Supporting information for:
"A bis-macrocylic peptide ligand mimicking the β-sandwich active site of multicopper oxidases"

Viet Thuc Dang[1], Alexander Drena[2], Joshua Telser[3], Lane Wofford[1], Andy I. Nguyen[1]*

[1] Department of Chemistry, University of Illinois Chicago, Chicago IL, 60607, United States
[2] Department of Chemistry, Northwestern University, Evanston, IL 60208, United States
[3] Department of Science, Health, and Pharmacy Chemistry, Roosevelt University, Chicago, IL 60605, United States*

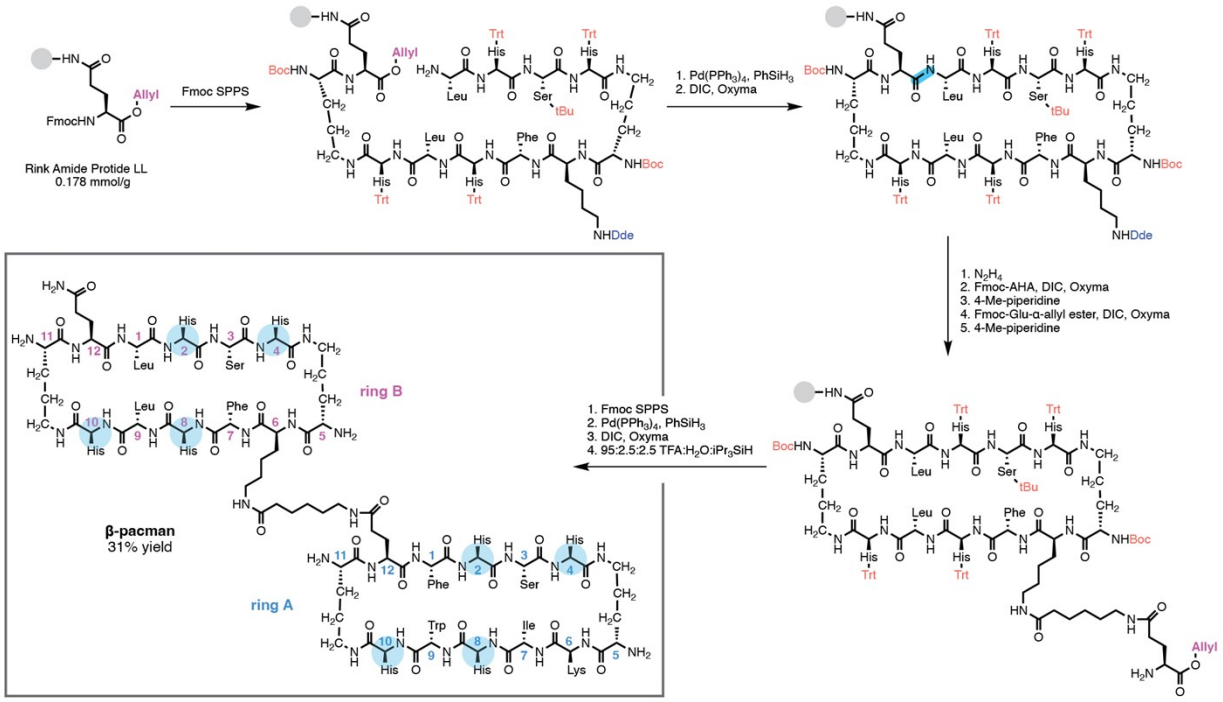
Corresponding author. *E-mail*: andyn@uic.edu

Table of Contents

I. General considerations

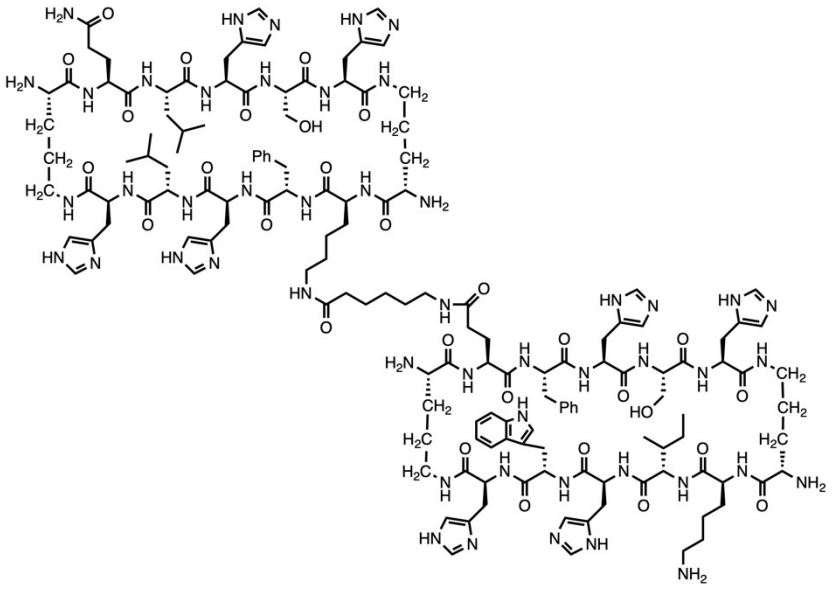
Trifluoroacetic acid (TFA), triisopropylsilane (TIPS), *N*,*N*'-diisopropylcarbodiimide (DIC), ethyl(hydroxyimino) cyanoacetate (Oxyma), Fmoc-L-Trp(Boc)-OH, Fmoc-His(Trt)-OH, Fmoc-L-Ser(tBu)-OH, Fmoc-L-Phe-OH, Fmoc-L-Ile-OH, *N*α-Boc-*N*δ-Fmoc-L-Orn-OH, Fmoc-L-Lys(Boc), Fmoc-L-Lys(Dde), Fmoc-L-Glu-α-allyl ester were purchased from Chem-Impex International Inc. ProTide Rink Amide LL resin (0.2 mmol/g) was purchased from CEM Corporation. Palladium tetrakis(triphenylphosphine), phenylsilane, and acetonitrile (MeCN) were purchased from Millipore Sigma. Dichloromethane (DCM) and 4-methyl-piperidine were purchased from Fisher. *N*, *N*-dimethylformamide (DMF), glycerol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and copper(II) chloride dihydrate was purchased from Sigma-Aldrich. Water was purified by Milli-Q purification system.

II. Synthesis and characterization



Scheme S1. Synthesis of β-pacman. The metal-coordinating His residues are highlighted in cyan.

The peptide was exposed to trifluoroacetic acid during the purification process (*vide infra*). Masses used for the preparation of samples from lyophilized peptide account for the presence of these trifluoroacetate counterions based on the number of functional groups with pK_a values greater than that of trifluoroacetic acid.



MW: 3156.67

MW + 13 trifluoroacetic acid: 4638.97

Resin loading determination

A known amount (~10 mg) of dried ProTide Rink Amide (LL) resin was transferred into a 1.5 mL microcentrifuge tube, and DMF (800 µL) was added and allowed to swell for 15 mins. Then, 4-methylpiperidine (200 µL) was added, and the mixture was allowed to stand at room temperature for 15 min. The solution above the resin (300 µL) was transferred to a 1 cm quartz cuvette and diluted with DMF (2700 µL). A UV-vis spectrum was recorded. The absorbance at 301 nm was used to calculate the resin loading (eq 1). The average resin loading determined from the four measurements is 0.178 ± 0.004 mmol/g (manufacturer lists 0.2 mmol/g).

$$L = \frac{(A_{301} \times V \times d)}{(\varepsilon \times w \times M)} \qquad \text{(eq S1)}$$

where L = resin loading (mmol/g)
A_{301} = absorbance at 301 nm
V = volume of sample (mL)
d = dilution factor = 10
ε = extinction coefficient of Fmoc = 7800 (mL $mmol^{-1}cm^{-1}$)
w = path length of cuvette (cm)
M = mass of resin used (g)

Preparation of amino acid solution

For a 0.1 mmol scale, 0.20 M of amino acid in DMF is used. 2.5 mL of this solution is used for each coupling step.

Preparation of Oxyma Pure (ethyl cyano(hydroxyimino)acetate) solution

For a 0.1 mmol scale, 1 M of Oxyma Pure in DMF is used. 0.5 mL of this solution is used for each coupling step.

Preparation of DIC (N,N'-Diisopropylcarbodiimide) solution

For a 0.1 mmol scale, 1 M of DIC in DMF is used. 1.0 mL of this solution is used for each coupling step.

Preparation of 4-methylpiperidine solution

For a 0.1 mmol scale, 20% v/v concentration of 4-methylpiperidine in DMF is used. 2.0 mL of this solution is used for each coupling step.

Scheme for automated synthesis

Peptides were synthesized on a 0.1 mmol scale using the CEM Liberty Blue automated peptide synthesizer. The solvent for all SPPS solutions was DMF unless otherwise stated. The prepared amino acid, Oxyma, and DIC concentrations were 0.20 M, 1.0 M, and 1.0 M respectively. The deprotection solution was 20% (v/v) 4-methylpiperidine in DMF.

1. ***Swelling:*** The resin was allowed to swell in 5 mL DMF at room temperature while being agitated with N_2 gas for 10 min, and then it was drained.

2. ***Single coupling 0.1 mmol scale:*** 2 mL of 4-methyl-piperidine in DMF (20% v/v) was added to the swelled resin and agitated with N_2 gas for 15 s at 90° C, and then it was drained. The resin was washed 4 times, each by adding 5 mL DMF, agitating with N_2 for 5 s, and then drained. 1.0 mL of 1.0 M DIC and 0.5 mL of 1.0 M Oxyma was added to the reaction vessel containing the resin followed by 2.5 mL of 0.20 M amino acid solution. The solution was allowed to warm to 75° C over the course of 15s and followed by an interval of 110 s being held at 90° C. N_2 gas was used to agitate the reaction vessel before being drained. The resin was washed 4 times, each by adding 5 mL DMF, agitating with N_2 for 5 s, and drained.

3. ***Double histidine coupling 0.1 mmol scale:*** 2 mL of 4-methyl-piperidine in DMF (20% v/v) was added to the swelled resin and agitated with N_2 gas for 15 s at 90° C, and then it was drained. The resin was washed 4 times each by adding 5 mL DMF, agitating with N_2 for 5 s, and drained. 1.0 mL of 1.0 M DIC and 0.5 mL of 1.0 M Oxyma were added to the reaction vessel followed by 2.5 mL of 0.20 M Fmoc-His(Trt)-OH solution. The solution was ramped to 50 °C over 120 s and held at 50° C for 480 s. The mixture was agitated with N_2 gas and then the reaction vessel was drained. Another round of Oxyma, DIC, and Fmoc-His(Trt)-OH were added as previously described. The resin was washed 4 times, each by adding 5 mL DMF, agitating with N_2 for 5 s, and drained.

4. ***Final Fmoc deprotection:*** The N-terminal amino acid was deprotected with 4 mL of 20% (v/v) 4-methyl-piperidine for 50 s then drained. The resin was then washed 4 times with 4 mL of DMF and drained.

5. ***Allyl deprotection:*** To prevent contaminating the automatic synthesizer with Pd, the allyl deprotection was carried out in a separate manual synthesis reaction vessel. The Fmoc-deprotected resin-bound peptide was transferred to a fritted jacketed reaction vessel heated to 35 °C, using DMF as a transfer medium. The DMF was drained from the reaction column and the resin was washed 4 times each by adding 5 mL DMF, agitating with N_2 for 5 s, and then drained. The resin was then washed 4 times each by adding 5 mL DCM, agitating with N_2 for 5 s, and then drained. 3 mL of 5 mM phenylsilane in DCM was added to the resin and allowed to react for 90 s with N_2 gas agitation. Then 1 mL of 25 mM $Pd(PPh_3)_4$ in DCM was added and the reaction was allowed to proceed for 810 s. The resin was washed 4 times, each by adding 5 mL DCM, agitating with N_2 for 5 s, and drained.

6. ***Lactamization:*** The allyl-deprotected resin-bound peptide was transferred back to the CEM automated synthesizer. 5 mL of 0.33 M DIC and 0.17 M HOBt in DMF solution was added to the swelled resin. After 120 s to warm up to 75 °C followed by 780 s held at 90 °C with N_2 gas agitation, the reaction vessel was drained. The resin was washed 4 times, each by adding 5 mL DMF, agitating with N_2 for 5 s, and drained. This procedure was repeated 2 additional times.

7. ***Dde deprotection:*** 4 mL of a 5% hydrazine in DMF solution was added to the swelled resin. After 15 s to warm up to 75 °C followed by 165 s held at 90 °C with N_2 gas agitation, the reaction vessel was drained. The resin was washed 4 times, each by adding 5 mL DMF, agitating with N_2 for 5 s, and drained. This procedure was repeated 2 additional times.

Table S1. β-Pacman synthesis

Step	Amino Acid	Specific AA Derivative	Cycle
1	N/A	N/A	swelling
2	Glu	Fmoc-L-Glu-**α-allyl ester**	single coupling
3	Orn	Boc-L-Orn(δ-Fmoc)-OH	single coupling
4	His	Fmoc-L-His(Trt)-OH	double histidine coupling
5	Leu	Fmoc-L-Leu-OH	single coupling
6	His	Fmoc-L-His(Trt)-OH	double histidine coupling
7	Phe	Fmoc-L-Phe-OH	single coupling
8	Lys	Fmoc-L-Lys(**Dde**)-OH	single coupling
9	Orn	Boc-L-Orn(δ-Fmoc)-OH	single coupling
10	His	Fmoc-L-His(Trt)-OH	double histidine coupling
11	Ser	Fmoc-L-Ser(tBu)-OH	single coupling
12	His	Fmoc-L-His(Trt)-OH	double histidine coupling
13	Leu	Fmoc-L-Leu-OH	single coupling
14	N/A	N/A	final deprotection
15	N/A	N/A	allyl deprotection
16	N/A	N/A	lactamization
17	N/A	N/A	Dde deprotection
18	ACA	Fmoc-aminohexanoic acid	single coupling
19	Glu	Fmoc-L-Glu-**α-allyl ester**	single coupling
20	Orn	Boc-L-Orn(δ-Fmoc)-OH	single coupling
21	His	Fmoc-L-His(Trt)-OH	double histidine coupling
22	Trp	Fmoc-L-Trp(Boc)-OH	single coupling
23	His	Fmoc-L-His(Trt)-OH	double histidine coupling
24	Ile	Fmoc-L-Ile-OH	single coupling
25	Lys	Fmoc-L-Lys(Boc)-OH	single coupling
26	Orn	Boc-L-Orn(δ-Fmoc)-OH	single coupling
27	His	Fmoc-L-His(Trt)-OH	double histidine coupling
28	Ser	Fmoc-L-Ser(tBu)-OH	single coupling
29	His	Fmoc-L-His(Trt)-OH	double histidine coupling
30	Phe	Fmoc-L-Phe-OH	single coupling
31	N/A	N/A	final deprotection
32	N/A	N/A	allyl deprotection
33	N/A	N/A	lactamization

Cleavage from solid support and global deprotection: The resin was transferred to a 5 mL fritted polypropylene cartridge and then washed with 5 mL DCM while being swirled by hand for 30 s and then drained by vacuum. The DCM wash was done twice more, after which the resin was air dried by pulling vacuum for 10 min. The resin was transferred to a scintillation vial, and a 10 mL cleavage cocktail of 95% TFA, 2.5% TIPS, and 2.5% water was added to the resin. The mixture was shaken for 3 h, and the filtrate was collected after filtering through a fritted polypropylene cartridge. The resin was washed with TFA (3 x 1 mL) followed by DCM washes (3 times 1 mL). The filtrate and washings were transferred into a round bottom flask and dried by a rotary evaporator to yield an oily residue.

Ether Precipitation: Diethyl ether (~20 mL) was added to the residue after global deprotection, and the mixture was sonicated to obtain a white precipitate, which was transferred to a centrifuge tube and separated from the liquid phase by centrifugation for 3.0 min at 3.0 RCF resulting in a white pellet at the bottom of the centrifuge tube. The liquid phase was then decanted and the pellet was washed twice more with ~20 mL of diethyl ether, each time breaking up the pellet by sonication, and the crude product was allowed to air dry. LC-MS of the crude product (Figure S1) shows β-pacman as the major species.

Reverse phase high performance liquid chromatography (RP-HPLC)

Preparative RP-HPLC was performed on a Waters Prep 2545 HPLC, equipped with a C18 (X-Bridge Peptide BEH 5μm, 19x 100 mm) column at a 17 mL/min flow rate monitored at a 214 nm wavelength. Crude samples were dissolved in water and filtered through a 0.2 μm filter before RP-HPLC purification. RP-HPLC was performed with water as the weak solvent and acetonitrile as the strong solvent. HPLC solvents were acidified with 0.1% trifluoracetic acid. Fractions were analyzed for the product using UPLC-MS. Fractions containing the product were dried by lyophilization. β-Pacman was purified using a 10-50% gradient over 30 min. The pure fractions were concentrated by rotary evaporation and lyophilized to yield 128 mg (31% yield) of β-pacman•13CF_3CO_2H.

LC-MS data

Ultra-performance liquid chromatography (UPLC-MS)

Mass spectroscopy was performed using a Waters Acquity H-Class PLUS system equipped with a C18 (Acquity UPLC BEHEH 1.7 μm) column and TUV detector. Low-resolution electrospray ionization data were collected with an inline Waters SQD2 single quadrupole detector.

High Resolution electrospray-ionization mass spectrometry

High-resolution data were acquired on the Waters Synapt G2-Si ESI/LC-MS.

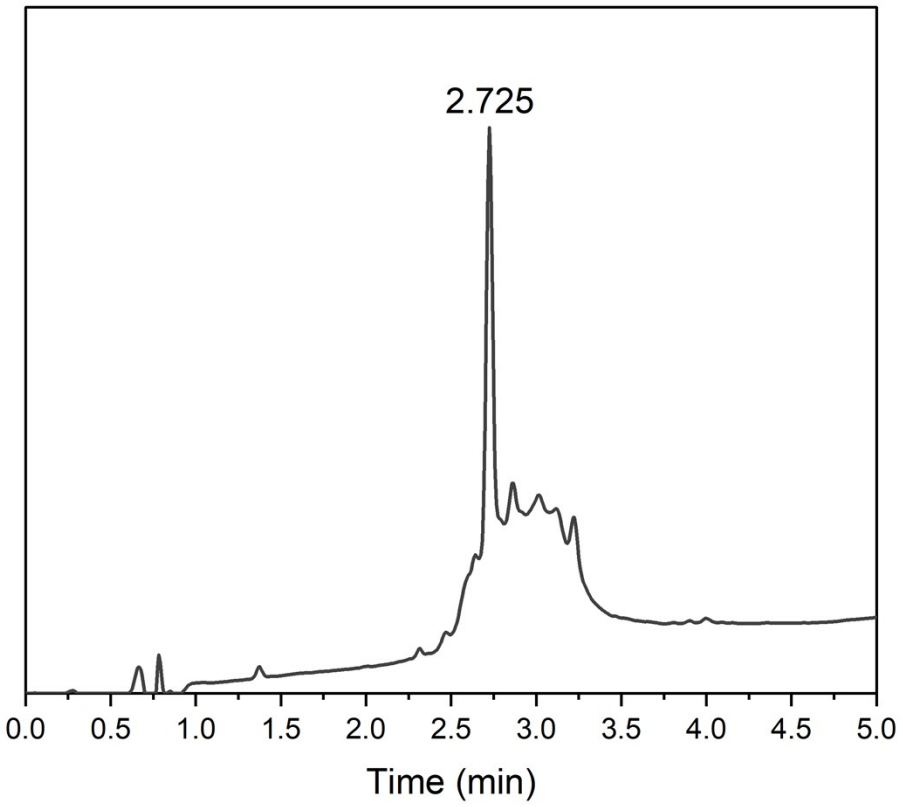


Figure S1. UPLC trace of crude β-pacman, 5-50% (MeCN/H_2O with 0.1% TFA) over 5 min. The denoted peak corresponds to β-pacman, identified by mass spectrometry.

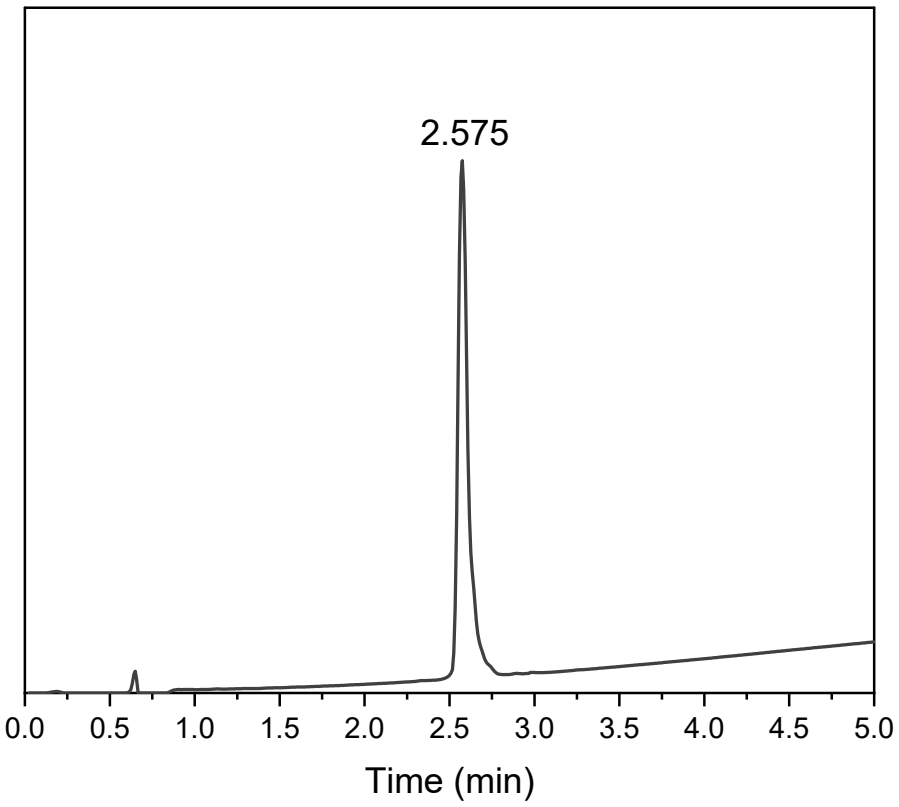


Figure S2. LC trace of purified β-pacman. Gradient: 5-50% (MeCN/H_2O with 0.1% TFA) over 5 min.

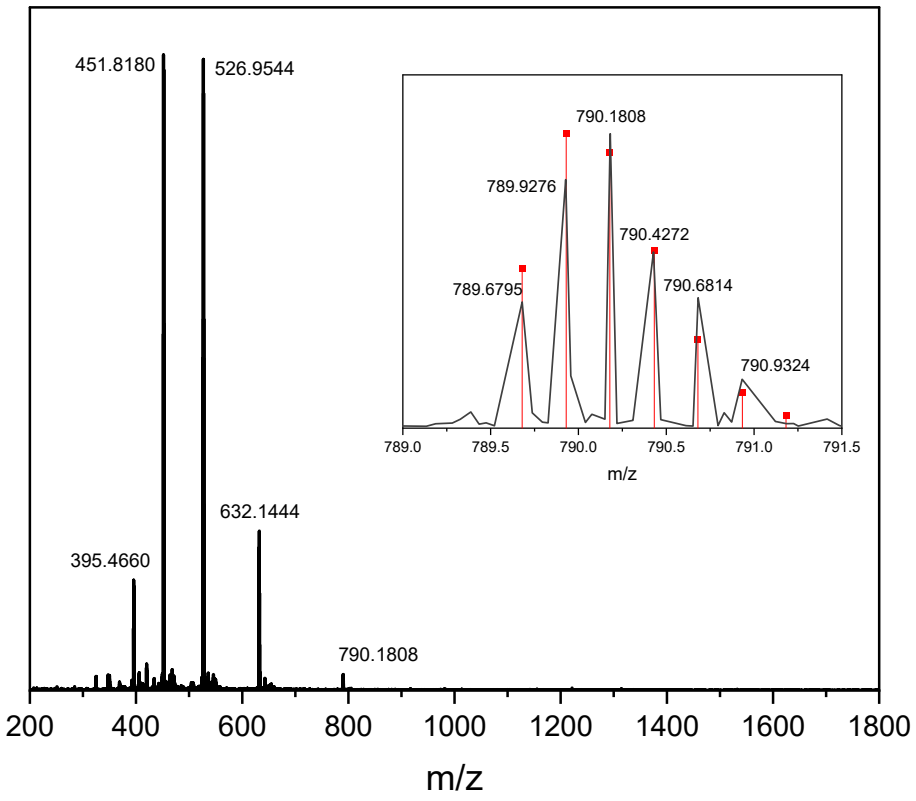


Figure S3. Mass spectrum of β-pacman. Graph showing (black) the mass spectrum of β-pacman with observed masses $[M+8H]^{8+}$ = 395.4660, $[M+7H]^{7+}$ = 451.8180, $[M+6H]^{6+}$ = 526.9544, $[M+5H]^{5+}$ = 632.1444, $[M+4H]^{4+}$ = 790.1808. The inset shows the simulated spectrum (red) for the +4 ion overlaid with the experimental data (black).

Nuclear magnetic resonance (NMR) spectroscopy

^{1}H 1D NMR spectra were measured on a Bruker Avance III 500 MHz console using Topspin3.7.0. Peptide samples were dissolved in 90% H_2O and 10% D_2O. The temperature was held at 280 K with a variable temperature module and the peaks are referenced to TMS. 1D ^{1}H water suppressed spectra were acquired using the Bruker parameter file ZGESGP having excitation sculpting for the water peak selection and suppression.[1] Typical spectra were acquired with a sweep width of 12 ppm, 32 scans, 3 second recycle delays and 1.5s of data acquisition. All spectra below were processed and analyzed with either Topspin3.7.0 or Mnova software.

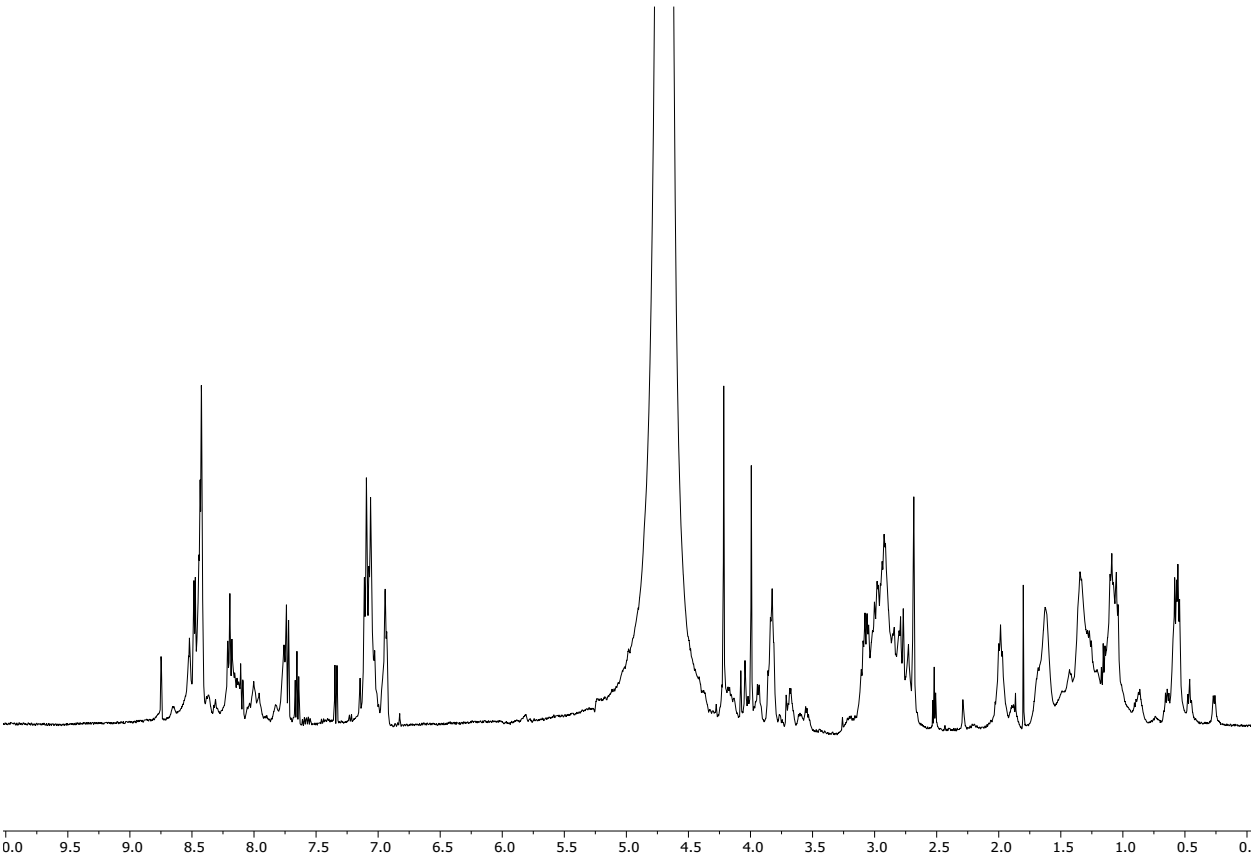


Figure S4. ^{1}H NMR spectrum of β-pacman. Sample dissolved in 10% D_2O and 90% H_2O at 280 K.

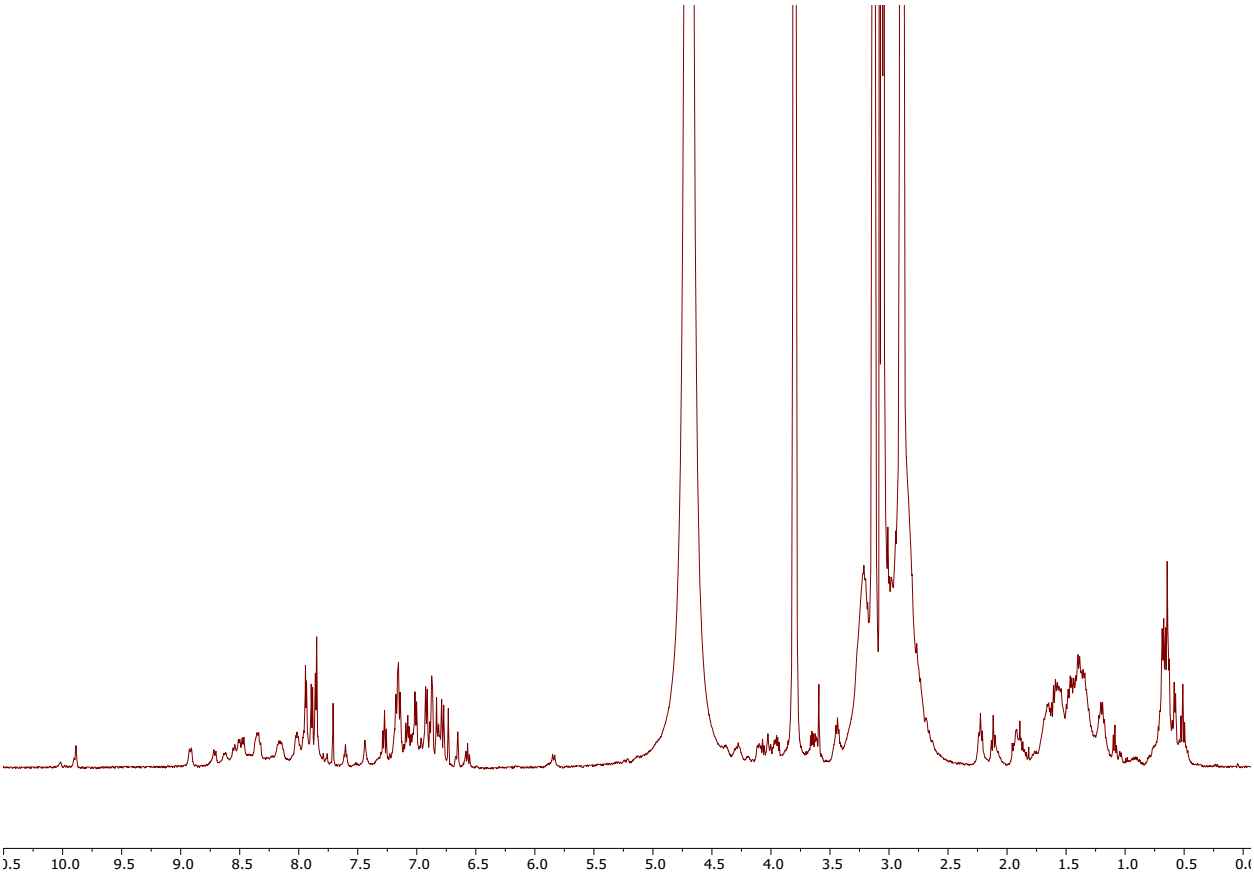


Figure S5. ^{1}H NMR spectrum of β-pacman. Sample dissolved in 0.1 M HEPES pH 7.0 with 10% D_2O and 90% H_2O at 280 K.

Diffusion NMR methods and processing

The diffusion NMR spectra were acquired using a stimulated echo with bipolar gradients and 3-9-19 water suppression element according to the Bruker pulse program (stebpgp1s19). Each 1D was recorded by linearly incrementing the PFG from 5-95% over 32 steps where the maximum applied gradient strength is 54.5 G/cm. The typical allowed diffusion time (big delta) Δ = d20 = 120 ms, δ for Eddy currents = 2 ms, 12 ppm sweep width, 32 steps, 16 scans per increment and 3 second recycle delay.[2] Each individual 1D DOSY spectrum, as a function of applied PFG strength, was processed in TOPSPIN and converted to text files using custom written AU programs within TOPSPIN. The text files were then used for manual integration routines written in C++ and fitted within GLOVE2 software according to eq S2. Jack-Knife / leave-one-out-method was used for the error analysis within GLOVE2. Fitted data were viewed with XMGRACE (XMGR) graphical plotter.[2] The diffusion data were fitted to the classic Stejskal and Tanner analytical equation for a stimulated-echo bipolar pulsed field gradient experiment (eq S2).[43]

$$I = I_0 \times e^{-D\gamma^2\delta^2 g^2\left(\Delta - \frac{\delta}{3}\right)} \qquad \text{(eq S2)}$$

where I = observed intensity
I_0 = reference intensity unattenuated
D = diffusion rate ($cm^2\ s^{-1}$)
g = applied Z-gradient strength ($G\ cm^{-1}$)
γ = gyromagnetic ratio of the observed nucleus
δ = gradient pulse duration (0.002 s)
Δ = Stejskal-Tanner diffusion delay (0.120 s)

The diffusion coefficient, D is roughly proportional to $M^{-\alpha}$, with α being a function of the molecular shape.[3] α is 1/3 for a perfect sphere and 1 for an infinite rod. Experimentally, it is shown that folded polypeptides have α ~ 0.39, close to the ideal value of 1/3, whereas unfolded chains have α ~ 0.58. Thus, $0.33 < \alpha < 0.58$ is reasonable for peptides. While **MC4H** has a higher aspect ratio than a perfect sphere, it is closer to a globular fold than a linear rod, and so we apply α = 1/3. Since spectroscopic data show all peptides in this work are folded, we assume that the mass ratios of peptides follow the relationship shown by eq S3. We can adjust eq. S3 to account for the mass differences between the reference and the sample to yield eq. S4.

$$\frac{D_1}{D_2} = \left(\frac{M_2}{M_1}\right)^{1/3} \qquad \text{(eq S3)}$$

where D_1 = diffusion rate of species 1 ($m^2\ s^{-1}$)
D_2 = diffusion rate of species 2 ($m^2\ s^{-1}$)
M_1 = molecular mass of species 1 ($g\ mol^{-1}$)
M_2 = molecular mass of species 2 ($g\ mol^{-1}$)

$$\langle O \rangle = \frac{\left(\frac{D_{ref}}{D_s}\right)^3 \times M_{ref}}{M_{mono}} \qquad \text{(eq S4)}$$

where D_{ref} = diffusion rate of the monomeric reference species ($m^2\ s^{-1}$)
D_s = diffusion rate of the sample species ($m^2\ s^{-1}$)

M_{ref} = molecular mass for a monomer of the reference species (g mol^{-1})
M_{mono} = expected mass for a monomer of the sample species (g mol^{-1})
$\langle O \rangle$ = average oligomeric state

Table S2. Oligomerization states of peptides determined by diffusion NMR spectroscopy

Sample	D (m^2/s • 10^{-10})	$\langle O \rangle$
trpzip2[4]	2.8	1.00
β-pacman	1.21	6.32

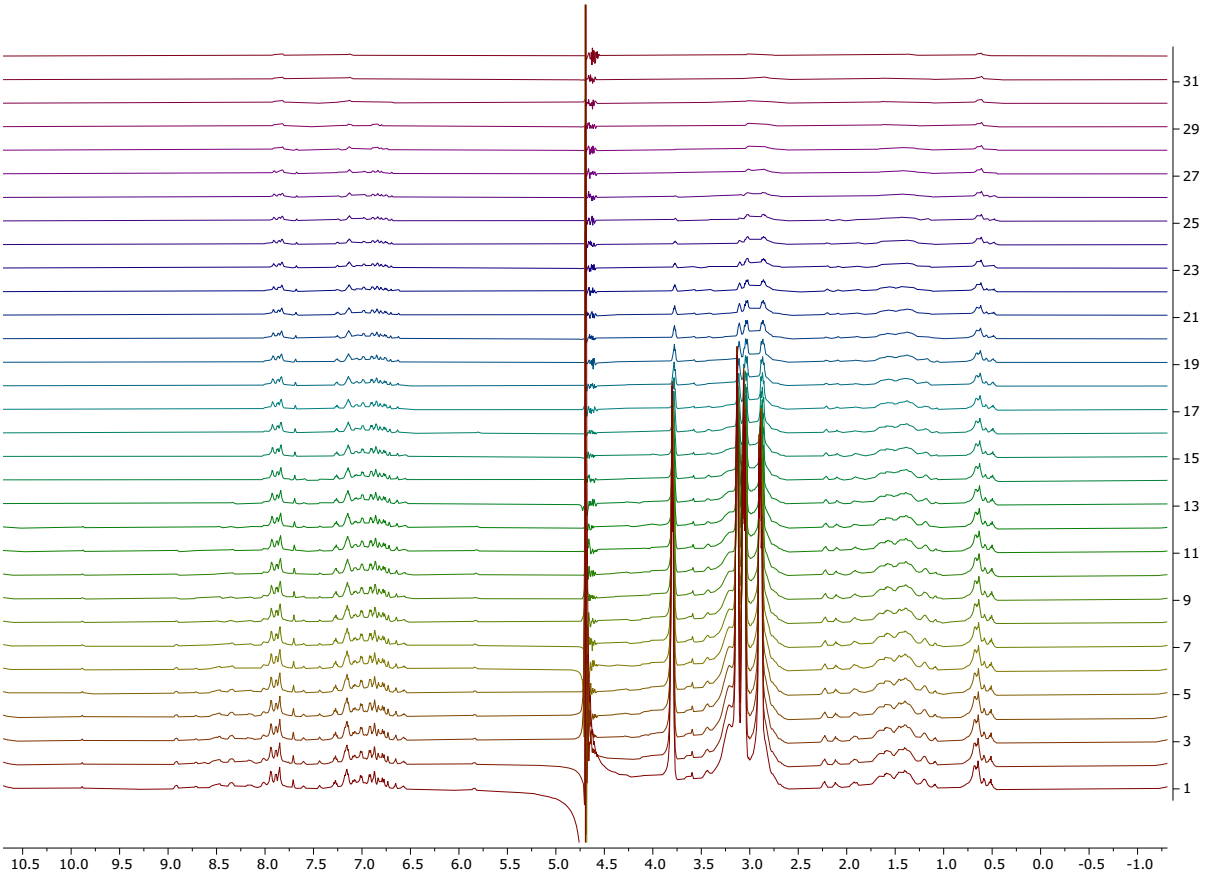


Figure S6. ^{1}H NMR spectra of β-pacman at different applied gradient strengths. Sample dissolved in 0.1 M HEPES pH 7.0 in 10% D_2O and 90% H_2O at 280 K.

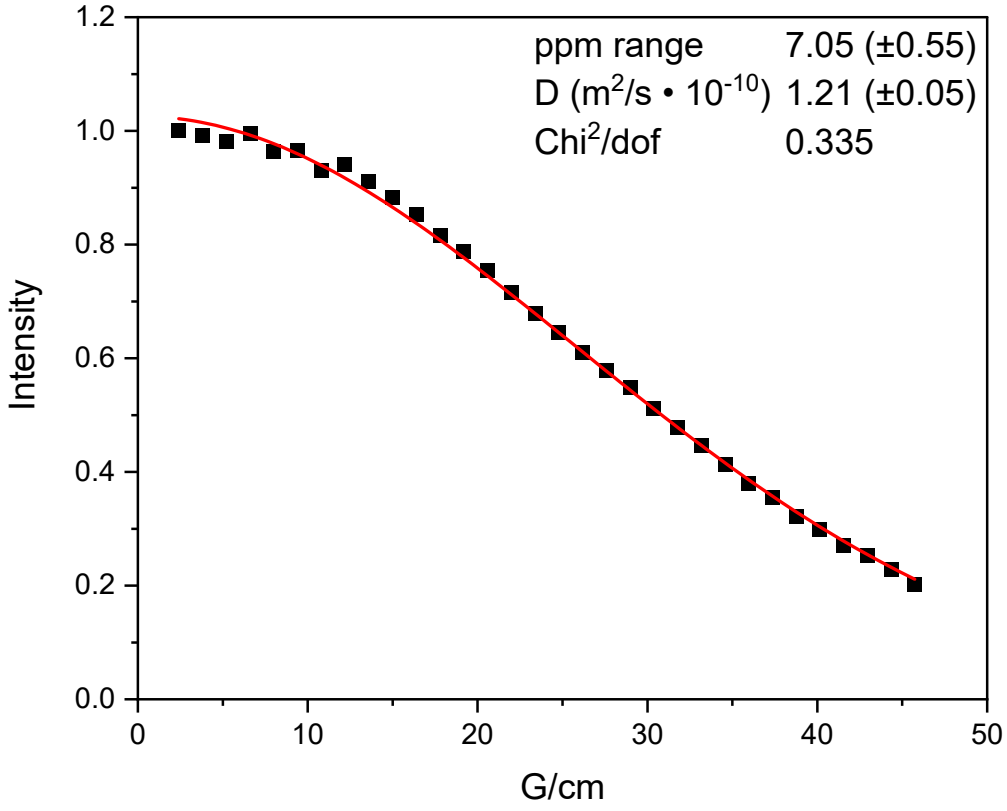


Figure S7. Fitting the integrated peaks for β-pacman from 6.50-7.60 ppm as a function of applied Z-gradient strength, using equation S2.

Circular dichroism spectroscopy

Spectra were collected on a Jasco J-1500 Circular Dichroism Spectrophotometer in a 1.0 mm pathlength quartz cuvette. The ellipticity of each sample was measured from 210–350 nm wavelength, with 5 acquisitions and at a scanning speed of 100 nm/min. The ellipticity (θ / mdeg) was converted to mean molar ellipticity (Φ / mdeg M^{-1} cm^{-1}) using the equation reported by Kelly et al (eq S6).[5]

$$\Phi = \frac{\theta \cdot M}{10 \cdot n \cdot l \cdot C} \qquad \text{(eq S6)}$$

θ is the ellipticity in mdeg, M is the molecular mass of the sample, n is the number of amide bonds in the structural formula, l is the optical path length in cm, and C is the concentration in mg/mL.

Titration of $CuCl_2$ into β-pacman. A 350 µL solution of 222 µM β-pacman in 10 mM HEPES pH 7.0 was prepared and loaded into a 1.0 mm quartz cuvette at 298 K. A 10 mM solution of $CuCl_2$ in 10 mM HEPES pH 7.0 was titrated into the β-pacman solution, 7.76 µL (1.0 eq) at a time.

Variable temperature CD.

A 350 µL solution of 222 µM β-pacman in 10 mM HEPES pH 7.0 was prepared and loaded into a 1.0 mm quartz cuvette. Data were collected at each 5-degree interval starting from 5 °C and ending at 90 °C. Temperatures were held for 20 s at each measured interval to allow for equilibration prior to each data collection. Using OriginPro, data were fit to a model for a two-state transition between a folded and unfolded state.[6]

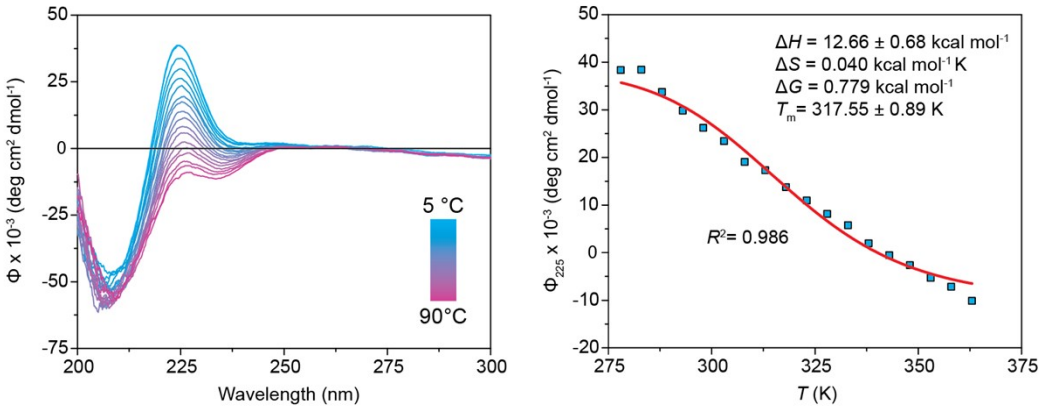


Figure S8. Variable temperature CD spectra and fitting.

UV–visible absorption spectroscopy
Solution-state samples were measured in quartz cuvettes with 1 cm pathlength. Data were collected on a Varian Cary 17.

Titration of β-pacman into $CuCl_2$. A 3.00 mL solution of 200 μM $CuCl_2$ in 100 mM HEPES pH 7.0 was prepared and loaded into a 1.0 cm quartz cuvette at 298 K. A 2.22 mM β-pacman solution in 100 mM HEPES pH 7.0 was titrated into the $CuCl_2$ solution, 27.1 μL (0.1 eq) at a time.

Isothermal Titration Calorimetry
Data were collected on a Malvern PEAQ-ITC at 25 °C. Analyte volume was 200 μL at a concentration of 0.10 mM. Titrants were prepared at 2.0 mM concentration. All samples were dissolved in 0.1 M HEPES buffer solution at pH 7.0. All peptide samples were prepared no more than 5 min before the experiment. $CuCl_2$ solution was prepared no more than 1 h before the experiment. Data fitting to a sequential 2-site binding model was performed using Malvern MicroCal software (Figure S9 and Table S3). Forward and reverse titrations, in other words, Cu into peptide and peptide into Cu, respectively, were performed to verify the stoichiometry, *n*.

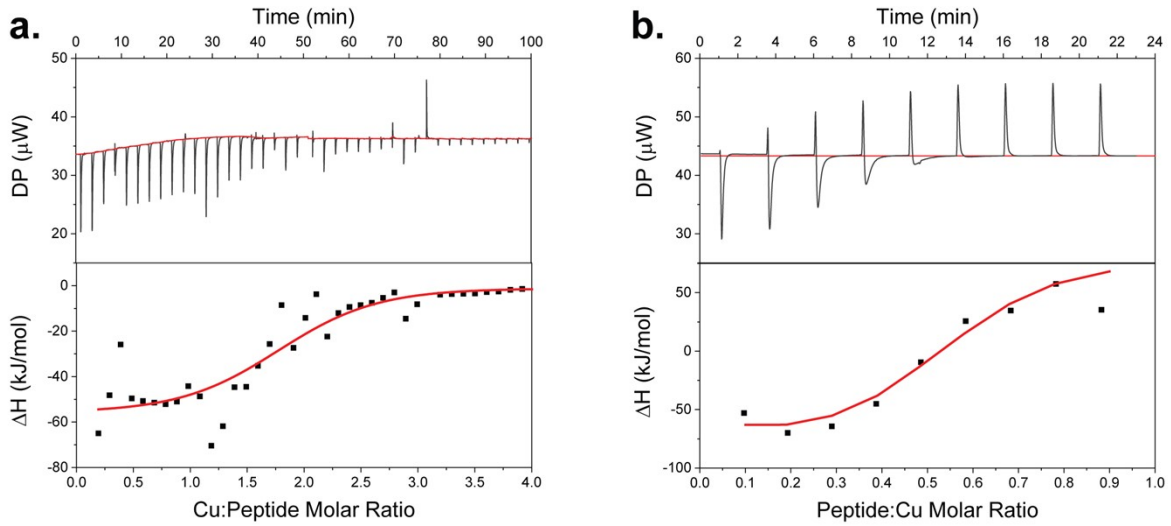


Figure S9. ITC measurements. (a) 0.1 mM β-pacman was titrated with 2.0 mM $CuCl_2$ and fitted with a sequential binding model (forward titration). (b) 0.1 mM $CuCl_2$ was titrated with 2.0 mM β-pacman and fitted with a sequential binding model (reverse titration).

Table S3. Thermodynamic parameters of Cu(II) binding to β-pacman obtained from ITC

Experiment	*n* (sites)	ΔG_1 (kJ/mol)	ΔH_1 (kJ/mol)	ΔS_1 (kJ/mol/K)	ΔG_2 (kJ/mol)	ΔH_2 (kJ/mol)	ΔS_2 (kJ/mol/K)
Cu into peptide	2.08	-35.0	-178	-0.47	-42.3	-20.1	0.074
Peptide into Cu	0.417	-35.6	-119	-0.28	-35.5	-15.1	0.068
Average		-35.3±0.4	-148.5±42	-0.375±0.1	-38.9±4.8	-17.6±3.5	0.071±0.004

Electron Paramagnetic Resonance Spectroscopy

EPR measurements were performed using a Bruker EMX X-band EPR equipped with a cold finger filled with liquid N_2 (77 K). Cu_2-β-pacman was prepared as a 1.07 mM solution in 0.1 M HEPES pH 7.0 buffer and 50% v/v glycerol was added and mixed thoroughly. The samples were then frozen by submersion in liquid N_2 until it formed a glassy solid. X-band EPR spectra were simulated using the program QPOWA originally from Belford and co-workers as modified by Telser.[7–10]

Cyclic Voltammetry

Electrochemical measurements were recorded using a Pine WaveDriver 200 potentiostat and Aftermath software. Protein-film voltammetry protocol was based on a procedure by Elliott and co-workers using an edge plane pyrolytic graphite electrode (PGE) (A=0.071 cm^2, BASi).[11] Between each use, the pyrolytic electrode was sonicated in water to remove any previous film and polished using 0.05 μm alumina solution, first against a Nylon polishing pad and then a sandpaper pad (1000 grit). The electrode was rinsed with ultrapure water and allowed to dry. A 2 mg/mL solution of multiwalled carbon nanotubes (MWCNT) in DMF was sonicated for 15 minutes and 15 μL of the solution was deposited on the PGE and allowed to dry overnight. Once dry, the electrode was rinsed with water to remove excess MWCNT and DMF. Then, 10 μL of the 15 mg/mL solution of Cu_2-β-pacman in 0.1 M HEPES was drop-casted on the MWCNT-modified PGE and allowed to air dry. The electrode was briefly rinsed with water and placed into a 3-electrode cell with a saturated Ag/AgCl reference electrode and a platinum counter electrode. A 0.1 M pH 7.0 HEPES buffer was used with 0.1 M Na_2SO_4 as a supporting electrolyte. All scans recorded were taken at 50 mV/s starting at open circuit potential. For scans under N_2, the solution was sparged with N_2 for 10 min before measurement. For scans under O_2, the solution was sparged with pure O_2 for 10 min before measurement.

Crystallization

Initial screening for crystallization conditions were performed using 96-well high throughput crystal screen kits purchased from Hampton Research and Molecular Dimensions (Table S4). Optimization of crystals were performed in 24-well plates using the hanging drop method. The condition that formed high-quality crystals is listed in Table S5.

Sitting drop (96-well)

High-throughput protein crystallization was carried out by a Tecan Freedom Evo 200. Crystallization plates contain 96 wells, with 3 sitting drop compartments per well. Sample of Cu_2-β-pacman was prepared as a 10 mg/mL in 0.1 M HEPES pH 7.0. 1 µL of sample and 1 µL crystallization were added to each compartment. Assembly of the crystallization plates was temperature controlled, and all completed plates were automatically sealed with an optically clear adhesive. Each screening kit contains 96 unique conditions in 96 deep-well blocks. High-throughput protein crystallization was performed for each peptide using kits shown on Table S4.

Table S4. High-throughput protein crystallization screening kits.

Manufacturer	Kit	Peptide concentration(s)
Hampton Research	Crystal Screen HT	10 mg/mL
Hampton Research	PEGRx Screen	10 mg/mL
Hampton Research	PEG/Ion Screen	10 mg/mL
Hampton Research	SaltRx Screen	10 mg/mL
Molecular Dimensions	MCSG-1 Screen	10 mg/mL
Molecular Dimensions	MCSG-2 Screen	10 mg/mL
Molecular Dimensions	MCSG-3 Screen	10 mg/mL
Molecular Dimensions	MCSG-4 Screen	10 mg/mL

Table S5. Crystallization condition that produced single crystals.

Sample	Sample Concentration	Buffer Composition
Cu_2-β-pacman	10 mg/mL	6.0 M ammonium nitrate 0.1 M BIS-TRIS propane pH 7.0

X-ray crystallography

Data were collected at 100 K the by the Life Sciences Collaborative Access Team at the BioMax beamline at MAX IV. Diffraction images for each data set were collected using an Eiger 2 16M detector. Data were processed with *XDS*. Cu_2-β-pacman was experimentally phased (using *Phaser*[12]) by single-wavelength anomalous diffraction (SAD) at the adsorption edge for Cu (1.362460 Å). *Coot*[13] was used to build the model that were then refined with *Phenix*.[14]

Table S6. SC-XRD data and refinement statistics for crystal structures. Statistics for the highest resolution shell are shown in parentheses.

Sample name	**Cu_2-β-pacman**
PDB ID	9PPU
Space group	$P4_12_12$
Cell constants (a, b, c, α, β, γ)	41.32 Å, 41.32 Å, 53.49 Å, 90.00°, 90.00°, 90.00°
Data Collection	
Wavelength (Å)	1.362460
Resolution (Å)	32.70 – 1.49 (1.543 – 1.49)
Total Observations	148327 (3336)
Unique Observations	14061 (624)
Redundancy	18.9 (5.3)
Completeness (%)	97.98 (80.41)
I/σ	19.31 (3.06)
R_{meas}	0.1286 (0.4084)
R_{merge}	0.1256 (0.3702)
Refinement	
$CC_{1/2}$	0.998 (0.905)
R_{free} (%)	19.1 (0.2130)
R_{work} (%)	15.5 (0.2091)
Avg. B Factor (Å^2)	19.63
RMS Bonds (Å)	0.004
RMS Angles (°)	0.95

Table S7. Dihedral angles for Cu_2-β-pacman from SC-XRD

Chain	Residue	Phi (°)	Psi (°)
A	Leu1	-74.5	149.1
A	His2	-139.0	142.6
A	Ser3	-100.2	131.9
A	His4	-122.7	72.4
A	Lys6	-105.2	167.5
A	Phe7	-126.7	134.2
A	His8	-120.1	141.3
A	Leu9	-96.3	120.4
A	His10	-125.9	119.5
A	Gln12	-142.2	159.2
B	Phe1	-76.2	144.0
B	His2	-129.2	139.0
B	Ser3	-105.5	135.3
B	His4	-114.8	45.3
B	Lys6	-71.7	139.2
B	Ile7	-114.9	131.6
B	His8	-124.2	149.7
B	Trp9	-100.4	121.1
B	His10	-128.6	128.2
B	Gln12	-163.8	171.1

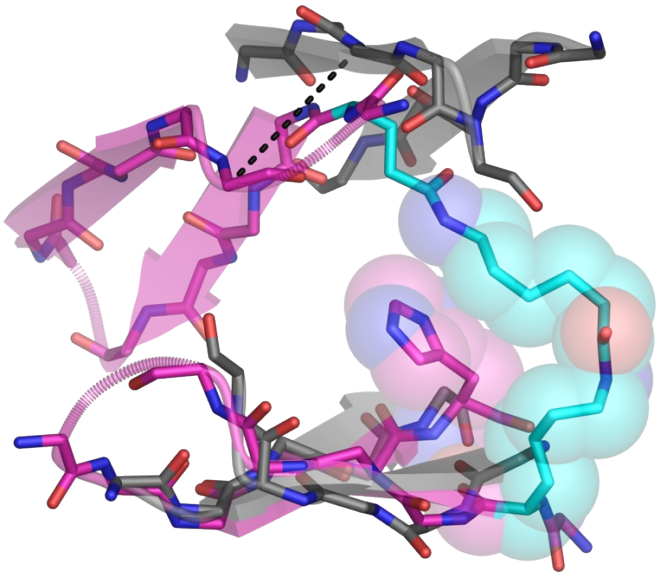


Figure S10. Folding of the aminohexanoic linker onto ring B/His4 (highlighted with spheres representation). The displacement of the design relative to laccase is shown with dashed black lines.

III. References

(1) Hwang, T. L.; Shaka, A. J. Water Suppression That Works. Excitation Sculpting Using Arbitrary Wave-Forms and Pulsed-Field Gradients. *Journal of Magnetic Resonance, Series A* **1995**, *112* (2), 275–279. https://doi.org/10.1006/jmra.1995.1047.

(2) Sugase, K.; Konuma, T.; Lansing, J. C.; Wright, P. E. Fast and Accurate Fitting of Relaxation Dispersion Data Using the Flexible Software Package GLOVE. *J Biomol NMR* **2013**, *56* (3), 275–283. https://doi.org/10.1007/s10858-013-9747-5.

(3) Augé, S.; Schmit, P.-O.; Crutchfield, C. A.; Islam, M. T.; Harris, D. J.; Durand, E.; Clemancey, M.; Quoineaud, A.-A.; Lancelin, J.-M.; Prigent, Y.; Taulelle, F.; Delsuc, M.-A. NMR Measure of Translational Diffusion and Fractal Dimension. Application to Molecular Mass Measurement. *J. Phys. Chem. B* **2009**, *113* (7), 1914–1918. https://doi.org/10.1021/jp8094424.

(4) Thuc Dang, V.; Engineer, A.; McElheny, D.; Drena, A.; Telser, J.; Tomczak, K.; Nguyen, A. I. Crystallography Reveals Metal-Triggered Restructuring of β-Hairpins. *Chemistry – A European Journal n/a* (n/a), e202402101. https://doi.org/10.1002/chem.202402101.

(5) Kelly, S. M.; Price, N. C. The Use of Circular Dichroism in the Investigation of Protein Structure and Function. *Curr Protein Pept Sci* **2000**, *1* (4), 349–384. https://doi.org/10.2174/1389203003381315.

(6) Greenfield, N. J. Using Circular Dichroism Collected as a Function of Temperature to Determine the Thermodynamics of Protein Unfolding and Binding Interactions. *Nat Protoc* **2006**, *1* (6), 2527–2535. https://doi.org/10.1038/nprot.2006.204.

(7) 21st Rocky Mountain Conference on Analytical Chemistry. In *21st Rocky Mountain Conference on Analytical Chemistry*; Rocky Mountain Conference, Ed.; Rocky Mountain Conference, 1979. https://doi.org/10.56902/RMCMR.1979.21.1.

(8) Belford, R. L.; Belford, G. G. Eigenfield Expansion Technique for Efficient Computation of Field-Swept Fixed-Frequency Spectra from Relaxation Master Equations. *The Journal of Chemical Physics* **1973**, *59* (2), 853–854. https://doi.org/10.1063/1.1680104.

(9) Duliba, E. P.; Hurst, G. C.; Belford, R. L. Nuclear Quadrupole Coupling in Octahedral Copper(II). EPR of Copper-Doped Magnesium Sulfate Heptahydrate-D2 (MgSO4.7D2O): A Test of Predictions. *Inorg. Chem.* **1982**, *21* (2), 577–581. https://doi.org/10.1021/ic00132a022.

(10) Telser, J. A Perspective on Applications of Ligand-Field Analysis: Inspiration from Electron Paramagnetic Resonance Spectroscopy of Coordination Complexes of Transition Metal Ions. *J. Braz. Chem. Soc.* **2006**, *17* (8), 1501–1515. https://doi.org/10.1590/S0103-50532006000800005.

(11) Walker, L. M.; Li, B.; Niks, D.; Hille, R.; Elliott, S. J. Deconvolution of Reduction Potentials of Formate Dehydrogenase from Cupriavidus Necator. *J Biol Inorg Chem* **2019**, *24* (6), 889–898. https://doi.org/10.1007/s00775-019-01701-1.

(12) McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. Phaser Crystallographic Software. *J Appl Cryst* **2007**, *40* (4), 658–674. https://doi.org/10.1107/S0021889807021206.

(13) Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K. Features and Development of Coot. *Acta Cryst D* **2010**, *66* (4), 486–501. https://doi.org/10.1107/S0907444910007493.

(14) Liebschner, D.; Afonine, P. V.; Baker, M. L.; Bunkóczi, G.; Chen, V. B.; Croll, T. I.; Hintze, B.; Hung, L.-W.; Jain, S.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R. D.; Poon, B. K.; Prisant, M. G.; Read, R. J.; Richardson, J. S.; Richardson, D. C.; Sammito, M. D.; Sobolev, O. V.; Stockwell, D. H.; Terwilliger, T. C.; Urzhumtsev, A. G.; Videau, L. L.; Williams, C. J.; Adams, P. D. Macromolecular Structure Determination Using X-Rays, Neutrons and Electrons: Recent Developments in Phenix. *Acta Cryst D* **2019**, *75* (10), 861–877. https://doi.org/10.1107/S2059798319011471.