



Cite this: DOI: 10.1039/c8nj00184g

Received 11th January 2018,
Accepted 11th April 2018

DOI: 10.1039/c8nj00184g

rsc.li/njc

2,2'-Bipyridine and hydrazide containing peptides for cyclization and complex quaternary structural control†

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A synthetic peptide containing two *N*_ε-methyl lysines (Ac-K(*N*_ε-Me)GYTGYTGK(*N*_ε-Me)D-OH) was alkylated with bipyridine (bipy) ligands substituted at the fifth (**MP-5**) and sixth (**MP-6**) positions, thereby creating Ac-K(*N*_ε-Me, *N*_ε-Bipy)GYTGYTGK(*N*_ε-Me, *N*_ε-Bipy)D-OH. Peptides with a bipyridine at the 6-position did not bind to Fe²⁺ and Zn²⁺. Peptides with a bipyridine at the 5-position bound these metals, and in the presence of one equivalent of a free bipy derivative folded into a macrocycle. Further, the free bipy derivative could also contain a cyclized peptide derived from hydrazone formation, resulting in a complex but controlled quaternary peptide structure.

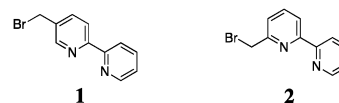
Introduction

Metal chelation is commonly found in nature for the control of protein quaternary structures.^{1,2} Nearly one-third of proteins have metal coordination sites to execute regulatory or enzymatic processes.³ Synthetic polypeptides that mimic this metal-mediated folding garner interest, because they can be used as therapeutics or novel materials capable of functioning under biotic and abiotic conditions.^{4,5} An advantage of synthetic structures is their ability to incorporate unnatural functionalities that act as recognition units, promote folding, or sequester small organic structures. Therefore, chemists introduce metal ligand coordination into peptides using both naturally occurring residues and non-canonical metal binding amino acids.⁶

The use of one pyridine ring, in conjunction with natural metal ligands, has led to interesting folding properties of synthetic peptides.⁷ Similarly, bipyridine (bipy) combines two binding sites, and is among the most widely used metal ligands. Synthesis of small molecules, polymers, and peptides containing this functional group to exploit their folding properties has been reported.^{8–12} These moieties facilitate helical formation of organic compounds, act as materials in photovoltaic/electronic devices, serve as molecular sensors, and are applied in luminescent devices. Bipyridines are also used extensively for binding Cu²⁺, Zn²⁺, and Fe²⁺, cations of biological interest.⁸ Ligand to metal stoichiometries of 2:1 and 3:1 are achieved with different

metals, and thus offer the ability to control peptide structures and aggregation states. Due to their utility, we sought a facile synthetic way for incorporating bipy ligands for controlled folding of synthetic polypeptides.

Alkylation of secondary amine residues was previously reported by our group as a method to efficiently incorporate boronic acids, and the use of a similar methodology to modify peptides with 2,2'-bipyridines is presented here.¹³ Brominated compounds **1** and **2** were synthesized for this purpose.



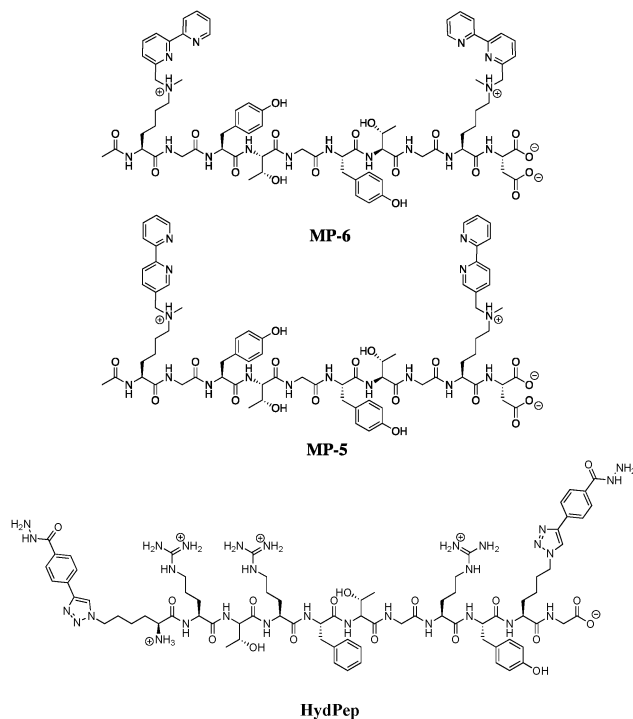
While natural amino acid building blocks are commercially available, most unnatural ones are not. If available, they are priced high, making them expensive to use for solid phase synthesis.^{14,15} Thus, when it comes to designing, synthesizing, and scaling up the production of commercially unavailable, synthetically challenging building blocks, solid-phase synthesis is a less attractive method. Despite these challenges, bipyridines have been studied and analysed for their peptide folding properties.³ The method introduced herein should further facilitate such studies because of its synthetic accessibility, reasonable yields, and inexpensive starting materials.

The alkylation approach previously described by our group was seen as a versatile means for synthesizing non-canonical amino acids.³ The commercial availability of alkyl bromide precursors and the synthetic ease to acquire brominated compounds makes the post-solid phase and alkylation-based strategies an attractive approach. From one sequence containing mono-methylated lysine

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† Electronic supplementary information (ESI) available: NMR, HRMS, and analytical HPLC. See DOI: 10.1039/c8nj00184g

residues, different side-chains can be incorporated, isolated, and studied for binding/folding properties. Two brominated bipyridines, compounds **1** and **2**, substituted at different positions, were used as a case study. We sought a straightforward approach to incorporate these unnatural ligands to the same synthetic peptide, followed by binding and folding studies. The hypothesis was that the methylated lysine would bind to a metal at a third site of a tridentate ligand when alkylated with **2** (ESI† Fig. S1). This served as further motivation for the alkylation strategy. Thus, **MP-5** and **MP-6** were synthesized. In addition, inspired by recent studies demonstrating that aldehyde and hydrazide containing peptides rapidly assemble into complex quaternary structures,¹⁶ we pursued even higher-ordered structuring by using a dihydrazide peptide, **HydPep**.



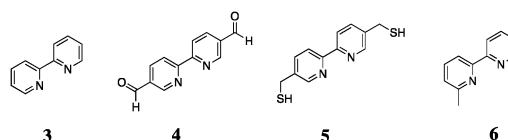
Results and discussion

Bipyridine ligands commonly participate in coordination stoichiometries of 3 : 1 for hexacoordinate metals because they are bidentate. Thus, peptides decorated with two bipy ligands modified at position 5 were postulated to form oligomeric species that would be difficult to characterize. On the other hand, peptides with two ligands modified at position 6 could satisfy the six-coordination requirements by acting as tridentate ligands (ESI† Fig. S1).

The sequence selected for **MP-5** and **MP-6** came from peptides previously studied by our group used for the cyclization of synthetic peptides.⁸ The combination of serine and threonine increased the hydrophilicity, while glycine minimized the steric repulsion. Acetylation of the N-terminus facilitated the alkylation of the ϵ -secondary amine residues. Furthermore, residues such as histidine, arginine, and cysteine, known to bind metal ions, were avoided.^{17,18} To characterize metals binding to the peptides, we used previously reported UV-Vis and ¹H-NMR spectroscopy titrations.

The concentration of the titrant at the point where the spectroscopic signals stop changing reports the stoichiometry of the binding/folding.¹⁹

Initially, to validate if our characterization methods would be effective for the peptide assemblies, we performed titrations with small molecules as the control species: 5-(bromomethyl)-2,2'-bipyridine (**1**), 2,2'-bipyridine (**3**), [2,2'-bipyridine]-5,5'-dicarbaldehyde (**4**), [2,2'-bipyridine]-5,5'-diylidimethanethiol (**5**), and 6-methyl-2,2'-bipyridine (**6**). Compounds **1**, **3**, and **4** coordinated with Fe²⁺ in a 3 : 1 stoichiometry. Compound **5** showed the same stoichiometry as zinc, however compound **6** did not readily bind (ESI†). This suggested that a methyl group at the 6-position introduced a steric hindrance to the metal complexation. The binding of compound **1** demonstrated that bulkier substituents, like bromide, could be tolerated for the ligands substituted at the 5-position (ESI†). As a result, **MP-5** was expected to readily bind to metals.



¹H-NMR spectra titrations were performed to determine the binding stoichiometries, and thereby the folding of **MP-5** and **MP-6**, upon addition of metals. After each addition of metals, successive NMR acquisitions tracked the alterations in the aromatic spectral region. Titrating Fe²⁺ with **MP-6** led to a change in color to a rusty red, but notable changes were not observed in the NMR spectra. Weakening of the signal intensity, however, was observed as the sample became more dilute (ESI†). This result suggested that no significant binding occurred.

In contrast, the NMR spectra of **MP-5** were quite sensitive to the addition of Fe²⁺ (Fig. 1). The spectra, however, were difficult to interpret. The signals remained at a constant ppm, but were at their broadest at 1 equivalent of metal. As the concentration of metal increased, additional peaks formed, suggesting that the peptides were oligomeric. With the addition of more metal, it is logical that the peptides break apart into singly bound residues.

A solution of Fe²⁺ was also titrated with **MP-6** and monitored by UV-vis spectrophotometry. No absorption change occurred, confirming that position 6 restricts metal binding, likely due to the steric hindrance. Therefore, despite a potential third coordination site introduced *via* an *N*_ε-methyl lysine, a 1 : 1 metal to peptide stoichiometry proved elusive (ESI†).

Literature accounts describing binding of metals with functionalized bipyridines and free 2,2'-bipyridine (bipy) have been described, particularly in the context of DNA binding with ruthenium centers.²⁰ Many metals, commonly of group VIII, bind three equivalents of bipy. Thus, to facilitate such a stoichiometry, mixtures of metals, peptides, and compound **3** were analyzed by UV/Vis spectroscopy. The aim was to study if free bipy would facilitate binding to **MP-6**. Despite the addition of a free bipy, **MP-6** did not demonstrate any binding (ESI†). Yet, an interpretable isotherm was acquired with this three-component ensemble for **MP-5** (Fig. 2).

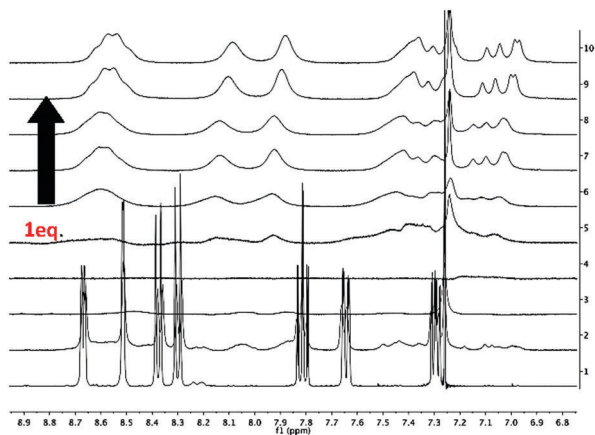


Fig. 1 **MP-5** (2.3 μmol) was dissolved in 600 μL of $\text{D}_2\text{O}:\text{MeOD}$ (1:1) (v/v). Iron triflate (4.6 μmol) was dissolved in 180 μL of a deuterated solvent. Arrow indicates an increase in the equivalents of the metal added: 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, corresponding to the spectra labelled 1 to 10, respectively.

These titration studies, repeated six times, confirmed a 1:1:1 stoichiometry, just as with the small-molecule titration studies (ESI^+). Furthermore, mass-spec studies revealed the mass of the cyclized peptide with Fe(II) , albeit missing the third bipy. Thus, a total of three stoichiometry was again observed, suggesting that aldehydes and thiols do not interfere with complexation. Loss of resolution of the peaks is due to dilution of the sample after each successive addition of iron.

Recently, our group reported a series of $^1\text{H-NMR}$ spectral studies confirming the orthogonality between four reversible covalent bonds and ligand binding: boronic acid esterification, thiol-conjugate addition, hydrazone formation, and terpyridine complexation with a metal cation.²¹ Combining peptides with unnatural functionalities that can participate in dynamic reversible covalent bonds is a means to induce folding of synthetic

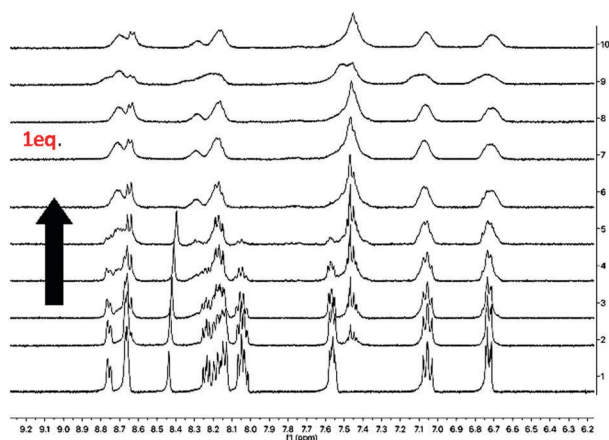


Fig. 2 **MP-5** and free bipy (2.3 μmol) were dissolved in 600 μL of $\text{D}_2\text{O}:\text{MeOD}$ (1:1) (v/v). Iron triflate (4.6 μmol) was dissolved in 180 μL of a deuterated solvent. Stoichiometry: (1:1:1). Arrow indicates an increase in the equivalents of the metal added: 0, 0.14, 0.28, 0.42, 0.56, 0.7, 0.84, 0.98, 1.12, 1.26, 1.4, corresponding to the spectra labelled 1 to 10, respectively.

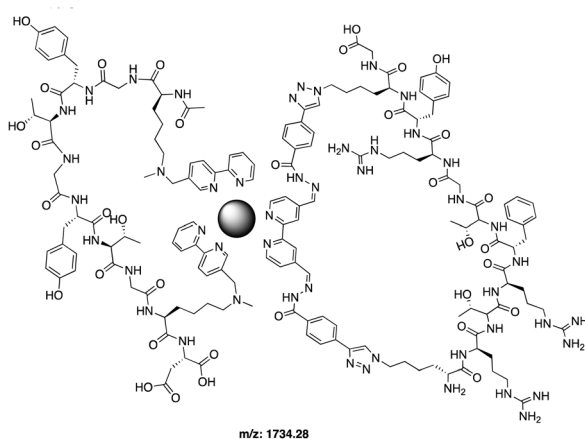


Fig. 3 Representative structure of quaternary control of **MP-5** and **HydBep** about Fe^{2+} with a predicted m/z value for $[\text{M}]^{2+}$.

peptides with non-canonical functionalities. It is the dynamic reversibility of these covalent bonds that make these reactions attractive in the studies presented here. These reactions can be considered as an intermediate between strong covalent bonds, which locks precursors into a specific arrangement, and of non-covalent interactions, which are often too dynamic to make discrete assemblies—unless they act cooperatively.²² We focused on hydrazone formation with compound **4** for the cyclic peptide ligand formation (Fig. 3). Compound **4** served as a convenient branching point for grafting further structural complexity using dynamic covalent bonds.^{18,23} Our confidence to induce quaternary structure formation of peptides was based on our small molecule studies of free bipyridine (ESI^+). With every small-molecule bipy ligand, **MP-5** consistently formed interpretable titrations indicating 1:1:1 stoichiometry (ESI^+). Thus, these results suggested the need for a third bidentate coordinating site to ensure one **MP-5** bound to only one metal center and a small-molecule ligand. Hence, if the third free-bipy ligand does not impose any steric encumbrance, such as having substitutions at the sixth position, a cyclized peptide containing a single bipy would readily coordinate about a metal center as the third ligand. Therefore, we chose **HydBep**.

Our first step was to induce cyclization of **HydBep** in the presence of **MP-5** to control non-specific interactions between the peptides. Compound **5** readily formed a cyclic peptide with **HydBep** (ESI^+), in aqueous mixtures at neutral pH, making it an ideal peptide partner for coordination with **MP-5**. Once cyclized, Fe(II) was titrated with this solution. The clean break occurring at 0.13 mM demonstrated complete complexation of **MP-5**, Fe^{2+} , and cyclized **HydBep** in a 1:1:1 ratio (Fig. 4a). To further confirm this stoichiometry, high-resolution mass spectrometry was performed. Mass spectrometry results for a cyclic **HydBep** complexed with the bipyridine-peptide along with an Fe(II) derivative (Fig. 4b).

Thus, a controlled stoichiometry of one equivalent of a bis(bipy) peptide, one *in situ* cyclized dihydrazide peptide with a small molecule dialdehyde, and one Fe(II) metal center led cleanly to a single large, complex, and folded peptide. Therefore, a strategy of combining metal coordination to induce

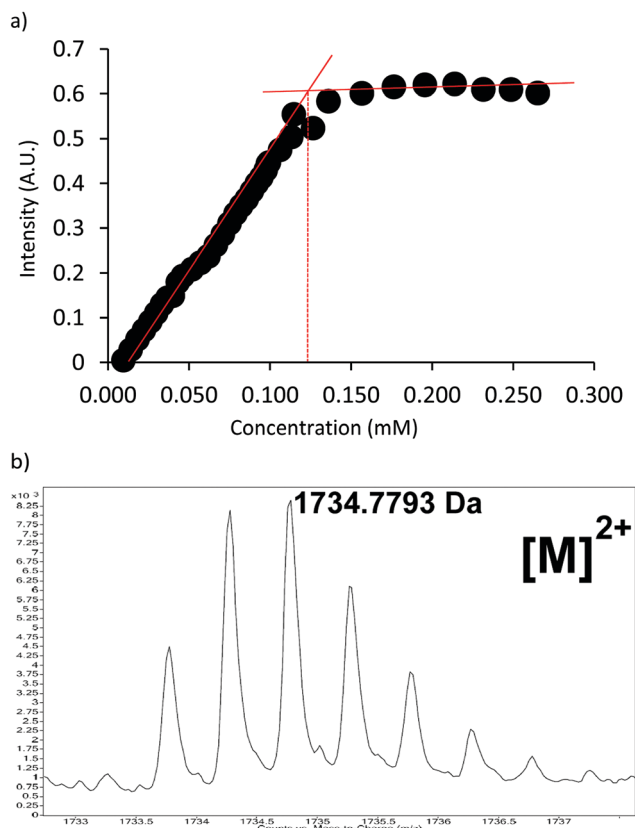


Fig. 4 (a) A titrant solution of Fe^{2+} (0.6 mM) in (MeOH : H₂O) (1 : 1) (v/v) was titrated to a solution of 0.1 mM cyclic **HydPep** and **MP-5**. Stoichiometry: (1 : 1 : 1). (b) Mass spec result for **MP-5** complexed with **HydPep**· $[M]^{2+}$.

peptide cyclization, combined with reversible covalent bonding, is a promising strategy to build complex artificial peptide quaternary structures.

Several stereochemical isomers of the assemblies presented herein will exist in solution. As with any octahedral complexes, meridional and facial, as well as delta and lambda twists, will be present. Quantifying and controlling the extent of these different species formed was not pursued in the work, but would be an added level of control to be considered in future studies.

Conclusions

Cyclization studies for **MP-6** and **MP-5** using 1H -NMR and UV-Vis titrations demonstrated that a free bipyridine is required for cyclic folding to occur. **MP-5** readily folded, but **MP-6** showed no affinity to iron or zinc. This suggested that steric hindrance at the 6-position impeded complexation. Compound **4** served as the necessary third bipyridine ligand that incorporated further complexity, demonstrating a straightforward means for forming higher order structures with synthetic peptides. The alkylation modification approach presented here, allowed for the facile creation of peptides modified with two bipyridine isomers. Future studies will include increasing the diversity of compounds formed by including other orthogonal reactions.

Experimental

General materials

For automated Fmoc amino solid-phase peptide synthesis, Gly, Thr(*t*Bu), and Tyr(*t*Bu) were purchased from P³ Biosystems. Fmoc-Lys(*N*_ε,Me)-OH was purchased from ChemPep, Inc. Fmoc-Lys(*N*₃)-OH was purchased from Chem-Impex International, Inc. Fmoc-Gly-Wang resin (0.62 mmol g⁻¹) was purchased from P³ Biosystems. *N,N'*-Diisopropylcarbodiimide (DIC) and Oxyma and (ethyl cyano(hydroxyimino)acetate) was purchased from Chem-Impex International, Inc. DMF and piperidine used for automated solid-phase peptide synthesis were purchased from Fisher Scientific and Sigma-Aldrich. Ammonium acetate and acetic anhydride was purchased from Fisher. Methacrolein and 1-[2-oxo-2-(2-pyridinyl)ethyl]iodide, 6-methyl-2,2'-dipyridyl, and [2,2'-bipyridine]-4,4'-dicarbaldehyde, and [2,2'-bipyridine]-5,5'-diylidimethanethiol[2,2'-bipyridine]-5,5'-diylidimethanethiol were purchased from Sigma-Aldrich. *N*-Bromo succinimide (NBS) and azobisisobutyronitrile (AIBN) were purchased from Acros Organics.

General instrumentation

A Liberty Blue microwave peptide synthesizer was used for solid-phase peptide synthesis. Preparative HPLC purification of the peptides was performed using an Agilent Zorbax SB-C18 Prep HT column 21.2 250 mm; 10 mL min⁻¹, 5–95% MeOH (0.1% FA) in 90 min. Analytical HPLC characterization of the peptides was performed using an Agilent Zorbax column 4.6 250 mm; 1 mL min⁻¹, 5–95% MeCN (0.1% TFA) in 35 min (RT). A Gemini C18 3.5 micron 2.1 50 mm was used for online separation; 0.7 mL min⁻¹, 5–95% MeCN (0.1% formic acid) in 12 min (RT). An Agilent Technologies 6530 Accurate Mass QTOF/MS and an AB Voyager-DE PRO MALDI-TOF were used for recording the high-resolution mass spectra of the purified peptides. The solvents used were of HPLC grade. A BioTek Cytation3 well plate reader and an Agilent Carey series spectrophotometer were used for UV-Vis titrations. A PowerEase Life technologies electrophoretic setup was used for gel separations of peptides and metallo-peptide complexes.

General procedure for peptide synthesis of MP-5 and MP-6

Synthesis of peptides was performed using standard settings using the Liberty Blue software, and using 1 M of DIC and 1 M oxyma used as coupling and bases, respectively. Amino acid solutions were prepared at 200 mM, except for Fmoc-Lys(*N*_ε,Me)-OH which was prepared at 100 mM. Each peptide made was capped using acetic anhydride incorporated into the automated synthesis. After the synthesis, resin was washed with glacial AcOH (5 mL, 3×), DCM (5 mL, 3×), and MeOH (5 mL, 3×). Resins were placed under vacuum overnight. The peptides were cleaved from the resin using trifluoroacetic acid (TFA), triisopropylsilane, and nanopure water (95:2.5:2.5) (4 h). TFA was evaporated and the remaining oil was precipitated with diethyl ether at 0 °C. No further purification of the crude peptide was performed.

HydPep

HydPep was prepared as reported in the literature.¹⁶

General procedure for alkylation of peptides

The peptides (1 eq.) were alkylated with 5-(bromomethyl)-2,2'-bipyridine (4.4 eq.) or 6-(bromomethyl)-2,2'-bipyridine (4.4 eq.) in a MeCN, H₂O, and MeOH (80 : 15 : 5) (v/v/v) solvent mixture, followed by addition of 100 µL of Hunig's base (DIPEA). The reaction was allowed to stir for 24 h at RT. The solution was purified by preparative HPLC. The purified samples were placed on a rotary evaporator to remove the organic solvent. The aqueous remnants were frozen at -70 °C and lyophilized overnight.

MP-6. Starting amount: (0.016 mmol). Yield: 38%. HRMS: (M + 2H)²⁺; calcd 748.35390, obs. 748.35630.

MP-5. Starting amount: (0.015 mmol). Yield: 25%. HRMS: (M + 2Na)²⁺; calcd 770.33580, obs. 770.33800.

Synthesis of brominated ligand precursors

Synthesis of 5-(bromomethyl)-2,2'-bipyridine was performed using a protocol reported in the literature.²⁴

Synthesis of 6-(bromomethyl)-2,2'-bipyridine was performed using a protocol reported in the literature.⁹

Synthesis of Fmoc-L-lysine(Me, Boc)-OH²². Mono-methylation at the N-position of lysine followed the indirect pathway²² of mono-benzoylation, then methylation *via* reductive amination with benzaldehyde and formaldehyde, respectively. The benzyl "protecting group" is then removed *via* palladium-catalyzed hydrogenolysis and replaced with a Boc protecting group for peptide synthesis. Whereas direct mono-methylation produces a mixture of di, mono, and non-methylated variants that are difficult to separate from each other, the indirect pathway primarily produces a mono-benzylated product, although di-benzylated and therefore non-benzylated still account for ~30% loss of yield. Methylation then produces ~70% mono-benzylated/mono-methylated product, with about equal percentages of di-methylated and di-benzylated products. These three products in the mixture are easy to separate, due to the hydrophobicity of the benzyl group. The mono-benzylated/mono-methylated intermediate is then subjected to debenzoylation with Pd/C/H₂ and *in situ* carbamoylation with Boc anhydride. Further details can be found in the ESI.[†]

Well-plate titration experiments¹⁹

Stock solutions of ligand peptides (0.6 mM), free bipy (0.2 mM), and Fe²⁺ (0.2 mM).

15 data points (15 wells) were taken for each experiment. To each well, 25 µL of metal and 25 µL of free bipy was introduced. Increasing amounts of peptide solution were introduced in 10 µL increments. The minimum was 0 µL solution introduced and the maximum was 150 µL introduced with all wells diluted to a total of 150 µL. Each well was scanned between 300–700 nm. Blanks were composed of MeOH : H₂O (1 : 1) (v/v).

Changes at 530 nm were monitored for titrations. A plot of concentration *vs.* change in absorption was drawn. Stoichiometry

was determined by plotting a line along the increasing changes in absorption and a different line where the data points plateaued. The intersection was used to determine the amount of peptide required for optimal binding.

Cuvette UV-Vis titration experiments

A titrant solution of Fe²⁺ (0.6 mM) containing 0.1 mM **MP-5** and cyclic **HydPep** was titrated to a solution containing 0.1 mM of **MP-5** and cyclic **HydPep**.

Changes at 530 nm were monitored. A plot of concentration *vs.* change in absorption was drawn. Stoichiometry was determined by plotting a line along the increasing changes in absorption and a different line where the data points plateaued. The intersection was used to determine the amount of metal required for optimal binding.

NMR titration experiments

NMR titrations were performed for both Zn²⁺ and Fe²⁺ to confirm the results of UV-Vis titration experiments. MP was dissolved to concentration in (2.3 µmol) 600 µL of D₂O : MeOD (1 : 1) (v/v). Zinc or iron triflate was dissolved to 180 µL (4.6 µmol) of deuterated solvent. 600 µL of the peptide solution was introduced to the NMR tube. NMR was performed initially before the incorporation of any metal. Incremental additions of metal solution (15 µL, 6×; 30 µL, 3×) were made. NMR experiment was performed immediately after each addition. Titration of unreacted bipy was performed in a similar fashion.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We gratefully acknowledge our funding sources: the National Institute of Health (5DP1GM106408), the Welch Foundation (F1515 to E. M. M.), the Defense Advanced Research Projects Agency (DARPA, N66001-14-2-4051), and the Welch Regents Chair to EVA (F-0046). We also gratefully acknowledge Dr Ian Riddington and Dr Kristin Suhr from the UT Austin Mass Spectrometry Facility.

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