolecularly or undergoes self assembly after folding. The CD spectra of tetraphilin 3 in the presence of sodium dodecyl sulfate micelles and dipalmitoylphosphatidylcholine vesicles indicates that this templated protein retains its  $\alpha$ -helical character suggesting that it is capable of penetrating lipid bilayers without significant structural perturbation.

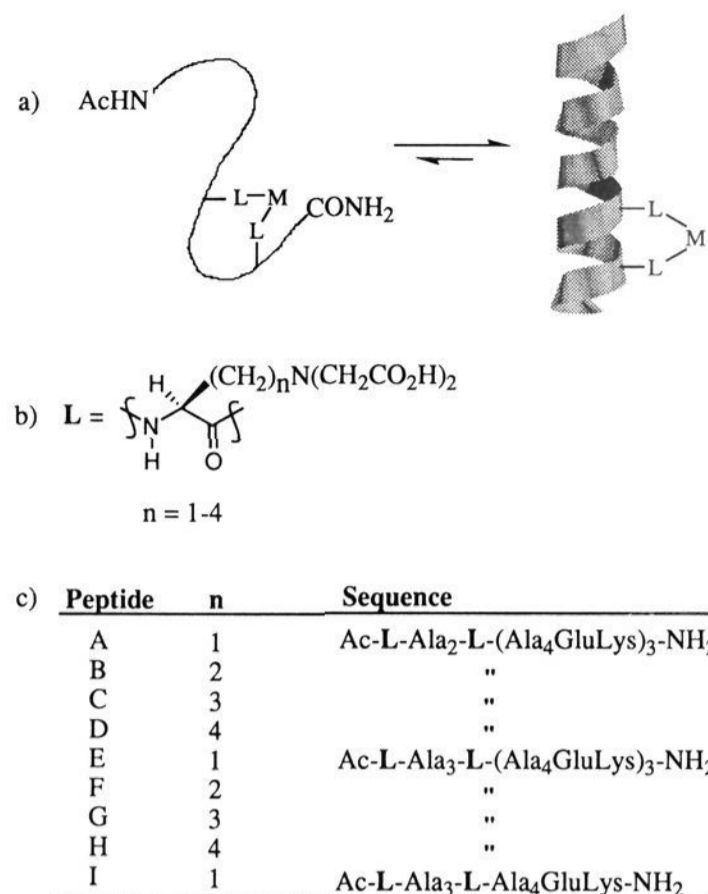
## B. Aminodiacetic Acid-Based Amino Acids

Another useful strategy for achieving folding in a relatively short polypeptide sequence employs metal complexation to stabilize the desired structure. Metal binding sites have been engineered into polypeptides using the side chains of naturally occurring amino acids (His, Cys) as ligands.<sup>37–45</sup> For example,  $\alpha$ -helical secondary structures have been stabilized through the formation of a metal-mediated cross-link employing the side chains of histidine or cysteine residues located at the *i* and *i* + 4 positions of an  $\alpha$ -helix as demonstrated by Arnold and Ghadiri.<sup>46–50</sup> In addition, DeGrado and Berg have studied the metal-mediated formation of tertiary and quaternary structure in peptides and proteins using engineered metal-binding sites.<sup>24,51–56</sup> In keeping with the focus of this section we will outline those examples involving unnatural amino acids or templates which bind to transition metal ions and stabilize helical structures.

Sasaki and Hopkins have stabilized, and in some cases, directed the folding of peptides into an  $\alpha$ -helical secondary structure by incorporating unnatural amino acids into the polypeptide sequence which serve as metal-binding ligands.<sup>57,58</sup> Kinetically labile metal-mediated cross-links are formed by complexing transition metals to two nonnatural aminodiacetic acid residues that are strategically incorporated into the peptide sequence. The formation of a metallo cross-link should facilitate the folding of an unordered peptide by lowering the conformational entropy of the unordered state and/or by stabilizing the folded conformation over the unfolded peptide (Figure 3a). Aminodiacetic acid side chains were chosen as the ligands because of their ability to bind many different transition metals with high affinity. By varying the structure of the aminodiacetic acid functionality, the distance between the peptide backbone and the metal binding site can be adjusted (Figure 3b).

The equilibrium between the unfolded polypeptide chain and the folded helix depends on the placement of the metal binding amino acids, their structure, and the metal used for complexation. The metal-binding amino acids were incorporated into a number of peptide sequences shown in Figure 3c. The sequences were designed to display helical structure in the absence of metal so that small conformational changes realized upon the addition of metal could be





**Figure 3.** (a) Metal-mediated formation of an  $\alpha$ -helix from a random coil (metal binding is thought to lower the entropy of the unordered state shifting the equilibrium towards the folded helix), (b) aminodiacetic acid residue responsible for metal binding (the distance between the metal binding site and the peptide backbone can be adjusted by varying the number ( $n$ ) of methylene units) and (c) peptides incorporating the ligating residue (L) at either the  $i$  and  $i + 3$  or the  $i$  and  $i + 4$  positions of a helical turn.

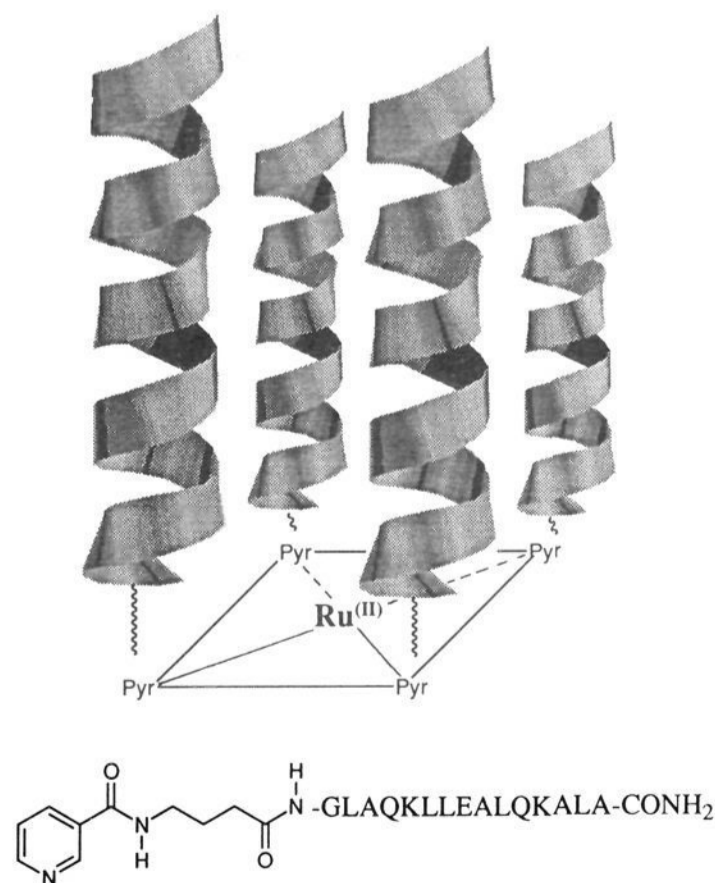
easily detected. In peptides **A–D**, two metal binding residues of varying structure are placed in what would be the  $i$  and  $i + 3$  positions of the folded helix, whereas in peptides **E–I** the ligating residues are placed at the  $i$  and  $i + 4$  positions. These spacings were evaluated because the typical  $\alpha$ -helix contains 3.6 residues per turn.<sup>59</sup> Several ions [Co(II), Ni(II), Cu(II), Zn(II), and Cd(II)] were tested with regard to their ability to form a cross-link which stabilizes the helix conformation. Several peptide/metal ion combinations yielded a stabilized  $\alpha$ -helical conformation: peptide **G** + Cu(II), **E** + Cd(II), **B** + Cu(II), and **F** + Co(II). Interestingly, the addition of Ni(II) to peptide **A** actually destabilized the existing helical structure. CD studies of control peptides with both ligating residues replaced with noncoordinating residues in the sequence, show that the addition of transition metal has little effect on the preexisting helix–coil equilibrium. In studies on peptides containing only one ligating residue, the addition of transition metal actually destabilizes the preexisting helical structure. These results indicate that the helix stabilization observed for the peptide/metal ion combinations discussed above is a result of metal-mediated conformational restriction as depicted in Figure 3a. In the most dramatic case, peptide **I** undergoes a random coil to helix transition upon the addition of Cd(II), indicating that peptides with little propensity to fold can be stabilized in a helical conformation by the addition of the appropriate metal ion. The metal-mediated formation of protein-like structure is not limited to the construction of small helical motifs but has evolved into an elegant ap-

proach applicable to the design of larger synthetic proteins.

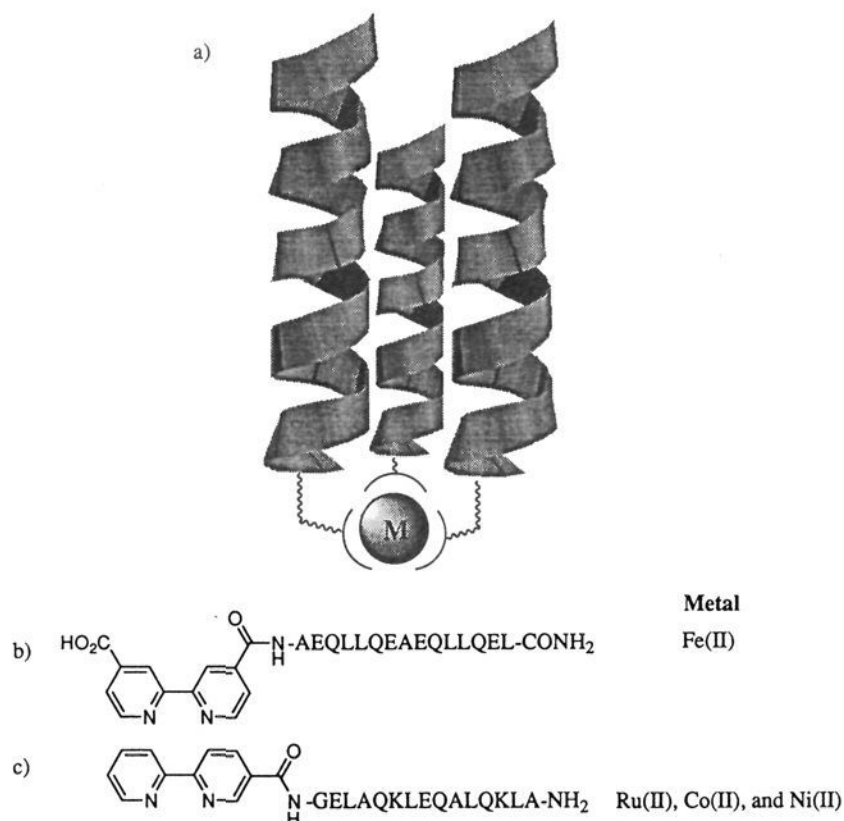
### C. Pyridine- and Bipyridine-Based Templates

Ghadiri and co-workers have demonstrated that unnatural pyridine-based metal binding amino acid residues can be incorporated into an  $\alpha$ -amino acid sequence to direct the folding of a four-helix bundle structure.<sup>60</sup> Amphiphilic peptide strands functionalized at their N-terminus with pyridyl functionalities were designed to fold into a water-soluble four-helix bundle complex upon Ru(II) complexation resulting in the exchange-inert metalloprotein shown in Figure 4. The major driving force for the folding of the four-helix bundle structure is proposed to be favorable interhelical hydrophobic interactions gained upon folding. The binding energy derived from the ruthenium complexation event should also contribute to the folding, but to a lesser extent. The complexation of Ru(II) to the peptides was established by UV spectroscopy as well as by atomic absorption spectroscopy. The molecular weight of the resulting metalloprotein was estimated by size exclusion chromatography and shown definitively by electrospray mass spectroscopy to be the desired four-helix bundle metalloprotein.

Circular dichroism spectroscopy reveals that the metalloprotein's structure is highly  $\alpha$ -helical and concentration independent, suggesting that the CD signal is a result of intramolecular formation of a monomeric four-helix bundle and not due to intermolecular association of helical bundles with undefined aggregation states. Guanidine hydrochloride denaturation studies employing CD as the spectroscopic probe resulted in the following thermodynamic values:  $\Delta G_{H_2O} = 5.6$  kcal/mol, and  $m = 1025$  cal mol<sup>-1</sup> M<sup>-1</sup>, indicating that the stability of the met-



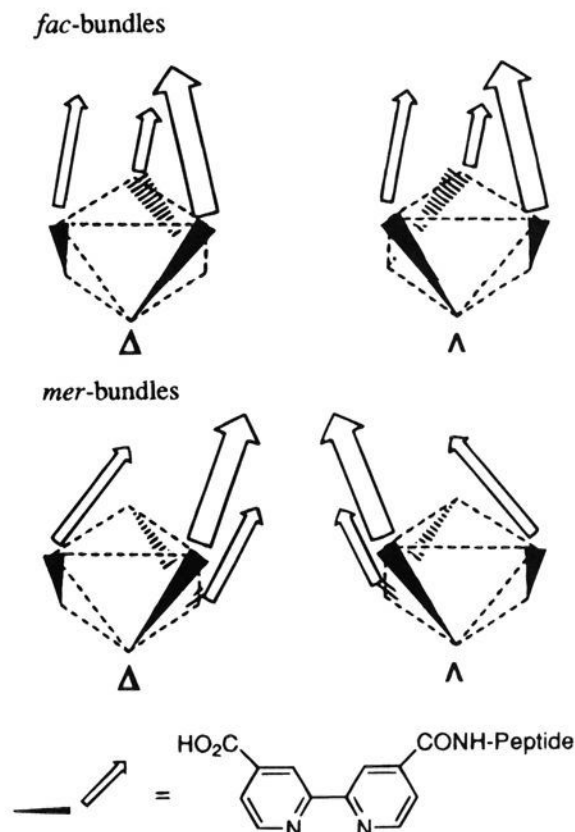
**Figure 4.** Complexation of four pyridine-functionalized amphiphilic peptides to exchange inert Ru(II) affords a 60-residue four-helix bundle ruthenium-metalloprotein. The sequence of the pyridine-functionalized peptide is also shown.



**Figure 5.** (a) Metal-assisted folding of bipyridine-functionalized peptide strands into a three-helix bundle metalloprotein (solid arcs represent the bipyridine–ligand portion of the peptides) and (b and c) sequence of the bipyridine-functionalized amphiphilic peptide strands along with the corresponding metal ion used for complexation.

alloprotein is comparable to other synthetic four-helix bundles such as the coproporphyrin-stabilized heli-chrome discussed above.

The formation of helical bundles via metal-mediated templating offers a viable method for controlling the aggregation state as well as for specifying the spatial arrangement of the helices. Three-helix bundles can be synthesized by metal complexation of peptides functionalized at their N-terminus with a bipyridine skeleton (Figure 5). Three bipyridine-functionalized peptides fulfill the octahedral coordination geometry of an appropriate metal, forming a stable complex and limiting the number of peptides composing the bundle. An example of this methodology was demonstrated simultaneously by the Ghadiri and Sasaki laboratories.<sup>61,62</sup> Sasaki and co-workers functionalized the 2,2'-bipyridine skeleton at the 4-position with an  $\alpha$ -amino acid sequence resulting in the amphiphilic bipyridine-functionalized peptide shown in Figure 5b.<sup>62</sup> Complexation of the resulting bipyridine-functionalized peptide to Fe(II) affords a parallel three-helix bundle metalloprotein which does not self-associate as discerned by gel filtration chromatography. CD spectroscopy reveals that the uncomplexed bipyridine-functionalized peptide exists in a partial  $\alpha$ -helical conformation. However, the helicity content increases by 50% upon addition of Fe(II) indicating that the tris-bipyridine complex is an efficient template for three-helix bundle formation in aqueous solution. The ability of the trisbipyridine template to direct the folding of peptides into stable three-helix bundles is highly sequence dependent. Changing the leucine residues composing the hydrophobic core of the bundle to less hydrophobic residues such as alanine, glutamine, or serine results in a significant decrease in  $\alpha$ -helical content. This result suggests that the main driving force for folding is derived from interhelical hydrophobic interactions as



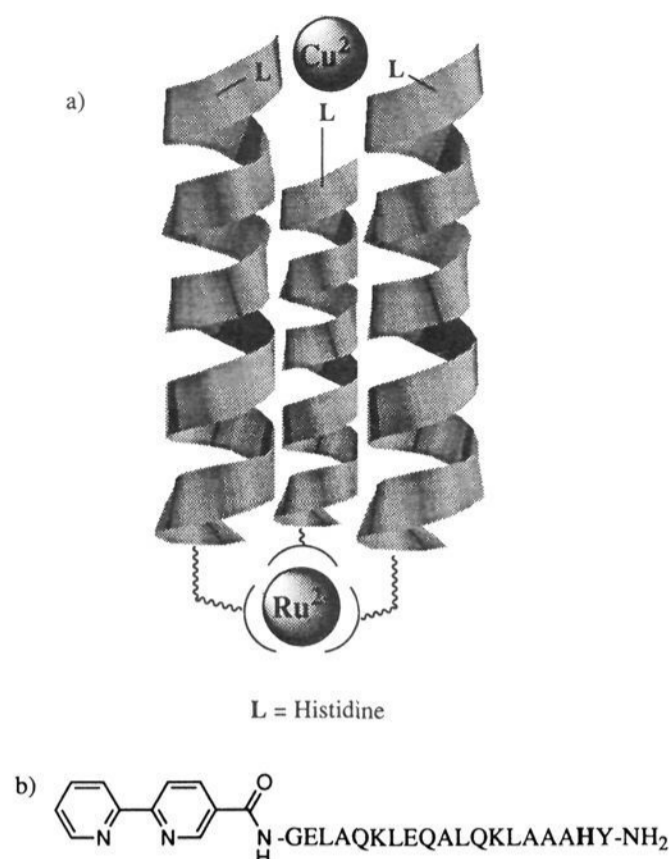
**Figure 6.** The complexation of Fe(II) to three bipyridine-functionalized peptide strands results in a three-helix bundle consisting of four possible energetically similar stereoisomers.

is the case in native proteins. Metal binding clearly plays a crucial role in controlling the number of helices forming the helix bundle and likely contributes to the free energy of folding by lowering the entropic penalties associated with the folding process.

Sasaki has shown that the bipyridine-functionalized peptides can adopt four possible stereoisomers about the iron atom denoted as fac- $\Delta$ , fac- $\Lambda$ , mer- $\Delta$ , and mer- $\Lambda$  in a 9.5:17:43:29 isomeric ratio, respectively (Figure 6).<sup>63</sup> Separation of these stereoisomers by RP-HPLC allows subsequent characterization of each isomer by NMR and CD spectroscopy. The spectroscopic data indicates that each isomer contains a high degree of  $\alpha$ -helical structure. Isolated isomers are able to slowly interconvert to an equilibrium mixture of all isomeric forms within a few hours. The authors conclude that each isomer is energetically similar and that this multiple collection of conformers resembles the molten globule state of a protein where the secondary structural elements are defined and the hydrophobic regions are interacting but are not packed into well-defined structure.<sup>64</sup>

Ghadiri and co-workers reported the complexation of bipyridine-functionalized amphiphilic peptides to Ni(II), Co(II), and exchange inert Ru(II) resulting in the folding of a stable 45-residue parallel three-helix bundle (Figure 5).<sup>61</sup> Size exclusion chromatography data is consistent with the formation of a trimer which does not self-associate. While the kinetically labile Ni(II) and Co(II) complexes could not be detected by FAB-MS, the kinetically inert Ru(II) trimer complex was amenable to observation by mass spectrometry. The use of kinetically inert complexes was a creative advance that facilitated more rigorous characterization of these complexes. The CD spectrum of an aqueous solution of the bipyridine-functionalized peptide (Figure 5c) at pH 6.4 in the absence of transition metal was consistent with partial helical structure. However, upon addition of

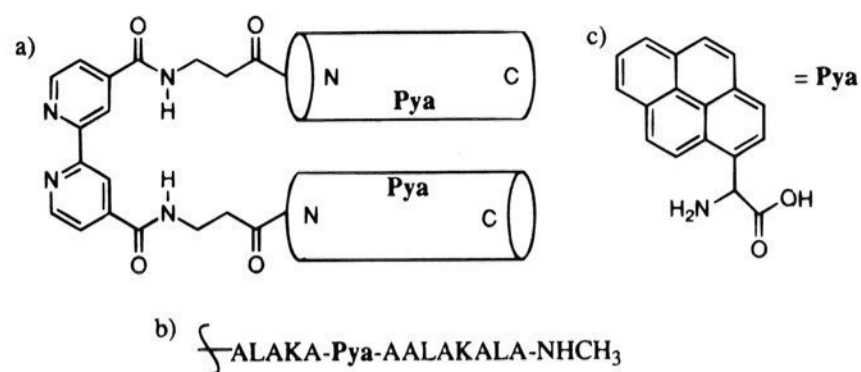




**Figure 7.** (a) The Ru(II)-directed folding of a three-helix bundle resulting in the formation of a moderate affinity type II copper binding site, and (b) the sequence of the bipyridine-functionalized amphiphilic peptide strand which contains a copper-binding histidine residue near the carboxy terminus.

transition metal ions, the CD signal increased by 40%, indicating that metal binding stabilizes the peptides in the desired three-helix bundle structure.

Metal-directed folding has also proven effective for the construction of binding sites in synthetic proteins. Ghadiri demonstrated that it is possible to construct a type II copper binding site in a synthetic protein by elaborating on the three-helix bundle Ru(II)-metalloprotein described above (Figure 7a).<sup>65</sup> A histidine residue was introduced into the sequence of the bipyridine-functionalized peptide near the C-terminus shown in Figure 7b. Histidine residues are commonly found in natural copper-containing proteins and coordinate Cu(II) through their imidazole side chains.<sup>66</sup> A tyrosine residue was also incorporated into the peptide sequence adjacent to the histidine as a spectroscopic probe to monitor Cu(II) binding. The fluorescence of tyrosine is known to be quenched efficiently by bound copper in a distance-dependent fashion. Ruthenium complexation by the bipyridine functionalities occurs with high chemoselectivity resulting in the formation of an exchange inert Ru(II)-three-helix bundle metalloprotein. Due to the metal-mediated folding process, the three histidines are positioned such that a moderate affinity type-II copper binding site is created at the C-terminus of the parallel helix bundle (Figure 7a). By monitoring the quenching of tyrosine fluorescence as a function of added copper, the authors conclude that the Ru(II)-templated metalloprotein is capable of binding Cu(II) with an apparent  $K_d$  of 0.30  $\mu\text{M}$ . Electrospray mass spectroscopy provides additional evidence for the formation of the desired heterodinuclear metalloprotein. A variant Ru(II)-metalloprotein where the histidine residues are replaced with alanine residues was prepared as a control to demonstrate that the quenching of tyrosine fluorescence is a result of copper binding.

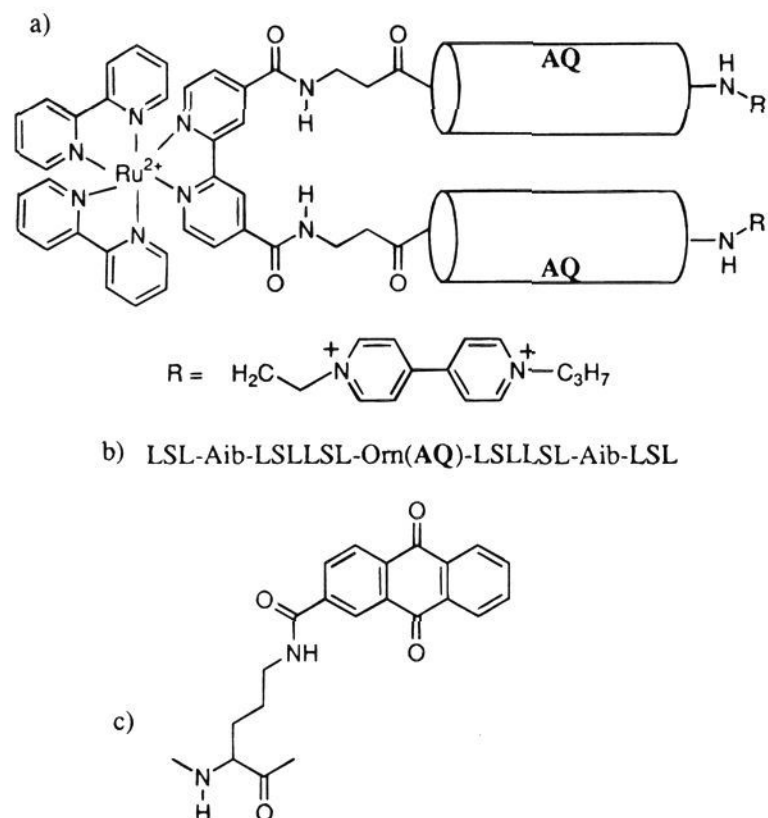


**Figure 8.** (a) Two-helix bundle formation facilitated by a bipyridine-based template, (b) amphiphilic sequence of the attached peptide, incorporating a 1-pyrenylalanine-based (Pya) residue, and (c) chemical structure of the Pya spectroscopic probe used to investigate the three-dimensional structure of the two-helix bundle.

The helicity of the copper-free Ru(II)-metalloprotein does not increase upon the addition of Cu(II) as discerned by CD spectroscopy. This suggests that the Ru(II)-metalloprotein provides a preorganized Cu(II) binding site, which according to UV-vis and EPR data, is capable of accommodating Cu(II) ions in either a square planar or tetragonal geometry. As an added bonus, guanidine hydrochloride denaturation experiments suggest that the structural stability of the Ru(II)-metalloprotein is further enhanced upon Cu(II) binding. The  $\Delta G_{\text{H}_2\text{O}}$  of the heterodimer metalloprotein is 3.5 kcal mol<sup>-1</sup>, which is 1.5 kcal mol<sup>-1</sup> greater than the copper-free Ru(II)-metalloprotein. This enhancement is presumably a result of the three-helix bundle being constrained at both its N-terminus by exchange inert Ru(II) complexation and at its C-terminus by exchange labile Cu(II) ion.

Nishino and co-workers have taken advantage of a bipyridine-based template functionalized at the 4- and 4'-positions with amphiphilic peptides to direct the folding of a two-helix bundle as depicted in Figure 8a.<sup>67</sup> In addition to the incorporation of the bipyridine-based residue, the  $\alpha$ -helical sequence (Figure 8b) also incorporates a 1-pyrenylalanine residue (Figure 8c) which is used to report on the three-dimensional structure of the resulting two-helix bundle using circular dichroism and fluorescence spectroscopy. The far-UV CD spectra of the two-helix bundle in water is indicative of a helical structure. The near-UV CD signal resulting from the interacting pyrene functionalities suggest that these chromophores are packed in the interior of the helical bundle in close proximity to one another. The fluorescence data also show that the two-helix bundle is denatured upon addition of methanol. The authors suggest that in aqueous solution, the bipyridine-based template directs the folding of the attached peptides such that a two-helix bundle is formed intramolecularly where the individual helices are arranged together with a right handed twist, exhibiting an angle between the helices of  $\sim 20^\circ$ .

An extension of this approach leads to the supersecondary structure depicted in Figure 9a, which was designed to penetrate lipid bilayers and act as an electron-transport system.<sup>68</sup> This supersecondary structure incorporates a potential photoinduced electron transfer triad composed of a ruthenium complex, an anthraquinone side chain (Figure 9b, c), and a viologen group. The CD spectra of this electron transfer synthetic protein in methanol and in sodium



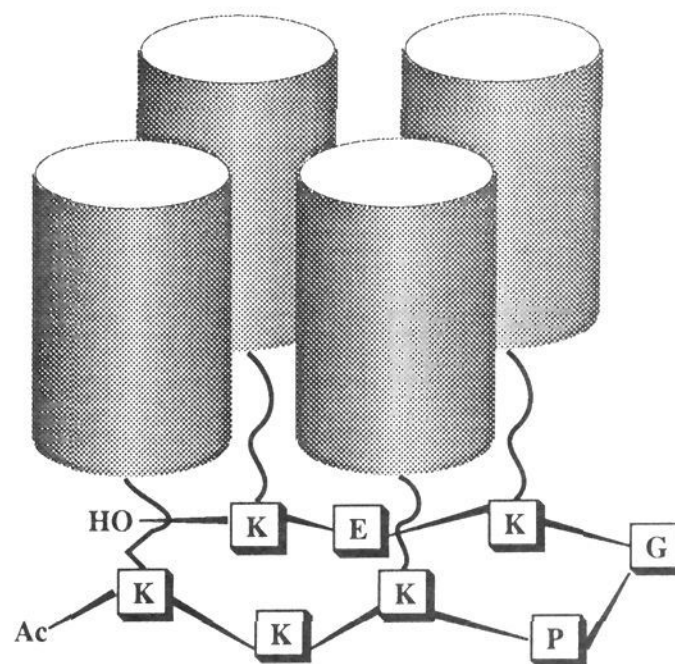
**Figure 9.** (a) A two-helix bundle whose folding is directed by a ruthenium-trisbipyridine complex (the structure incorporates a photo-induced electron-transfer triad consisting of a ruthenium complex, anthraquinone, and viologen functionalities), (b) the amphiphilic sequence, containing an anthraquinone-functionalized ornithine, designed to penetrate lipid bilayers, and (c) chemical structure of the anthraquinone-functionalized ornithine.

dodecyl sulfate micelles is indicative of an  $\alpha$ -helical structure. The synthetic protein was introduced into egg yolk lecithin vesicles for subsequent conformational and electron transport studies. Circular dichroism studies indicate that the structure of the synthetic protein embedded in lecithin vesicles is nonhelical and electron transport studies show that it is not an effective electron transport system, which is likely to be a result of its undefined conformation in the lipid bilayer.

#### D. Template-Assembled Synthetic Protein Approach

Another method for lowering the conformational entropy of the unordered state relative to the folded state of the desired structure involves using small cyclic and acyclic peptides as templates for assembling supersecondary and tertiary structures. Mutter and co-workers have shown that the folding of unordered peptide strands into a desired supersecondary structure can be accomplished by attaching peptide sequences to cyclic and acyclic templates through the side chains of the template.<sup>69-74</sup> The template-assembled synthetic protein (TASP) approach has been used to construct several related helical structures. The formation of a four-helix bundle depicted in Figure 10 is one example of the utility of this approach.

A common feature of the templates utilized in the TASP approach is the incorporation of lysine residues in the template sequence. Peptides are typically covalently attached at their carboxy termini to the  $\epsilon$ -amino groups of lysine residues in the template (Figures 10 and 11). Acyclic peptide-based templates containing a -Pro-Gly- dipeptide fragment are proposed to exist in a low-energy  $\beta$ -hairpin conformation


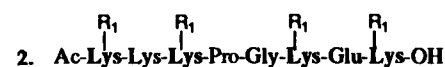

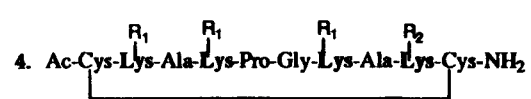
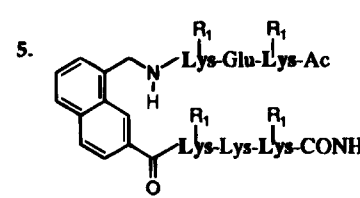
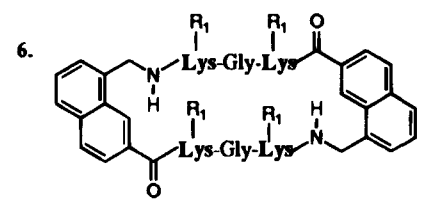


**Figure 10.** Schematic representation of a template-assembled synthetic protein (TASP). In this case, the folding of a four-helix bundle is directed via the attachment of four amphiphilic peptides to an acyclic peptide-based template. The peptides are covalently attached at their carboxy terminus to the  $\epsilon$  nitrogens of template lysine side chains.

with the -Pro-Gly- fragment serving as the  $i + 1$  and  $i + 2$  residues of a type II  $\beta$ -turn.<sup>75-78</sup> The structure of the template as depicted in Figure 10 positions the lysine side chains perpendicular to the plane of the template and is expected to preorganize the structure to direct the folding of attached peptide sequences. In an effort to further constrain the conformational mobility of the template, cyclic peptide-based templates were synthesized which incorporate disulfide bonds<sup>79-83</sup> as well as conformationally restricted nonnatural amino acids.<sup>84-86</sup> It has been shown that four-helix bundles resulting from conformationally constrained cyclic templates are more stable than those resulting from acyclic templates.<sup>87</sup> The molecular weight of these TASP assembled proteins has been confirmed by size exclusion chromatography and in some cases plasma desorption and laser desorption mass spectroscopy. Circular dichroism spectroscopy along with a limited amount of NMR spectroscopy has been used to demonstrate the structural integrity of these template-assembled synthetic proteins.

Tomich and Montal have used the TASP approach to synthesize synthetic pore proteins (synporins) by attaching four 23-residue peptides to an acyclic template (Figure 11, template 3).<sup>78,88</sup> The sequence of these four peptides, -EKMSTAISVLLANAV-FLLLTSNR-, is based on a segment of the *Torpedo californica* acetylcholine receptor (AcChoR)  $\delta$  subunit. AcChoR is a pentamer composed of four glycoprotein subunits ( $\alpha_2\beta\gamma\delta$ ) which forms a ligand-activated transmembrane cation channel. A 23-residue segment of the  $\delta$  subunit alone (sequence shown above) is capable of forming cation-selective channels in lipid bilayers via the putative self-assembly of conductive oligomers. However, controlling the aggregation state of the peptides which is essential in the formation of discrete conductive oligomers is difficult. By appending four of these peptide segments to a template, Tomich and Montal can effectively control the aggregation number of the peptide segments



Template	Sequence attached to template	Structural Type	Reference
1. 	$R_1 = \text{AELE-Aib-LEA-Aib-LAE-Ac}$ $R_2 = \text{VTVTVT-Ac}$	$\beta$ -sheet / Helix	69, 70
2. 	a) $R_1 = \text{GLKK-Aib-AN-Aib-AAT-Aib-AD-NH}_2$ b) $R_1 = \text{LKLANALATAAD-NH}_2$	4-Helix Bundle 4-Helix Bundle	80 68, 69
3. 	a) $R_1 = \text{RQSTLLLFVAQALLVSIATSMKE-NH}_2$ b) $R_1 = \text{RQSTLLLFVAQALLVAIATSMKE-NH}_2$ c) $R_1 = \text{YFALSALFSILVCPTIFNIVYFL-NH}_2$ d) $R_1 = \text{ESLIVDIISGIVILFDFVNWPD-NH}_2$ e) $R_1 = \text{KGF MQMGIVAYIFFLMVILLAVY-NH}_2$	4-Helix Bundle 4-Helix Bundle 4-Helix Bundle 4-Helix Bundle 4-Helix Bundle	71 71 71 81 81
4. 	a) $R_1=R_2 = \text{GLKALAEKLAKELAE-NH}_2$ b) $R_1 = \text{GLKALAEKLAKELAE-NH}_2$ $R_2 = \text{GLEAMAKEFAEKLAKL-NH}_2$ c) $R_1=R_2 = \text{GLKALAEKLAKELAELE-NH}_2$ d) $R_1=R_2 = \text{GTQLLAKAKRTAGDLLE-Ac}$	4-Helix Bundle 4-Helix Bundle 4-Helix Bundle 4-Helix Bundle	74, 75, 76 73, 76 75 72, 73
5. 	$R_1 = \text{GLKK-Aib-AN-Aib-AAT-Aib-AD-NH}_2$	4-Helix Bundle	80
6. 	$R_1 = \text{GLKK-Aib-AN-Aib-AAT-Aib-AD-NH}_2$	4-Helix Bundle	73, 77, 79, 80

**Figure 11.** Acyclic and cyclic peptide-based templates where the lysine attachment sites are shown in bold. Also, the sequences of attached peptide strands are shown

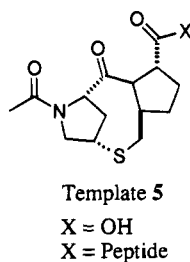
along with the resulting supersecondary structure after template-assisted folding takes place (sequences are written left to right from the carboxy to amino terminus).



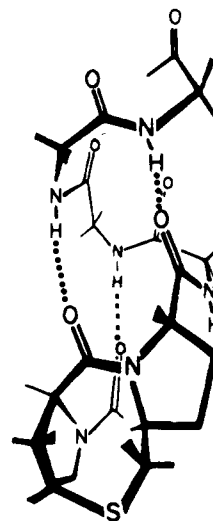
forming the cation selective channel. The resulting 101 residue synporin composed of an acyclic TASP peptide forms a four-helix bundle that inserts into lipid bilayers to form an ion channel that is specific for cations. Transitions between the closed and open states occur on the millisecond time scale. A derivative of the local anesthetic lidocaine (Qx-222) was shown to block the flow of current through the synporin, just as it does with native AcCohR channels.<sup>78</sup>

### E. Nucleating Helix Mimics

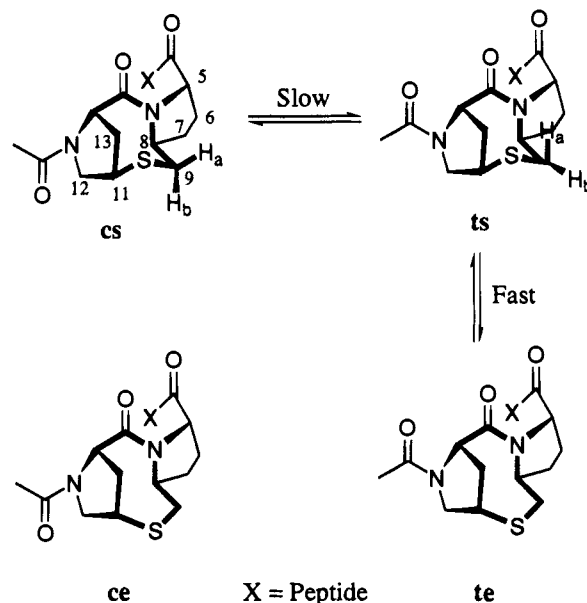
The  $\alpha$ -helical-promoting templates discussed thus far have mainly been used to control the aggregation state of helical bundles. The isolated amphiphilic peptides attached to these templates have a propensity to self-assemble into helical bundles and usually adopt a helical bundle structure in the absence of the template. The templates do contribute to the folding free energy changes in that they lower the conformational entropy of the unordered state; however, self-assembly of the amphiphilic structures provides a significant driving force for folding owing to the hydrophobic effect. Kemp and co-workers have developed a novel helix nucleator which promotes the folding of an attached polypeptide sequence affording a monomeric  $\alpha$ -helix in aqueous solution.<sup>7</sup> The Kemp N-terminal helix template (**5**) based on (2*S*,5*S*,8*S*,11*S*)-1-acetyl-1,4-diaza-3-keto-5-carboxy-10-thia-tricyclo[2.8.1.0]tridecane has proven useful to evaluate the Zimm–Bragg parameters which characterize helix propagation.<sup>89,90</sup>



Template **5**, when covalently attached to the N-terminus of a peptide sequence operates as an  $\alpha$ -helical nucleator by positioning its three carbonyl functionalities in an orientation mimicking the pitch and spacing of a right-handed  $\alpha$ -helix.<sup>91,92</sup> The oriented carbonyl groups of the template are capable of forming hydrogen bonds with the amide NH protons of the attached  $\alpha$ -amino acid sequence (Figure 12). The capacity of template **5** to function is dependent on its ability to adopt a nucleation-competent conformation in aqueous solution. NMR studies indicate that **5** can exist in four possible conformations (Figure 13).<sup>93</sup> A crystal structure of the free acid of **5** indicates that it adopts a conformation in the solid state in which the acetamide group is in a cis conformation (c) and the sulfur atom is in an “up” orientation (s) relative to the template molecule affording the (cs) conformer. This conformer is incapable of propagating helix structure because the acetamide carbonyl is unavailable for hydrogen bonding to the attached peptide sequence. Also, conformational analysis by NMR in  $\text{CDCl}_3$  shows that **5**



**Figure 12.** A template **5** nucleated  $\alpha$ -helix where the template is in a nucleation-competent conformation with the orientation of its three carbonyl groups mimicking the pitch and spacing of those found in a right-handed  $\alpha$ -helix. These carbonyls serve as hydrogen-bond acceptors for the amide protons of the attached peptide strand.



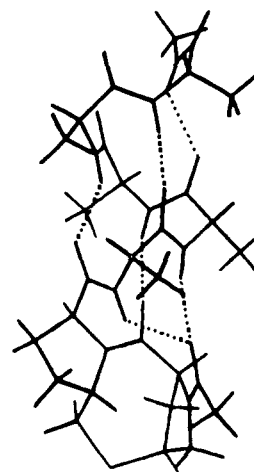
**Figure 13.** The four possible conformations of template **5** in aqueous solution. The (cs) conformer which is unable to nucleate helical structure, due to the cis orientation of the acetamide carbonyl, is in slow equilibrium with the (ts) conformer which is also unable to support structure. However, the (ts) conformer is in fast equilibrium with the (te) conformer, where the carbonyls are positioned such that hydrogen bonds can form with the amide protons of an attached peptide strand therefore nucleating helix formation. The (ce) conformer has not been detected and would be expected to be nonnucleating since the acetamide carbonyl is in a cis orientation.

exists in the (cs) conformation, but in other solvents such as DMF, a slow equilibrium on the NMR time scale exists between the (cs) and the (ts) conformers. These conformers are identical except that the acetamide group is now in a trans orientation. The authors indicate that the (ts) conformer is also incapable of propagating helix formation because the carbonyls are not properly arranged to form stable hydrogen bonds with the attached peptide (not

evident from the figure). However, the (ts) conformer is in rapid equilibrium on the NMR time scale with the (te) conformer. In this conformation, the acetamide group is in a trans orientation (t) and the sulfur atom is in a down position (e) relative to the template molecule affording the (te) conformer. Molecular mechanics suggests that the (te) conformer is slightly less stable than the (ts) conformer. However, the carbonyls are positioned such that stable hydrogen bonds can form with the amide protons of an attached peptide strand resulting in the formation of helical structure. The (ce) conformer (Figure 13), which has not been observed, would be incapable of helix propagation due to the cis orientation of the acetamide functionality.

Template **5** when appended to peptide strands not only provides a structural framework capable of propagating helix structure, but also serves as a spectroscopic reporter of helix formation. The detection of helical structure can be accomplished in two ways: (1) Since both cis conformers [i.e. (cs) and (ce)] are unable to support helical structure and only the trans (te) conformer nucleates helix formation, the (trans/cis) ratio of **5** should be directly proportional to the extent of helix formation. This ratio is determined by monitoring the relative shifts of the protons located at C-12 which are influenced by the orientation of the acetamide functionality (Figure 13). (2) The shift in equilibrium between the trans non-helical (ts) and helical (te) conformers can be detected by monitoring the relative shifts of the C-9 and C-13 protons as well as changes in the vicinal coupling constant at the C-8,C-9 bond. In addition, when template **5** adopts primarily the (te) conformation, an NOE between a proton at C-13 and C-9-H<sub>a</sub> is observed (Figure 13).

The (2*S*,5*S*,8*S*,11*S*)-1-acetyl-1,4-diaza-3-keto-5-carboxy-10-thiatricyclo[2.8.1.0]tridecane template was incorporated into the peptide derivatives **5**-(L-Ala)<sub>*n*</sub>-OtBu where *n* = 1–6 and **5**-Sar-(L-Ala)<sub>*n*</sub>-OtBu where *n* = 1–4 (Sar is an abbreviation for sarcosine, i.e. *N*-methylglycine). NMR studies of **5**-(L-Ala)<sub>*n*</sub>-OtBu (*n* = 1–6) in CDCl<sub>3</sub> or CD<sub>3</sub>CN indicate that the (trans/cis) ratio increases with increasing chain length which suggests a significant length dependence on helical stability. Also, NMR data demonstrates that under these conditions any **5** in which the acetamide group is in the trans orientation, exists primarily as the helical promoting (te) conformer. NMR studies of **5**-Sar-(L-Ala)<sub>*n*</sub>-OtBu (*n* = 1–4) in CDCl<sub>3</sub> indicate that there is no apparent length dependence on the (trans/cis) ratio with an increase in chain length and that template **5** is locked in the nonnucleating (cs) conformation. These results indicate that in nonpolar solvents, the ability of **5** to nucleate  $\alpha$ -helical structure is dependent both on chain length and the amino acid at the template-helix interface. Residues like Sar which do not have the capability to hydrogen bond to the template prevent template-induced nucleation. NMR studies of **5**-(L-Ala)<sub>*n*</sub>-OtBu (*n* = 1–6) in hydrogen-bonding solvents such as DMF and water, show that the (trans/cis) ratio is effected less by chain length and that the template never fully adopts either of the trans conformations [i.e. (ts) or (te)]. Also, any **5** in



**Figure 14.** A line drawing of a template-nucleated helix. The template is the (te) conformation and the nucleated helix adopts a structure described by the authors as a  $3_{10}$ - $\alpha$ -helix hybrid where first two residues adjacent to template **5** forms a bifurcated hydrogen bond with the acetamide carbonyl.

which the acetamide group does adopt a trans conformation, exists mainly in the nonnucleating (ts) conformation.

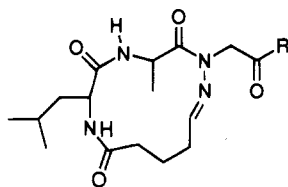
The conformation of the peptide portion of these derivatives was investigated by solvent- and temperature-dependent NMR spectroscopy which indicate that under conditions where template **5** exists in the (cs) conformation, the attached peptide sequence adopts an ensemble of random coil states. However, intrahelical and interhelical template NOEs consistent with a template-nucleated helix structure are observed in CDCl<sub>3</sub> and DMSO when template **5** exists in the (te) conformation. The authors suggest that the conformation of the helix is best thought of as a hybrid of a  $3_{10}$  and an  $\alpha$ -helix where bifurcated hydrogen bonding exists between the acetamide carbonyl and the amide protons of the first two residues (Figure 14). This structural deviation from a classical  $\alpha$ -helix is not unexpected in that structural variety within the helix motif is sometimes observed in helices composed of short sequences. The structural stability of the resulting nucleated-helices was also investigated by NMR and was found to be dependent on solvent conditions. In relatively nonpolar solvents such as CDCl<sub>3</sub> or CD<sub>3</sub>CN, the helices were very stable and little conformational fraying was observed. However, in aprotic dipolar solvents such as DMF or DMSO, the helices formed are relatively unstable and mobile.

Classical equilibrium models for helix folding dictate that the folding of a peptide existing as an ensemble of random conformations into a structurally well-defined helix mainly involves two parameters.<sup>94</sup> The first is an initiation parameter,  $\sigma$ , which measures the difficulty in forming a nucleating-competent conformation capable of helix propagation. The second is a propagation parameter, (*s*), which is an equilibrium constant that measures the ease of adding an unordered residue to the growing helix. Earlier observations suggest that (*s*) is dependent on the relative strengths of hydrogen bonds formed between amide functional groups within the peptide and those formed between the residues and compet-

ing solvent molecules. The (*s*) values reported for residues in helical peptides have considerable variability in large part because this parameter can be influenced by the initiation parameter,  $\sigma$ . The initiation parameter, in turn, is thought to be dependent on the sequence of the amino acid residues defining the nucleation site. However, isolation of these two parameters is made possible through the use of template **5** and offers a unique opportunity to measure the true propagational propensities [(*s*) values] of individual amino acid residues.

Peptides derivatives incorporating **5** were synthesized to evaluate the (*s*) parameter for the amino acid alanine.<sup>95</sup> Peptides **5**-(L-Ala)<sub>*n*</sub>-Lys-NH<sub>2</sub> (*n* = 3–8) and **5**-Gly-(L-Ala)<sub>*n*</sub>-Lys-NH<sub>2</sub> (*n* = 2–8) were prepared incorporating a C-terminal lysine residue which aids in solubility and allows the experiments to be conducted in water. Both peptide derivatives show a length-dependent increase in helical structure as expected. Using classical helix-coil transition analysis, Kemp utilizes this length dependence to calculate (*s*) values for alanine. Analysis of both peptide derivatives yields an (*s*) value of 0.95, supporting earlier reports of 1.06 reported by Scheraga.<sup>96</sup>

Satterthwait and co-workers have also developed a helix nucleator which promotes the folding of an attached polypeptide chain affording a monomeric  $\alpha$ -helix in deuterated trifluoroethanol.<sup>97</sup> The following cyclic template

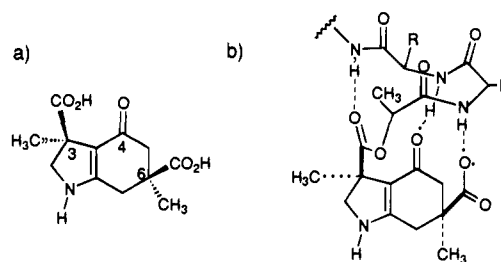


Hydrazone-ethylene based cyclic template  
R = -NH-L-alanine-(L-glutamic acid- $\gamma$ -ethyl ester)<sub>4</sub>-ethyl ester-NH<sub>2</sub>

is designed to mimic one turn of an  $\alpha$ -helix and incorporates a hydrazone-ethylene bridge (N–N=CH–CH<sub>2</sub>–CH<sub>2</sub>) as a covalent replacement for a backbone hydrogen bond (N–H...O=CR–NH) between the *i* and *i* + 4 residues of an  $\alpha$ -helix. Structural studies on the template alone employing NMR spectroscopy suggest that the template adopts a “relaxed helical turn” conformation where the carbonyl oxygen atoms are located on the same face of the template but lack the pitch of a naturally occurring  $\alpha$ -helical turn. NMR studies on a pentapeptide attached to the template suggest that the template serves as a nucleation site for  $\alpha$ -helix formation in the pentapeptide portion. However, further studies are needed to define the interactions between the attached peptide strand and the cyclic template as well as the conformation of the template when incorporated into a peptide sequence.

Bartlett and co-workers have reported four diastereomers of a hexahydroindol-4-one diacid template shown below which was designed as a hydrogen-bonding scaffolding for the nucleation of  $\alpha$ -helical structure in attached  $\alpha$ -amino acid sequences.<sup>134</sup> Peptides are linked at their N-terminus via an amide bond to L-lactic acid which is attached to the carboxyl at position 3 of the template employing an ester

linkage. The carboxylate at C-6, the carbonyl of the vinylogous amide and the ester carbonyl at C-3 are designed to mimic the pitch and orientation of three carbonyls in an  $\alpha$ -helical conformation. In addition, the carboxylate at C-6 should electrostatically stabilize the helix by interacting with the helix dipole. The *S,S* diastereomer of the template induces approximately 50% helical character in the peptide -Glu-Ala-Leu-Ala-Lys-Ala-NH<sub>2</sub> linked as a C-3 ester through the L-lactic residue in aqueous solution at 0 °C as discerned by far-UV CD. High-resolution structural studies should provide further insight into the mechanism of helix induction.



a) (*s-s*)  $\alpha$ -helical template b) intended conformation as  $\alpha$ -helix template

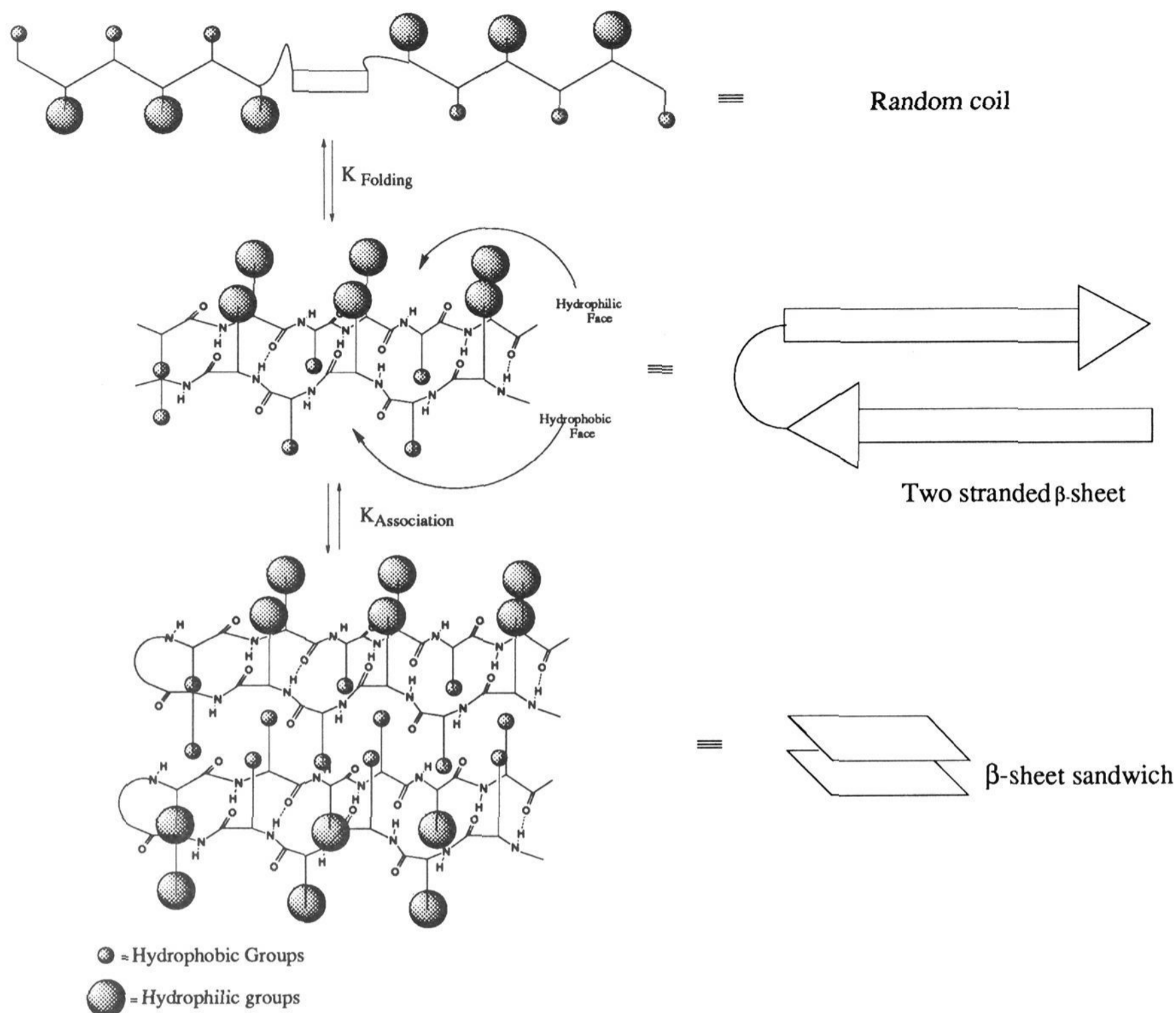
### III. Templates That Induce $\beta$ -Sheet Folding

#### A. Nucleating Strand Mimics

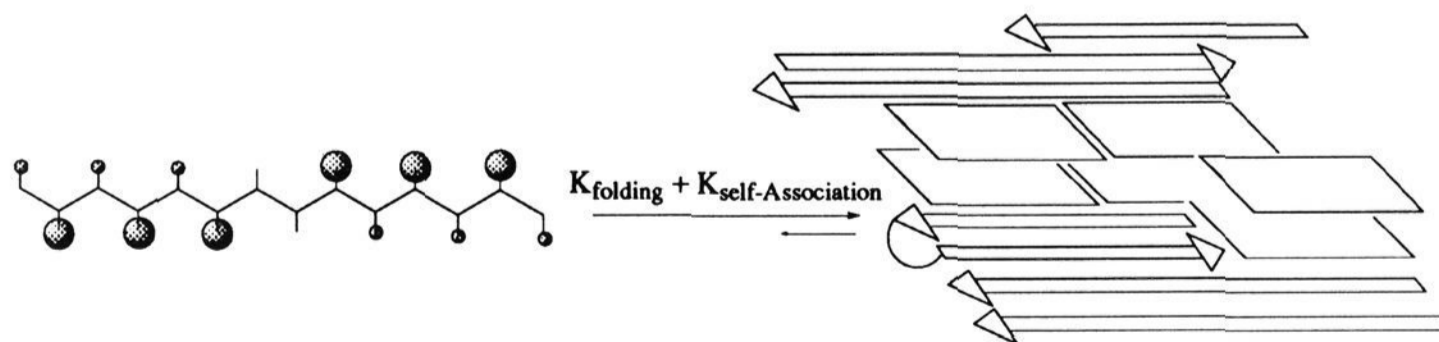
Results from the literature indicate that consensus  $\beta$ -turn sequences and  $\beta$ -turn mimetics which promote chain reversal and intramolecular hydrogen bonding are not sufficient to nucleate  $\beta$ -sheet formation in sequences that are known to adopt  $\beta$ -sheet structure.<sup>98–103</sup> These failures, while not definitive evidence, indicate that local conformational propensities are only part of what is required to achieve  $\beta$ -sheet folding. The folding of peptide fragments into  $\beta$ -sheet structure has been accomplished in our laboratory by using  $\beta$ -turn mimics which promote favorable hydrophobic interactions between the  $\beta$ -turn mimetic and the attached peptide strands in addition to promoting chain reversal and intramolecular hydrogen bonding.<sup>104,105</sup> The hypothesis that tertiary interactions are also required for  $\beta$ -sheet formation is consistent with recent work from the Berg, Kim, and Regan laboratories which indicate that the thermodynamic propensity of a given residue to adopt a  $\beta$ -sheet structure is much more context dependent than analogous  $\alpha$ -helical propensities.<sup>106–109</sup> This context dependence suggests that a  $\beta$ -sheet structure may be best thought of as having elements of both secondary and tertiary structure particularly in the case of sheets having complex topologies. In other words, long-range residue–residue side chain interactions appear to be much more important in sheet folding than in  $\alpha$ -helix formation. Also, emerging protein biophysical data suggest that the folding of predominantly  $\beta$ -sheet proteins is slow relative to predominantly helical folds consistent with rate-limiting tertiary structure formation.<sup>98,110–15</sup> Moreover, it is well established that intermolecular  $\beta$ -sheet formation is a common motif by which proteins form well-defined quaternary structures, further empha-



### A. Ideally, Intramolecular Folding Precedes Self-Association Affording a Homogenous $\beta$ -Sheet Structure



### B. Often Folding and Self-Association Have Comparable Rates Which Leads to a Heterogeneous $\beta$ -Sheet

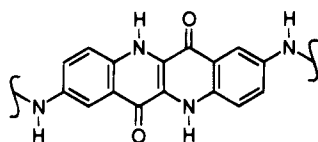


**Figure 15.** The folding of small peptides into structurally well-defined  $\beta$ -sheet motifs is often hampered by competitive intramolecular folding and self-assembly which leads to the formation of heterogeneous high molecular weight  $\beta$ -sheet quaternary structures.

sizing the importance of long-range interactions in sheet formation. These observations suggest that helices and sheets fold differently. For the sake of this discussion we will refer to  $\beta$ -sheets as either tertiary or quaternary structures.

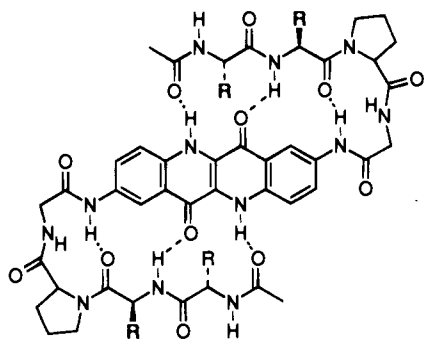
Model  $\beta$ -sheet systems have proven very difficult to prepare in part because their folding is complex and in part because they have a propensity to self-

associate into high molecular weight  $\beta$ -sheet quaternary structures (Figure 15). Therefore, the incorporation of a template into an  $\alpha$ -amino acid sequence to induce intramolecular  $\beta$ -sheet folding is appealing. The utility of templates to nucleate  $\beta$ -sheet folding was first demonstrated by Kemp and his colleagues by incorporating an epindolidione skeleton into a nonapeptide sequence.<sup>116,117</sup>

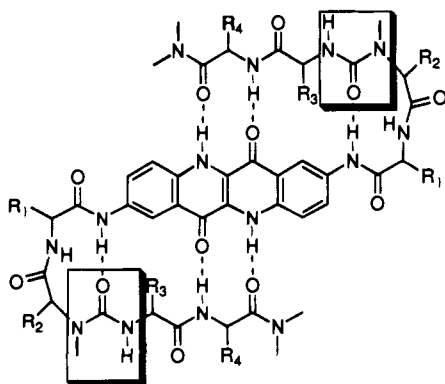


Epindolidione Skeleton

The rigid epindolidione template mimics the central strand of a  $\beta$ -sheet by appropriately orienting three hydrogen bonds to enforce an extended conformation on the attached peptide chains. The strand-strand side chain interactions which typically stabilize a  $\beta$ -sheet structure are not possible in this system due to the planarity of the epindolidione central strand mimic. To enhance the likelihood of  $\beta$ -sheet nucleation in the peptide chimera, the Kemp group also employed sequences such as Pro-D-Ala in the turn region proximal to the epindolidione skeleton to favor the reverse turn conformation. A chain reversal in this portion of the molecule is critical for  $\beta$ -sheet nucleation by the epindolidione skeleton. The attachment of  $\alpha$ -amino acid sequences directly to the amino functional groups of the skeleton leads to the formation of a parallel  $\beta$ -sheet mimic (Figure 16). The Kemp group also demonstrated that antiparallel  $\beta$ -sheets models could be prepared by the incorporation of two urea moieties into the peptidomimetic (Figure 17).<sup>118</sup> The parallel  $\beta$ -sheet model system proved to be monomeric in DMSO and adopts a well-defined  $\beta$ -sheet conformation as discerned from the



**Figure 16.** The attachment of peptide strands to the epindolidione amino groups affords a parallel  $\beta$ -sheet mimic. Sequences such as Pro-D-Ala are often incorporated proximal to the epindolidione skeleton in order to enforce a reverse turn conformation on the attached peptide which is critical for  $\beta$ -sheet formation.

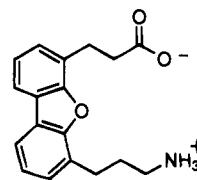


**Figure 17.** The incorporation of two urea moieties (high-lighted) into the peptide portion of the  $\beta$ -sheet mimetic affords an epindolidione-directed antiparallel  $\beta$ -sheet mimic.

temperature dependence of the amide NH chemical shifts, the  $^3J_{\text{CH}_\alpha\text{-NH}}$  coupling constants which estimate the  $\phi$  dihedral angle and the observation of NOEs characteristic of a  $\beta$ -sheet. This study demonstrates that the epindolidione strand mimic/nucleator in combination with consensus  $\beta$ -turn sequences together are sufficient to achieve  $\beta$ -sheet folding in DMSO. The importance of both the turn sequence and the strand mimic supports the hypothesis that both local conformational propensities and long-range interactions are required for  $\beta$ -sheet nucleation. Kemp and co-workers have recently completed an extensive study on water-soluble epindolidione-tetrapeptide conjugates which adopt an antiparallel  $\beta$ -sheet conformation both in the solid state and in solution.<sup>119</sup> This elegant work demonstrates that templated  $\beta$ -sheet nucleation is achievable and has set the stage for further developments in this area.

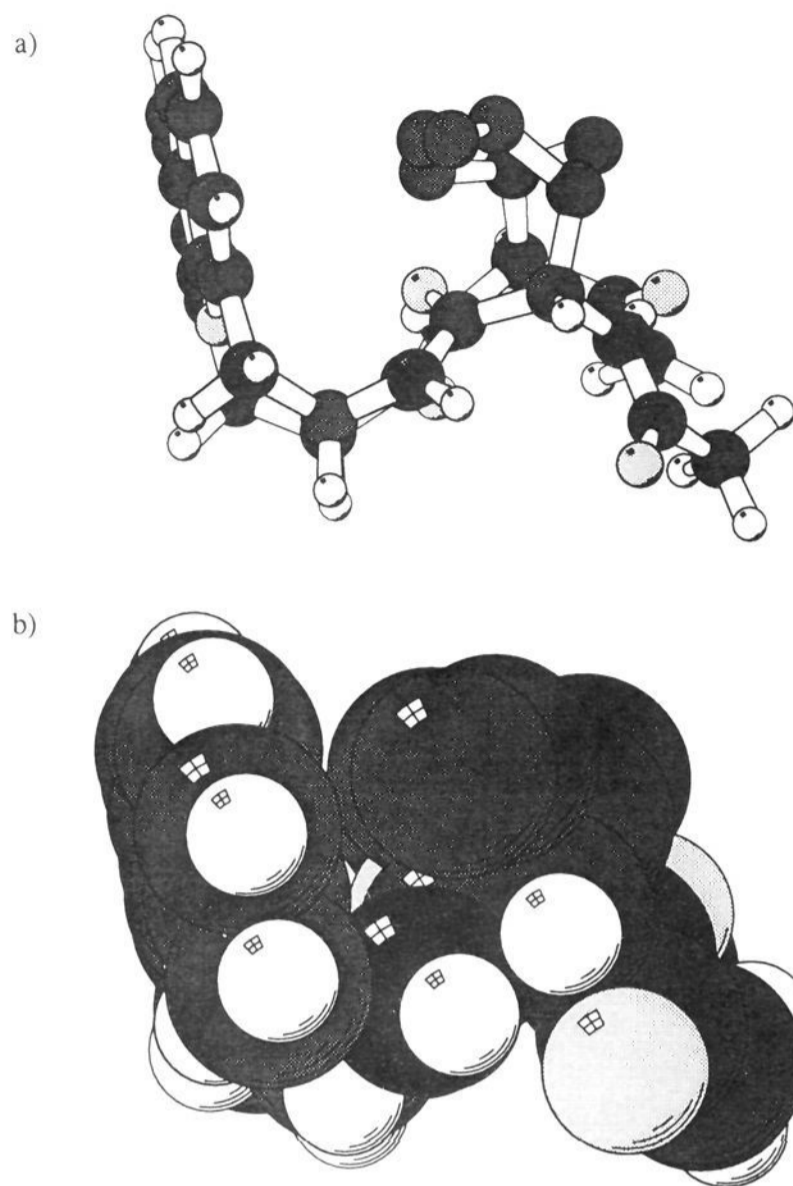
## B. Nucleating Turn Mimics

Our laboratory has approached the challenge of achieving  $\beta$ -sheet folding in relatively small peptides using a strategy that is complementary to the Kemp approach. Instead of employing a  $\beta$ -strand mimic/template as a nucleator, we have prepared a  $\beta$ -turn template that replaces the  $i + 1$  and  $i + 2$  residues of a  $\beta$ -turn. We have not attempted to prepare  $\beta$ -turn mimics which resemble authentic  $\beta$ -turns, instead we have concentrated on molecules that will reverse the peptide chain, promote intramolecular hydrogen bonding and mediate hydrophobic tertiary interactions which should promote  $\beta$ -sheet nucleation. The tertiary clustering of hydrophobic side chains during the early stages of protein folding (i.e. hydrophobic cluster formation) is emerging as one of the common themes by which a linear amino acid sequence can limit the large number of possible folding pathways to a few in order to ultimately arrive at the desired fold.<sup>120,121</sup> Therefore, it is reasonable to expect that a conformationally rigid turn mimic would be able to function as a sheet nucleator provided that intramolecular interactions in addition to hydrogen bonding were employed to propagate the conformational bias of the nucleator to the attached  $\alpha$ -amino acid sequence. The 4-(2-aminoethyl)-6-dibenzofuranpropionic acid residue (**6**) shown below was designed to facilitate intramolecular hydrogen bonding between the amides of the flanking  $\alpha$ -amino acid residues:<sup>122-124</sup>



4-(2-aminoethyl)-6-dibenzofuranpropionic acid template (**6**)

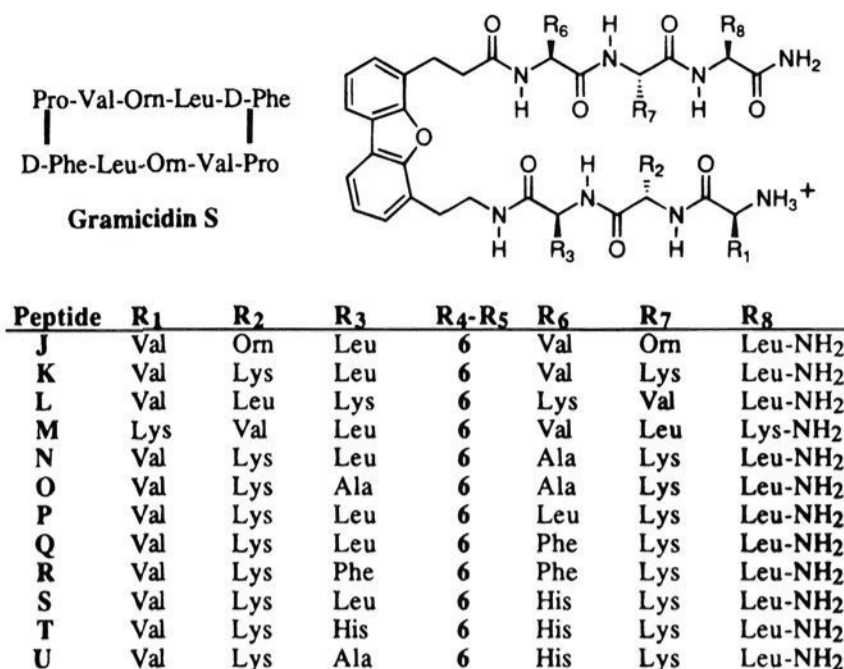
In addition, this unnatural amino acid replacement for the  $i + 1$  and  $i + 2$  residues of a  $\beta$ -turn can adopt a conformation that promotes the formation of a hydrophobic cluster composed of the hydrophobic side



**Figure 18.** The 4-(2-aminoethyl)-6-dibenzofuranpropionic acid residue adopts a hydrophobic cluster conformation in aqueous solution where favorable hydrophobic interactions exist between the dibenzofuran skeleton and the hydrophobic side chains of the  $\alpha$ -amino acid residues flanking the  $\beta$ -turn template: (a) Ball and stick representation of the hydrophobic cluster conformation clearly showing the perpendicular conformation of the ethyl fragments relative to the aromatic skeleton and (b) CPK representation of the hydrophobic cluster. Note that the van der Waals surfaces of the dibenzofuran skeleton and the flanking leucine residues pack tightly to form the cluster (protons have been left off the  $\alpha$ -amino acid side chains for clarity).

chains of the flanking  $\alpha$ -amino acids and the dibenzofuran skeleton.<sup>104</sup> The  $-\text{CH}_2\text{CH}_2-$  fragment that attaches the amino and carboxyl functional groups to the dibenzofuran skeleton allows this residue to adopt a "perpendicular conformation" where the dibenzofuran skeleton is oriented orthogonally to the plane of the  $\beta$ -sheet defined by the hydrogen bonds between the attached  $\alpha$ -amino acid residues. The "perpendicular conformation" adopted by residue **6** allows the side chains of flanking hydrophobic  $\alpha$ -amino acids to interact with the dibenzofuran skeleton (Figure 18). This hydrogen-bonded hydrophobic cluster is sufficient to nucleate a wide variety of sequences to fold into a  $\beta$ -sheet (hairpin) conformation in aqueous solution.

Acyclic analogs of the cyclic peptide gramicidin S were studied initially because gramicidin S adopts a well-defined  $\beta$ -sheet structure in the portion of the sequence between the  $\beta$ -turns. Linear gramicidin S analogs are prepared conceptually by excising one of the two D-Phe-Pro dipeptide units (i.e. the  $i + 1$  and  $i + 2$  residues of a  $\beta$ -turn) and replacing the other D-Phe-Pro fragment with residue **6** (Figure 19).<sup>104,123</sup>



**Figure 19.** Heptapeptides **J–U** incorporating the 4-(2-aminoethyl)-6-dibenzofuranpropionic acid residue (**6**) are conceptually based on the antibiotic gramicidin S where one of the turn regions (D-Phe-Pro) is excised and the other is replaced with template **6**; R =  $\alpha$ -amino acid side chain.

A series of linear heptapeptides incorporating the dibenzofuran-based residue were initially characterized by analytical equilibrium ultracentrifugation and by far- and near-UV circular dichroism spectroscopy. The heptapeptides are monomeric and exhibit far-UV CD spectra consistent with a mixture of random coil and  $\beta$ -sheet structure. Interestingly, those peptides which adopt a partial  $\beta$ -sheet structure by far-UV CD always exhibit a near-UV CD spectra implying that hydrophobic cluster formation is critical for a  $\beta$ -sheet structure nucleated by the dibenzofuran-based residue. In fact, it has been shown that residue **6** is capable of  $\beta$ -sheet nucleation only when flanked by hydrophobic amino acid residues (e.g. Val, Leu, Phe, etc.) or by a hydrophobic residue and a His residue having a charged imidazole side chain.<sup>125</sup> The His residue improves solubility and contributes to the stabilization of a nucleation competent cluster through a putative  $\pi$ -cation-like interaction. NMR studies show that the diastereotopic methyl groups on the flanking Val and Leu residues are shifted significantly upfield due to their placement in the ring current of the dibenzofuran skeleton in the hydrophobic cluster. In addition, characteristic NOEs are observed between the protons of the flanking  $\alpha$ -amino acid side chains and the aromatic protons on dibenzofuran skeleton composing the hydrophobic cluster. NMR characterization of heptapeptide **K** in acetate buffer at pH 4.5 using 2D-NMR methods (temperature coefficients,  $^3J_{\text{CH}_\alpha\text{-NH}}$  coupling constants, and NOEs) reveals a fluctuating  $\beta$ -sheet structure proximal to the dibenzofuran-based amino acid and disorder at the N- and C-termini of the peptide.<sup>104</sup> Disorder at the termini is expected in small isolated helices and sheets. Interestingly, these peptides do not fold cooperatively as discerned from linear circular dichroism changes as a function of denaturant concentration.

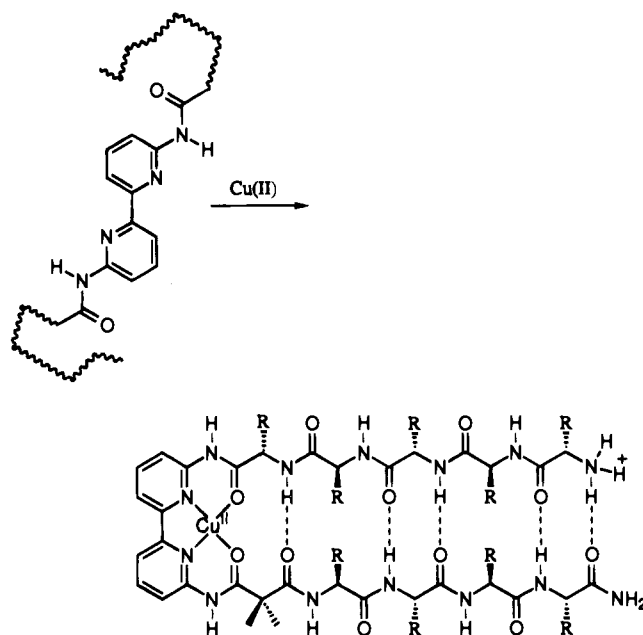
In an effort to produce a dibenzofuran-nucleated  $\beta$ -sheet that folds cooperatively and adopts a well-defined structure we have increased the length of the linear chain to 13 residues. The sequence chosen K-V-K-V-K-V-**6**-V-K-V-K-V-K-NH<sub>2</sub> (peptide **V**) has a



charge of +7 at pH 4.5 and as a result is not expected to fold. However, the hydrogen-bonded hydrophobic cluster composed of -V-6-V- is formed under these conditions according to the NMR and near-UV CD criteria described above. This cluster is poised and waiting to nucleate folding in the remainder of the peptide sequence once the solution conditions allow the strands to interact to form a two-stranded anti-parallel  $\beta$ -sheet. Conditions that allow strand collapse include increasing the ionic strength or pH of the medium, which attenuate or remove respectively, the unfavorable charge-charge repulsions between the strands allowing the  $\beta$ -sheet to fold. Analogs of peptide **V** having the  $\epsilon$ -amino groups of two lysine side chains trifluoroacetylated fold in a pH-independent fashion indicating that sequences having a charge density of  $< +5$  are capable of folding. The hydrophobic cluster in peptide **V** is present under all types of solution conditions, even in 8 M urea solution at pH 4, indicating how robust this nucleation site is. Similar hydrophobic clusters present in the 434-repressor are also stable in concentrated urea solutions and have been proposed to nucleate folding in this protein since the residues composing the cluster also interact in the folded structure.<sup>120,121</sup>

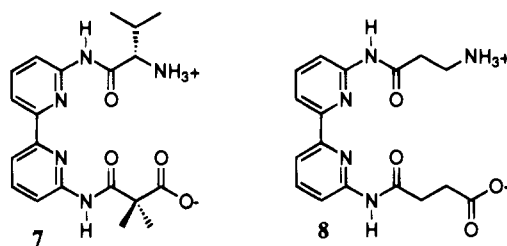
After intramolecular folding, peptide **V** dimerizes due to its amphiphilicity and laterally self-assembles into fibrils via intermolecular  $\beta$ -sheet interactions between the dimers. In these systems, the intramolecular folding and self-association equilibria are linked. This has made it impossible to find solution conditions which permit folding but which disfavor self-assembly. It cannot be stated with certainty that intramolecular folding precedes self-assembly in peptide **V** although recent success in uncoupling folding from self-assembly by incorporating two N-methylated amino acids in place of Lys residues in peptide **V** makes this likely. Interestingly, these peptides are monomeric in aqueous solution as discerned from analytical equilibrium ultracentrifugation studies and have the spectroscopic signatures of well-defined  $\beta$ -sheet structures. A <sup>15</sup>N-labeled peptide is under study using multidimensional NMR in order to fully characterize its structure which will be reported in due course. The ability to nucleate folding in  $\alpha$ -amino acid sequences incorporating the 4-(2-aminoethyl)-6-dibenzofuranpropionic acid residue (**6**) coupled with the ability to control the self-assembly via the incorporation of N-methylated amino acids allows for the reliable *de novo* design of predominantly  $\beta$ -sheet folds.

Other templates for  $\beta$ -sheet nucleation designed to replace the  $i + 1$  and  $i + 2$  residues of a  $\beta$ -turn have also been prepared in our laboratory. The 6,6'-bis-(acylamino)-2,2'-bipyridine-based amino acids are designed to promote  $\beta$ -sheet folding upon the addition of Cu(II) ions (Figure 20).<sup>105</sup> The design hypothesis is that in the absence of added metal ion, the peptide would exist as an ensemble of conformations most having a transoid bipyridine conformation. Upon addition of Cu(II), the ligand substructure of the amino acid would be stabilized in the cisoid conformation promoting  $\beta$ -sheet folding in the attached  $\alpha$ -amino acid sequence. Two bipyridine-based amino acid residues **7** and **8** (shown below) were prepared



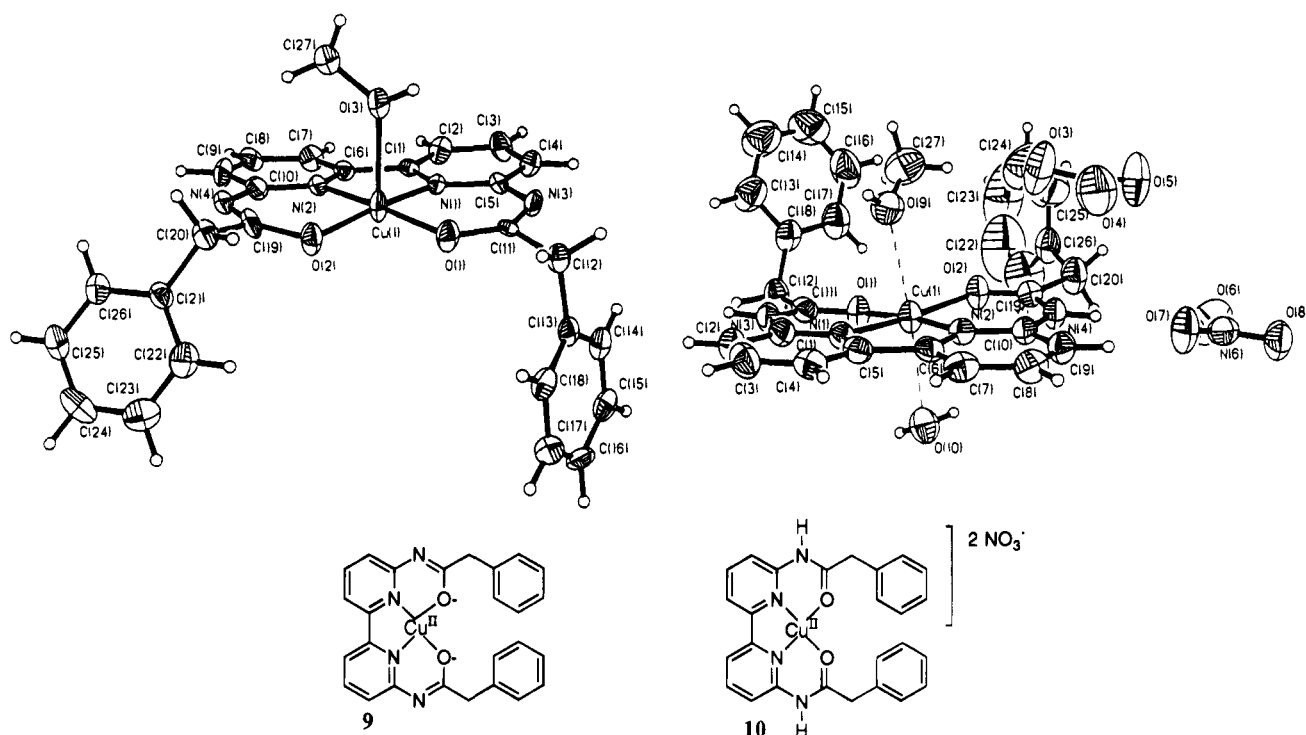
**Figure 20.** Envisioned transoid to cisoid bipyridine conformational equilibrium shift mediated by Cu(II) binding within peptides incorporating residues **7** and **8** is expected to nucleate  $\beta$ -sheet folding; R =  $\alpha$ -amino acid side chain.

in order to evaluate their ability to nucleate  $\beta$ -sheet structure. The residues differ in the length of the linker intervening between the peptide attachment sites and the metal binding portion of the template. The bipyridine-based residue **8**, having a  $-\text{CH}_2\text{CH}_2-$  fragment attaching the ligand substructure to the remainder of the peptide, was expected to promote the formation of a hydrophobic cluster when flanked by hydrophobic amino acid residues in an analogous manner to template **6** (Figure 18). It is possible that hydrophobic clustering alone or hydrophobic clustering in combination with Cu(II) binding would be sufficient for  $\beta$ -sheet nucleation. Residue **7** was prepared to probe the ability of metal binding alone to nucleate  $\beta$ -sheet folding.



**6,6'-bis(acylamino)-2,2'-bipyridine-based amino acids**

The bipyridine-based ligand portions of residues **7** and **8** bind Cu(II) in a square-planar geometry as discerned from X-ray crystallographic studies of model compounds **9** and **10** (Figure 21). The  $pK_a$  of the amide NH's is such that at high pH they are removed affording a neutral complex but at pH's  $< 6$  they are retained affording a complex with a +2 charge. The distance between the benzylic carbons ( $\sim 5.2$  Å) in the model compounds is similar to the distance between the strands of a naturally occurring  $\beta$ -sheet ( $\sim 4.8$  Å). Therefore, residues **7** and **8** should function as effective  $\beta$ -sheet nucleators when incor-

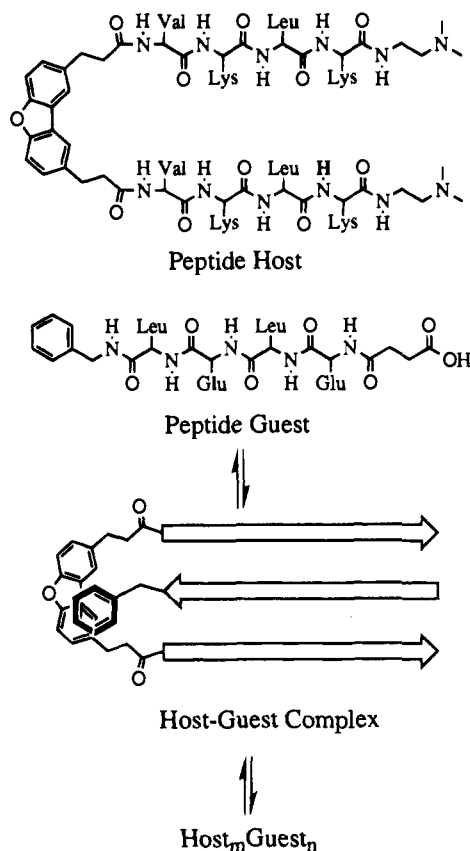


**Figure 21.** ORTEP depictions of the crystal structures of neutral Cu(II) complex **9** and charged (+2) Cu(II) complex **10** which were designed to model the ligand substructure of peptides containing bipyridine-based residues **7** and **8** in the presence of Cu(II) under alkaline and acidic conditions, respectively. The crystal structures of the model complexes suggest that residues **7** and **8** when incorporated into peptide sequences should bind Cu(II) in a square-planar geometry effecting  $\beta$ -sheet structure formation in the attached peptides.

porated into appropriate  $\alpha$ -amino acid sequences. Residues **7** and **8** were incorporated into amphiphilic sequences resulting in peptide-derivatives K-V-T-V-K-7-K-V-T-V-K-NH<sub>2</sub> (peptide **W**) and K-V-T-V-K-8-V-K-V-T-V-K-NH<sub>2</sub> (peptide **X**). These peptides appear to bind Cu(II) in a manner analogous to model compounds **9** and **10** as discerned from the nearly identical UV spectra of the model complexes and the peptides as a function of pH. Peptide **W** exists as an ensemble of unordered conformations in aqueous solution in the absence of Cu(II). However, upon the addition of Cu(II), peptide **W** adopts  $\beta$ -sheet structure indicating that residue **7** is capable of nucleating  $\beta$ -sheet structure upon Cu(II) binding. The behavior of peptide **X**, which has two carbons intervening between the ligand substructure and the amino acid functional groups, is markedly different. This peptide is capable of folding and self-associating in the absence of Cu(II). Apparently the hydrophobic cluster, which stabilizes the cisoid bipyridine conformation, is capable of nucleating folding in the absence of Cu(II). The addition of Cu(II) appears to further stabilize the  $\beta$ -sheet structure as determined by an increase in ellipticity at 220 nm in the far-UV CD, however this apparent increase in structure could be caused by a change in the aromatic contribution to the far-UV CD upon Cu(II) binding. Current efforts to uncouple folding from self-assembly appear successful, again using the N-methylation strategy, and will be reported in due course.

In an attempt to understand  $\beta$ -sheet quaternary structure formation in aqueous buffers we have designed a peptidomimetic host that binds a peptide guest affording a three-stranded  $\beta$ -sheet structure that self-assembles (Figure 22).<sup>126</sup> This system in the

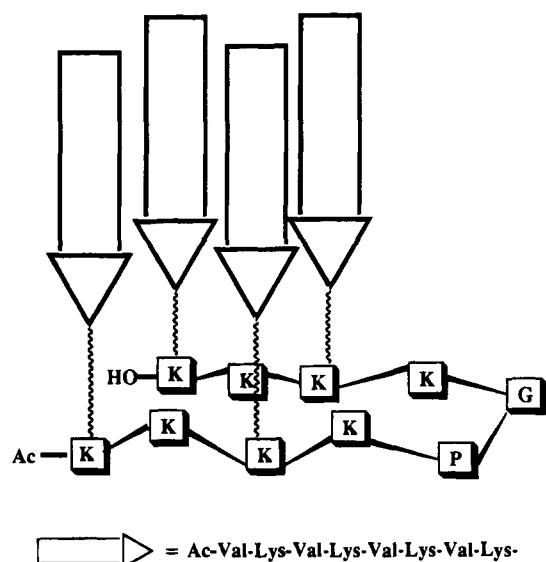
simplest sense mimics the binding and self-assembly properties exhibited by most receptors. The host sequence, which is composed of a 2,8-dibenzofurandiyl-bis(3-propanoic acid) residue flanked by  $\alpha$ -amino acid sequences containing alternating hydrophobic and cationic residues, is selective for anionic guests having an amphiphilic periodicity of 2. The dibenzofuran diacid template was envisioned to separate the two covalently attached peptide strands by  $\sim 10$  Å on average, allowing the guest to bind between the strands of the host molecule. The C-terminal aromatic group in the peptide guest is thought to initiate the binding event allowing the hydrophobic and oppositely charged side chains of the host molecule and the guest peptide to interact resulting in an amphiphilic antiparallel  $\beta$ -sheet structure. When studied in isolation the host and the guest are unstructured; however, when mixed at  $\mu$ M concentrations the complex yields a three stranded  $\beta$ -sheet structure that subsequently self-assembles. In order to be certain that the host binds to the guest, prior to self-assembly, the host was covalently linked to a chromatography column and the apparent binding constant ( $510 \mu$ M) was determined by high-performance affinity chromatography. Immobilizing the host on the column via an amide linkage derived from the  $\epsilon$ -amino group of Lys ensures that the host-guest complex will not self-associate. The host is very selective with regard to the peptides that it is capable of binding, i.e. it does not bind to most tetrapeptides. There is a strong electrostatic component as well as significant hydrophobic component which contributes to the binding free energy as discerned from the ongoing binding studies with various guest analogs. The formation of a stable three-stranded  $\beta$ -sheet



**Figure 22.** Structure of the host molecule containing a 2,8-dibenzofurandiylbis(3-propanoic acid) residue and the guest peptide. The specificity of the binding event results from favorable hydrophobic interactions as well as electrostatic interactions resulting from the formation of the host-guest complex. After the initial binding event takes place, the complex further self-associates to form soluble  $\beta$ -sheet quaternary structure.

quaternary structure which does not aggregate is currently in development.

Mutter and colleagues have reported a parallel  $\beta$ -sheet sandwich protein using the template-assembled synthetic protein (TASP) approach described above for helical bundles. The template peptide Ac-Lys-Lys-Lys-Lys-Pro-Gly-Lys-Lys-Lys-Lys may adopt a  $\beta$ -hairpin conformation placing the side chains of lysines 1, 3, 8, and 10 (in bold) on the same side of the putative  $\beta$ -sheet structure adopted by the template. Parallel  $\beta$ -strand sequences Ac-Val-Lys-Val-Lys-Val-Lys-Val-Lys were attached to the  $\epsilon$ -amino groups of lysines 1, 3, 8, and 10 by solid-phase peptide synthesis affording the TASP assembled parallel  $\beta$ -sandwich structure shown in Figure 23.<sup>74</sup> This peptide undergoes a coil to sheet transition at pH 12 as discerned by the far-UV CD signature for  $\beta$ -sheet structure (minimum at 218 nm), however the characterization of this peptide by NMR or by ultracentrifugation has not been reported. An analogous template Ac-Lys-Lys-Lys-Lys-Pro-Gly-Lys-Lys-Lys-Lys- $\epsilon$ -6-amino-hexanoic acid-OH was prepared as a scaffold for a  $\beta$ -barrel structure. Parallel  $\beta$ -strand sequences Ac-Val-Lys-Val-Lys-Val-Lys-Val-Lys were attached to the  $\epsilon$ -amino groups of all lysines in the sequence affording a putative 8-stranded  $\beta$ -barrel folding motif as discerned from far-UV CD and FT-IR methods at pH 10.<sup>70</sup> Characterization of this peptide by NMR or X-ray crystallography and by



**Figure 23.** Schematic representation of a template-assembled synthetic protein (TASP). In this case, the folding of a parallel  $\beta$ -sandwich is directed via the attachment of four peptide sequences to an acyclic peptide-based template. The peptides are covalently attached at their carboxy terminus via an amide linkage to the  $\epsilon$  nitrogens of template lysine side chains.

equilibrium ultracentrifugation has not been reported and is required to establish the structure of this putative  $\beta$ -barrel structure. A template-assembled synthetic protein composed of both a 4-helix bundle and a  $\beta$ -barrel-like structure has also been reported by Mutter and colleagues.<sup>70</sup> The four-helix bundle assembles on one face of the template and the  $\beta$ -sheet sandwich on the other. The monomeric nature and the far-UV CD spectrum of the peptide is consistent with the proposed structure.

Several cyclic peptides incorporating templates have been prepared to demonstrate that they are good replacements for the  $i + 1$  and  $i + 2$  residues of  $\beta$ -turns. Even though we have concentrated predominantly on acyclic peptides in this review, these templates may prove useful for the nucleation of sheet structure in acyclic peptides.<sup>127-129</sup> In addition, Gellman and Nowick have recently reported templates which are in the early stages of development that may prove useful in achieving  $\beta$ -sheet folding.<sup>130,131</sup>

#### IV. Templates That Induce Other Types of Structures

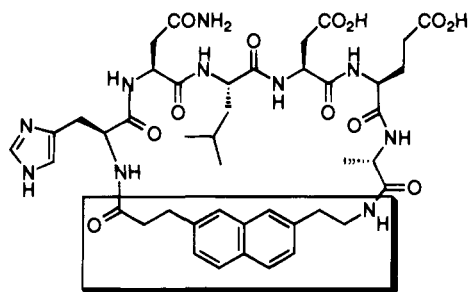
##### A. Olson $\Omega$ -Loop

Sarabu and co-workers have employed a naphthalene-based amino acid to control the conformation of a cyclic peptide to afford an  $\Omega$ -loop conformation.<sup>132</sup> Interleukin-1 $\alpha$  (IL-1 $\alpha$ ) is a cytokine released by macrophages and is responsible for triggering inflammatory and immunological responses by binding to the receptors on lymphocytes. Residues 41-48 of IL-1 $\alpha$  adopts a solvent exposed  $\Omega$ -loop which was thought to play an important role in its biological activity. Several acyclic loop peptides were tested for their binding affinities to the IL-1 receptor.<sup>133</sup> However, these peptides lacked affinity for the receptor



presumably due to their inability to fold into the desired bioactive conformation. A peptidomimetic which adopts the  $\Omega$ -loop conformation should be an effective antagonist for the IL-1 $\alpha$  inflammatory response, which should have important pharmaceutical implications.

Shown below is peptidomimetic **11**, designed to mimic the 41–48  $\Omega$ -loop. The peptidomimetic contains a 7-(2-aminoethyl)-2-naphthalenepropanoic acid spacer group (boxed) that positions the termini of the attached sequence a distance of 10.94 Å apart. This is the approximate distance found separating the ends of the  $\Omega$ -loop in IL-1 $\alpha$ . The three dimensional structure of **11**, determined by COSY and NOESY NMR experiments in DMSO along with molecular modeling, confirmed that this peptidomimetic adopts an  $\Omega$ -loop conformation, which is similar to that conformation found in IL-1 $\alpha$ . However, biological assays concluded that **11** was not an effective antagonist indicating that perhaps the  $\Omega$ -loop of IL-1 $\alpha$  is not solely responsible for receptor recognition and activation.



Compound 11

## V. Conclusions and Perspective

A variety of templates have been incorporated into peptides and studied as folding nucleators or are evolving in that direction. This approach looks very promising for the *de novo* design of proteins having a defined structure. The ability to realize the full potential of templated folding depends on our ability to determine the strengths and limitations of each template. Each templated peptide developed must be characterized as extensively as possible. Ideally each peptide should be characterized by mass spectrometry (sequence verification), by analytical equilibrium ultracentrifugation (solution molecular weight), by far- and near-UV CD as well as IR (secondary structural probe), and by NMR (identify tertiary contacts by NOEs,  $^3J_{\text{CH}_\alpha\text{-NH}}$  coupling constants characteristic of the  $\phi$  angles as well as employing amide exchange data and temperature coefficients which evaluate hydrogen bonding) and/or if possible by X-ray crystallography. From our perspective the challenges in this endeavor are to rigorously characterize each system prepared and to design evolved tertiary structures that achieve defined packing arrangements as opposed to molten globule-type structures. The most difficult challenge ahead is to design templated proteins that possess tertiary structures with defined enzymatic activity, which is what the evolution of this approach combined with advances in diversity generation and screening should allow.

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