Metallopeptide Design: Tuning the Metal Cation Affinities with Unnatural Amino Acids and Peptide Secondary Structure

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Abstract: The ability to tune the metal binding affinity of small peptides through the incorporation of unnatural multidentate α-amino acids and the preorganization of peptide structure is illustrated. Herein, we describe the exploitation of a family of α-amino acids that incorporate powerful bidentate ligands (bipyridyl and phenanthrolyl groups) as integral constituents of the residues' side chains. The residues involved are the 6-, 5-, and 4-substituted (*S*)-2-amino-3-(2,2′-bipyridyl)propanoic acids (**1**, 6Bpa; **2**, 5Bpa; **3**, 4Bpa), (*S*)-2-amino-3-(1,10-phenanthrol-2-yl)-propanoic acid (**4**, Fen), and a novel neocuproine-containing α-amino acid, (*S*)-2-amino-3-(9-methyl-1,10-phenanthrol-2-yl)propanoic acid (**5**, Neo). Within this family of amino acids, variations in metal binding due to the nature of the ring system (2,2′-bipyridyl or 1,10-phenanthrolyl) and the point of attachment to the amino acid β-carbon are observed. Additionally, the underlying peptide architecture significantly influences binding for peptides that include multiple metal-ligating residues. These differences in affinity arise from the interplay of ligand type and structural preorganization afforded by the peptide sequence, resulting in dissociation constants ranging from 10^{-3} to $<10^{-6}$ M for Zn^{II}. These studies illustrate that significant control of metal cation binding affinity, preference, and stoichiometry may be achieved through the use of a wide variety of native and unnatural metal-coordinating amino acids incorporated into a polypeptide architecture.

Introduction

Recent efforts in the *de novo* design of metalloproteins have focused on the assembly of polypeptides with defined structural and potentially functional properties.[1,2] The production of peptidyl motifs designed to detect Zn^{II} in solution emphasizes the importance of tuning the metal binding affinity of such constructs.[3] The ability to modulate metal binding affinity is essential in order to operate such peptide-based sensors over a wide metal cation concentration range. Furthermore, it is necessary for the peptide to preferentially bind the metal cation of interest to exploit such peptides in sensor applications. One approach to the construction of these devices involves the use of novel multidentate metal-binding residues.[4−9] An advantage to the utilization of such residues in small peptidyl constructs is that enhanced metal binding affinities may be achieved. Whereas nature exploits the protein architecture to carefully position the metal-binding side chain functionalities within metalloprotein systems, unnatural metal-chelating amino acids simplify the task by simultaneously orienting more than one ligating atom. Additionally, the chemical synthesis of small peptides generally affords milligram quantities of material and allows the incorporation of the unnatural amino acids at any position in the primary sequence.

The bidentate heteroaromatic ligands 2,2′-bipyridine and 1,-10-phenanthroline are among the best known coordination agents.[10] These multidentate nitrogen ligands generally afford high binding affinities for metal cations due to the chelation effect and the π-accepting ability of these moieties.[11] While alkyl substitution at the 6,6′-positions of 2,2′-bipyridine and the 2,9-positions of 1,10-phenanthroline is expected to raise the basicity of the donor nitrogens, substituted ligands generally exhibit weaker metal binding affinities than the parent heterocycle due to steric effects. Additionally, alkyl substitution can affect the metal binding preference and complex stoichiometry. α-Amino acids featuring these bidentate ligands as side chains can therefore be exploited to control the metal cation binding affinities and preferences of peptides incorporating these residues.

Herein, we describe the metal-binding properties of several peptides that incorporate 2,2′-bipyridine, 1,10-phenanthroline, or 2,9-dimethyl-1,10-phenanthroline (neocuproine) functionalities. The syntheses and peptide incorporation of the 6-, 5-, and 4-substituted (*S*)-2-amino-3-(2,2′-bipyridyl)propanoic acid (**1**, 6Bpa; **2**, 5Bpa; **3**, 4Bpa) have been previously reported.[6] The utility of these residues has been highlighted in the semisynthesis of cytochrome *c* mutants with enhanced electron transfer properties.[12] Additionally, the residue (*S*)-2-amino-3-(1,10-phenanthrol-2-yl)propanoic acid (**4**, Fen) has been used

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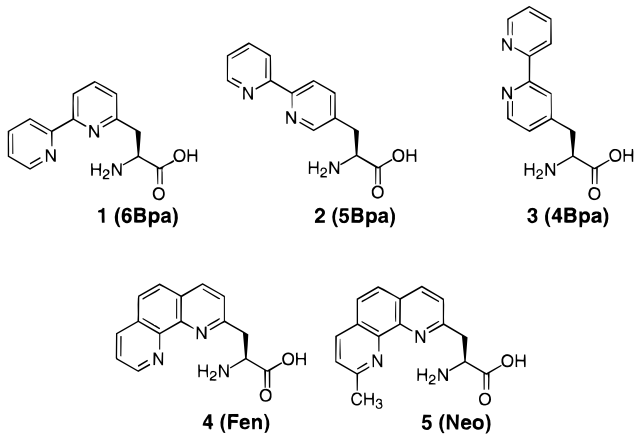
to provide a spectroscopic reporter group in an iterative design process that resulted in the formation of a folded, metal-independent $\beta\beta\alpha$ motif.[13] The syntheses of Fen (**4**) and the novel neocuproine-containing α-amino acid, (*S*)-2-amino-3-(9-methyl-1,10-phenanthrol-2-yl)propanoic acid (**5**, Neo), are also presented in this paper. These syntheses afford multigram quantities of N^{α}-Fmoc-protected α-amino acids for incorporation into polypeptides. A key step in the syntheses of both the Fen and Neo residues involves an enzymatic resolution with alkaline protease.[6a,14] These unnatural amino acids are completely compatible with peptide assembly using standard Fmoc-based solid phase methodology and do not require side chain protection.[15] Therefore, these bidentate unnatural residues with enviable inherent metal-binding properties can be readily incorporated into small peptides.



Naturally occurring metalloproteins frequently form metal binding sites with multiple residues to achieve the high affinity and selectivity for their native metal cations. Therefore, the incorporation of more than one metal-ligating amino acid in designed metallopeptides could enhance the affinity and selectivity of such constructs. In order for two residues to cooperatively participate in the formation of a high-affinity metal binding site, it is advantageous to place the coordinating side chain functionalities in close proximity. One of the most direct methods to achieve this placement would be to situate the key residues flanking a preorganized polypeptide motif such as a reverse turn. The chain reversal afforded by a β-turn would place residues at either end of the sequence proximal to each other and promote the cooperative binding of metal cations.[16] Judicious placement of the residues is essential to situate the two metal binding side chains on the same face of the β-hairpin motif (Figure 1).[17] Molecular modeling studies reveal that two metal-binding amino acids flanking a four-residue turn-forming peptide sequence places the metal coordinating moieties on the same face of the motif and in close proximity. The type II β-turn has been studied extensively,[18−20] and therefore different peptide sequences with varying turn-forming propensities were utilized to connect the two metal-binding residues. The metal-binding affinity can therefore be varied by changing the extent of structural preorganization of the motif required for high-affinity and cooperative binding between two ligating residues.

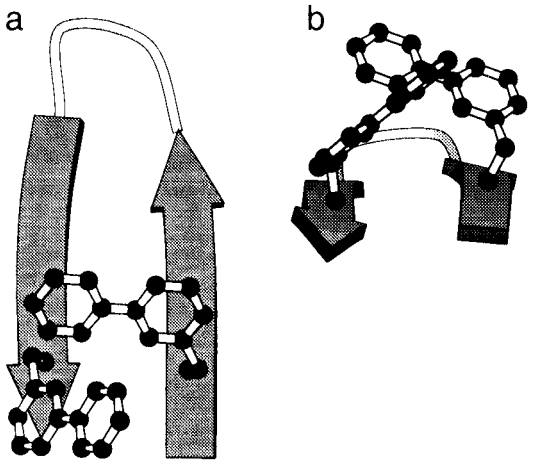


Figure 1. (a) Schematic diagram of the β-hairpin motif designed to bring two 6Bpa residues into close proximity (generated using the program MOLSCRIPT[17]). (b) An edge-on view of the same model.

Several hexapeptides incorporating the unnatural metal-chelating amino acids described above were synthesized, and their metal-binding properties were investigated. Peptides containing only one bidentate residue and those with two bidentate amino acids display different affinities for metal cations, indicating cooperative ligation in the latter. Furthermore, constructs containing both a bidentate residue and a naturally occurring metal-binding residue (Asp or His) were prepared and exhibited varying metal-binding affinities. Two sequences, -Val-Pro-D-Ser-Phe- and -Thr-Pro-D-Ala-Val- with different type II β-turn propensities were used to connect the metal-binding residues to probe the influence of structural preorganization on metal binding affinity.[19,21,22] In light of the successful utilization of a type II′ turn as a structural nucleation element in a designed $\beta\beta\alpha$ motif,[13] an additional peptide containing the sequence -Val-D-Pro-Ser-Phe-, which was predicted to form a type II′ β-turn, was also investigated. For all the peptides studied, the *N*-termini were capped with an acetyl group and the *C*-termini were capped with an amide in order to avoid potential peptide conformational changes due to electrostatic interactions and competing metal cation coordination effects involving the free carboxyl or amino termini.

Results

Synthesis of N^{α}-Fmoc-(*S*)-2-amino-3-(1,10-phenanthrol-2-yl)propanoic Acid (Fmoc-Fen) and N^{α}-Fmoc-(*S*)-2-amino-3-(9-methyl-1,10-phenanthrol-2-yl)propanoic Acid (Fmoc-Neo). The syntheses of the two racemic amino acids were accomplished using a modified version of the Sørenson method[23] from the corresponding chloromethyl derivatives. Esterification of the amino acids in acidic methanol produced the amino acid methyl esters (**6** and **7**)[24] ready for enzymatic resolution. Stereoselective hydrolysis of the α-amino acid methyl esters was achieved using the enzyme alkaline protease (Scheme 1).[6a,14] The hydrolysis afforded the L-enantiomer with high stereoselectivity (>98% ee). The absolute configuration of the resulting

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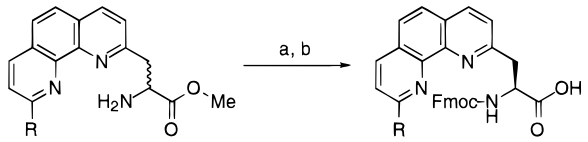
(24) Experimental details for the preparation of compounds **6** and **7** are described in the Supporting Information.

Scheme 1. Synthesis of Fmoc-Protected Fen and Neo α-Amino Acids



6 R = H
7 R = CH_3
8 R = H
9 R = CH_3

[a] Alkaline protease, tBuOH, 10% $NaHCO_3$, room temperature. [b] Fmoc-OSu, dioxane, bicarbonate solution, room temperature.

amino acids was established by the specificity of the enzyme to hydrolyze only the L-amino acid ester and the convention of the elution profile for the CrownPak CR (+) chiral column. Furthermore, the change of optical rotation upon lowering the pH for Neo followed the Clough−Lutz−Jirgenson rule (pH 8.8, $[\alpha]_D^{25}$ +80.3; pH 0, $[\alpha]_D^{25}$ 8.5),[25] which further defines the configuration of the stereocenter. Subsequent protection of the L-amino acid with *N*-9-fluorenylmethylsuccinimidyl carbonate (Fmoc-OSu) yielded the fully protected phenanthroline amino acid derivatives (**8** and **9**), which were used directly for solid phase peptide synthesis. All steps leading to both α-amino acids are amenable to large-scale synthesis.

Dissociation Constants of the Peptide−M^{II} Complexes. The hexapeptides are designated according to the residues present at positions 1 and 6. Standard three-letter abbreviations are employed for natural residues, while 6Bpa, 5Bpa, and 4Bpa are used to designate the unnatural α-amino acids **1**, **2**, and **3**, and Fen and Neo are used for residues **4** and **5**, respectively. The prime (′) designates that the type II′ turn sequence -Val-D-Pro-Ser-Phe- connects the first and last residues. The subscript w indicates the connecting sequence -Thr-Pro-D-Ala-Val-, which displays a limited tendency to form a turn.[22] Lack of any additional notation would refer to the intervening sequence -Val-Pro-D-Ser-Phe- which exhibits stronger turn propensity.[19,21]

Bpa-Containing Peptides. The peptides $4BpaPhe_w$ (Ac-4Bpa-Thr-Pro-D-Ala-Val-Phe-NH_2), $5BpaPhe_w$ (Ac-5Bpa-Thr-Pro-D-Ala-Val-Phe-NH_2), and $6BpaPhe_w$ (Ac-6Bpa-Thr-Pro-D-Ala-Val-Phe-NH_2) were synthesized and studied. Additionally, peptides 6Bpa6Bpa (Ac-6Bpa-Val-Pro-D-Ser-Phe-6Bpa-NH_2) and $6Bpa6Bpa_w$ (Ac-6Bpa-Thr-Pro-D-Ala-Val-6Bpa-NH_2), which both contain two 6Bpa residues, were also investigated. The metal cation titrations of these Bpa-containing peptides were monitored by absorption spectroscopy (UV−Vis) as illustrated in Figure 2. The $\pi-\pi^*$ transition of the bipyridyl moiety displays a distinct red-shift upon chelation of metal cations. Clean isosbestic points were observed, supporting a two-state model equilibrium. Binding isotherms were obtained from such experiments. The apparent dissociation constants for the peptide−M^{II} complexes were determined to be the free metal cation concentration when half of the peptide was bound. The apparent dissociation constants of the peptide−M^{II} complexes for the Bpa-containing peptides are summarized in Table 1.

Fen- and Neo-Containing Peptides. Peptides $FenPhe_w$ (Ac-Fen-Thr-Pro-D-Ala-Val-Phe-NH_2) and NeoPhe (Ac-Neo-Val-Pro-D-Ser-Phe-Phe-NH_2) were synthesized and evaluated to assess the inherent chelation properties of the residues Fen and Neo, respectively. Additionally, five hexapeptides were designed to probe the effect upon metal binding by delivering an auxiliary, natural metal-binding residue that may act cooperatively with the Fen or Neo bidentate residue (Table 2). The

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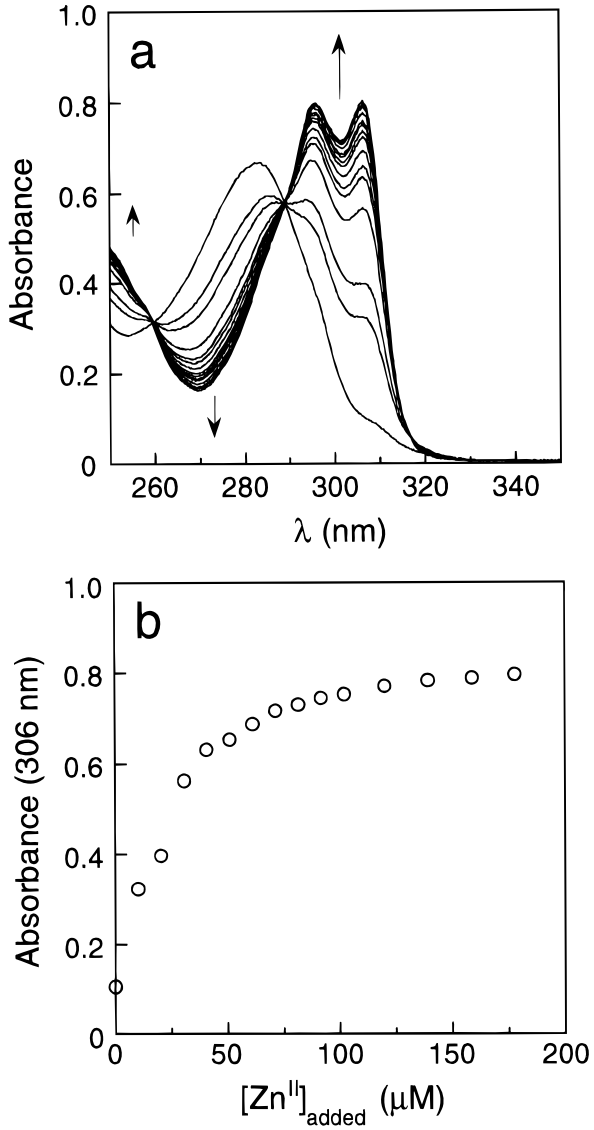

Figure 2. (a) Absorption spectra of 52 μM $4BpaPhe_w$ upon addition of $ZnCl_2$ up to 178 μM at pH 6.8−7.0 in 0.2 M NaCl. (b) The binding isotherm obtained from the data shown in panel a at 306 nm.

Table 1. Apparent Dissociation Constants (μM) for Bpa-Containing Peptide-M^{II} Complexes[a]

peptide	sequence	Zn^{II}	Co^{II}
$4BpaPhe_w$	Ac-4Bpa-Thr-Pro-D-Ala-Val-Phe-NH_2	22	ND[b]
$5BpaPhe_w$	Ac-5Bpa-Thr-Pro-D-Ala-Val-Phe-NH_2	19	ND[b]
$6BpaPhe_w$	Ac-6Bpa-Thr-Pro-D-Ala-Val-Phe-NH_2	2700	1100
$6Bpa6Bpa_w$	Ac-6Bpa-Thr-Pro-D-Ala-Val-6Bpa-NH_2	960	110
6Bpa6Bpa	Ac-6Bpa-Val-Pro-D-Ser-Phe-6Bpa-NH_2	16	7

[a] Metal cation titrations performed at pH 7 in 0.2 M NaCl. [b] Dissociation constants not determined due to stoichiometry of 2:1 peptide:metal.

metal cation titrations of the Fen- and Neo-containing peptides were monitored by UV−Vis spectroscopy (Figures 3 and 4). Similar to the Bpa-containing peptides, a distinct red-shift of the $\pi-\pi^*$ transition for the phenanthrolyl moiety was observed upon binding metal cations. Thus, UV−Vis spectroscopy was used to monitor the metal cation titrations. Clean isosbestic points were observed for all the titrations except for experiments performed on peptide NeoPhe, in which essentially no change in the absorption spectrum was observed upon the addition of Zn^{II} or Co^{II}. For this peptide only, metal cation titrations were monitored by circular dichroism (CD) spectroscopy, since distinct changes in the near-UV region of the CD spectra

Table 2. Apparent Dissociation Constants (μM) of Fen- and Neo-Containing Peptide-M^{II} Complexes[a]

peptide	sequence	Zn^{II}	Co^{II}
$FenPhe_w$	Ac-Fen-Thr-Pro-D-Ala-Val-Phe-NH_2	26.8	7.91
$FenHis_w$	Ac-Fen-Thr-Pro-D-Ala-Val-His-NH_2	1.50	2.48
FenHis	Ac-Fen-Val-Pro-D-Ser-Phe-His-NH_2	0.65	0.60
FenHis′	Ac-Fen-Val-D-Pro-Ser-Phe-His-NH_2	0.96	1.57
FenAsp	Ac-Fen-Val-Pro-D-Ser-Phe-Asp-NH_2	8.28	3.91
NeoPhe	Ac-Neo-Val-Pro-D-Ser-Phe-Phe-NH_2	110[b]	300[b]
NeoHis	Ac-Neo-Val-Pro-D-Ser-Phe-His-NH_2	0.69	5.45

[a] Metal cation titrations performed in 0.05 M HEPES buffer at pH 8.25. [b] Values obtained from titrations performed in 0.01 M Tris buffer at pH 8.25 monitored by circular dichroism (CD) spectroscopy.

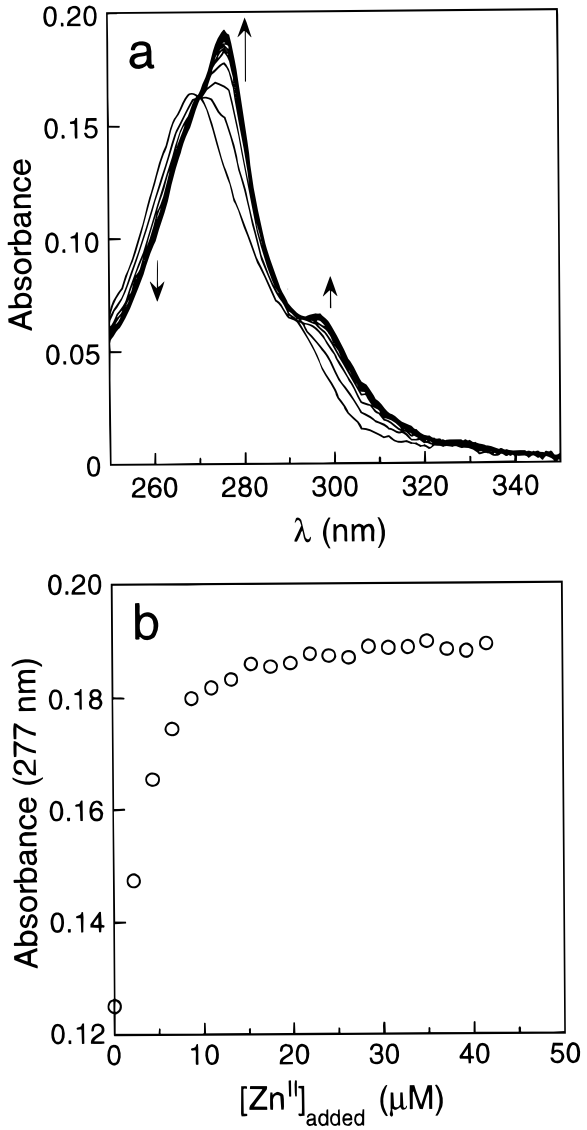

Figure 3. (a) Absorption spectra of 7.85 μM FenHis upon addition of $ZnCl_2$ up to 42 μM in 0.05 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer at pH 8.25. (b) The binding isotherm obtained from the data shown in panel a at 277 nm.

attributable to the neocuproine moiety were observed (Figure 5). Calculations employing an iterative method based on the Scott equation[26] were performed to obtain the apparent dissociation constants for the peptide−M^{II} complexes (Table 2). The final iteration from a typical analysis is shown in Figure 6, in which $[M^{II}]_{free}/b\Delta A$ is plotted against $[M^{II}]_{free}$ and the apparent dissociation constant is given by *y*-intercept/slope ($[M^{II}]_{free}$ = free M^{II} concentration in solution, where *b* is the path length of cuvette and ΔA is the change in absorbance at the wavelength of interest).

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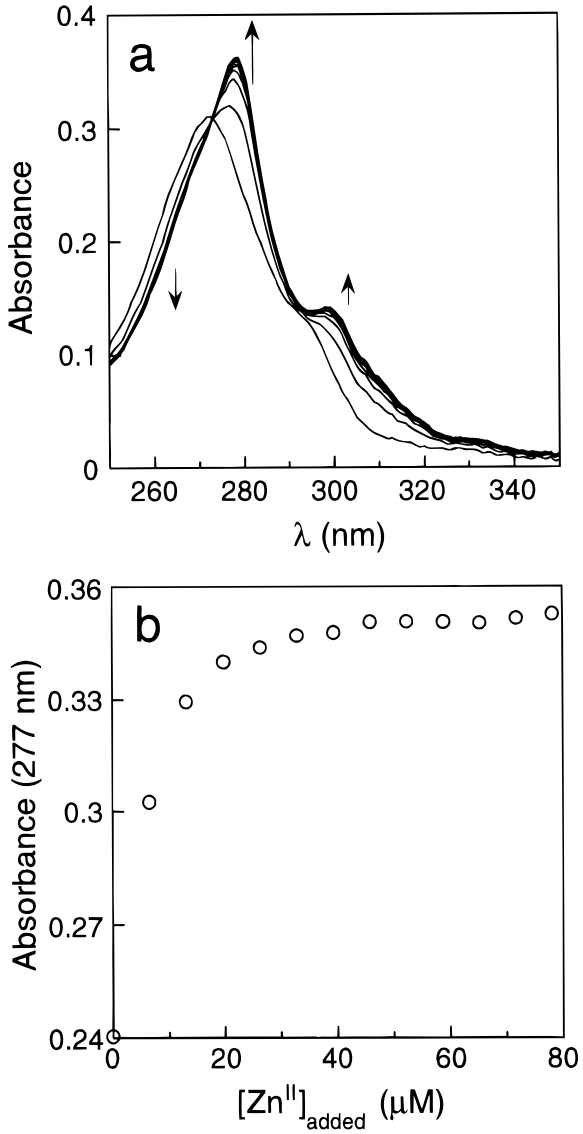

Figure 4. (a) Absorption spectra of 12.8 μM NeoHis upon addition of $ZnCl_2$ up to 123 μM in 0.05 M HEPES buffer at pH 8.25. (b) The binding isotherm obtained from the data shown in panel a at 280 nm.

Discussion

The N^{α}-Fmoc-protected Fen and Neo residues have been synthesized and incorporated into several peptides. This further expansion in the repertoire of α-amino acid building blocks enables the construction of a wide variety of novel metal-binding peptides. Additionally, these residues facilitate the formation of metal-binding sites with specific ligand type and geometry. These multidentate amino acids provide avid metal binding within short peptides and are also useful as structural probes due to their rich spectroscopic properties.[13]

Bpa-Containing Peptides. Peptides $4BpaPhe_w$, $5BpaPhe_w$, and $6BpaPhe_w$ each contain only one metal-binding Bpa residue. These peptides differ only at the point of attachment to the β-carbon of the bipyridyl-containing amino acid (4Bpa, 5Bpa, and 6Bpa). Since the 4 and the 6 isomers have previously behaved differently in the construction of bipyridyl-alanine cytochrome *c* mutants,[12] the various isomers were investigated in peptides with the same underlying sequence to evaluate their individual metal-binding characteristics more carefully. The

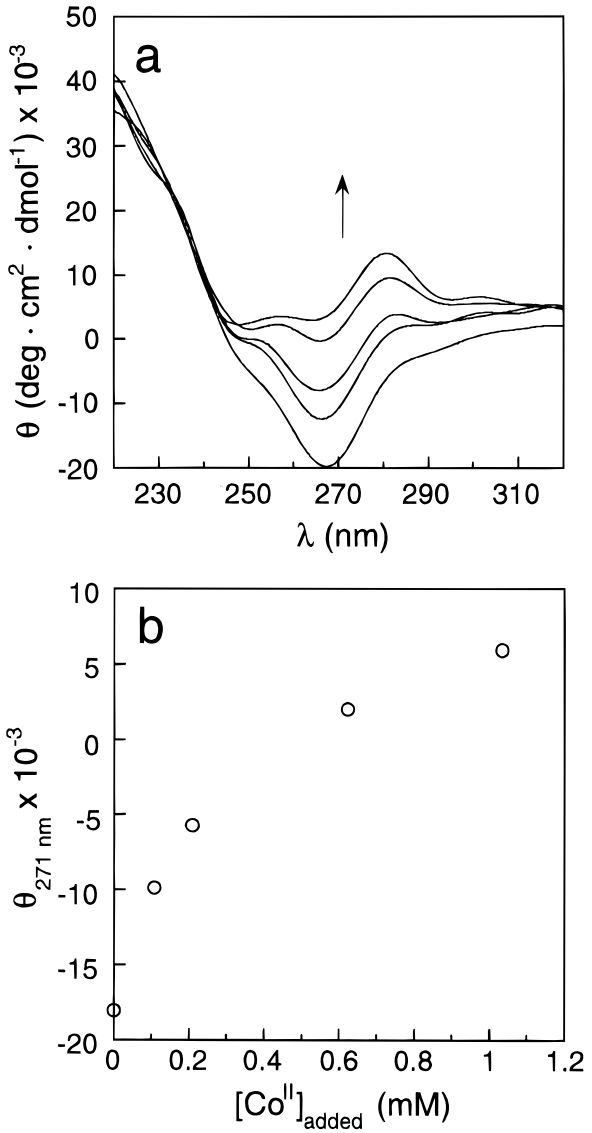

Figure 5. (a) Circular dichroism spectra of 66 μM NeoPhe upon addition of $CoCl_2$ up to 1.0 mM in 0.01 M Tris buffer at pH 8.25. (b) The binding isotherm obtained from the data shown in panel a at 271 nm.

dissociation constants of the peptide−Zn^{II} complexes for 6BpaPhe$_w$, 5BpaPhe$_w$, and 4BpaPhe$_w$ exhibit the overall trend 6BpaPhe$_w$ > 5BpaPhe$_w$ ∼ 4BpaPhe$_w$ (Table 1). Thus, the peptide 6BpaPhe$_w$ shows the lowest affinity for Zn^{II} among the three peptides. The steric hindrance of the *ortho* substitution presumably results in the larger dissociation constant for peptide 6BpaPhe$_w$. As the substitution of the residues 5Bpa and 4Bpa does not interfere with the metal-binding process, the peptides 5BpaPhe$_w$ and 4BpaPhe$_w$ exhibit similar affinities for Zn^{II}. Unlike all the other peptide−M^{II} complexes studied in this paper, the stoichiometries of the peptide−Co^{II} complexes for 5BpaPhe$_w$ and 4BpaPhe$_w$ were determined to be 2:1, reflecting the unencumbered chelation by these residues. Thus, a change in the point of attachment of the bipyridyl ring to the amino acid results in alteration of both the complex stoichiometry and the metal binding affinity.

Two bidentate metal-chelating residues were positioned at opposite ends of peptides 6Bpa6Bpa and 6Bpa6Bpa$_w$. The intervening sequences were designed to adopt a β-turn structure with different turn-forming propensities. The sequence -Val-Pro-D-Ser-Phe- has been shown to exhibit more turn character than -Thr-Pro-D-Ala-Val- by CD and NMR spectroscopy.[19,21,22]

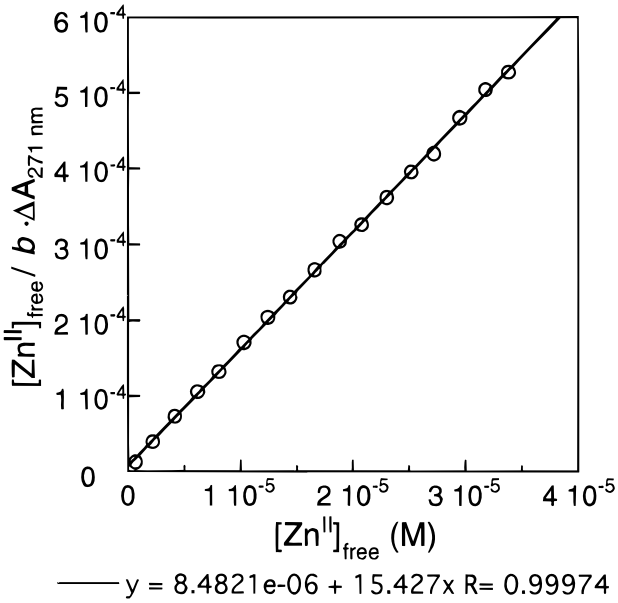

Figure 6. Final analysis of the data obtained from Figure 3 where 7.85 μM FenHis is titrated with $ZnCl_2$ up to 42 μM. The ratio of the y-intercept and slope derived from the linear fit gives the dissociation constant of 0.55 μM.

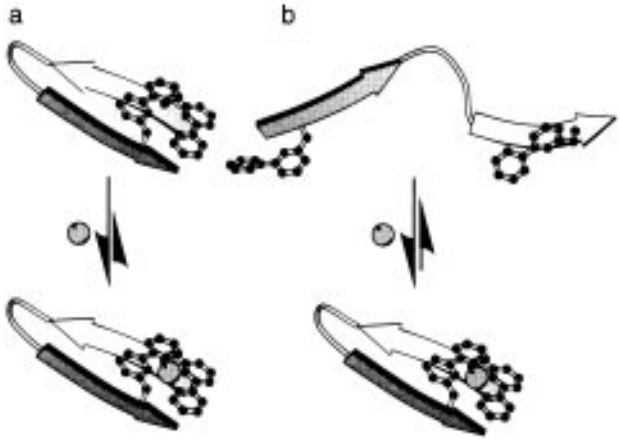

Figure 7. Schematic diagram of the metal binding event for (a) the hexapeptide 6Bpa6Bpa incorporating a structurally preorganized intervening sequence with high turn propensity and (b) the hexapeptide 6Bpa6Bpa$_w$ with a less structured connecting sequence (generated using the program MOLSCRIPT[17]).

The affinities of peptides 6Bpa6Bpa$_w$ and 6Bpa6Bpa for Zn^{II} and Co^{II} were significantly stronger than those observed for peptide 6BpaPhe$_w$ (i.e., lower dissociation constants), indicating the cooperative participation of both bipyridyl moieties as ligands (Table 1). This participation of a second 6Bpa residue enhances the metal binding affinity by two orders of magnitude. The peptide 6Bpa6Bpa, which contains an intervening sequence with higher type II β-turn propensity, exhibits stronger binding for both Zn^{II} and Co^{II} than the weak turn-forming peptide 6Bpa6Bpa$_w$. This result strongly suggests that the connecting residues can serve to bring the two metal-chelating residues into close proximity and therefore play a role in the process of binding metal cations (Figure 7). Thus, through the control of the peptidyl structure, the metal-binding affinity of a peptide for a specific metal cation can be modulated by 60-fold.

Fen- and Neo-Containing Peptides. Peptides FenPhe$_w$ and NeoPhe were analyzed to determine the inherent metal-binding properties of the bidentate residues. The peptide FenPhe$_w$ has a stronger affinity for Zn^{II} and Co^{II} than peptide NeoPhe (Table 2). This suggests that the steric effect of the 9-methyl group

of Neo interferes with metal chelation. This trend parallels that observed for the parent ligands 2-methyl-1,10-phenanthroline (Zn^{II} complex, $K_D = 11.0\ \mu M$; Co^{II} complex, $K_D = 7.94\ \mu M$) and 2,9-dimethyl-1,10-phenanthroline (neocuproine) (Zn^{II} complex, $K_D = 79.4\ \mu M$; Co^{II} complex, $K_D = 63\ \mu M$).[27] However, the peptide NeoPhe preferentially binds Zn^{II} over Co^{II} . This result is unusual given the opposite trends observed for Bpa and Fen peptides as well as the parent ligand neocuproine. This reversal in binding specificity is most likely due to the steric bulk introduced by the peptide. However, the participation of functional groups other than the Neo side chain cannot be completely ruled out.

Peptides $FenHis_w$, FenHis, FenHis′, and FenAsp exhibit stronger metal-binding affinities than the parent "Fen only" peptide, $FenPhe_w$ (Table 2). Thus, the auxiliary naturally occurring coordinating residues (His or Asp) incorporated within these peptides must be actively participating in the metal-binding event. Furthermore, the greater metal-binding affinity of peptide FenHis as compared to peptide $FenHis_w$ suggests that the sequence with higher turn propensity promotes a higher metal-binding affinity, which is consistent with the results obtained for peptides 6Bpa6Bpa and $6Bpa6Bpa_w$. Additionally, FenHis′ binds Co^{II} and Zn^{II} with affinities similar to FenHis. In this regard, the type II′ turn does not appear to contribute a significant advantage over the type II turn in preorganizing the metal binding site.

Comparison between peptides NeoPhe and NeoHis illustrates that incorporation of the auxiliary metal-binding residue, histidine, results in a 160-fold increase in affinity for Zn^{II} (Table 2). Additionally, the peptide NeoHis was anticipated to have diminished metal-binding properties as compared to the analogous Fen-containing peptide due to the steric crowding of a bound metal cation by the 9-methyl group near a nitrogen donor atom. While this was demonstrated for the Co^{II} binding affinities of peptides NeoHis and FenHis, the K_D of the Zn^{II} complexes for the two peptides were comparable. In addition, there was 1 order of magnitude preference for NeoHis to bind Zn^{II} over Co^{II}. This result highlights the potential for tuning the metal-binding affinities and selectivities of short peptide motifs for future metal cation sensor designs by judicious selection of the unnatural bidentate residue and auxiliary naturally occurring metal-ligating amino acid. Since Co^{II} is known to favor octahedral geometry due to ligand field stabilization while Zn^{II} has no specific geometrical preference,[2,28] it is possible that the steric effect of the 9-methyl group interferes with the formation of an octahedral complex and results in the increased K_D for the peptide$-Co^{II}$ complex. On the other hand, Zn^{II} does not prefer a specific geometry and therefore exhibits similar metal-binding properties for FenHis and NeoHis. Note that the differences in metal-binding preferences between FenHis and NeoHis must be ascribed to a single methyl group.

Summary. Several guidelines for the design of short metallopeptides with high metal-binding affinity can be derived from this study. First, the novel chelating amino acids presented generally bind metal cations with higher affinity than naturally occurring metal-binding residues due to the bidentate nature of the side chain functionality. The selection of unnatural chelating amino acid can affect both affinity and stoichiometry of the metal complex for the designed peptide. The metal-binding affinity for the hexapeptides with bipyridyl-containing amino acids follows the trend 4Bpa ∼ 5Bpa > 6Bpa. However, the stoichiometry of the peptide$-Co^{II}$ complexes for peptides $4BpaPhe_w$ and $5BpaPhe_w$ are 2:1. In contrast, high affinity and 1:1 stoichiometry can be achieved through the incorporation of the phenanthrolyl-containing amino acids (Fen, Neo). Second, further modulation of the metal-binding affinity can be accomplished via the incorporation of auxiliary metal-binding amino acids (6Bpa, Asp, or His). The participation of an auxiliary residue is exemplified by the 160-fold enhancement in Zn^{II} binding affinity for NeoHis over NeoPhe. Third, control of the peptide structure can be exploited to bring two metal-binding residues into close proximity and facilitate the metal-binding process. Peptide 6Bpa6Bpa, which contains a sequence with high turn propensity connecting two 6Bpa residues, exhibits a 80-fold higher affinity for Zn^{II} than peptide $6Bpa6Bpa_w$, which has a considerably less structured intervening sequence.

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Conclusion

The ability to tune the metal-binding affinity of designed peptides has been demonstrated in this study by exploiting three different features: selection of unnatural bidentate residue, incorporation of auxiliary metal-binding amino acid, and control of structural preorganization of the peptide architecture. Dissociation constants ranging from 10^{-3} to $<10^{-6}$ M for Zn^{II} have been achieved by altering these three features. The combination of a variety of bidentate amino acids with the structural preorganization afforded by a peptidyl template, such as a reverse turn, results in the ability to modulate the metal-binding affinity of metallopeptides. The availability of the novel chelating α-amino acids and the guidelines for their utilization derived from this study can be exploited in future metalloprotein and peptide-based sensor design.

Experimental Section

General. All reagents were obtained from Aldrich Chemical Co. and used without further purification. Protease Type VIII (Subtilisin Carlsberg, Bacterial, CAS Registry No. 9014-01-1, 11.6 units/mg) was purchased from Sigma Chemical Co. and stored at 4 °C. All synthetic intermediates were stored at 4 °C after purification. ^1H and ^{13}C NMR spectra were recorded on a General Electric QE-300 at 300 MHz for ^1H NMR, 75 MHz for ^{13}C.

CrownPak CR Column Analysis. Characterization was obtained through HPLC analysis using a CrownPak CR(+) column (J. T. Baker), on an Isco Model 2350 HPLC equipped with an Isco Model V^4 absorbance detector using the conditions as described. 2-Amino-3-(1,10-phenanthrol-2-yl)propanoic acids and methyl esters: flow rate 1.0 mL/min; 25 °C, monitored at 278 nm; eluent 10% MeOH/0.1 M $HClO_4$. Methyl esters: D 11.50 min, L 12.25 min; amino acids: D 7.75 min, L 8.25 min. 2-Amino-3-(9-methyl-1,10-phenanthrol-2-yl)-propanoic acids and methyl esters: flow rate 0.6 mL/min; 25 °C, monitored at 280 nm; eluent 10% MeOH/0.1 M $HClO_4$. Methyl esters: D 15.73 min, L 18.10 min; amino acids: D 11.87 min, L 12.61 min.

***N*$^\alpha$-(9-Fluorenylmethyloxycarbonyl)-(*S*)-2-amino-3-(1,10-phenanthrol-2-yl)propanoic Acid (8).** A solution of racemic 2-amino-3-(1,-10-phenanthrol-2-yl)propanoic acid methyl ester[24] (**6**, 3.6587 g, 16.0 mmol) was dissolved in 45 mL of *tert*-butanol and added to 445 mL of 10% $NaHCO_3$. A sample of alkaline protease (125 mg, 800−2000 units) in 120 mL of 0.2 M $NaHCO_3$ was added to the amino acid methyl ester (8 mmol) in a 1-L Erlenmeyer flask. The mixture was rotated at 120−200 rpm and monitored by HPLC at 20-min intervals. When the ratio of the D-amino acid methyl ester to the L-amino acid methyl ester was >50:1 (about 40 min), the mixture was extracted with $CHCl_3$ (6 × 150 mL). The aqueous phase was reduced in volume to remove any chloroform and then lyophilized yielding a mixture of amino acid and carbonate salts. The combined organic layers were dried over Na_2SO_4, and the solvent was evaporated. HPLC analysis of the aqueous phase showed a 92−94% ee of the L-amino acid based on conventions

of the elution profiles on the CrownPak(+) column and the specificity of alkaline protease. The amino acid/carbonate salt mixture was dissolved in 25 mL of 10% Na_2CO_3. The 9-fluorenylmethylsuccinimidyl carbonate (1.2 equiv) was dissolved in 15 mL of dioxane and added dropwise to the amino acid solution. The reaction mixture was shaken periodically for 1.5 h, then transferred to a separatory funnel, and diluted with 100 mL of water. This mixture was washed with ether (4 × 60 mL) and transferred to a 250-mL Erlenmeyer flask. After being cooled to 0 °C and adjusted to pH <2 with concentrated HCl, the precipitate was isolated by centrifugation and washed with water (3 × 100 mL). The residue was transferred to a round bottom flask with methanol (200 mL). The methanol was removed under reduced pressure and replaced with toluene (80 mL). The resulting toluene/water mixture was removed under reduced pressure. The residue was rubbed with ether to obtain a solid that was dried in vacuo to yield the Fmoc-protected amino acid (85% based on the amount of L-amino acid methyl ester). mp = 187 °C (dec); HRMS: calculated $[MH]^+$ for $C_{30}H_{24}N_3O_4$ [490.1767]; observed [490.1749]; TLC (SiO_2; 4:1 $CHCl_3$:MeOH; UV); R_f = 0.55; ^{1}H NMR (DMSO-d_6) δ: 3.45 (m, 1H), 3.65 (m, 1H), 4.10 (m, 3H), 4.40 (m, 1H), 4.80 (d, 2H, J = 5.8 Hz), 7.05 (m, 2H), 7.35 (m, 2H), 7.50 (m, 2H), 7.68 (d, 1H, J = 7.2Hz), 7.80 (d, 1H, J = 7.6 Hz), 7.93 (m, 2H), 8.10 (m, 2H), 8.64 (d, 1H, J = 8.3Hz), 9.00 (d, 1H, J = 4.6 Hz), 9.25 (d, 1H, J = 4.85 Hz); ^{13}C NMR (DMSO-d_6) δ: 47.5, 55.1, 66.6, 121.0, 124.3, 125.0, 126.0, 126.1, 127.2, 127.3, 127.4, 128.0, 128.6, 128.8, 129.6, 137.2, 137.6, 141.6, 144.7, 144.8, 150.9, 159.4, 174.2; IR (mineral oil mull) cm^{-1} : 3307, 1690, 1601, 1537, 1454, 1372, 1337, 1261, 1150, 1102, 1078, 1037, 850, 761, 732; $[\alpha]_D^{25}$ −67.9° (c = 0.35, DMSO); UV (MeOH, nm) λ_{max} = 299 (11 000), 268 (32 000), 224 (38 900).

***N*-(9-Fluorenylmethoxycarbonyl)-(*S*)-2-amino-3-(9-methyl-1,10-phenanthrol-2-yl)propanoic Acid (9).** A solution of racemic 2-amino-3-(9-methyl-1,10-phenanthrol-2-yl)propanoic acid methyl ester[24] (**7**, 0.5733 g, 2.04 mmol) was dissolved in 20 mL of *tert*-butanol and added to 200 mL of 10% $NaHCO_3$. A sample of alkaline protease (55.4 mg, 400−1000 units) was added to the amino acid methyl ester in a 1-L Erlenmeyer flask. The mixture was rotated at 150 rpm and monitored by HPLC at 20-min intervals. When the ratio of the D-amino acid methyl ester to the L-amino acid methyl ester was >50:1 (about 40 min), the mixture was extracted with $CHCl_3$ (6 × 150 mL). The aqueous phase was reduced in volume to remove any $CHCl_3$ and then lyophilized to yield a mixture of amino acid and carbonate salts. HPLC analysis of the aqueous phase showed a 98% ee of the L-amino acid based on conventions of the elution profiles on the CrownPak(+) column and the specificity of alkaline protease. The resulting product L-Neo had $[\alpha]_D^{25}$ +80.3 at pH 8.8 and $[\alpha]_D^{25}$ +8.5 at pH 0, which is consistent with the Clough−Lutz−Jirgenson rule, confirming the configuration of the stereocenter. The amino acid/carbonate salt mixture was suspended in 200 mL of water. The 9-fluorenylmethylsuccinimidyl carbonate (0.5508 g, 1.63 mmol) was dissolved in 20 mL of dioxane and added dropwise to the amino acid solution. The reaction mixture was stirred at room temperature for 1.5 h. To the reaction, a solution of 9-fluorenylmethylsuccinimidyl carbonate (0.3431 g, 1.02 mmol) dissolved in 15 mL of dioxane was added dropwise. After being stirred overnight, this mixture was washed with ether (4 × 100 mL). After being cooled to 0 °C and adjusted to pH < 2 with concentrated HCl, the precipitate was isolated by centrifugation and washed with water (3 × 100 mL). The residue was transferred to a round bottom flask with methanol (150 mL). The methanol was removed under reduced pressure and replaced with toluene (80 mL). The resulting toluene/water mixture was removed under reduced pressure. The residue was rubbed with ether to yield a solid that was dried in vacuo (0.4726 g, 92% yield based on the amount of L-amino acid methyl ester). mp = 131.4−132.7 °C (dec.); HRMS: calculated for $[MH]^+$ $C_{31}H_{26}N_3O_4$ [504.1923]; observed [504.1931]; TLC (SiO_2; 4:1 $CHCl_3$:MeOH; UV); R_f = 0.36; ^{1}H NMR (DMSO-d_6) δ: 22.76 (s, 3H), 3.45 (m, 1H), 3.55 (m, 1H), 4.18 (m, 3H), 4.61 (m, 1H), 7.15 (t, 1H, J = 7.4 Hz), 7.21 (t, 1H, J = 7.4 Hz), 7.35 (m, 3H), 7.61 (m, 2H), 7.70 (d, 1H, J = 8.2 Hz), 7.84 (d, 2H, J = 7.6 Hz), 7.90 (s, 2H), 8.17 (d, 1H, J = 8.3 Hz), 8.36 (d, 1H, J = 8.2 Hz), 8.40 (d, 1H, J = 8.3 Hz); ^{13}C NMR (DMSO-d_6) δ: 40.8, 47.0, 47.2, 54.2, 66.2, 120.5, 120.6, 125.6, 126.3, 127.3, 127.5, 127.6, 128.0, 128.1, 137.5, 141.2, 144.4, 156.6, 158.7, 173.7; IR (KBr) cm^{-1} : 3316, 3063, 3043, 2946, 1728, 1694, 1538, 1446, 1372, 1261, 1222, 1149, 1105, 1080, 1046, 857, 759, 740; $[\alpha]_D^{25}$ −71.4°.

Table 3. Calculated and Observed High-Resolution Mass Values for the Synthesized Hexapeptides

peptide	formula	calcd $[MH^+]$	obsd $[MH^+]$
4BpaPhe$_w$[a]	$C_{41}H_{53}N_9O_8$	800.4095	800.4055
5BpaPhe$_w$[a]	$C_{41}H_{53}N_9O_8$	800.4095	800.4057
6BpaPhe$_w$[a]	$C_{41}H_{53}N_9O_8$	800.4095	800.4153
6Bpa6Bpa$_w$[a]	$C_{45}H_{55}N_{11}O_8$	878.4313	878.4285
6Bpa6Bpa[a]	$C_{50}H_{57}N_{11}O_8$	940.4470	940.4459
FenPhe$_w$[a]	$C_{43}H_{53}N_9O_8$	824.4095	824.4117
FenHis$_w$[a]	$C_{40}H_{51}N_{11}O_8$	814.4000	814.3983
FenHis[a]	$C_{45}H_{53}N_{11}O_8$	876.4156	876.4140
FenAsp[a]	$C_{43}H_{51}N_9O_{10}$	854.3837	854.3841
FenHis′[b]	$C_{45}H_{53}N_{11}O_8$	898.3976[c]	898.4031[d]
NeoPhe[b]	$C_{49}H_{57}N_9O_8$	900.4408	900.4422
NeoHis[b]	$C_{46}H_{55}N_{11}O_8$	890.4302	890.4313

[a] Peptides synthesized on the automated peptide synthesizer. [b] Peptides synthesized on macrocrown supports. [c] Calculated $[MNa^+]$. [d] Observed $[MNa^+]$.

Peptide Synthesis. All peptides were synthesized on a 0.084−0.168 mmol scale by solid phase methods using N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) amino acid with BOP/HOBT-activated ester chemistry on a Milligen 9050 automated peptide synthesizer, except for the Neo-containing peptides and FenHis′. These peptides were synthesized on a macrocrown support purchased from Chiron Mimotopes. Commercially available starting materials and reagents were purchased from MilligenBiosearch, EM Science, NovaBiosearch, or Aldrich Chemical Co.

Solid Phase Peptide Synthesis. Fmoc-PAL-PEG-PS resin (0.21 mmol/g) was used to afford carboxyl terminus primary amides. Typical protocols for coupling a residue involved 30−90 min coupling cycles with 3 or 4 equiv of amino acid. However, for the bipyridyl and phenanthrolyl amino acids, a double coupling protocol was employed, where 2.5 equiv was used for the first coupling followed by a similar acylation with 1.5 equiv of the residue with 60-min coupling times for each. Activated esters were formed in situ using benzotriazol-1-yloxy−tris(dimethylamino)−phosphoniumhexafluorophosphate (BOP), 1-hydroxybenzotriazole (HOBt), and *N*-methylmorpholine or diisopropylcarbodiimide (DIPCDI), HOBt, and diisopropylethylamine (DIEA). Due to poor solubility of the bipyridyl and phenanthrolyl amino acid-activated esters, the amino acid ester solutions were filtered manually through a 0.45-μm nylon syringe filter (Gelman Scientific) prior to manual injection onto the column. Deprotection of FMOC-protected amine groups were performed using either a 7-min 20% piperidine/dimethylformamide (DMF) wash or a 5-min 2% 1,7-diazabicyclo[5,4,0]-undec-7-ene (DBU)/DMF wash. Peptides were *N*-acylated on the resin using either 7 equiv of acetic anhydride and 7 equiv of triethylamine (TEA) or 21 equiv of acetic anhydride and 5 equiv of triethylamine in 3 mL of DMF for 2 h. The resin was then washed with DMF and dichloromethane and lyophilized overnight.

The peptides were then deprotected and cleaved from the resin by treatment with trifluoroacetic acid (TFA)/phenol/H_2O/thioanisole/ethanedithiol (82.5:5:5:5:2.5) or TFA/thioanisole/ethanedithiol/anisole (90:5:3:2) for 2 h.[29] The resin was filtered and washed with TFA, and the combined filtrates were concentrated to 2 mL and precipitated with ether/hexane 1:1 or 2:1 (20 mL). The supernatant was decanted, and the peptides were triturated with ether/hexane 1:1 (5 × 20 mL) or ether/hexane 2:1 (3 × 20 mL). The peptides were lyophilized overnight and purified by reverse-phase C_{18} HPLC. All peptides were confirmed by mass spectrometry (Table 3).

Peptide Synthesis on Macrocrowns. A typical full coupling cycle involves the following steps; a 30 min soak in 20% (v/v) piperidine/DMF suspended in DMF followed by a 5 min wash with DMF and three rinses with methanol. After air drying for 10 min and soaking in DMF 5 min, the pin is submitted to the coupling cocktail. Typical couplings were performed for 2 h with Fmoc-protected amino acid

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pentafluorophenyl esters and HOBt at 80 mM concentration each with 450 μL of activated amino acid ester solution per pin. After coupling,the pins were then washed once with DMF and three times with methanol. The pins were then air dried for 10 min to complete a full cycle. The residues Neo and Fen were coupled with 3 equiv of amino acid and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) /1-hydroxy-7-azabenzotriazole (HOAt)/ DIEA mediated chemistry in *N*-methylpyrrolidinone (NMP).[30−32] These cycles were repeated until the peptide was complete. Peptides were *N*-acetylated on the solid support using 450 μL of DMF/Ac_2O/TEA (43:1:1) in place of a typical coupling solution. The peptides synthesized were deprotected and removed from the solid support by treatment with trifluoroacetic acid (TFA)/phenol/H_2O/thioanisole/ ethanedithiol (82.5:5:5:5:2.5) for 4 h.[29] The resulting solution was concentrated to 2 mL volume and precipitated with ether/hexane 1:1. The supernatant was decanted, and the peptides were triturated with ether/hexane 1:1 (5 × 10 mL). The peptides were lyophilized overnight and purified by reverse-phase C_{18} HPLC. The peptides were confirmed by mass spectrometry (Table 3).

Absorption Spectroscopy (UV−Vis) Studies. The absorption spectra were obtained on a Shimadzu UV-160U in 10-mm path length cuvettes. The concentrations of the peptide solutions were determined spectrophotometrically using the characteristic absorption band of the bipyridyl (λ_{max} = 285 nm, ϵ_{max} = 12800) or phenanthrolyl (Fen, λ_{max} = 268 nm, ϵ_{max} = 20890; Neo, λ_{max} = 274 nm, ϵ_{max} = 24150) moiety. The aqueous solutions of known peptide concentrations were then titrated with standard metal cation solutions at room temperature. For the metal cation titration performed on bipyridyl-containing peptides, the pH of the solution was held within the range pH = 7.0−7.5 through small additions of 0.1 N NaOH or 0.1 N HCl in the presence of 200 mM NaCl. For the peptides containing Fen or Neo, the titrations were performed in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer, pH 8.25. The distinct red-shift of the bipyridyl or phenanthrolyl $\pi-\pi^*$ transition upon metal-binding cations was monitored by absorption (UV−Vis) spectroscopy. With peptide concentrations ranging from 1 to 500 μM, the metal-binding constants were determined by calculations carried out using KaleidaGraph version 3.0 (Abelbeck Software, CA). A minimum of three titrations were performed for each peptide−metal cation complex. The binding isotherms were assumed to follow a two-state equilibrium when well-defined isosbestic points associated with the addition of metal cations were observed. The dissociation constants (K_D) were obtained using a iterative process based on the Scott equation:[26]

$$\frac{b[\mathrm{M}]}{\Delta A} = \frac{[\mathrm{M}]}{[\mathrm{Pep}]_t} + \frac{K_D}{[\mathrm{Pep}]_t \Delta\epsilon}$$

where b is the cell path length, [M] is the free metal cation concentration, ΔA is the change in absorbance due to complex formation, $[\mathrm{Pep}]_t$ is the total peptide in solution, and $\Delta\epsilon$ is the change in molar absorptivity due to complex formation. Initial estimations of [M] were achieved by using data where $[\mathrm{M}]_{total} \gg [\mathrm{Pep}]_t$. An analysis was performed where $b[\mathrm{M}]/\Delta A$ was plotted against [M]. The $\Delta\epsilon$ thus generated was used to calculate [M] more accurately ($[\mathrm{M}] = [\mathrm{M}]_{total} - (\Delta A/\Delta\epsilon)$) for every iteration until $\Delta\epsilon$ converges. When the $\Delta\epsilon$ converged, the dissociation constants (K_D) were calculated from the last iterations as

$$K_D = \frac{y\text{-intercept}}{\text{slope}}$$

Circular Dichroism Spectroscopy Studies. Circular dichroism spectra were recorded on a Jasco J600 circular dichroism spectrometer. The concentration of the peptide solutions was determined spectrophotometrically using the characteristic absorption band of the phenanthrolyl (Neo, λ_{max} = 274 nm, ϵ_{max} = 24 150) moiety. The spectra werebaseline corrected and noise reduced using the Jasco J600 System software and were then further analyzed using KaleidaGraph version 3.0 (Abelbeck Software). Metal cation titrations were performed with peptide concentrations ranging from 65 to 70 μM in 0.01M Tris buffer (pH 8.25) in a 1-mm path length cuvette. The change in CD signature where the phenanthrolyl moiety absorbs was monitored, and the binding isotherms were thus obtained. The dissociation constants were calculated as described in the UV−Vis experiments.

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Supporting Information Available: Text description of the experimental details for the syntheses of compounds **6** and **7** with the ^{1}H NMR spectra of the new compounds **6**−**9**, **12**, and **13** (11 pages). See any current masthead page for ordering information and Internet access instructions.

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