Synthesis and Efficacy of Square Planar Copper Complexes Designed to Nucleate β-Sheet Structure

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Abstract: The synthesis of four 6,6'-bis(acylamino)-2,2'-bipyridine-based amino acids **1a–c** and **2** are described. These residues, when coordinated to Cu(II), are designed to replace the i + 1 and i + 2 residues of a β-turn. Amino acids **1a–c** and **2** were incorporated into several different peptides to evaluate their efficacy as β-sheet nucleators. Matrix assisted laser desorption mass spectroscopy and UV spectroscopy reveal that peptides incorporating these residues bind Cu(II) ions under alkaline and acidic conditions with a 1:1 binding stoichiometry. In an effort to predict the geometry of the metal binding site of peptides containing β-turn mimics **1a–c** and **2**, three model compounds, **18**, **19**, and **20**, were prepared, and their crystal structures were determined. The crystal structure of 6,6'-bis(phenylacetamide)-2,2'-bipyridine (**18**) suggests that the bipyridine rings of peptides containing these residues should exist in a transoid conformation in the absence of Cu(II) ions and other stabilizing forces. The crystal structures of neutral (deprotonated) Cu(II) complex **19** and 2+ charged (protonated) Cu(II) complex **20** suggest that peptides containing residues **1a–c** and **2** bind Cu(II) ions under alkaline and acid conditions resulting in a cisoid bipyridine ring conformation with a nearly perfect square planar geometry about the copper atom. Spectroscopic studies on peptides incorporating residue **1b** indicate that this residue is capable of nucleating an antiparallel β-sheet conformation upon binding a single Cu(II) ion in basic aqueous buffer. Peptides incorporating residue **2** behave differently than those containing residue **1b** in that they are capable of adopting an antiparallel β-sheet conformation either in the absence or presence of Cu(II) ions. The chemical structure of residue **2** is such that the cisoid nucleating conformation may be stabilized by hydrophobic interactions in the absence of transition metal binding.

The mechanisms of β-sheet folding are not well understood at this time; however, this area is receiving the attention of several laboratories.[1] Protein β-sheet structures are stabilized by long range residue–residue interactions instead of the localized i, i+4 residue–residue interactions characteristic of α-helices, suggesting that sheets are best thought of as tertiary structures.[1g] Moreover, the topologies of β-sheet structures are complex and involve at least two peptide strands interacting with one another in an extended conformation.[2] Studies on the folding of all β-proteins suggest that the mechanism of β-sheet folding may differ significantly from that of α-helical folding. The rate of α-helical folding in peptides and proteins is at least an order of magnitude faster, in general, than that of β-sheet folding in proteins indicating that helices and sheets fold differently.[1r–u] Moreover, peptide fragments of the β-sandwich protein plastocyanin show no tendency to fold under conditions where the protein is folded, unlike analogous studies on predominantly α-helical proteins (e.g., myohemerythrin) where peptide fragments of these proteins have a tendency to adopt a helical secondary structure.[1r,s] It appears that a peptide which exists in a β-sheet conformation in the context of a larger sequence does not adopt a β-sheet structure in the absence of the surrounding sequence.[1r] This observation and others suggest that hydrophobic clustering or similar tertiary interactions are required, in addition to local conformational propensities, for β-sheet folding.[1gw,3]

In principle, a β-turn structure could be envisioned to nucleate β-sheet folding. However, the conformational propensities of established β-turn sequences do not appear to be sufficient to effect sheet folding.[1r,4] Because of these difficulties, our laboratory has pursued the design of unnatural aromatic amino acids that nucleate the formation of β-sheet structure in a

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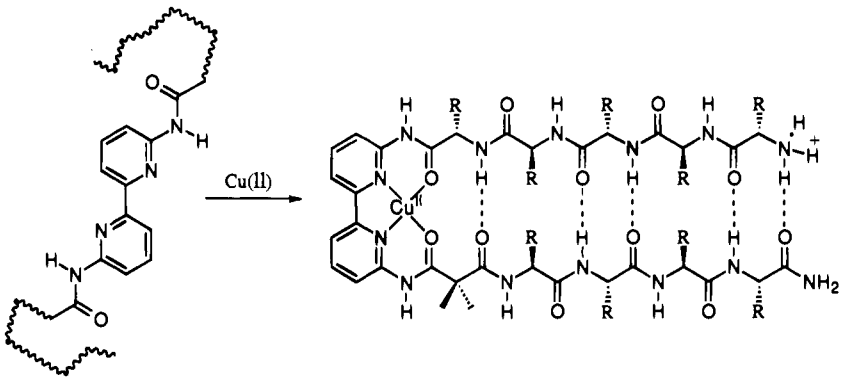
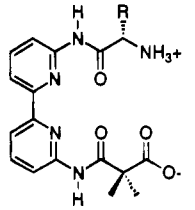


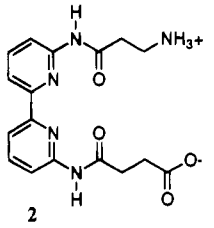
Figure 1. Envisioned transoid to cisoid conformational change mediated by Cu(II) binding within peptides incorporating residues **1a–c** and **2** is expected to nucleate β-sheet folding; R = α-amino acid side chain.

predominantly α-amino acid sequence in an effort to form a well-defined β-sheet.[1w,3b,5] The concept of using conformationally restricted molecules with the appropriate dimensions and geometry to increase the stability of a given conformation in peptides was demonstrated in the 1970s by Hirschmann and his colleagues.[6] Since then, the design and synthesis of several β-turn analogs have been reported.[1cdv,7] The majority of these turn mimetics have been prepared for medicinal chemistry purposes, however, a subset of these has been employed to study β-sheet structure.[1w,3a,b,5,7h]

Another useful strategy for studying secondary structures is to direct the folding of an α-amino acid sequence or stabilize existing secondary structure within polypeptides by metal complexation. Metal binding sites have been engineered into peptides and proteins using the side chains of naturally occurring amino acids or unnatural metal binding residues as ligands.[8] For example, Arnold, Ghadiri, Hopkins, and Sasaki have stabilized α-helical secondary structures through the formation of a metal-mediated cross link in a folded peptide where aminodiacetic acid ligands or the side chains of histidine or cysteine residues located in the i and i+4 positions of an α-helix were ligated to metal ions to constrain the intervening chain effecting α-helical stabilization.[9] In addition, DeGrado and Berg have studied the metal-mediated formation of tertiary and quaternary structure in peptides and proteins via engineered metal-binding sites.[10] Unnatural bipyridine-based metal binding amino acid residues have also proven useful for stabilizing secondary structures in short peptides. Imperiali has incorporated two bipyridine-based (s)-α-amino-2,2'-bipyridine-6-propanoic acid residues at the amino and carboxy termini of a reverse turn (β-type II) sequence. The resulting peptide was found to bind Cu(II) with high affinity due to the preorganization of the peptide in a turn conformation.[11] Ghadiri and Sasaki demonstrated that pyridine and bipyridine-based metal binding residues can also be used in the metal-mediated self-assembly of tertiary and quaternary structure. Pyridyl functionalities as well as bipyridine-based amino acids were covalently attached to amphiphilic α-helical segments and complexed to Fe(II), Co(II), Ni(II), and exchange inert Ru(II), effecting the self-assembly of stable four and three-helix bundle metalloproteins.[12] Despite recent advances in strategies employing metal complexation to stabilize or nucleate secondary structure, there have been no reports of the use of amino acids which can chelate metals and upon binding nucleate β-sheet structure formation. The goal of the work described here is to develop a bipyridine-based amino acid that will bind Cu(II) in a square planar geometry enforcing a reverse turn conformation which effects β-sheet folding. The incorporation of one of these residues into an α-amino acid sequence will provide the means of affording a folded antiparallel β-sheet upon Cu(II) binding, Figure 1.



1a, R = H
1b, R = isopropyl
1c, R = isobutyl



2

Incorporation of the bipyridine-based amino acid residues **1a–c** and **2** into an α-amino acid sequence should serve to reverse the polypeptide chain direction with a minimal entropic penalty upon Cu(II) binding. The 6,6'-bis(acylamino)-2,2'-bipyridine-based ligands, initially prepared by Shiraishi, were reported to form square planar complexes with Cu(II) and Co(II) to yield a protonated (2+) complex under acidic conditions

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and a neutral (deprotonated) complex under alkaline conditions based on IR, UV, and EPR spectroscopic data.[13] These bipyridine-based ligands were able to selectively bind Cu(II) in the presence of other transition metals. Therefore, Cu(II) seemed to be the logical choice to nucleate β-sheet structure in peptides composed of residues **1a−c** or **2**.[14] The ligand substructure of peptides containing these residues is expected to bind Cu(II) in a cisoid square planar conformation, which has the appropriate dimensions to nucleate β-sheet folding in the attached peptide strands.

Residues **1a−c** and **2** differ in that $-CH_2CH_2-$ fragments were incorporated into **2** to allow a possibly favorable hydrophobic interaction to take place between the aromatic bipyridine rings and the hydrophobic side chains of the α-amino acids flanking residue **2**. Phenethyl compounds are known to adopt a low energy conformation wherein the plane of the aromatic ring is perpendicular to the C−C bond in the $-CH_2CH_2-$ fragment. This conformation may allow the hydrophobic bipyridine rings to interact with the side chains of hydrophobic α-amino acid residues via the "hydrophobic effect" to form a hydrophobic cluster, Figure 2. This type of hydrophobic interaction is critical for the nucleation of β-sheet structure in peptides incorporating a 4-(2-aminoethyl)-6-dibenzofuranpropionic acid residue.[1w,3a,b] Residues **1a−c** and **2** were incorporated into amphiphilic peptides of varying chain length and sequence to assess the affects of structural variation on the ability of these peptidomimics to nucleate β-sheet structure.

Results

Synthesis of Cu(II) Binding β-Turn Mimics Based on Residues 1a−c. The synthesis of Boc-protected analogs of residues **1a−c** (i.e., **8**, **10**, and **12**) are outlined in Figure 3. Due to the lack of reactivity of the aniline functionality in **6**, amino acid analogs were prepared in which this group was functionalized with a Boc-protected α-amino acid prior to their use in solid phase peptide synthesis. The synthesis of these Boc-protected analogs employ a common intermediate, namely 6,6′-diamino-2,2′-bipyridine **(5),** prepared previously by our laboratory.[15] Improvements on the original synthesis of diamine **5** were necessary to obtain preparative quantities of this compound.[16] The variation in amide bond forming procedures

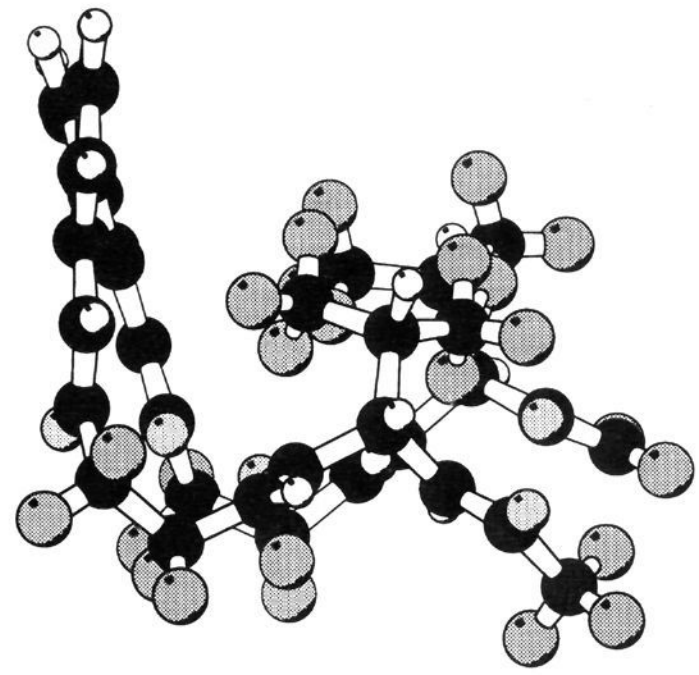

Figure 2. Ball and stick representation of CH_3CO-Val-**2**-Val-$CONH_2$ in the absence of Cu(II) depicting the putative hydrophobic cluster conformation mediated by a cisoid conformation within **2** which appears to be stabilized by favorable hydrophobic interactions between the aromatic bipyridine rings and the hydrophobic side chains of the α-amino acids flanking **2**. The structure shown was constructed in the anticipated conformation and energy minimized using Discover.

used in the synthesis of the compounds described within is a result of yield optimization and ease of product isolation.

The synthesis of analogs **8**, **10**, and **12** commences with the monoacylation of **5** employing monoethyl-2,2-dimethyl malonate utilizing 2-chloro-1-methylpyridinium iodide activation to afford the ethyl ester **6** in 40% yield.[17] Boc-protected glycine, valine, and leucine were coupled to **6** employing BOP activation to afford the Boc-protected ethyl esters **7**, **9**, and **11**, respectively, in 83%, 59%, and 47% yield, Figure 3. Removal of the ethyl ester protecting groups by basic hydrolysis yielded the Boc-protected amino acids **8**, **10**, and **12** in 51%, 88%, and 90% yields, respectively. The overall yields of **8**, **10**, and **12** from 6,6′-dibromo-2,2′-bipyridine are 12%, 15%, and 12%, respectively.

Synthesis of Cu(II) Binding β-Turn Mimic Based on Residue 2. The Cu(II) binding β-turn mimic **2** is prepared most conveniently as a Boc-protected dipeptide where the carboxy terminus of residue **2** was converted into an amide derived from the α-amino acid valine affording **15**, Figure 4. Peptidomimetic **15** was incorporated into solid phase peptide synthesis as though it were a normal α-amino acid. Functionalization of the carboxy terminus of **2** was necessary in order to avoid intramolecular succinimide ring formation resulting from the 6-amido nitrogen attacking the activated carboxy terminus during the coupling of Boc-protected **2** to a resin supported peptide chain. The synthesis of the Boc-protected **15**, outlined in Figure 4, commences by monoacylating **5** with Boc-β-alanine employing DCC activation at room temperature to afford the Boc-protected amine **13** in 41% yield. The anilino functionality in **13** is then acylated with the pentafluorophenyl ester **17** in benzene to afford the Boc-protected methyl ester **14** in 86% yield. Removal of the methyl ester protecting group by basic hydrolysis yielded the Boc-protected amino acid **15** in 75% yield. The overall yield of **15** from 6,6′-dibromo-2,2′-bipyridine is 19%. The synthesis of the pentafluorophenyl ester **17** begins with the ring opening of succinic anhydride by the methyl ester of valine hydrochloride to yield methyl ester **16** in 99% yield. Finally

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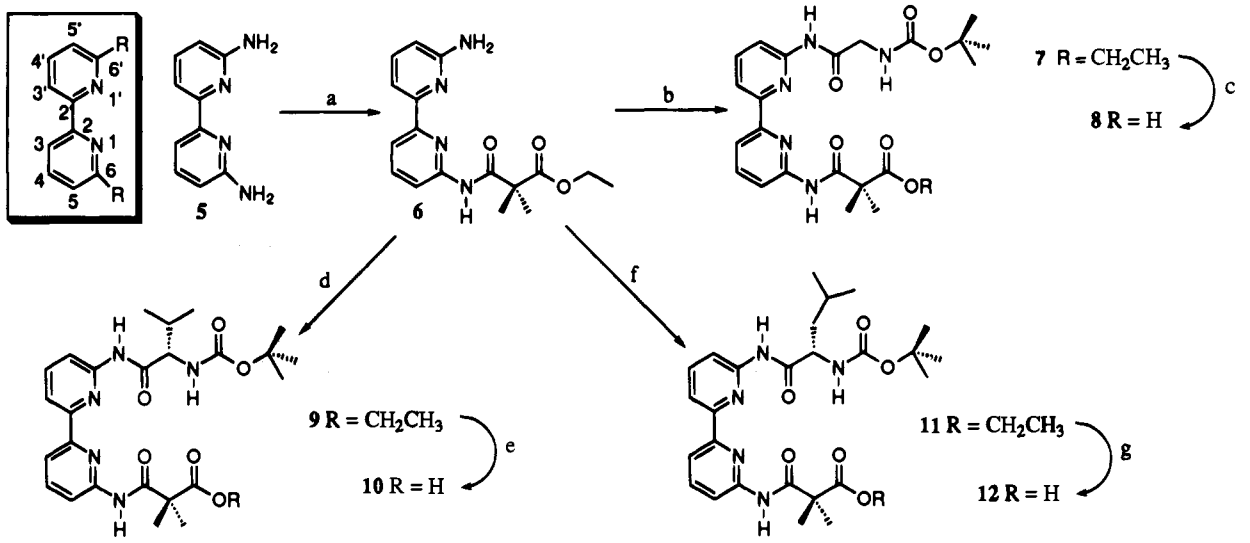


Figure 3. Outline of the synthesis of analogs **8**, **10**, and **12** corresponding to residues **1a**–**c**. Reaction conditions: (a) 1.0 equiv of monoethyl-2,2-dimethyl malonate, 1.05 equiv of 2-chloro-1-methylpyridinium iodide, 2.4 equiv of TEA, $CHCl_3$, initially heated to reflux and then allowed to cool to room temperature, 3.5 h, 40%; (b) 4 equiv of N-Boc-glycine, 4 equiv of BOP, 5.4 equiv of DIEA, CH_2Cl_2, room temperature, 48 h, 83%; (c) 2.1 equiv of NaOH, ethanol, 0 °C, 1.5 h, 51%; (d) 3 equiv of N-Boc-Valine, 3 equiv of BOP, 5 equiv of DIEA, CH_2Cl_2, room temperature, 30 h, 59%; (e) 2.1 equiv of NaOH, ethanol, 0 °C, 2.5 h, 88%; (f) 10 equiv of N-Boc-Leucine, 10 equiv of BOP, 15 equiv of DIEA, CH_2Cl_2, DMF, room temperature, 96 h, 47%; (g) 2.1 equiv of NaOH, ethanol, 0 °C, 3 h, 90%.

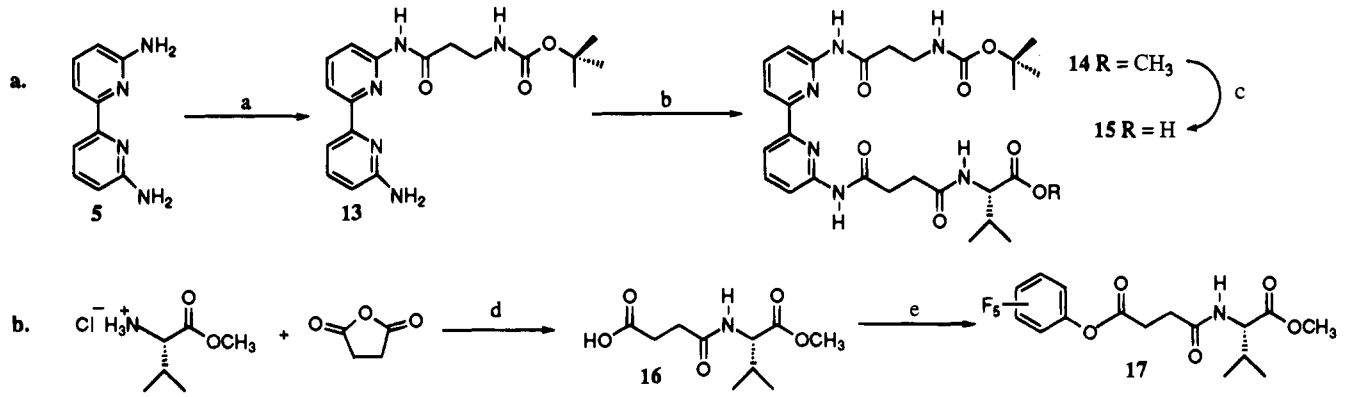
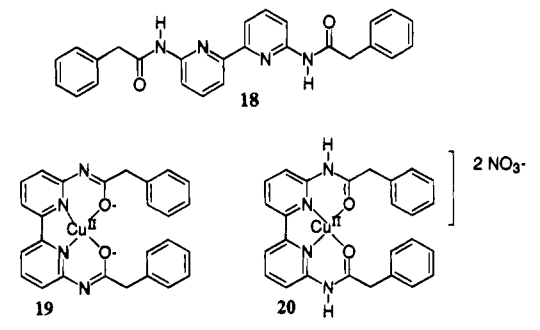


Figure 4. Outline of the synthesis of Boc-protected dipeptide **15** based on residue **2**. Reaction conditions: (a) 1 equiv of DCC, 1 equiv of Boc-β-alanine, CH_2Cl_2, DMF, room temperature, 23 h, 41%; (b) 2.98 equiv of **17**, 1.09 equiv of DIEA, benzene, reflux, 17 h, 86%; (c) 10 equiv of LiOH, methanol, room temperature, 2 h, 75%; (d) 2 equiv of DIEA, $CHCl_3$, room temperature, 3 h, 99%; (e) 1.05 equiv of pentafluorophenol, 1.05 equiv of EDCI, CH_2Cl_2, initially at 0 °C and then allowed to warm to room temperature, 20 h, 80%.

esterification of **16** with pentafluorophenol employing EDCI activation furnishes the intermediate **17** in 80% yield.



Synthesis of Phenylacetamide Derivative 18 and Copper Complexes 19 and 20. In order to demonstrate that the bipyridine rings in residues **1a**–**c** and **2** exist in a transoid conformation in the absence of Cu(II), phenylacetamide derivative **18** was synthesized, and crystals suitable for X-ray diffraction were grown. The synthesis is effected by the diacylation of diamine **5** with phenylacetic acid employing BOP activation to produce **18** in 77% yield. To gain insight into the Cu(II) binding geometry of residues **1a**–**c** and **2** under alkaline and acidic conditions, the deprotonated and protonated Cu(II) complexes **19** and **20**, respectively, were prepared, and crystals suitable for X-ray diffraction were grown. The deprotonated Cu(II) complex **19** was synthesized by treating **18** with 2 equiv of copper hydroxide at 67 °C in THF to afford **19** in 51% yield. Addition of 1.05 equiv of copper nitrate hemipentahydrate to **18** in methanol at room temperature afforded the $Cu(II)\cdot 2NO_3^-$ complex **20** in 58% yield.

Single Crystal X-ray Analysis. Crystals of 6,6'-bis(phenylacetamide)-2,2'-bipyridine (**18**) were obtained from DMSO-d_6 by allowing a routine 5 mm NMR sample to sit at room temperature overnight to afford colorless rodlike crystals having a monoclinic space group $P2_1/n$.[18] The bipyridine rings exist in a transoid conformation in the absence of Cu(II) as depicted in the ORTEP representation of **18**, Figure 5. The structure of **18** demonstrates that all of the atoms occupy the same plane with the exception of the atoms that compose the phenyl groups. It is interesting to note that the heterocyclic nitrogen atom, N(1), and the carbonyl carbon, C(6), exist in a trans conformation with respect to the N(2)–C(5) bond, indicating that the solid state structure supports earlier structural proposals originating

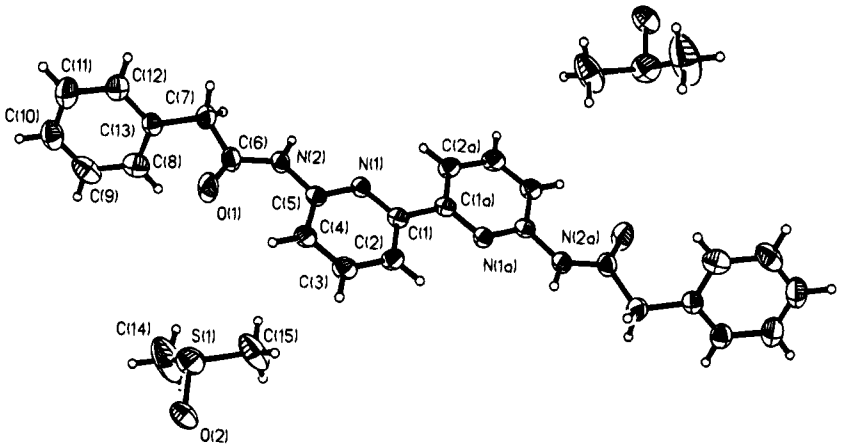


Figure 5. An ORTEP depiction of 6,6'-bis(phenylacetamide)-2,2'-bipyridine (**18**). Note that the bipyridine rings exist in a transoid conformation and that the heterocyclic nitrogen atom N(1) and the carbonyl carbon C(6) exist in a trans conformation with respect to the N(2)−C(5) bond.

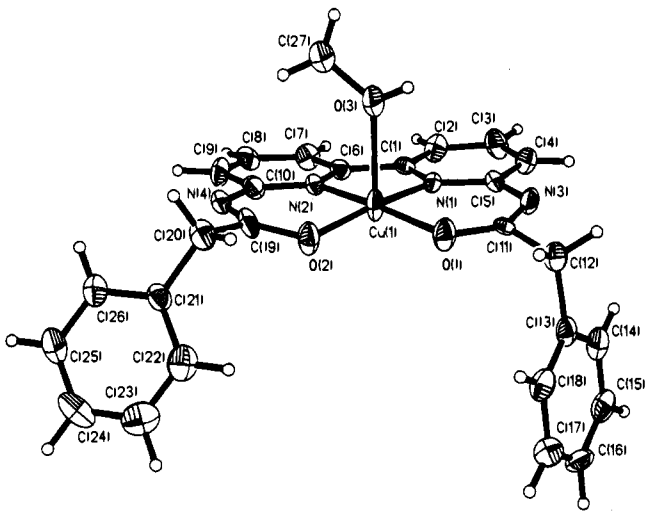


Figure 6. An ORTEP depiction of deprotonated copper complex **19**. Note that the bipyridine rings are in a cisoid conformation and that the amide protons have been removed affording a nearly perfect square planar neutral complex.

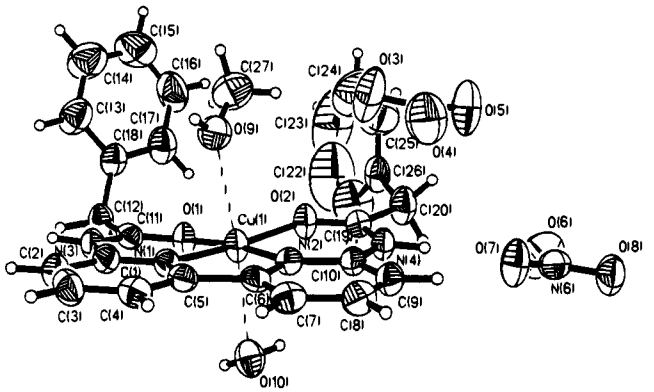


Figure 7. An ORTEP depiction of protonated copper complex **20**. Note that the bipyridine rings exist in a cisoid conformation and that the amide protons are intact affording a slightly distorted square planar (2+) charged complex.

from spectroscopic data.[13b] The crystal structure of **18** suggests that in absence of Cu(II), the bipyridine rings of peptides incorporating residues **1a**−**c** and **2** should exist in a transoid conformation, projecting the attached peptide strands in opposite directions. However, it is conceivable that secondary interactions such as hydrophobic cluster formation or hydrogen bonding could stabilize the cisoid conformation of **1a**−**c** or **2** in a polypeptide.

Crystals of the neutral Cu(II) complex **19** were obtained from reagent grade methanol by allowing a saturated solution of **19** to slowly evaporate over 48 h to afford blue plates having a triclinic space group $P1$.[18] The structure of **19** depicted in the ORTEP representation (Figure 6) clearly demonstrates that under alkaline conditions 6,6'-bis(phenylacetamide)-2,2'-bipyridine (**18**) is a tetradentate ligand which binds Cu(II) in a square planar geometry. The bipyridine rings adopt a cisoid conformation with respect to one another. The pK_a of the amide protons is such that under these conditions, they are removed affording a neutral complex. The absence of counter ions in the crystal structure as well as high resolution FAB mass spectroscopic data corroborate the formation of a deprotonated neutral complex. The angle between the plane containing atoms Cu(1), N(2), C(10), N(4), C(19), and O(2) and the plane occupied by atoms Cu(1), N(1), C(5), N(3), C(11), and O(1) is only 2.6°. An angle of 0° is indicative of perfect square planar geometry, whereas an angle of 90° defines tetrahedral geometry. The distance between C(11) and C(19) (5.26 Å) is slightly greater than the distance between the strands of a typical antiparallel β-sheet (4.85 Å).[19]

Crystals of the Cu(II) complex **20** were obtained from reagent grade methanol by allowing a saturated solution of **20** to slowly evaporate over 72 h to afford blue plates having a triclinic space group $P1$.[18] The structure of **20** depicted by the ORTEP representation in Figure 7 demonstrates that under acidic conditions, **18** binds Cu(II) in a square planar fashion. The presence of nitrate counter ions observed in the crystal structure and high resolution FAB mass spectroscopic data indicates the presence of the amide protons leading to a charged (2+) complex. The fifth and sixth Cu(II) coordination sites are occupied by a water molecule and a methanol molecule in this complex. Interestingly, the nitrate counter anions form a hydrogen bonding network where two oxygens of a nitrate ion hydrogen bond independently to the amide protons of adjacent complexes, e.g., (amide−NH−O−NO−O−HN−amide) (not shown in figure). A similar evaluation of planarity used for structure **19** results in an angle between the plane containing atoms Cu(1), N(1), C(1), N(3), C(11), and O(1) and the plane containing atoms Cu(1), N(2), C(10), N(4), C(19), and O(2) of 8.1° indicating that complex **20** is slightly less square planar when compared to complex **19**. The distance between C(11)

(18) Hahn, T. *International Tables for X-ray Crystallography*; D. Reidel Publishing Co.: Dordrecht, Holland, 1987; Vol. A, pp 39, 101−709.

(19) (a) Pauling, L.; Corey, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **1953**, *39*, 253. (b) Reppart, W. J.; Gallucci, J. C.; Lundstedt, A. P.; Gerkin, R. E. *Acta Crystallogr.* **1984**, *C40*, 1572.

Peptide A	I-T-V-K-**1c**-K-L-K-V-NH_2
Peptide B	I-T-V-S-**1c**-K-L-K-V-NH_2
Peptide C	L-S-V-K-V-**1a**-V-T-L-K-V-NH_2
Peptide D	K-V-T-V-K-**1b**-K-V-T-V-K-NH_2
Peptide E	K-V-K-V-K-V-**2**-V-K-V-K-V-K-NH_2
Peptide F	K-V-T-V-K-V-**2**-V-K-V-T-V-K-NH_2
Peptide G	K-V-T-V-K-V-**G-G**-V-K-V-T-V-K-NH_2
Peptide H	V-O-L-**2**-V-O-L-NH_2

Figure 8. Sequence of peptides **A**–**D** containing residues **1a**–**c**, peptides **E**, **F**, and **H** containing residue **2**, and control peptide **G**.

and C(19) (5.20 Å) is close but slightly greater than the intrastrand distance required by an antiparallel β-sheet. The crystal structures of **19** and **20** suggest that peptides incorporating residues **1a**–**c** and **2** should bind Cu(II) in a square planar geometry under alkaline and acidic conditions positioning the peptide strands in an antiparallel orientation with an intrastrand distance of ~5 Å.

Synthesis of Peptides Incorporating Residues 1a–c and 2. Peptides **A**–**H** were synthesized on benzhydrylamine resin following procedures described in detail previously, Figure 8.[1w] Briefly, t-Boc synthesis procedures were employed using the BOP reagent for carbonyl activation of the α-amino acids.[20] The putative nucleating residues **1a**–**c** and **2** were incorporated into the growing peptide chain as compounds **8**, **10**, **12**, and **15**, respectively employing DIC/HOBT activation. Peptides **A**–**H** were deprotected and cleaved from the benzhydrylamine resin employing high-HF and purified by reverse phase C_{18} HPLC.[21] The primary structures of peptides **A**–**H** were confirmed by nominal resolution matrix assisted laser desorption mass spectroscopy.[22]

Analysis of Cu(II) Binding. In order to demonstrate that peptides incorporating residues **1a**–**c** and **2** bind Cu(II) under acidic and alkaline conditions, the electronic spectra of **18**, deprotonated Cu(II) complex **19**, and protonated Cu(II) complex **20** were recorded and used as standards, Figure 9a. The electronic spectra of **18** (THF) shows a characteristic absorption at 308 nm with an extinction coefficient of 19 731 M^{-1} cm^{-1} due to the bipyridine chromophore. The deprotonated Cu(II) complex **19** (THF) shows a decrease in absorption at 308 nm and the appearance of two new absorption bands centered at 378 nm and 398 nm. The electronic spectra of protonated Cu(II) complex **20** (methanol) shows that these two absorption bands are blue-shifted to 334 and 349 nm, relative to those of **19**. Additional absorptions due to d-d transitions were observed at 588 nm for **19** and at 645 nm for **20**, Figure 9a (inset).

As expected, peptides incorporating the putative β-sheet nucleators **1a**–**c** and **2** exhibit electronic spectra upon Cu(II) binding under alkaline and acidic conditions that are very similar to the spectra recorded for the model compounds **19** and **20**, respectively. The UV spectra of peptide **F** is shown as a function of Cu(II) concentration and pH, Figure 9b, and is representative of the electronic spectra recorded for the other peptides (data not shown). The electronic spectra of peptide **F** in the absence of Cu(II) at pH 6 closely resembles that of **18**, showing the characteristic absorption of the bipyridine chromophore at 307 nm. The UV spectra of peptide **F·Cu(II)** at pH 9 closely resembles that of the deprotonated Cu(II) complex **19**, showing a decrease in absorption at 307 nm and the appearance of two new absorption bands at 377 and 400 nm. The titration of peptide **F** at pH 6 with 0.5 and 1.0 equiv of Cu(II) (50 and 100 μM, respectively) results in electronic spectra similar to the protonated Cu(II) complex **20**, showing absorption bands at 335 and 350 nm. The presence of isobestic points centered at 325, 290, and 260 nm in the titration of **F** with Cu(II) at pH 6 suggests a simple two-state equilibrium, i.e., apopeptide in equilibrium with Cu(II)-bound peptide. Also, the spectra of peptide **F** in the presence of 1 equiv of Cu(II) at pH 6 and 9 does not change upon further addition of Cu(II) suggesting that peptides incorporating the putative Cu(II) binding β-sheet nucleators **1a**–**c** and **2** bind Cu(II) with a 1:1 stoichiometry.

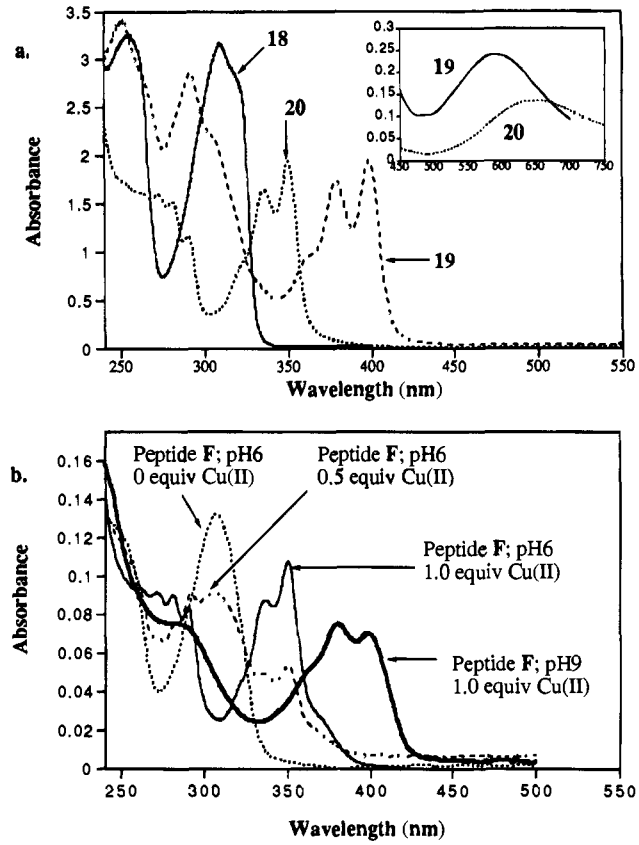


Figure 9. (a) UV spectra of 6,6'-bis(phenylacetamide)-2,2'-bipyridine (**18**), deprotonated Cu(II) complex **19**, and protonated Cu(II) complex **20**. The inset shows the d-d transitions for **19** and **20**. The sample concentration was 1.6 mM for **18** in THF and 1.3 mM for **19** and **20** in THF and methanol, respectively. (b) UV spectra of peptide **F** at pH 6 as a function of Cu(II) concentration. The electronic spectra of peptide **F** at pH 9.0 in the presence of 1 equiv of Cu(II) is also shown. The sample concentration was 100 μM in 10 mM borate buffer containing 75 mM NaCl for pH 9 and 10 mM acetate buffer containing 75 mM NaCl for pH 6.

Matrix assisted laser desorption ionization (MALDI) mass spectroscopy was employed to corroborate the 1:1 binding stoichiometry of Cu(II) to peptides **D**, **E**, and **F**. Solutions of peptides **D**, **E**, and **F** in methanol in the presence of excess Cu(II) (6.6 equiv) resulted in mass spectra which showed almost exclusively the $[M + Cu(II)]^+$ peaks for the corresponding peptides. Minor peaks assignable to small amounts of apopeptide $[M + H]^+$ and $[M + 2Cu(II)]^+$ were also detected.

Peptides incorporating residues **1a**–**c** and **2** bind Cu(II) in a 1:1 fashion under alkaline and acidic conditions as discerned by UV analysis and MALDI mass spectroscopy. Under basic conditions, these residues appear to bind Cu(II) in an analogous

(20) Barany, G.; Merrifield, R. B. In *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1980; Vol. 2, pp 3–254.
(21) Tam, J. P.; Merrifield, R. B. In *The Peptides; Analysis, Synthesis and Biology*; Udenfriend, S., Meienhofer, J., Eds.; Academic Press: New York, 1987; pp 185–244.
(22) (a) Chait, B. T.; Kent, S. B. H. *Science* **1992**, *257*, 1885. (b) Kinsel, G. R.; Preston, L. M.; Russell, D. R. *Biol. Mass Spec.* **1994**, *23*, 205.

manner to the deprotonated Cu(II) complex **19**, forming a neutral square planar complex with the 6,6'-amide protons being removed. Under acidic conditions, residues **1a–c** and **2** appear to bind Cu(II) like the protonated Cu(II) model complex **20**, forming a charged (2+), slightly distorted square planar complex with the amide protons present.

Assessment of the Efficacy of Nucleators 1a–c and 2 To Nucleate Folding in Simple Peptides by CD Spectroscopy. The propensity of residues **1a–c** and **2** to nucleate β-sheet formation upon the addition of Cu(II) in aqueous medium was assessed with peptides of varying chain length and sequence employing far UV-CD spectroscopy. In general, the peptide sequences (Figure 8) were chosen to have an amphiphilic periodicity of two, such that the hydrophobic and hydrophilic residues are segregated on opposite sides of the plane of the folded β-sheet.[23] The residues making up these amphiphilic sequences were chosen based on their statistical preference for adopting an extended or β-strand conformation and knowledge derived from previous sequences used by our laboratory for evaluating the efficacy of dibenzofuran-based amino acids as β-sheet nucleators.[1bgnw,3ab,5cd,24] Amphiphilic peptides like peptides **A–F** are expected to dimerize upon folding in order to bury their hydrophobic surface from exposure to aqueous solution.[2b,23]

A series of amphiphilic nona- and undecapeptides containing residues **1a–c** were prepared and evaluated, Figure 8. The far UV-CD spectra of peptides **A–D** at pH 9.5 in the absence of Cu(II) are shown in Figure 10a. The spectra exhibit minima centered at 203 nm which are assignable to random coil conformation indicating that these residues are not capable of nucleating β-sheet structure in peptides **A–D**. However, upon addition of 1 equiv of Cu(II), the CD spectrum of peptide **D** exhibits a minimum at 220 nm and a maximum around 200 nm, which is strongly indicative of β-sheet structure, Figure 10b. The CD spectra of peptides **A·Cu(II)** and **C·Cu(II)** indicate that these peptides exist in a random coil conformation. It is not clear at this time why these sequences do not fold. The CD spectra of peptide **B·Cu(II)** was unattainable due to aggregation under the conditions employed, suggesting that this sequence is self-assembling into a large macromolecular β-sheet structure after intramolecular folding. Figure 10c shows the CD spectra of peptide **D** at pH 9.5 as a function of added Cu(II). Upon addition of 0.5 equiv of Cu(II), the CD spectra exhibits two minima at 203 and 220 nm, which are assignable to random coil and β-sheet contributions, respectively. Addition of 1 equiv of Cu(II) results in a CD spectrum indicative of a fully developed β-sheet structure, implying that Cu(II) binding by residue **1b** is necessary for the nucleation of β-sheet folding in peptide **D**. Addition of more than 1 equiv of Cu(II) results in peptide denaturation, probably due to Cu(II) binding to the peptide backbone (*vide infra*). The capacity of peptide **D·Cu(II)** to adopt β-sheet secondary structure is highly pH dependent. The pH dependent structural transition from random coil to β-sheet occurs in the presence of Cu(II) at pH 9.5, where most of the intrastrand charge repulsions originating from the lysine side chains have been neutralized.

The inability of undecapeptide **C·Cu(II)** to fold may be a result of the Gly fragment composing **1a**, which provides the chain with too many conformational possibilities enabling the random coil conformation to have an energetic advantage over the folded state. Further studies on peptide analogs of peptides **D** and **C** should allow us to further understand their ability or

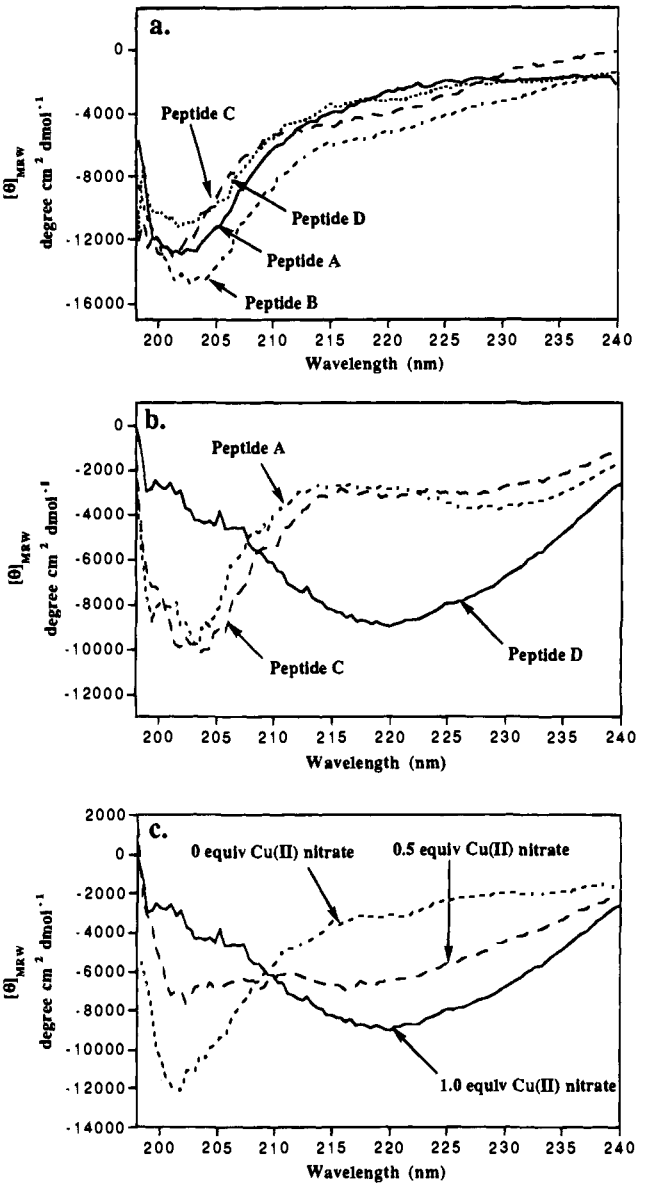


Figure 10. (a) Far-UV CD spectra of peptides **A–D** in the absence of Cu(II) at pH 9.5. (b) Far-UV CD spectra of peptides **A**, **C**, and **D** in the presence of 1 equiv of Cu(II) at pH 9.5. (c) Far-UV CD spectra of peptide **D** as a function of Cu(II) concentration at pH 9.5. The sample concentrations were 175 μM for peptides **A**, **B**, **D**, and 125 μM for **C** in 10 mM borate buffer containing 75 mM NaCl.

inability to fold. The studies carried out thus far demonstrate that residue **1b·Cu(II)** has the ability to promote folding provided an appropriate α-amino acid sequence is chosen. Residue **1b·Cu(II)** does not appear to have the ability to promote hydrophobic cluster formation as a result of its rigid extended structure, suggesting that intramolecular strand–strand hydrogen bonding and hydrophobic interactions are mainly responsible for achieving folding.

Residue **2·Cu(II)** was evaluated as a potential nucleator because it may effect sheet formation via hydrogen bonding as well as through hydrophobic cluster formation mediated by residue **2·Cu(II)**. Residue **2** incorporates $-CH_2CH_2-$ fragments which connect the bipyridine-based metal binding portion of the amino acid and the attached peptide strands. The design premise is that these ethyl fragments will allow a favorable hydrophobic interaction to take place between the bipyridine rings of **2** and the hydrophobic side chains of the flanking α-amino acid residues, Figure 2. Peptide **E** is based on a repeating (-Lys-Val-)$_n$ sequence in which residue **2** is flanked

(23) Kaiser, E. T.; Kezdy, F. J. *Science* **1984**, *223*, 249.
(24) (a) Chou, P. Y.; Fasman, G. D. *J. Mol. Biol.* **1977**, *115*, 135175. (b) Barbier, B.; Caille, A.; Brack, A. *Biopolymers* **1984**, *23*, 2299.

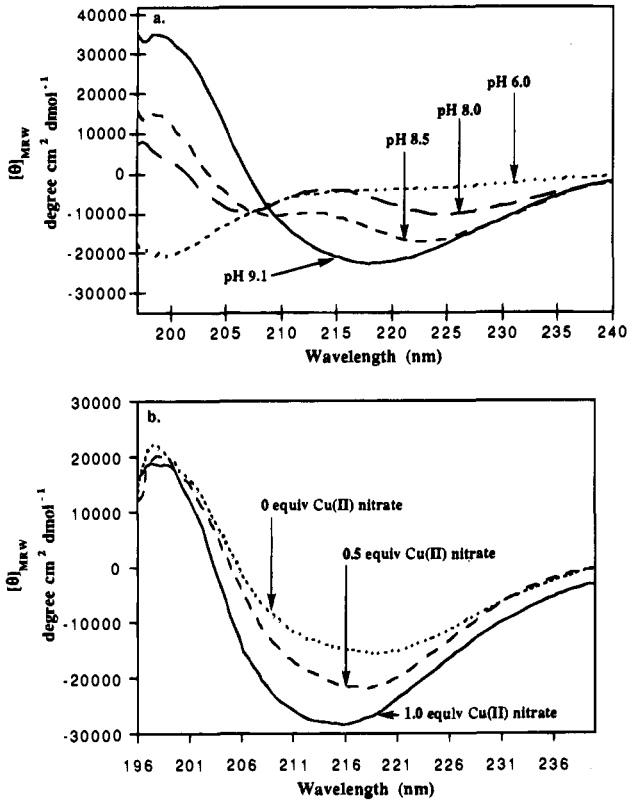


Figure 11. (a) Far-UV CD spectra of peptide **F** as a function of pH in the absence of Cu(II). (b) Far-UV CD spectra of peptide **F** as a function of Cu(II) concentration at pH 9.0. The sample concentration was 100 μM for **F** in 10 mM acetate buffer at pH 6.0 and 10 mM borate buffer for pHs 8.0, 8.5, 9.0, and 9.1 containing 75 mM NaCl.

with Val residues enabling a putative hydrophobic cluster to form between the bipyridine rings of **2** and the Val side chains. Residue **2** was also incorporated into peptide **F** which has a sequence similar to that of peptide **D**, except that **1b** was replaced with -V-**2**-V-. Interestingly, the CD spectra of peptides **E** and **F** demonstrate that peptides containing residue **2** behave differently than those containing residues **1a**−**c** in the absence of added Cu(II). The pH dependent CD spectra of peptide **F** (Figure 11a) indicates that residue **2** is capable of effecting β-sheet structure formation at pH 9.1 in the absence of Cu(II). This behavior was not observed with peptide **D** which contains residue **1b**. It is presumed that folding in peptides **E** and **F** is made possible in the absence of Cu(II) through hydrophobic cluster formation, Figure 2. Hydrophobic cluster formation could stabilize the cisoid bipyridine conformation which is necessary to afford a nucleation competent conformation. Unfortunately, we have not been able to demonstrate that the bipyridine rings of peptides **E** and **F** are cisoid in the absence of Cu(II) at pH 9.1 due to the lack of a reliable spectroscopic probe. It is possible that these peptides are adopting a β-sheet structure via intermolecular interactions, however, this seems unlikely (*vide infra*). The CD spectra of peptide **F** at pH 9.0 as a function of Cu(II) concentration (Figure 11b) exhibits an increase in the minimum at 216 nm demonstrating that **F** binds Cu(II) ions, apparently resulting in the stabilization of the existing β-sheet structure.

Peptide **E** is similar to peptide **F** in sequence except that the two threonine residues in peptide **F** have been replaced by two hydrophilic lysine residues. The CD spectra of peptide **E** as a function of pH (Figure 12a) indicates that this peptide also forms β-sheet structure in the absence of Cu(II), but at a higher pH (9.5), consistent with the higher charge density in peptide **E**. The CD spectra of peptide **E**•**Cu(II)** as a function of pH (Figure

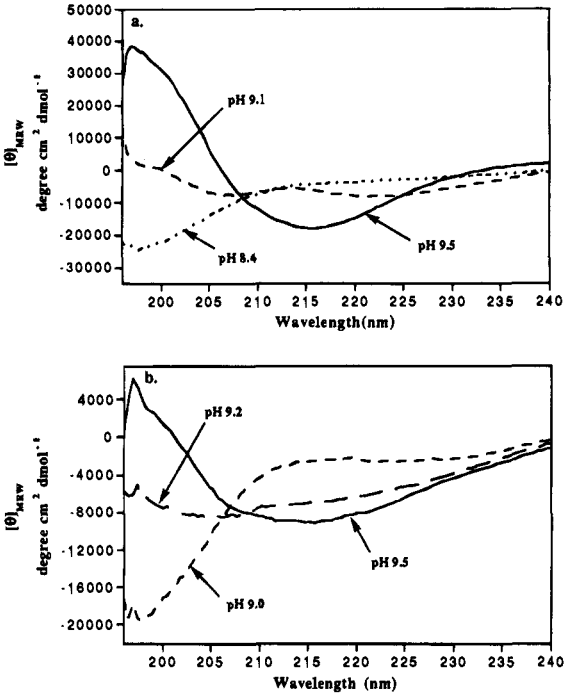


Figure 12. (a) Far-UV CD spectra of peptide **E** as a function of pH in the absence of Cu(II). (b) Far-UV CD spectra of peptide **E** in the presence of 1 equiv of Cu(II) as a function of pH. The sample concentration was 90 μM for **E** in 10 mM borate buffer containing 75 mM NaCl.

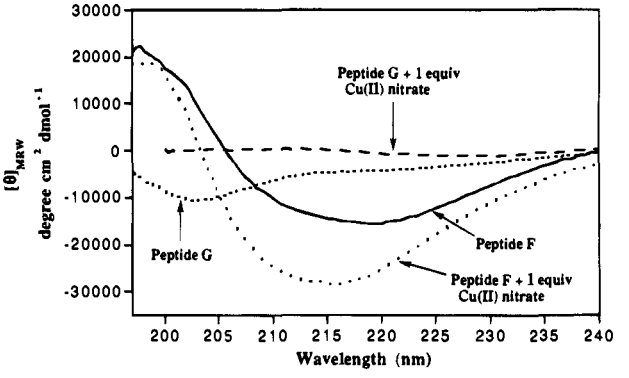


Figure 13. Far-UV CD spectra of peptides **F** and **G** as a function of Cu(II) concentration. The sample concentrations were 100 μM for **F** and 200 μM for **G** in 10 mM borate buffer containing 75 mM NaCl at pH 9.0.

12b) demonstrates that this peptide can also adopt a β-sheet conformation at high pH.

Peptide **G** was prepared in order to demonstrate that the α-amino acid portion of peptides **D** and **F**, alone, are not sufficient to adopt a β-sheet structure and that residue **1b**•**Cu(II)** in peptide **D** and residue **2** in peptide **F** in the presence or absence of Cu(II) are critical for their folding. The dipeptide residue -G-G- was substituted for residue **2** in peptide **F** to afford peptide **G** to understand the role that the amphiphilic α-amino acid sequence plays in the folding of peptide **F**. Similarly, -V-G-G-V- was substituted for residue **1b** to probe the importance of the α-amino acid sequence in the folding of peptide **D**, again via peptide **G**. The CD spectra of peptide **G** in the absence of Cu(II) at pH 9.0 (Figure 13) is that expected of a random coil conformation. If peptide **G** is treated with 1 equiv of Cu(II) at pH 9.0, the CD signal is strongly attenuated due to nonspecific binding of Cu(II) to the peptide backbone. For comparison, the CD spectra of peptide **F** at pH 9.0 in the absence and presence of 1 equiv of Cu(II) is shown. The inability of peptide

G to fold under alkaline conditions in the absence or presence of Cu(II) suggests that the α-amino acid sequence in peptides **D** and **F** are not capable of folding without assistance from residue **1b·Cu(II)** or residue **2** in the presence or absence of Cu(II). Interestingly, if more than 1 equiv of Cu(II) is added to peptides **D** and **F**, the intensity of the β-sheet CD signal decreases significantly (data not shown), demonstrating that the 6,6'-bis(acylamino)-2,2'-bipyridine-binding site has been saturated and that the additional Cu(II) begins to bind to the peptide backbone resulting in denaturation. The CD studies on peptide **G** demonstrate that residues **1b** and **2** play an essential role in nucleating β-sheet structure in peptides **D** and **F**.

Heptapeptide **H** was prepared to gauge the extent of structure in a smaller peptide containing residue **2**. The CD spectra of heptapeptide **H** indicates an ensemble of random conformations exists under alkaline conditions in either the absence or in the presence of 1 equiv of Cu(II). This was not a total surprise owing to the low stability of heptapeptides incorporating the 4-(2-aminoethyl)-6-dibenzofuranpropionic acid nucleating residue.[1w,5c] The inability of peptide **H** to adopt a well-defined conformation demonstrates an apparent length dependence on the folding process in peptides containing residue **2**.

Quaternary Structure Determination. Analytical sedimentation equilibrium ultracentrifugation was employed to assess the aggregation state of peptides **D**, **E**, and **F** under conditions where β-sheet structure is observed by circular dichroism. Sedimentation equilibrium studies on peptide **D·Cu(II)** (pH 9.5) indicate that this peptide adopts a soluble highly associated β-sheet quaternary structure. Peptide **D** appears to undergo intramolecular folding mediated by Cu(II) binding, followed by the self-association of the monomeric β-sheets. Sedimentation equilibrium studies on peptides **E·Cu(II)** and **F·Cu(II)** at pH 9.5 and 9.0, respectively, indicate that these peptides also form soluble β-sheet quaternary structures, presumably as a result of Cu(II)-mediated intramolecular folding followed by self-association.

Sedimentation equilibrium studies on peptides **E** and **F**, in the absence of Cu(II) at pH 9.5 and 9.0, respectively, also reveal that peptides containing residue **2** form soluble β-sheet quaternary structures. Apparently, peptides **E** and **F** can fold intramolecularly in the absence of Cu(II) because the bipyridine rings adopt a putative cisoid conformation stabilized by hydrophobic cluster formation. Intramolecular folding appears to be followed by self-association resulting in the formation of a soluble β-sheet quaternary structure. Alternatively, the bipyridine rings could remain in a transoid conformation under conditions where β-sheet structure is observed. The formation of β-sheet structure in this scenario would be a result of intermolecular association of the peptide strands attached to residue **2**. This possibility seems unlikely because peptide **D**, which has an α-amino acid sequence analogous to that of peptide **F**, does not form intermolecular β-sheet structure under identical conditions.

Discussion

The incorporation of metal binding sites into peptides and proteins has several useful applications in structural and functional biochemistry as well as in protein purification.[8-12,25] The stability of α-helical and β-turn secondary structures have been enhanced by the complexation of transition metal ions to engineered metal binding sites.[9,26] Herein, bipyridine-based amino acids **1a-c·Cu(II)** and **2·Cu(II)** were designed to replace the i+1 and i+2 residues of a β-turn and, in so doing, to test the feasibility of metal-mediated β-sheet nucleation.

Single crystal X-ray analysis of model compound **18** suggests that in the absence of Cu(II), the bipyridine rings of residues **1a-c** and **2** should exist in a transoid conformation in the absence of additional interactions such as hydrophobic cluster formation or hydrogen bonding which could stabilize the cisoid bipyridine conformation. The transoid conformation projects the attached peptide strands in opposite directions making it very difficult for them to associate in an intramolecular fashion. The crystal structures of the neutral Cu(II) complex **19** and charged (2+) Cu(II) complex **20** strongly suggest that residues **1a-c** and **2** bind Cu(II) stoichiometrically under acidic and alkaline conditions in aqueous media, affording square planar complexes. Ultraviolet studies and MALDI mass spectroscopy data fully support the 1:1 Cu(II) binding stoichiometry of peptides incorporating residues **1a-c** and **2**. The resulting cisoid conformation within the bipyridine substructure upon Cu(II) complexation orientates the peptides strands flanking **1a-c** and **2** in an antiparallel fashion with a distance between the strands of ~5 Å.

Circular dichroism studies of peptides incorporating residues **1a-c·Cu(II)**, **2·Cu(II)**, and **2** demonstrate that the ability of these residues to nucleate β-sheet structure is highly dependent on the chain length and the α-amino acid sequence. Peptides **D**, **E**, and **F** were able to fold into a β-sheet conformation in the presence of Cu(II) under alkaline conditions. A circular dichroism study of peptide **D** clearly demonstrates that the ability of residue **1b** to nucleate β-sheet structure at pH 9.5 is dependent on Cu(II) binding. In the absence of Cu(II), peptide **D** exists in a random coil conformation.

The nucleating characteristics of residue **2** are distinct from residue **1b** in that peptide **F** exists in an associated β-sheet conformation in the absence of Cu(II) at pH 9.0. The CD signal assignable to β-sheet structure is enhanced upon the addition of 1 equiv of Cu(II) which can be attributed to additional stabilization of the existing β-sheet structure. Alternatively, Cu(II) binding to the bipyridine system could attenuate the aromatic contribution to the far-UV CD signal (which is usually positive) leading to an apparent increase in the β-sheet minimum.[5c,27] The structure of residue **2** makes it possible to form a hydrophobic cluster composed of the bipyridine rings and the hydrophobic side chains of the α-amino acid residues flanking **2**. It may be that hydrophobic clustering stabilizes the cisoid conformation in the absence of Cu(II) which results in the folding of peptides composed of **2**. Experiments are currently underway to investigate this possibility.[28]

Analytical sedimentation equilibrium ultracentrifugation studies on peptides **D·Cu(II)**, **E·Cu(II)**, and **F·Cu(II)** under alkaline conditions indicate that these peptides form soluble β-sheet quaternary structures. A model of the folding pathway which is in agreement with the spectroscopic data presented is as follows. In the absence of Cu(II), peptide **D** adopts a random coil conformation because the bipyridine rings exist in a transoid conformation. Upon Cu(II) complexation, peptide **D** intramolecularly folds as a result of the square planar cisoid conformation within the bipyridine substructure affording a folded β-sheet

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(28) Near-UV CD experiments of peptides incorporating residue **2** which probe the chiral environment of the bipyridine chromophore are of limited utility in that the chromophore could exist in a chiral environment resulting from the putative hydrophobic cluster or from the soluble β-sheet quaternary structure formed after intramolecular folding takes place.

structure. The resulting monomeric β-sheet then self-associates to form a soluble β-sheet quaternary structure via intermolecular β-sheet formation. It is unlikely, but possible, that the binding of Cu(II) to residue **1b** effects intermolecular β-sheet folding. This scenario seems even less tenable when the inability of peptides **A·Cu(II)** and **C·Cu(II)** to fold is considered. All data on peptide **D·Cu(II)** suggests that intramolecular folding precedes self assembly.

Sedimentation equilibrium studies on peptides **E** and **F** in the absence of Cu(II) at pH 9.5 and 9.0, respectively indicate that the β-sheet structure observed under these conditions is also highly associated. There are two likely folding pathways which are possible in the absence of Cu(II). The first involves a transoid−cisoid isomerization of the bipyridine rings allowing intramolecular folding to take place. The cisoid conformation of the bipyridine rings could be stabilized by the formation of a hydrophobic cluster. After folding, the structured peptides could then self-associate to form a soluble highly extended β-sheet structure. Alternately, the β-sheet structure observed in peptides **E** and **F**, in the absence of Cu(II), could result from intermolecular association of the α-amino acid portions of peptides incorporating residue **2** in a transoid geometry. However, this mode of intermolecular assembly is not consistent with the observation that peptide **D** is incapable of adopting a sheet structure in the absence of Cu(II). Peptides **D** and **F** have similar α-amino acid sequences and differ with regard to whether residues **1b** or **2** are incorporated. It seems unlikely that residue **1b** in the transoid conformation would prevent self-assembly if the α-amino acid portion of the sequence has a propensity to do so. This hypothesis is further supported by peptide **G**'s inability to fold.

Conclusion

An X-ray crystal structure of the ligand portion of residues **1a−c** and **2** demonstrates that these residues can bind Cu(II) in basic aqueous solution affording neutral square planar complexes with the appropriate geometry to support an antiparallel β-sheet structure. Peptide **D** is able to fold at pH 9.5 in a Cu(II) dependent fashion indicating that **1b·Cu(II)** is essential for folding. All evidence supports Cu(II)-mediated intramolecular folding followed by self-association. Residue **2** functions to nucleate sheet folding both in the absence and presence of Cu(II). Folding in the absence of Cu(II) may be facilitated by hydrophobic cluster formation. Efforts currently underway to prepare monomeric and dimeric intramoleculary folded peptides containing **1b·Cu(II)** and **2·Cu(II)** will be reported in due course.

Experimental Section

General Methods and Material. The tetrahydrofuran (THF) and benzene used were distilled from sodium/benzophenone ketyl, while dichloromethane (CH_2Cl_2) was distilled from calcium hydride. Triethylamine (TEA) and diisopropylethylamine (DIEA) were refluxed over ninhydrin, distilled, and distilled again from calcium hydride under N_2. Valine-methyl ester hydrochloride was prepared by refluxing 1 equiv of valine and 10 equiv of thionylchloride in 30 mL of anhydrous methanol. Hydrazine monohydrate and hexadecyltributylphosphonium bromide were purchased from Lancaster and used without further purification. Dicyclohexylcarbodiimide (DCC), anhydrous *N,N*-dimethylformamide (DMF), succinic anhydride, 1-(3-ethylcarbodiimide hydrochloride) (EDCI), phenylacetic acid, 2-chloro-1-methylpyridinium iodide, and copper(II) nitrate hemipentahydrate were purchased from Aldrich and used without further purification. The appropriate side chain protected Boc-amino acids and BOP were purchased from Advanced Chemtech and used without further purification. The pentafluorophenol which was purchased from PCR Fluoroorganics and other reagents were used without further purification. Monoethyl-2,2-dimethyl malonate was prepared according to Krapcho *et al.*[17] Copper hydroxide was prepared by titrating a 85 mM solution of copper(II) nitrate hemipentahydrate in water with 1 N NaOH.[30] Flash chromatography was performed as described by Still.[31] Melting points were determined using a Mel-temp apparatus and are uncorrected. Routine NMR spectra were recorded on a Varian XL-200E spectrometer. FT-IR data were collected on a Galaxy 5000 spectrometer. UV data were collected on a Milton Roy spectronic 3000 diode array or a Varian Cary 2200 spectrophotometer. Preliminary examination and data collection for the X-ray diffraction studies were performed on a Siemens R3m/V X-ray diffractometer (oriented graphite monochromator; MoKα $\lambda = 0.710\,73$Å radiation). Mass determinations were carried out on a VG-70S double focusing high-resolution mass spectrometer. Preparative HPLC was carried out on a dual pump system equipped with Altex 110A pumps and a 420 gradient programmer. The column employed was a Waters RCM Delta Pak C_{18} (15 μM, 300 Å, 25 × 100 mm) attached to a Knauer 86 variable-wavelength detector set at 254 nm. Solvent A was composed of 95% water, 5% acetonitrile (Fisher, Optima grade), and 0.2% TFA. Solvent B was composed of 5% water, 95% acetonitrile, and 0.2% TFA. The synthesis of diamine **5** was accomplished by improving on a published procedure.[15] Briefly, 6,6'-dibromo-2,2'-bipyridine was refluxed in hydrazine monohydrate to afford 6,6'-dihydrazine-2,2'-bipyridine (**3**). Oxidation of **3** with sodium nitrite yields 6,6'-diazide-2,2'-bipyridine (**4**). Reduction of **4** with sodium borohydride affords diamine **5**. Experimental details can be found below.

Synthesis of 6,6'-Dihydrazine·2,2'-bipyridine (3). The 6,6'-dibromo-2,2'-bipyridine used in these studies was prepared according to the published procedure by Garber and Rillema.[29a,b] A 250 mL, round-bottomed flask equipped with a reflux condenser was charged with 6,6'-dibromo-2,2'-bipyridine (5.0 g, 15.9 mmol) and hydrazine monohydrate (120 mL, 3.85 mol). This stirred suspension became a clear solution after 10 h of heating at reflux. Cooling the reaction solution slowly results in the formation of yellow needles. The crystals were isolated by filtration, washed with water, and air dried to afford 3.25 g (15.0 mmol, 95%) of **3**; mp 206−208 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 7.6−7.5 (m, 4H, Ar-3,3',4,4' *H*), 7.4 (s, 2H, N*H*), 6.68 (dd, $J = 7.1$ and 1.8 Hz, 2H, Ar-5,5' *H*), 4.16 (s, 4H, NH_2);^{13}C NMR (DMSO·d_6, 200 MHz) δ 161.5, 153.9, 137.7, 109.7, 106.9; EIMS *m/z* (M^+) calcd 216.1123, obsd 216.1124.

Synthesis of 6,6'-Diazide·2,2'-bipyridine (4). A 1 L round-bottomed flask was charged with dihydrazine **3** (5.73 g, 26.5 mmol) and 143 mL of 12 M HCl. The reaction mixture was cooled with an ice bath to 5 °C and stirred. The flask was then equipped with an addition funnel containing a solution of $NaNO_2$ (56 g, 0.81 mol) in 278 mL of water which was added slowly and carefully. After the addition, the foaming mixture was stirred at 5 °C for 1 h, allowed to warm to room temperature, and then stirred for an additional 6 h. The reaction mixture was reduced to 1/3 of its original volume under reduced pressure, and the resulting solution was made basic (pH 10−11) with 10% NaOH. The resulting white precipitate was collected and washed with copious amounts of water, affording 6.11 g (25.7 mmol, 97%) of **4** after drying under high vacuum: mp 193−194 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.52 (dd, $J = 7.7$ and 2.3 Hz, 2H, Ar-3,3' *H*), 8.0−8.2 (m, 4H, Ar-4,4',5,5' *H*); ^{13}C NMR (DMSO-d_6, 200 MHz) δ 148.4, 133.0, 127.7, 120.7, 117.5; EIMS *m/z* (M^+) calcd 238.0715, obsd 238.0715.

Synthesis of Diamine 5. A 100 mL, three-necked, round-bottomed flask equipped with an addition funnel and reflux condenser was charged with hexadecyltributylphosphonium bromide (1.1 g, 2.16 mmol), diazide **4** (750 mg, 3.15 mmol), and 27 mL of toluene. This stirred solution was held at 100 °C, while a solution of $NaBH_4$ (1.3 g, 34.3 mmol) in 10 mL of water was added dropwise. After addition,

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the reaction was allowed to proceed for 8 h at 100 °C. The reaction mixture was then cooled to room temperature, and the aqueous and organic phases were separated. The organic phase was extracted with 10% HCl (3 × 20 mL). The acidic aqueous phase was then made basic by dropwise addition of NH_4OH and extracted with chloroform (4 × 25 mL). The combined chloroform extracts were dried (Na_2SO_4) and concentrated to yield 467 mg (2.52 mmol, 80%) of **5** as a light yellow solid. Crystals suitable for X-ray diffraction were obtained by recrystallization from an absolute ethanol/CH_2Cl_2 mixture and were subsequently washed with diethyl ether: mp 185−186 °C; ^{1}H NMR (acetone-d_6, 200 MHz) δ 7.57 (dd, J = 7.9 and 0.9 Hz, 2H, Ar-3,3'*H*), 7.42 (t, J = 7.9 Hz, 2H, Ar-4,4'*H*), 6.49 (dd, J = 7.9 and 0.9 Hz, 2H, Ar-5,5'*H*), 5.33 (s, 4H, NH_2); ^{13}C NMR (acetone-d_6, 200 MHz) δ 160.2, 158.1, 139.4, 110.5, 109.5; EIMS *m/z* (M^+) calcd 186.0905, obsd 186.0903.

Synthesis of Ethyl Ester 6. A flame dried 250 mL, round-bottomed flask equipped with a reflux condenser was charged with monoethyl-2,2-dimethyl malonate (4.0 g, 25.2 mmol), diamine **5** (4.7g, 25.2 mmol), 2-chloro-1-methylpyridinium iodide (6.75 g, 26.4 mmol), 193 mL of chloroform, and TEA (8.42 mL, 60.4 mmol). The reaction mixture was heated at reflux and stirred under argon until the mixture became homogeneous. The solution was then cooled to room temperature and stirred an additional 3.5 h, and then it was transferred to a 500 mL separatory funnel along with 125 mL of 10% HCl. The aqueous and organic phases were separated, and the hydrochloride salt of **6** which precipitated from the chloroform extract was collected by filtration and suspended in 150 mL of CH_2Cl_2. The suspension was transferred to a 500 mL separatory funnel, washed with 10% NaOH (1 × 100 mL), dried (Na_2SO_4), and concentrated to yield **6** as a light yellow oil. The chloroform mother liquor was evaporated, and the resulting dark yellow oil was dissolved in 150 mL of CH_2Cl_2, washed with 10% NaOH (1 × 100 mL), dried (Na_2SO_4), and evaporated to yield an oil which was flash chromatographed on silica (ethyl acetate/hexanes/NH_4OH, 44: 56: 1) to afford additional **6**. Total isolated yield of **6** was 3.3 g (10.0 mmol, 40%): ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.82 (s, 1H, Ar-6-N*H*CO-), 8.19 (d, J = 8.0 Hz, 1H, Ar-5*H*), 8.03 (d, J = 8.0 Hz, 1H, Ar-3*H*), 7.78 (t, J = 8.0 Hz, 1H, Ar-4*H*), 7.67 (d, J = 8.0 Hz, 1H, Ar-3'*H*), 7.54 (t, J = 8.0 Hz, 1H, Ar-4'*H*), 6.55 (d, J = 8.0 Hz, 1H, Ar-5'*H*), 4.55 (s, 2H, Ar-NH_2), 4.25 (q, J = 7.2 Hz, 2H, $-CH_2CH_3$), 1.59 (s, 6H, $-C(CH_3)_2$), 1.31 (t, J = 8.0 Hz, 3H, $-CH_2CH_3$); ^{13}C NMR (acetone-d_6, 200 MHz) δ 174.2, 171.2, 159.9, 155.7, 154.1, 151.7, 139.4, 138.5, 116.9, 113.8, 110.3, 109.5, 61.9, 51.6, 23.5, 14.1; mass spectrum (FAB, poly(ethylene glycol) (PEG)/thioglycerol matrix) *m/z* 329.1642 [(M + H)$^+$, calcd for $C_{17}H_{21}N_4O_3$, 329.1613].

Synthesis of Ethyl Ester 7. A 100 mL, round-bottomed flask was charged with ethyl ester **6** (470 mg, 1.43 mmol), *N*-Boc-glycine (1.0 g, 5.73 mmol), BOP (2.53 g, 5.73 mmol), and 10 mL of anhydrous CH_2Cl_2. This solution was stirred under N_2, and 1.34 mL (7.69 mmol) of DIEA was added via syringe. After 48 h, the reaction solution was transferred to a 125 mL separatory funnel, washed with 5% $NaHCO_3$ (3 × 20 mL), 1 M citric acid (1 × 20 mL), water (1 × 20 mL), and dried (Na_2SO_4). The CH_2Cl_2 extract was evaporated to yield a clear oil which was flash chromatographed on silica (ethyl acetate/hexanes/NH_4OH 1:1:0.002) to afford 570 mg (1.18 mmol, 83%) of **7** as a white solid: mp 144−146 °C; ^{1}H NMR (acetone-d_6, 200 MHz) δ 9.56 (s, 1H, Ar-6-N*H*−), 9.35 (s, 1H, Ar-6'-N*H*−), 8.30−7.85 (m, 6H, Ar-3,3',4,4',5,5'*H*), 6.41 (bs, 1H, $-NHCO_2-$), 4.23 (q, J = 7.2 Hz, 2H, $-OCH_2CH_3$), 4.05 (bd, 2H, $-COCH_2NH-$), 1.60 (s, 6H, $-C(CH_3)_2$), 1.48 (s, 9H, -$C(CH_3)_3$), 1.27 (t, J = 8.0 Hz, 3H, $-CH_2CH_3$); ^{13}C NMR (acetone-d_6, 200 MHz) δ 188.6, 185.2, 174.1, 171.3, 169.4, 154.4, 151.9, 139.6, 116.8, 116.7, 114.5, 114.3, 79.3, 61.9, 51.6, 45.3, 28.3, 23.5, 14.0; mass spectrum (FAB, PEG/thioglycerol matrix) *m/z* 486.2354 [(M + H)$^+$, calcd for $C_{24}H_{32}N_5O_6$, 486.2353].

Synthesis of Acid 8. A 25 mL, round-bottomed flask was charged with ethyl ester **7** (783 mg, 1.61 mmol) and 14 mL of absolute ethanol. The reaction solution was stirred at 0 °C and 3.38 mL of 1 N NaOH was added via pipet. After 1.5 h of stirring, the reaction solution was transferred to a separatory funnel along with 47 mL of cold water, and 1 M citric acid was added until the aqueous solution remained acidic (pH 4). This solution was extracted with ethyl acetate (3 × 15 mL), and the organic phase was washed with water (1 × 25 mL), dried (Na_2SO_4), and evaporated to yield 374 mg (0.82 mmol, 51%) of **8** as a white solid: mp 190−191 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 10.50 (s, 1H, Ar-6-N*H*−), 10.13 (s, 1H, Ar-6'-N*H*-), 8.20−7.90 (m, 6H, Ar-3,3',4,4',5,5'*H*) 7.12 (bt, 1H, $-NHCO_2-$), 3.84 (bd, 2H, -$COCH_2NH$-), 1.49 (s, 6H, $-C(CH_3)_2$), 1.39 (s, 9H, $-C(CH_3)_3$); ^{13}C NMR (DMSO-d_6, 200 MHz) δ 174.9, 171.7, 169.2, 155.9, 153.3, 153.2, 151.4, 151.2, 139.1, 116.0, 115.9, 114.3, 113.7, 78.0, 50.5, 43.7, 28.1, 23.1; mass spectrum (FAB, PEG/thioglycerol matrix) *m/z* 458.2045 [(M + H)$^+$, calcd for $C_{22}H_{28}N_5O_6$, 458.2039].

Synthesis of Ethyl Ester 9. A 100 mL, round-bottomed flask was charged with ethyl ester **6** (520 mg, 1.58 mmol), *N*-Boc-valine (1.03 g, 4.75 mmol), BOP (2.10 g, 4.75 mmol), and 75 mL of anhydrous CH_2Cl_2. This solution was stirred under N_2, and 1.38 mL (7.93 mmol) of DIEA was added via syringe. After 30 h, the reaction solution was transferred to a 125 mL separatory funnel, washed with 5% $NaHCO_3$ (3 × 20 mL), 1 M citric acid (1 × 20 mL), water (1 × 20 mL), and dried (Na_2SO_4). The chloroform extract was evaporated to yield an oil which was flash chromatographed on silica (ethyl acetate/hexanes 95:5) to afford 491 mg (0.93 mmol, 59%) of **9** as a white solid: mp 178−180 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.92 (s, 1H, Ar-6-N*H*−), 8.59 (s, 1H, Ar-6'-N*H*−), 8.23−8.12 (m, 2H, Ar-5,5'*H*), 8.01 (dd, J = 7.7 and 2.5 Hz, 2H, Ar-3,3'*H*), 7.80 (t, J = 7.9 Hz, 2H, Ar-4,4'*H*) 5.20 (d, J = 8.4 Hz, 1H, $-NHCO_2-$), 4.27 (q, J = 7.1 Hz, 2H, -OCH_2-CH_3), 4.18 (m, 1H, -$CHCH(CH_3)_2$), 2.27 (m, 1H, $-CH(CH_3)_2$), 1.59 (s, 6H, $-C(CH_3)_2$), 1.45 (s, 9H, $-C(CH_3)_3$), 1.31 (t, J = 7.0 Hz, 3H, $-OCH_2CH_3$), 1.02 (m, 6H, $-CH(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 200 MHz) δ 174.9, 171.3, 171.0, 156.4, 154.3, 151.3, 150.9, 139.7, 117.6, 114.5, 81.0, 62.3, 51.2, 31.0, 28.5, 23.8, 19.6, 17.9, 14.2; mass spectrum (FAB, PEG/thioglycerol matrix) *m/z* 528.2841 [(M + H)$^+$, calcd for $C_{27}H_{38}N_5O_6$, 528.2822].

Synthesis of Acid 10. A 50 mL, round-bottomed flask was charged with ethyl ester **9** (932 mg, 1.76 mmol) and 17 mL of absolute ethanol. The reaction solution was stirred at 0 °C and 3.71 mL of 1 N NaOH was added via pipet. After 2.5 h of stirring, the reaction solution was transferred to a separatory funnel along with 43 mL of cold water, and 1 M citric acid was added until the aqueous solution remained acidic (pH 4). This solution was extracted with ethyl acetate (3 × 15 mL), and the organic phase was washed with water (1 × 25 mL), dried (Na_2SO_4), and evaporated to yield 768 mg (1.54 mmol, 88%) of **10** as a white solid: mp 169−170 °C; ^{1}H NMR (acetone-d_6, 200 MHz) δ 9.68 (s, 1H, Ar-6-N*H*-), 9.42 (s, 1H, Ar-6'-N*H*-), 8.30−7.81 (m, 6H, Ar-3,3',4,4',5,5'*H*), 6.20 (bd, 1H, $-NHCO_2-$), 4.29 (bt, 1H, $-COCHCH(CH_3)_2-$), 2.25 (m, 1H, $-CH(CH_3)_2$), 1.61 (s, 6H, $-C(CH_3)_2-$), 1.42 (s, 9H, $-C(CH_3)_3$), 1.05 (m, 6H, $-CH(CH_3)_2$); ^{13}C NMR (acetone-d_6, 200 MHz) δ 175.7, 174.8, 172.1, 171.5, 154.4, 154.3, 152.0, 151.9, 139.7, 139.6, 117.0, 116.9, 114.5, 79.2, 73.4, 51.2, 31.2, 28.3, 23.6, 19.6, 18.0; mass spectrum (FAB, PEG/thioglycerol matrix) *m/z* 500.2510 [(M + H)$^+$, calcd for $C_{25}H_{34}N_5O_6$, 500.2509].

Synthesis of Ethyl Ester 11. A 100 mL, round-bottomed flask was charged with ethyl ester **6** (1.69 g, 5.15 mmol), *N*-Boc-leucine·H_2O (12.8 g, 51.5 mmol), BOP (22.7 g, 51.5 mmol), 14 mL of anhydrous CH_2Cl_2, and 9 mL of DMF. This solution was stirred under N_2 and 13.5 mL (77.5 mmol) of DIEA was added via syringe. After 96 h, the reaction solution was transferred to a 250 mL separatory funnel, washed with 5% $NaHCO_3$ (3 × 25 mL), 1 M citric acid (1 × 25 mL), and water (3 × 25 mL), and dried (Na_2SO_4). The CH_2Cl_2 extract was evaporated to yield a clear oil which was flash chromatographed on silica (ethyl acetate/hexanes, 1: 9) to afford 1.31 g (2.42 mmol, 47%) of **11** as a white solid: mp 165−167 °C. ^{1}H NMR (acetone-d_6, 200 MHz) δ 10.06 (bs, 1H, Ar-6-N*H*−), 9.60 (bs, 1H, Ar-6'-N*H*-), 8.33−7.90 (m, 6H, Ar-3,3',4,4',5,5'*H*), 6.50 (bd, 1H, $-NHCO_2-$), 4.49 (m, 1H, $-CHCH_2CH(CH_3)_2-$), 4.27 (q, J = 7.1 Hz, 2H, $-OCH_2CH_3$), 1.86 (m, 1H, $-CH(CH_3)_2$), 1.75 (m, 2H, $-CH_2CH(CH_3)_2$), 1.61 (s, 6H, $-C(CH_3)_2$), 1.45 (s, 9H, $-(CH_3)_3$), 1.28 (t, J = 7.5 Hz, 3H, $-OCH_2CH_3$), 1.00 (d, J = 6.4 Hz, 6H, $-CH(CH_3)_2$); ^{13}C NMR (acetone-d_6, 200 MHz) δ 175.0, 173.9, 172.2, 157.1, 155.3, 155.2, 153.0, 152.8, 140.3, 140.2, 117.7, 117.5, 115.2, 115.1, 79.9, 62.4, 55.2, 52.1, 41.9, 41.5, 28.8, 25.7, 23.9, 23.7, 22.1, 14.5; mass spectrum (FAB, PEG/thioglycerol matrix) *m/z* 542.2971 [(M + H)$^+$, calcd for $C_{28}H_{40}N_5O_6$, 542.2978].

Synthesis of Acid 12. A 100 mL, round-bottomed flask was charged with ethyl ester **11** (1.31 g, 2.42 mmol) and 24 mL of absolute ethanol. The reaction solution was stirred at 0 °C, and 5.0 mL of 1 N NaOH

was added via pipet. After 3 h, the reaction solution was transferred to a separatory funnel along with 47 mL of cold water, and 1 M citric acid was added until the aqueous solution remained acidic (pH 4). This solution was extracted with ethyl acetate (3 × 25 mL), and the organic phase was washed with water (1 × 25 mL), dried (Na_2SO_4), and evaporated to yield 1.11 g (2.16 mmol, 90%) of **12** as a white solid: mp 102−104 °C; ^{1}H NMR (acetone-d_6, 200 MHz) δ 9.64 (s, 1H, Ar-6-N*H*−), 9.47 (s, 1H, Ar-6'-N*H*−), 8.21 (dd, J = 8.1 and 1.1 Hz, 2H, Ar-3,3'*H*), 8.03 (d, J = 7.2 Hz, 2H, Ar-5,5'*H*), 7.94−7.84 (m, 2H, Ar-4,4'*H*), 6.40 (bd, 1H, −N*H*CO_2−), 4.42 (m, 1H, −C*H*CH_2CH-$(CH_3)_2$−), 1.82 (m, 1H, −C*H*$(CH_3)_2$), 1.72 (m, 2H, −CH_2C*H*$(CH_3)_2$), 1.59 (s, 6H, −C$(CH_3)_2$), 1.41 (s, 9H, −C$(CH_3)_3$), 0.97 (m, 6H, -CH-$(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 200 MHz) δ 176.6, 172.0, 171.0, 155.9, 153.6, 153.5, 150.8, 150.7, 139.0, 138.9, 117.0, 114.1, 77.2, 54.1, 50.6, 41.2, 28.3, 24.8, 23.7, 23.0, 21.8; mass spectrum (FAB, PEG/thioglycerol matrix) m/z 514.2678 [(M + H)$^+$, calcd for $C_{26}H_{36}N_5O_6$, 514.2666].

Synthesis of Amine 13. A 250 mL, round-bottomed flask equipped with a rubber septum was charged with dicyclohexylcarbodiimide (2.2 g, 10.75 mmol), anhydrous CH_2Cl_2 (100 mL), Boc-β-alanine (2.0 g, 10.75 mmol), diamine **5** (2.0 g, 10.75 mmol), and 3 mL of anhydrous DMF. This solution was stirred under N_2 for 23 h. The reaction mixture was concentrated under reduced pressure, and the resulting oily solid was suspended in 50 mL of chloroform. The suspension was allowed to sit at 4 °C overnight after which the insoluble urea byproduct was filtered away. The resulting filtrate was concentrated to yield a yellow oil which was dissolved in 10 mL of DMSO and purified by preparative HPLC employing a linear gradient from 20% to 30% solvent B over 30 min. The resulting solution was concentrated to remove the acetonitrile and the remaining aqueous fraction was made basic with 5% $NaHCO_3$ (pH 8) and extracted with ethyl acetate (3 × 30 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated to yield 1.59 g (4.45 mmol, 41%) of **13** as a light yellow oil. ^{1}H NMR (acetone-d_6, 200 MHz) δ 9.61 (s, 1H, Ar-6'-N*H*CO−), 8.20 (d, J = 8.3 Hz, 1H, Ar-5'*H*), 8.05 (d, J = 7.7 Hz, 1H, Ar-3'*H*), 7.80 (t, J = 7.7 Hz, 1H, Ar-4'*H*), 7.52 (m, 2H, Ar-3,4*H*), 6.60 (d, J = 7.6 Hz, 1H, Ar-5*H*), 6.12 (s, 1H, −CH_2N*H*CO−), 5.55 (s, 2H, Ar-N*H*$_2$), 3.46 (m, 2H, −C*H*$_2$NHCO−), 2.73 (t, J = 5.5 Hz, 2H, −COC*H*$_2$-CH_2−), 1.39 (s, 9H, -C$(CH_3)_3$); ^{13}C NMR (acetone-d_6, 200 MHz) δ 171.2, 159.9, 156.4, 155.5, 154.3, 152.0, 139.1, 138.4, 116.5, 113.8, 110.3, 109.3, 78.6, 37.7, 37.2, 28.4; mass spectrum (FAB, PEG/NBA matrix) m/z 358.1879 [(M + H)$^+$, calcd for $C_{18}H_{24}N_5O_3$, 358.1879].

Synthesis of Methyl Ester 14. A dry 25 mL, round-bottomed flask equipped with a reflux condenser and rubber septum was charged with amine **13** (30 mg, 0.084 mmol), methyl ester **17** (100 mg, 0.25 mmol), DIEA (16.1 uL, 0.092 mmol), and 4 mL of anhydrous benzene. The reaction solution was heated at reflux and stirred under N_2 for 17 h. The product precipitated from solution upon cooling to 5 °C. The solid was collected and washed with diethyl ether and dried under high vacuum for 2 h to afford 41 mg (0.072 mmol, 86%) of **14**; mp 213−215 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 10.61 (s, 1H, Ar-N*H*-$CO(CH_2)_2CO$−), 10.51 (s, 1H, Ar-N*H*$CO(CH_2)_2$NH−), 8.2−7.8 (m, 6H, Ar-3,3',4,4',5,5'*H*), 6.85 (bt, 1H, −N*H*CO_2−), 3.59 (s, 3H, CO_2CH_3), 3.22 (m, 2H, -CH_2NHCO_2−), 2.74 (m, 2H, −CH_2CH_2-NHCO_2−), 2.60 (m, 4H, −COC*H*$_2$C*H*$_2$CO_2CH_3), 1.39 (s, 9H, −C$(CH_3)_3$); ^{13}C NMR (DMSO-d_6, 200 MHz) δ 172.7, 170.9, 170.5, 155.4, 153.4, 153.3, 151.4, 139.0, 115.6, 113.8, 113.7, 77.5, 51.3, 39.2, 36.8, 36.7, 30.7, 28.1; mass spectrum (FAB, PEG/glycerol/thioglycerol matrix) m/z 571.2878 [(M + H)$^+$, calcd for $C_{28}H_{39}N_6O_7$, 571.2880].

Synthesis of Acid 15. A 100 mL, round-bottomed flask was charged with methyl ester **14** (0.97 g, 1.7 mmol), 37 mL of methanol, and 17 mL of 1 N LiOH. The reaction solution was stirred for 2 h and then transferred to a separatory funnel along with 50 mL of ethyl acetate. Citric acid (1 M) was added until the aqueous layer remained acidic (pH 4). The aqueous and organic phases were separated, and the aqueous phase was extracted with ethyl acetate (2 × 25 mL). The combined ethyl acetate extracts were washed with water (1 × 50 mL), dried (Na_2SO_4), and concentrated under reduced pressure to yield an oil which was crystallized from acetone. The white solid was collected, washed with diethyl ether, and dried under high vacuum to afford 713 mg (1.28 mmol, 75%) of **15**; mp 134−136 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 12.52 (bs, 1H, −CO_2*H*), 10.51 (s, 2H, Ar-N*H*−), 8.22−7.85 (m, 6H, Ar-3,3',4,4',5,5'*H*), 7.82 (bd, 1H, −CON*H*C$_\alpha$H), 6.84 (bt, 1H, −CH_2N*H*CO_2−), 4.20 (m, 1H, −NHC$_\alpha$*H*CO_2H), 3.29 (m, 2H, −C*H*$_2$NH−), 2.82−2.44 (m, 7H, −COC*H*$_2$C*H*$_2$CO, −C*H*$_2$CH_2NH, −C*H*$(CH_3)_2$), 1.29 (s, 9H, −C$(CH_3)_3$), 0.85 (m, 6H, −C$(CH_3)_2$); ^{13}C NMR (DMSO-d_6, 200 MHz) δ 173.4, 171.8, 170.9, 155.9, 153.7, 151.9, 151.8, 139.3, 116.0, 115.9, 114.1, 114.0, 77.9, 65.2, 36.9, 36.7, 31.9, 30.2, 30.1, 28.5, 19.4, 18.3; mass spectrum (FAB, PEG/glycerol/thioglycerol matrix) m/z 557.2739 [(M + H)$^+$, calcd for $C_{27}H_{37}N_6O_7$, 557.2724].

Synthesis of Methyl Ester 16. A dry 250 mL, round-bottomed flask was charged with succinic anhydride (1 g, 9.99 mmol) and 100 mL of chloroform. This mixture was allowed to stir until most of the anhydride dissolved (15 min). Valine-methyl ester hydrochloride (1.67 g, 9.99 mmol) was then added along with 1.74 mL (9.98 mmol) of DIEA, and the solution was stirred for 1 h. Additional DIEA (1.74 mL, 9.98 mmol) was added, and the reaction was allowed to proceed for 2 additional h. The chloroform was removed under reduced pressure to yield an oil which was dissolved in 50 mL of ethyl acetate. This solution was transferred to a separatory funnel and 5% HCl was added with gentle swirling (in order to avoid an emulsion) until the aqueous phase remained acidic (pH 4). The organic and aqueous phases were separated, and the aqueous phase was extracted with ethyl acetate (2 × 25 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated to yield **16** as a clear oil which was dried under high vacuum: 2.29 g (9.91 mmol, 99%); ^{1}H NMR (acetone-d_6, 200 MHz) δ 9.02 (bs, 1H, −CO_2*H*), 7.26 (d, J = 7.2 Hz, 1H, -N*H*-), 4.42 (dd, J = 8.7 and 5.7 Hz, 1H, −NHC*H*CO−), 3.62 (s, 3H, −CO_2CH_3), 2.55 (m, 4H, −COC*H*$_2$CO−), 2.09 (m, 1H, $(CH_3)_2$C*H*−), 0.85 (m, 6H, $(CH_3)_2$−); ^{13}C NMR (acetone-d_6, 200 MHz) δ 176.2, 173.8, 173.7, 58.5, 52.7, 31.9, 31.4, 30.3, 19.7, 18.6; EIMS m/z (M$^+$) calcd 231.1107, obsd 231.1120.

Synthesis of Methyl Ester 17. A flame dried 100 mL, round-bottomed flask equipped with a rubber septum was charged with pentafluorophenol (0.4 g, 2.17 mmol), methyl ester **16** (478 mg, 2.07 mmol), and 10.3 mL of anhydrous CH_2Cl_2. This solution was stirred under N_2 at 0 °C, and 1-(3-ethylcarbodiimide hydrochloride) (0.42 g, 2.17 mmol) was added as a solid under a blanket of N_2. The reaction solution was stirred at 0 °C for an additional 10 min and then allowed to warm to room temperature where it was stirred for an additional 20 h. The solution was transferred to a separatory funnel and washed with 5% HCl (3 × 10 mL) and then water (2 × 10 mL). The CH_2Cl_2 extract was dried (Na_2SO_4) and concentrated to yield an oil which was triturated with hexanes to yield a white solid. The solid was recrystallized from a hexanes/ethyl acetate mixture to yield 0.66 g (1.65 mmol, 80%) of **17**; mp 81−82 °C. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 6.29 (d, J = 7.5 Hz, 1H, −N*H*−), 4.59 (dd, J = 5.0 and 8.9 Hz, 1H, −NHC*H*CO−), 3.71 (s, 3H, −CO_2CH_3), 3.03 (m, 2H, −C*H*$_2$CONH−), 2.68 (t, J = 7.5 Hz, 2H, −C*H*$_2$CH_2CONH−), 2.14 (m, 1H, −C*H*$(CH_3)_2$), 0.89 (m, 6H, $(CH_3)_2$−); ^{13}C NMR ($CDCl_3$, 200 MHz) δ 173.1, 170.9, 169.5, 57.5, 52.4, 31.5, 30.7, 28.8, 18.9, 17.9; mass spectrum (FAB, PEG/NBA matrix) m/z 398.1031 [(M + H)$^+$, calcd for $C_{16}H_{17}NO_5F_5$, 398.1026].

Synthesis of Diphenylacetamide 18. A dry 250 mL, round-bottomed flask equipped with a rubber septum was charged with phenylacetic acid (1.17 g, 8.6 mmol), BOP (3.8 g, 8.6 mmol), and 168 mL of anhydrous CH_2Cl_2. The solution was stirred under N_2, while DIEA (2.43 mL, 13.9 mmol) was added via syringe. After 20 min of additional stirring, diamine **5** (400 mg, 2.15 mmol) was added as a solid under a blanket of N_2, and the reaction solution was stirred for 24 h. The product, which precipitated during the course of the reaction, was collected and washed with dichloromethane as well as diethyl ether and dried under vacuum to afford 700 mg (1.65 mmol, 77%) of **18**; mp 268−270 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 10.79 (s, 2H, Ar-N*H*−), 8.20−7.85 (m, 6H, Ar-3,3',4,4',5,5'*H*), 7.46−7.20 (m, 10H, Ph-*H*), 3.79 (s, 4H, −COC*H*$_2$−); ^{13}C NMR (DMSO-d_6, 200 MHz) δ 170.5, 153.8, 151.9, 139.4, 136.1, 129.5, 128.6, 126.9, 116.3, 114.1, 43.3; IR (KBr) 3242 (br), 1656, 1590, 1542, 1500, 1441, 1376, 1318, 1297, 1073, 800, 722, 695 cm^{-1}; EIMS m/z (M$^+$) calcd 422.1743, obsd 422.1735.

Synthesis of Deprotonated Copper Complex 19. A 250 mL, round-bottomed flask equipped with a reflux condenser was charged

with **18** (300 mg, 0.71 mmol), $Cu(OH)_2$ (138 mg, 1.42 mmol), and 143 mL of anhydrous THF. The reaction was heated at reflux and stirred under N_2 for 6 h. The hot mixture was filtered in order to remove excess $Cu(OH)_2$, and the resulting green solution was concentrated and dried under high vacuum to yield a purple solid which was recrystallized from methanol to afford 174 mg (0.36 mmol, 51%) of **19** as blue needles. Crystals suitable for X-ray diffraction were obtained by slow evaporation of a saturated solution of **19** in methanol: mp 224−226 °C; IR (KBr) 3367, 3083, 3059, 3027, 1602, 1565, 1507, 1469, 1415, 1250, 1173, 1016, 964, 803, 737, 697; UV (THF) 250.5 (26 153), 290.9 (21 846), 378.4 (13 292), 398.6 (14 938), 588.3 (184); mass spectrum (FAB/PEG matrix) *m/z* 484.0973 [(M + H)$^+$, calcd for $C_{26}H_{21}N_4O_2Cu$, 484.0961].

Synthesis of Protonated Copper Complex 20. A 50 mL, round-bottomed flask was charged with **18** (150 mg, 0.35 mmol) and 19.2 mL of methanol. The reaction mixture was stirred at room temperature, and $Cu(NO_3)_2 \cdot 2.5\ H_2O$ (86.8 mg, 0.37 mmol) was added as a solid. The reaction was stirred until it became a homogenous blue solution (20 min), and then it was concentrated to yield a blue solid which was recrystallized from water at 4 °C to afford 126 mg (0.21 mmol, 58%) of **20** as blue needles. Crystals suitable for X-ray diffraction were obtained by slow evaporation of a saturated solution of **20** in methanol: mp 247−249 °C dec; IR (KBr) 3432, 2854, 1621, 1550, 1384, 1307, 1186, 1025, 800, 743; UV (methanol) 272.4 (12 231), 281.3 (11 615), 290.1 (8923), 334.7 (12 615), 349.3 (14 923), 645.3 (103) mass spectrum (FAB, PEG/NBA matrix) *m/z* 485.1035 [calcd for $C_{26}H_{22}N_4O_2Cu$, 485.1039].

Synthesis of Peptides. Manual solid-phase peptide synthesis was carried out by employing the benzhydrylamine (BHA) resin available from Advanced Chemtech having a loading of 0.6 mequiv/g for peptides **A** and **B**, 0.7 mequiv/g for peptides **C**, **D**, **F**, and **G**, and 0.55 mequiv/g for peptide **H**. The dichloromethane (CH_2Cl_2), isopropyl alcohol (IPA), and dimethylformamide (DMF) used were reagent grade. DMF was stored over 4-Å molecular sieves to reduce the primary and secondary amine impurities. Side-chain protected Boc-amino acids were purchased from Advanced Chemtech. Trifluoroacetic acid (TFA) was purchased from PCR Fluorochemicals and was used as a 25−50% solution in CH_2Cl_2 containing 1% thioanisole (Aldrich) as a scavenger. Diisopropylethylamine (DIEA) was dried as described earlier.

The BHA resin was swelled in CH_2Cl_2 for 2−3 h and washed with 12% DIEA (2 × 5 min) and CH_2Cl_2 (4 × 1 min) prior to use. The first amino acid was loaded onto the resin by shaking 3 equiv of Boc-protected amino acid, 3 equiv of BOP, and 5 equiv of DIEA in CH_2Cl_2 containing 10−15% DMF for 4 h. The resin was washed with DMF (2 × 1 min), CH_2Cl_2 (2 × 1 min), IPA (2 × 1 min), and CH_2Cl_2 (3 × 1 min). The completion of the reaction was monitored by the Kaiser ninhydrin test. If the result was slightly positive, the remaining unreacted amino groups on the resin were then capped by acetylation. The following cycle was used for each coupling: TFA prewash (25−50% TFA × 1 min), TFA deprotection (25−50% TFA × 40 min), CH_2Cl_2 (2 × 1 min), IPA (2 × 1 min), CH_2Cl_2 (3 × 1 min), preneutralization (12% DIEA × 1 min), neutralization (12% DIEA × 10 min), CH_2Cl_2 (4 × 1 min), coupling (3 equiv of amino acid, 3 equiv of BOP, 5 equiv of DIEA in CH_2Cl_2 containing 10−15% DMF for 2−5 h), DMF (2 × 1 min), CH_2Cl_2 (2 × 1 min), IPA (2 × 1 min), and CH_2Cl_2 (3 × 1 min). Each coupling step was monitored by the Kaiser ninhydrin test. In the case of peptides containing the 6,6'-bis-(acylamino)-2,2'-bipyridine-based amino acids, the 1-hydroxybenzotriazole (HOBt) active ester was employed to incorporate amino acids **8**, **10**, **12**, and **15** into the respective peptide. The coupling was performed in the following manner: 2 equiv of amino acid was preactivated in a separate reaction flask under N_2 by stirring 2 equiv of diisopropylcarbodiimide (DIC) and 2 equiv of HOBT in CH_2Cl_2 containing 10% DMF at 0 °C for 20 min. This solution was added to the resin which contained enough CH_2Cl_2 to swell the resin and 1.1 equiv of DIEA.

After the completion of each sequence, the resin bound peptides were treated with TFA to remove the Boc-protecting group, dried under vacuum, and finally cleaved from the resin and side chain deprotected by HF.[21] All peptides were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOFMS).[22]

Summary of Purification and Characterization of Peptides A−H.

Peptide A ($C_{65}H_{111}N_{17}O_{12}$). Purification by preparative C_{18} HPLC employing a linear gradient from 15% to 35% solvent B over 35 min: overall yield 32%; MALDI-TOFMS (MH)$^+$ calcd 1323.7, obsd 1323.8.

Peptide B ($C_{62}H_{104}N_{16}O_{13}$). Purification by preparative C_{18} HPLC employing a linear gradient from 0% to 30% solvent B over 5 min: overall yield 8%; MALDI-TOFMS (MH)$^+$ calcd 1282.6, obsd 1282.6.

Peptide C ($C_{68}H_{113}N_{18}O_{15}$). Purification by preparative C_{18} HPLC employing a linear gradient from 0% to 30% solvent B over 3 min followed by isocratic elution at 30% B for 10 min: overall yield 10%; MALDI-TOFMS (MH)$^+$ calcd 1423.8, obsd 1423.9.

Peptide D ($C_{72}H_{124}N_{20}O_{15}$). Purification by preparative C_{18} HPLC employing a linear gradient from 15% to 30% solvent B over 30 min: overall yield 45%; MALDI-TOFMS (MH)$^+$ calcd 1510.9, obsd 1511.1.

Peptide E ($C_{83}H_{146}N_{24}O_{15}$). Purification by preparative C_{18} HPLC employing isocratic conditions of 20% B for 30 min: overall yield 41%; MALDI-TOFMS (MH)$^+$ calcd 1721.2, obsd 1721.2.

Peptide F ($C_{79}H_{136}N_{22}O_{17}$). Purification by preparative C_{18} HPLC employing isocratic conditions of 20% B for 30 min: overall yield 65%; MALDI-TOFMS (MH)$^+$ calcd 1667.1, obsd 1667.1.

Peptide G ($C_{66}H_{123}N_{19}O_{16}$). Purification by preparative C_{18} HPLC employing isocratic conditions of 30% B for 30 min: overall yield 50%; MALDI-TOFMS (MH)$^+$ calcd 1439.8, obsd 1439.8.

Peptide H ($C_{49}H_{81}N_{14}O_9$). Purification by preparative C_{18} HPLC employing isocratic conditions of 20% B for 30 min: overall yield 37%; MALDI-TOFMS (MH)$^+$ calcd 1011.3, obsd 1011.3.

UV−Vis Studies. The UV spectra were collected on either a Milton Roy spectronic 3000 diode array or a Varian Cary 2200 spectrophotometer using 1 and 10 mm quartz cells. The peptide samples were prepared from stock solutions in water and diluted to the appropriate concentration with either 10 mM acetate or 10 mM borate buffers containing 75 mM NaCl. Cu(II) was introduced by preparing a stock solution of copper nitrate hemipentahydrate in water and adding the appropriate volume to each sample via pipet. UV samples of compound **18** and Cu(II) complexes **19** and **20** were prepared by weight. All samples were allowed to equilibrate for 20 min at 25 °C, and all spectra are corrected for buffer or solvent contributions.

Circular Dichroism Studies. CD spectra were collected on a Jasco J-600 spectropolarimeter using a 1 mm quartz cell. The samples were prepared from stock solutions in water and diluted to the appropriate concentration with desired buffer. The concentrations of peptides **A**−**F** and **H** were determined by UV spectroscopy at 307 nm (ϵ = 15 608 $cm^{-1}\ M^{-1}$), while the concentration of peptide **G** was calculated based on its mass. The samples were allowed to equilibrate at 25 °C over 10 h and degassed prior to performing the CD experiments. CD data were collected at 25 °C using a scan speed of 20 nm/min, a time constant of 0.5 s, and a band width of 1 nm. All spectra were corrected for copper and buffer contributions and are reported in units of mean residue ellipticity.[32] The data from the Jasco spectrometer was imported into the Macintosh version of KaleidaGraph and processed.

Matrix Assisted Laser Desorption Mass Spectroscopy. Nominal mass spectra were collected on a matrix assisted laser desorption ionization (MALDI) mass spectrometer constructed by Dave Russell and co-workers.[22b] The matrix employed was 4-nitroaniline and gramacidin S was employed to externally calibrate the instrument. Nominal masses of peptides **A**−**H** were obtained from 1 mg/mL solutions of the peptides in methanol. Nominal masses of peptides **D**, **E**, and **F** in the presence of excess Cu(II) nitrate (6.6 equivalence) were obtained by incubating 100 μM solutions of peptide in methanol with Cu(II) nitrate introduced from a 0.0165 M stock solution of Cu(II) nitrate in methanol for 15 min. Observed masses for **D·Cu(II)**, **E·Cu(II)**, and **F·Cu(II)** were 1573.3, 1784.1, and 1729.0, respectively, and are consistent with the calculated values: [peptide **D** + Cu(II)]$^+$, 1573.4; [peptide **E** + Cu(II)]$^+$, 1783.8; [peptide **F** + Cu(II)]$^+$, 1729.6).

Sedimentation Equilibrium Analytical Ultracentrifugation. The apparent molecular weights of peptides **D**, **E**, and **F** were obtained from data collected on a Beckman XL-A Analytical Ultracentrifuge equipped with absorption optics. A double sector cell equipped with a 12 mm Epon centerpiece and sapphire windows was used for the experiments described within. Samples were loaded into the cell using a blunt end micro-syringe resulting in a column height of approximately 3 mm. The samples were prepared from stock solutions in water and diluted

to the appropriate concentration with 10 mM borate buffer containing 75 mM NaCl. The concentrations of peptides **D**, **E**, and **F** were determined by UV spectroscopy at 307 nm (ϵ = 15 608 cm^{-1} M^{-1}). In a typical experiment, an initial absorbance scan is performed at 23 °C with a rotor speed of 3000 rpm. The rotor speed is then increased to 42 000 rpm where sedimentation equilibrium is established. A complete loss of the initial absorbance is indicative of a highly associated system.

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Supplementary Material Available: A listing of bond lengths, bond angles, and coordinates as well as isotropic and anisotropic displacement factors characteristic of the solid state structure of 6,6'-bis(phenylacetamide)-2,2'-bipyridine (**18**), neutral Cu(II) complex **19**, and (2+) charged Cu(II) complex **20** (9 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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