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Peptide Tertiary Structure Nucleation by Side-Chain Crosslinking with Metal Complexation and Double "Click" Cycloaddition

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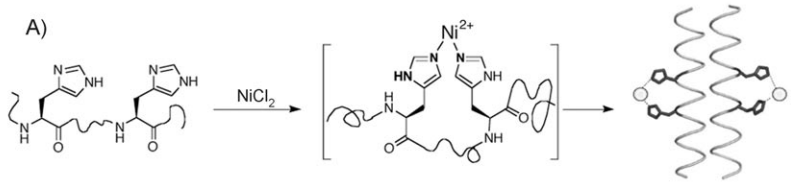
Stabilized helical peptides show potential as therapeutics capable of modulating protein function through enhanced helix–protein binding.[1] Helix-turn stabilization may be accomplished though covalent side-chain crosslinking on one face of the helix, in the *i* and *i*+4 or *i*+7 positions,[2–4] or replacement of a main-chain hydrogen bond with a covalent linker.[3,5,6] We report herein a new helix-nucleating[7] *i* and *i*+4 crosslinking strategy based on copper-catalyzed azide–alkyne [3+2] "click" cycloaddition,[8] and demonstrate the ability of this method and metal complexation to restore coiled-coil dimerization in a folding-incompetent sequence crippled by two helix-breaking glycine residues. Elegant applications of azide–alkyne cycloaddition chemistry in peptide conjugation[9] and structure stabilization[10] are known in the literature; this design strategy complements known intramolecular methods for structure nucleation, such as ring-closing metathesis (RCM),[2,5,11] and allows convergent installation of functional groups pendant to the bis-alkyne linker that could be used to modulate peptide binding, targeting, and membrane permeability.

We studied 21-residue sequences derived from the GCN4 leucine zipper[12] in which the central heptad contains glycine residues in the *c* and *e* helix positions and crosslinking residues (X) in the *b* and *f* (*i* and *i*+4) positions, leaving the hydrophobic core residues in the *a* and *d* positions intact as isoleucine and leucine, respectively. Metal complexation with *i* and *i*+4 X = His residues is known to induce monomeric helix folding,[4,13] though it has not been previously demonstrated to restore structure in peptides containing two glycine residues. We postulated that the bis-triazole product of a double [3+2] cycloaddition between *i* and *i*+4 azidoalanine (Az)[14] residues and a bis-alkyne could yield a nonlabile covalent linker isosteric with *i* and *i*+4 His–His metal complex (Figure 1 A). We pre-



B)

gabcdefg-abcdefg-abcdefg

Az=

(**1**) ABA-RIKQLEEK-I**H**GLG**H**K-IEELEKK-NH_2

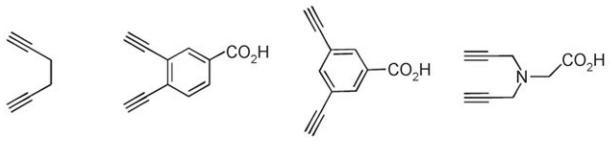
(**2**) ABA-RIKQLEEK-I**Az**GLG**Az**K-IEELEKK-NH_2

(**3**) ABA-RIKQLEEK-I**Az**GLG**S**K-IEELEKK-NH_2

(**4**) ABA-RIKQ<u>K</u>EEK-I**X**G<u>A</u>G**X**K-<u>E</u>EELEKK-NH_2

(**5**) ABA-R<u>KI</u>Q<u>EL</u>EK-I**X**GLG**X**<u>E</u>-<u>EKIEKKL</u>-NH_2

C)



6 **7** **8** **9**

D)

Peptide	Linker	Adduct	State (CD)	T_m / °C	# Helices
1	--	--	unfolded	--	--
2	--	--	unfolded	--	--
1	$NiCl_2$	--	helical	50	2
2	**6**	**6a**	helical	40	2
2	**7**	**7a**	unfolded	--	--
2	**8**	**8a**	insoluble	--	--
2	**9**	**9a**	helix-coil	40	1, 2

Figure 1. A) Strategies for helix structure nucleation with *i* and *i*+4 crosslinking by using metal complexation (top) or double-click cycloaddition (bottom). a) Diazidoalanine peptide was treated in aqueous buffer with bis alkynes **6**–**9**, sodium ascorbate, $CuSO_4$, and bathophenanthroline disulfonic acid or on resin in DMF with CuI and DIEA. B) Peptide sequences **1**–**5** used in this study, with helical wheel positions shown above, here X = Az or His and ABA = acetamidobenzoate ($\varepsilon_{270} = 18\,000\ M^{-1}cm^{-1}$). Dimerization-inhibiting mutations in **4** and **5** are underlined. C) Bis-alkyne linkers **6**–**9**. D) Properties of stabilized and unstabilized peptides.

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pared peptides **1** and **2** using standard Fmoc solid-phase synthesis; they (i.e., **1** and **2**) may be crosslinked in the central heptad by Ni^{2+} complexation with histidine and bis-alkyne cyclization with Az, respectively (Figure 1B). Indeed, the circular dichroism (CD) spectrum of **1** exhibits a saturable increase in helicity upon treatment with $NiCl_2$, which can be reversed with EDTA treatment (see Figure 3, below). While the free peptide is a completely unfolded monomer, the nickel-complexed peptide melts cooperatively with a T_m of 46 °C, and is found by analytical ultracentrifugation (AUC) to be dimeric (Figure 1D); this indicates the ability of side-chain crosslinking to restore tertiary structure even in sequences containing two centrally placed glycine residues.

We sought to use copper-catalyzed click chemistry to similarly induce secondary[15] and tertiary structure by linking azidoalanines in peptide **2** with bis alkyne linkers **6**–**9** (Figure 1C). These intermolecular cyclizations were successful with both resin-bound protected peptide in DMF with CuI/*N*,*N*-diisopropylethylamine (DIEA) and in aqueous buffer with a copper(I) catalyst[16] (Figure 1A). Click adducts were subjected to Staudinger reduction conditions with PPh_3 (on resin) or tris(2-carboxyethyl)phosphine hydrochloride (in aqueous solution) to confirm ring closure; while starting material **2** reacted with phosphine to yield the expected reduction and other products, all adducts remained unchanged; this is consistent with conversion of azide to triazole. Additionally, MS-MS peptide fragmentation patterns were indicative of cyclic "double-click" products (Figure 2). Fragmentation of the bisalkyne-treated diazido peptides yielded all possible peptide bond mass fragments except for those putatively joined by a bis-triazole linker, which should not fragment under these conditions. The identical experiment with the click adduct to a monoazide peptide **3**, in which the C-terminal Az residue was replaced with serine, yielded all possible peptide mass fragments. Notably, the observed ratio of ion intensities of the 4+ parent ion relative to the common mass fragment y1 is much higher in the spectrum of cycloadduct of **2** (Figure 2B) relative to the cycloadduct of **3**. This is consistent with double cycloaddition to **2** that results in the coalescence of ion intensities of four possible distinct mass fragments into a single, more intense peak.

Interestingly, though linkers were used in large excess on resin, the bis-adduct was never observed, and diazide **2** could be near quantitatively converted on resin to the double cycloadduct (determined as described above); this suggests that the second click reaction to close the ring is much faster than the first, despite the 23- or 24-atom ring closure required. Similarly efficient peptide ring closures have been observed when using RCM; this suggests that the peptide backbone might greatly reduce the entropic cost of cyclization through templating effects or hindered amide bond rotations.[2,11] Cyclization in aqueous buffer also yielded the desired product in acceptable (40–50%) isolated yields, though bis-adducts were observed when the linker was used in moderate excess. The more rigid unsaturated linkers **7** and **8** gave more bis-adducts than linkers **6** and **9**, which have single bonds connecting the two alkynes. The functional effect of ring-closure by double cycloaddition was observed by comparing the folding properties of monoazide peptides in which one AzAla residue was replaced by serine. These negative-control peptides are also readily coupled with **6**–**9**, but remain unfolded under all conditions, like diazido starting material **2** (Figure 3). Commercially available hexa-1,5-diyne (linker **6**) induced the most significant change in helicity upon double cycloaddition, nucleating helical structures in the central heptad with sufficient efficiency to restore dimerization to the folding-crippled **2**. Like the nickel complex of **1**, the hexa-1,5-diyne adduct peptide (**6a**) melts cooperatively ($T_m = 40$ °C) but dimerizes independently of nickel concentration (Figure 3). Linkers **7**–**8** were used to probe the steric requirements of side-chain constraint. When the ethyl spacer of hexa-1,5-diyne **6** is replaced by the rigid two-carbon linker of the *ortho*-diethynylbenzoic acid linker **7**, the folded state is not favored. The *meta*-diethynylbenzoic acid **8** and dipropargylated glycine linker, **9** both extend the chain by one atom, with a rigid unsaturated linker and more flexible saturated linker, respectively. The cycloadducts of diazidopeptide **2** with **7** (**7a**) and **9** (**9a**) exhibit random-coil signatures, while **8a** is insoluble in aqueous solvent. Interestingly, treatment of **9a** with $NiCl_2$ increases the helicity of the system; this suggests the formation of a new nickel binding site, presumably composed of the triazoles, tertiary amine, and carboxylate from the ligated linker. The dependence of ellipticity on nickel concentration indicated tighter nickel binding by adduct **9a** ($K_d = 10^{-6}$ M) than by dihistidine peptide **1** ($K_d = 10^{-4}$ M); this is consistent with a more organized binding site in the cycloadduct than in the open-chain peptide **1**. Indeed, the double cycloadduct of propargyl alcohol with **2** installs the two triazole moieties in a noncyclic product, and this peptide remains unfolded even in the presence of nickel chloride, thus indicating the importance of cyclization (Supporting Information). However, AUC measurements indicated a monomer–dimer equilibrium for **9a** under all conditions; this suggests that metal complexation does not completely restore dimerization. This result stands in contrast to the enhanced helicity and dimerization found upon crosslinking with **6** to obtain **6a**. It is possible that the additional atom in linker **9** actually prevents the formation of a helical turn, and metal binding induces a contraction of the cyclic binding site that allows partial, but incomplete folding and dimerization. The weak dimerization and partially helical signature in **9a** suggests that, similar to the parent "uncrippled" coiled coils, helix folding and oligomerization in these systems are coupled.[17] Peptides **4** and **5** are dimerization-inhibited by charged residues at the dimerization interface or scrambled coiled-coil sequences, respectively. These soluble peptides (X = Az or His) retained random-coil CD signatures upon metal complexation with $NiCl_2$ and were insoluble upon cycloaddition, consistent with coupled folding and assembly; this is likely accentuated by the presence of two helix-breaking glycines in the sequence.

We have thus demonstrated a new methodology for helix structural nucleation using double azide–alkyne [3+2] cycloadditions to form *i* and *i*+4 constrained peptide sequences. This chemistry is sufficiently robust to allow intermolecular cyclization with few byproducts, thus setting the stage for convergent functionalization and structure nucleation. This technolo-

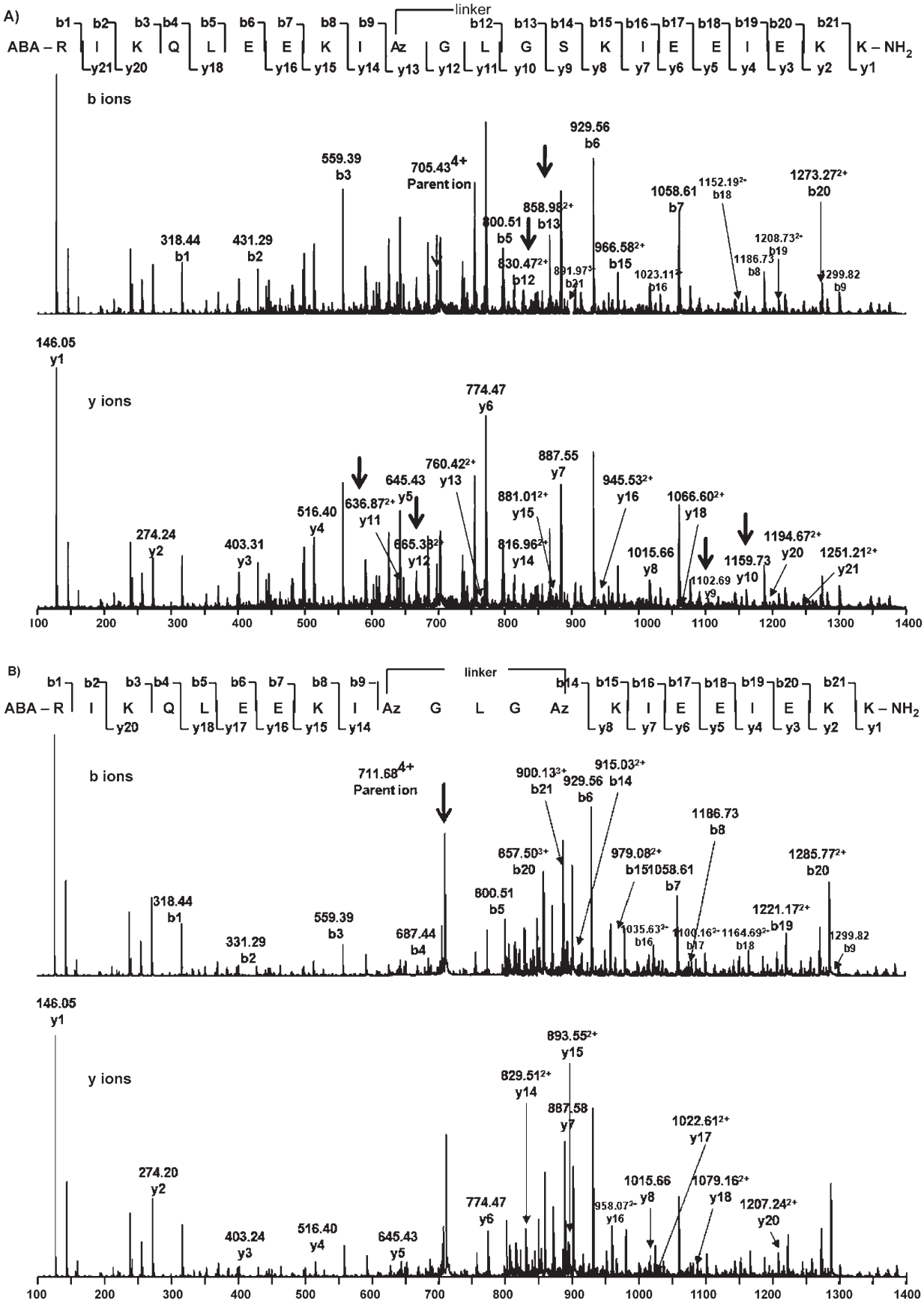

Figure 2. Representative MS–MS data indicating fragmentation at the peptide bonds of the adduct of linker **6** to A) peptide **3** and B) peptide **2** (**6a**). Arrows indicate diagnostic C-terminal y-fragment ions, N-terminal b-fragment ions, and g-parent ions (4+).

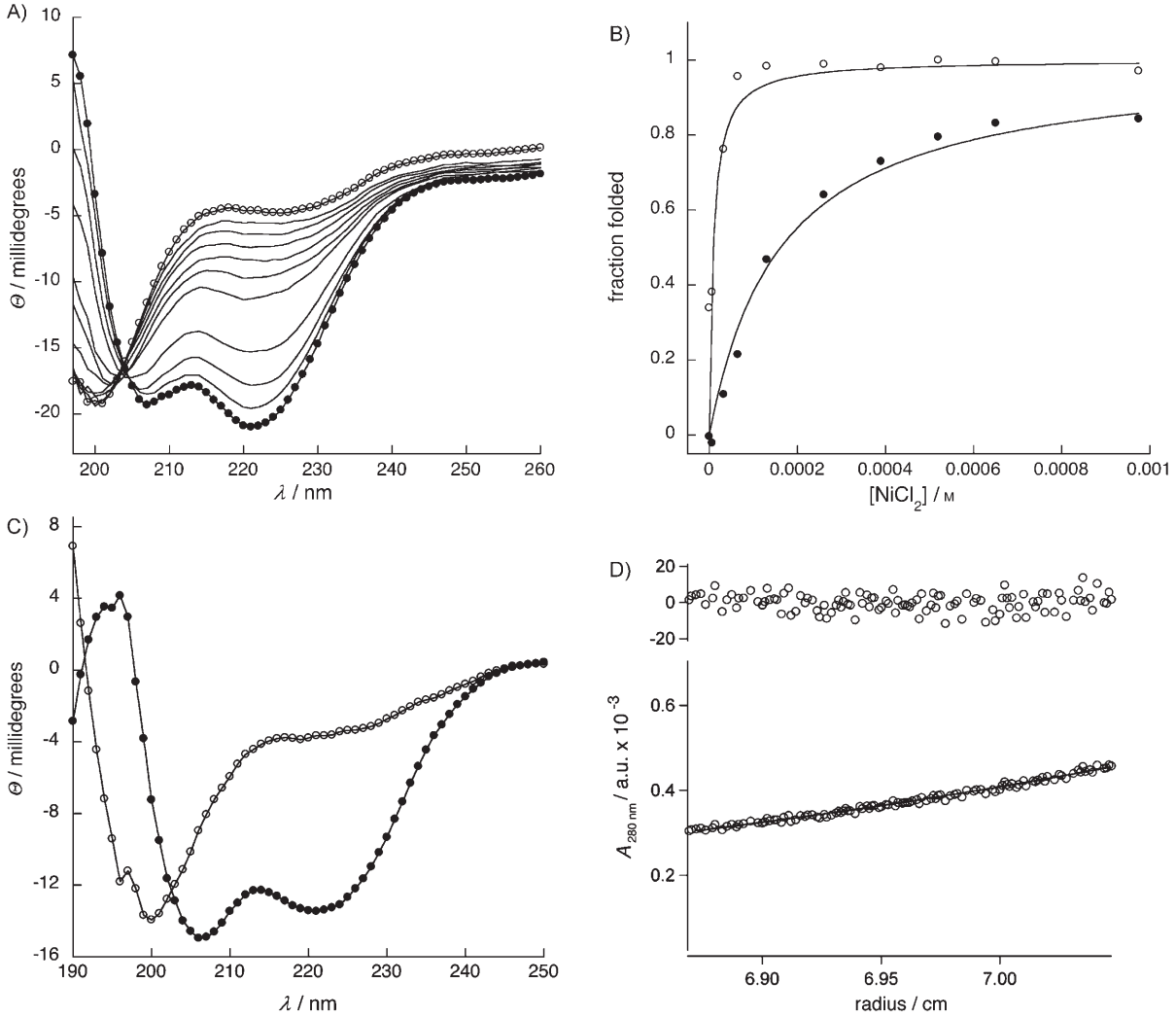

Figure 3. A) Nickel-dependent folding of **1**, from 0 (○) to 100 μM $NiCl_2$ (●), with intermediate concentrations shown in black. B) Binding isotherms of $NiCl_2$ to peptide **1** (●) and peptide **9a** (○), fitted to a 1:1 binding model. C) CD spectra of the cycloadduct of hexa-1,5-diyne linker **6** to mono(Az) mutant **3** (○) and cycloadduct **6a** (●). D) A typical sedimentation profile of hexa-1,5-diyne cycloadduct **6a** equilibrated at 25000 rpm. Data (○) are fitted to the solid curve with residuals shown on top.

gy could be useful as a means of synthesizing new building blocks for peptide-based materials[18] or preparing stabilized helical peptides capable of binding protein interfaces and altering biological function. These studies are currently underway in our laboratory.

Experimental Section

Peptide synthesis: Peptides were synthesized on an AAPPTEC Apex 396 SPPS synthesizer by using a Fmoc strategy. The synthesis was performed on 0.34 or 0.7 mmol g^{-1} Rink amide resin. Crude peptides were purified by reversed-phase HPLC on C18 preparative columns (Higgins Analytical and Phenomenex). Stock solutions of peptides were prepared in deionized water. Peptide concentrations were measured in pure water by using the UV absorbance of ABA (4-acetamidobenzoic acid) at 270 nm ($\varepsilon_{270} = 18000\ M^{-1}cm^{-1}$).

Azide–alkyne double [3+2] cycloaddition in aqueous buffer: All solutions were degassed by argon sparge prior to use. Alkyne (1.5 equiv) was dissolved in Tris-buffered saline (TBS, 10 mM Tris, 110 mM NaCl, pH 8.5) with diazidopeptide **2**. Separately, $CuSO_4$ (4 equiv) was treated with sodium ascorbate (50 equiv) and bathophenanthroline disulfonate ligand (7.2 equiv). The copper solution was vortexed and immediately added to the peptide solution to yield a final peptide concentration of 3.62 mM. The red-brown mixture was stirred under argon for 2.5–12 h (40–50% HPLC isolated yield).

Azide–alkyne double [3+2] cycloaddition on solid support: Peptide resin was preswelled with DMF and treated with hexa-1,5-diyne (50 equiv, 5.15 M in pentane), CuI and DIEA (3 equiv each in CH_3CN) were added to the resin to give a final alkyne concentration of 0.5 M. The resin was shaken overnight under Ar and treated again with CuI/DIEA for 4–6 h following DMF wash, without additional bisalkyne linker. The peptide was cleaved from the resin by using TFA/TIS/H_2O (95:2.5:2.5) and purified by HPLC. Only the cyclic product was observed.

Circular dichroism: CD spectra were obtained from an AVIV Model 202 spectropolarimeter and corrected for background and dilution effects. Wavelength scans were taken at 25 °C in 1 nm intervals, with 3.0 s averaging time, 2 min equilibration time. $NiCl_2$ was

added in 5, 10, 15 and 20 μM increments. Normalized data were plotted as fraction folded (Q) vs. nickel concentration [Ni^{2+}], then fitted to the mass action law: $Q = [Ni^{2+}]/(K_d + [Ni^{2+}])$.

Quadrupole time of flight MS–MS: Accurate molecular weight and further detailed sequence information of these peptides were determined on a Micromass Q-Tof II apparatus (Micromass, Wythenshawe, United Kingdom) equipped with an orthogonal electrospray source (Z-spray) and operated in positive-ion mode. For external mass calibration, NaI was used over the m/z range 200 to 2500. The peptides were dissolved in H_2O/CH_3OH/HAc (50:50:2.5) and directly infused into the electrospray source at a 2 μL min^{-1} flow rate. To achieve the optimal electrospray, capillary voltage was set at 3000 V, the source temperature was 150 °C, and the cone voltage was 60 V. The first quadrupole, Q1, was set to pass ions between 200 and 2500 m/z. The target ion was isolated and fragmented within the second quadrupole by adding a voltage of between 20 and 40 V. The fragment ions were then analyzed in the time-of-flight tube. Data were acquired in continuum mode until well-averaged data were obtained.

Analytical ultracentrifugation: Apparent molecular masses of peptides were determined by sedimentation equilibrium on a Beckman ProteomeLab™ XL-I ultracentrifuge. Purified peptides (25 μM) were analyzed at three different $NiCl_2$ concentrations (0, 100, and 200 μM) in Tris (10 mM) containing NaCl (50 mM) at pH 7.1. The peptides were equilibrated at three rotor speeds (25 000, 32 000, and 45 000 rpm) for 24 and 30 h at 20 °C. Absorbance scans at 270 and 280 nm were fitted to Equation 1, which describes the equilibrium sedimentation of a homogeneous single ideal species:

$$\mathrm{Abs}(r) = A' \exp[H \cdot M(x^2 - x_0^2)] + B \quad (1)$$

where $\mathrm{Abs}(r)$ = Absorbance at radius r, A' = absorbance at reference radius x_0, $H = (1 - \bar{v}\rho)\cdot\omega^2/2RT$, with $\bar{v}$ = partial specific volume of the peptide, ρ = solvent density, ω = angular velocity in radians/second, M = apparent molecular weight, and E = blank absorbance. Data were fitted by using Igor Pro v5.03 and partial specific volumes, and solution densities were calculated by using the program SEDNTERP.

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