Supporting Information for

Discovery and Characterization of a Disulfide-Locked C_2-Symmetric Defensin Peptide

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This supporting information includes:

Abbreviations

AUC – Analytical ultracentrifugation

CD – Circular dichroism

CFU – Colony forming unit

EDT – 1,2-Ethanedithiol

DIPEA – *N,N*-Diisopropylethylamine

DTDP – 2,2′-dithiodipyridine

HD5 – Human α-defensin 5

$HD5_{red}$ – Reduced human α-defensin 5

$HD5_{ox}$ – Oxidized human α-defensin 5

HD5-CD – Human α-defensin 5 covalent dimer

HSQC – Heteronuclear single quantum coherence

GuHCl – Guanidine hydrochloride

IPTG – Isopropyl-β-D-thiogalactopyranoside

LB – Luria Broth

NOESY – Nuclear Overhauser effect spectroscopy

OD – Optical density

SDS – Sodium dodecyl sulfate

SE – Sedimentation equilibrium

SV – Sedimentation velocity

TCEP – *Tris*(2-carboxyethyl)phosphine

TFA – Trifluoroacetic acid

TIS – Triisopropylsilane

TOCSY – Total correlation spectroscopy

TRACT – TROSY for rotational correlation times

TROSY – Transverse relaxation optimized spectroscopy

TSB – Trypticase soy broth

YPD – Yeast extract peptone dextrose

Experimental Section

Materials and General Methods. All solvents, reagents, and chemicals were purchased from commercial suppliers and used as received unless noted otherwise. Deuterated water (D_2O), [*U*-^{15}N]-ammonium chloride, and [*U*-^{13}C]-glucose were purchased from Cambridge Isotopes (Cambridge, MA). All aqueous solutions, buffers, and NMR samples were prepared with Milli-Q water (18.2 MΩcm^{-1}) that was passed through a 0.22-μm filter before use. HD5 was either overexpressed[S1] as a His_6-fusion peptide in *E. coli* BL21(DE3) or synthesized by Fmoc solid-phase peptide synthesis as described below. The His_6-tag was cleaved and the reduced ($HD5_{red}$) and oxidized ($HD5_{ox}$) forms of the 32-residue native peptide were obtained and purified as previously described (Table S4).[S1] Formation of HD5-CD is independent of the source (i.e. bacterial overexpression or synthetic) of peptide. ^{15}N-$HD5_{red}$ and ^{13}C,^{15}N-$HD5_{red}$ were obtained following overexpression of the His_6-fusion peptides in defined minimal media as previously described and the oxidized forms were obtained by oxidative folding as previously described.[S2]

General Instrumentation. Analytical and semi-preparative high-performance liquid chromatography (HPLC) were performed on an Agilent 1200 instrument equipped with a thermostated autosampler set at 4 °C and thermostated column compartment set at 20 °C, and a multi-wavelength detector set at 220 and 280 nm (500 nm reference wavelength). Preparative HPLC was performed using an Agilent PrepStar 218 instrument outfitted with an Agilent ProStar 325 dual-wavelength UV-Vis detector set at 220 and 280 nm. A Clipeus C18 column (5-μm pore, 4.6 x 250 mm, Higgins Analytical, Inc.) set at a flow rate of 1 mL/min was employed for all analytical HPLC experiments. A ZORBAX C18 column (5-μm pore, 9.4 x 250 mm, Agilent Technologies, Inc.) set at a flow rate of 5 mL/min was employed for all semi-preparative-scale HPLC purifications. A Luna 100 Å C18 LC column (10-μm pore, 21.2 x 250 mm, Phenomenex) operated at 10 mL/min was utilized for all preparative-scale HPLC purification. HPLC-grade acetonitrile (MeCN) and HPLC-grade trifluoroacetic acid (TFA) were purchased from either EMD or Alfa Aesar. For all HPLC separations, solvent A was 0.1% TFA/H_2O and solvent B was 0.1% TFA/MeCN. These solvents were passed through a 0.22-μm filter prior to use. High-resolution

mass spectrometry was performed with an Agilent LC/MS system comprised of an Agilent 1260 series LC system outfitted with an Agilent Poroshell 120 EC-C18 column (2.7-μm pore size) and an Agilent 6230 TOF system housing an Agilent Jetstream ESI source. LC/MS-grade MeCN containing 0.1% formic acid and LC/MS-grade water containing 0.1% formic acid were obtained from Fluka. For all LC/MS analyses, solvent A was 0.1% formic acid/H_2O and solvent B was 0.1% formic acid/MeCN. The samples were analyzed by using a gradient of 5–95% B over 5 min with a flow rate of 0.4 mL/min. The MS profiles were analyzed and deconvoluted by using Agilent Technologies Quantitative Analysis 2009 software version B.03.02. A BioTek Synergy HT plate reader outfitted with a calibrated BioTek Take3 Multi-Volume Plate was employed for determining peptide concentration. The peptide stock solution concentrations were regularly quantified by using the calculated extinction coefficients for the peptides (Table S1). Solution and buffer pH values were verified by using either a Mettler Toledo S20 SevenEasy pH meter or a HANNA Instruments HI 9124 pH meter equipped with a microelectrode. All anaerobic experiments were performed using Ar-purged buffers and under a N_2 atmosphere in a glovebox (Vacuum Atmospheres, Co.).

Materials and General Methods for the Fmoc Solid-Phase Peptide Synthesis of $HD5_{red}$. All Fmoc-protected amino acids used for solid-phase peptide synthesis were obtained from Aapptec. Fmoc-Cys(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Tyr(tBu)-OH, along with the psuedoproline dipeptides[S3] Fmoc-Ala-Thr($^{\Psi Me,Me}$pro)-OH, Fmoc-Ile-Ser($^{\Psi Me,Me}$pro)-OH and Fmoc-Leu-Ser($^{\Psi Me,Me}$pro)-OH, were employed. Fmoc-Arg(Pbf)-NovaSyn®TGA resin (0.20 mmol/g) was purchased from EMD Chemicals. All coupling reagents were obtained from AK Scientific, Inc. Piperidine and trifluoroacetic acid (TFA) were obtained from Alfa Aesar. Ethanedithiol (EDT), triisopropylsilane (TIS) and *N,N*-diisopropylethylamine (DIPEA) were purchased from Sigma Aldrich. A custom-made 25-mL glass reaction vessel outfitted with a medium porosity frit and a "T"-bore for N_2 gas bubbling was purchased from Chemglass Life Sciences and employed for all syntheses.

Fmoc Solid-Phase Peptide Synthesis of $HD5_{red}$. The solid-phase synthesis of HD5 was performed by employing procedure described for HD5[$Ser^{5,20}$][S1] with modifications to improve efficiency and the $HD5_{red}$ yield as detailed below. Fmoc-deprotection was performed with 20% piperidine in DMF solution (3 x 5 min). All amino acids were coupled to the resin using Fmoc-amino acid (10 equiv) activated with HATU (10 equiv), HOAt (10 equiv), and DIPEA (20 equiv) except for Fmoc-Cys(Trt)-OH (4 equiv) and pseudoprolines (4 equiv). Each coupling reaction was agitated for 10-15 min at room temperature with N_2 bubbling. For coupling cysteine, Fmoc-Cys(Trt)-OH (4 equiv) was dissolved in a CH_2Cl_2/DMF (1:1) mixture containing HATU (4 equiv), HOAt (4 equiv) and TMP (4 equiv) as a mild base instead of DIPEA to minimize racemization.[S4] Each amino acid was coupled at least twice. Residues identified as difficult to couple during trial syntheses were the Arg residues as well as Ser_{15}, Glu_{21}, Cys_{30} and Cys_{31}. The major side products in initial syntheses were identified to be truncation peptides (ISGRLYRLCR, LSGVCEISGRLYRLCR) with a cysteine deletion at the C-terminus (Table S3). After each coupling step, thorough washing of the resin with CH_2Cl_2 was performed (3 x 3 min). The capping of unreacted N-termini by 1-acetylimidazole was omitted. After coupling the last amino acid, the N-terminal Fmoc-group was removed and the resin was thoroughly washed with CH_2Cl_2 (3 x 3 min). After drying the resin *in vacuo*, 20 mL of cold cleavage mixture, TFA/H_2O/TIS/EDT (94:2.5:1:2.5), was added and reaction was agitated with N_2 bubbling for 3 h, and the cleavage mixture was drained and the filtrate collected. To the resin, another 15 mL of fresh cleavage mixture was added and reaction was agitated for 30 min. The filtrates were combined and concentrated under N_2 to a final volume of 5 mL. The crude peptide was precipitated from the cleavage mixture using 30 mL of pre-cooled diethyl ether (−20 °C). The resulting mixture was centrifuged (3500 rpm x 4 °C for 20 min) and the supernatant was removed. The pellet was then dissolved in 0.1 M acetic acid and acetonitrile mixture until fully dissolved and lyophilized to obtain the crude peptide. The synthesis on a 0.0345-mmol scale, following cleavage and global deprotection, afforded 80 mg of crude peptide (64% yield). The crude peptide was reduced with TCEP and purified by preparative HPLC (10–60% B over 30

min, 10 mL/min) to obtain 19 mg (15% overall yield) of $HD5_{red}$. Representative HPLC traces are provided in Figure S1.

Analytical-Scale Folding Assays in the Presence of GSH/GSSG. $HD5_{red}$ (25 μM) or $HD5_{ox}$ (25 μM) was incubated in buffers with defined redox potentials prepared by using reduced glutathione (GSH) and glutathione disulfide (GSSG) (75 mM HEPES, pH 7.0, anaerobic). The redox potentials of the buffers spanned the –275 to –235 mV range. For analytical assays, each 100-μL solution was prepared in the glove box and incubated at room temperature (ca. 22 °C) in the glove box for 36 h. At this time point, a 10-μL aliquot of 6% aqueous TFA was added to each sample. The acidified samples were removed from the glove box, centrifuged (13,000 rpm x 10 min, 4 °C), and analyzed by HPLC (10–60% B over 30 min).

Preparative-Scale Folding of HD5-CD in the Presence of GSH/GSSG. HD5-CD was routinely prepared on multi-milligram scales by incubating $HD5_{red}$ (50 μM) or $HD5_{ox}$ (50 μM) at pH 7.4 in 75 mM HEPES buffer (Chelex-treated) containing 12 mM GSH and 0.48 mM (−316 mV) or 0.12 mM (−334 mV) GSSG, respectively, with gentle rocking at room temperature (ca. 22 °C) under anaerobic conditions for 48 h. At this time, the solutions were acidified by addition of 5% aqueous TFA (1:10 v/v), removed from the glovebox, centrifuged (13,000 rpm x 10 min, 4 °C), and purified by preparative HPLC (10–45% B in 25 min). These conditions provide HD5-CD and $HD5_{ox}$ as a 3:1 or 5:1 mixture starting from either $HD5_{red}$ or $HD5_{ox}$, respectively.

Analytical-Scale Oxidative Folding Assays in the Presence of Additives. To determine the effect of cations on HD5-CD formation, analytical-scale folding assays were performed under anaerobic conditions at pH 7.4 in 75 mM HEPES buffer (Chelex-treated) containing 12 mM GSH and 0.48 mM (for $HD5_{red}$) or 0.12 mM (for $HD5_{ox}$) GSSG. Each additive was introduced at the start of the assay and the resulting mixtures were analyzed by HPLC after 48 h incubation at room temperature (*vide supra*). Samples containing either $HD5_{red}$ (25 μM) or $HD5_{ox}$ (25 μM) were prepared with the following concentrations of additive: NaCl, 0, 10, 50, and 100 mM; $CaCl_2$, 0, 1, 5, and 10 mM; $MgCl_2$, 0, 1, 5, and 10 mM. To study the effect of a denaturant on HD5-CD formation, solutions of $HD5_{red}$ (25 μM) or $HD5_{ox}$ (25 μM) were prepared

with buffers containing 0, 0.25, 0.50, 1.0, and 2.0 mM GuHCl. To determine the effect of HD5 concentration on HD5-CD formation, the same experiment was performed but the concentrations of $HD5_{ox}$ and $HD5_{red}$ were varied (5, 10, 25, 50, and 100 μM) and no additives were included.

Thiol Quantification Assays. Quantification of free thiol residues was performed by using 2,2′-dithiodipyridine (DTDP) and a GSH standard curve as described previously.[S1] The final peptide concentrations were 2–4 μM for $HD5_{red}$ or 6–7 μM for $HD5_{ox}$ and HD5-CD.

Circular Dichroism Spectroscopy. Peptides were dissolved in 10 mM sodium phosphate buffer, pH 7.0 (20 μM, 280 μL) and placed in a 1-mm path-length quartz CD cell (Hellma). The CD spectra were collected at 25 °C scanning a 260–190 nm range at 1 nm intervals (5 s averaging time, three independent scans per wavelength). The data from the three scans were averaged and the buffer reference was subtracted. For experiments performed in the presence of 10 mM SDS, the peptide solution was prepared as above, a 2.8 μL aliquot of 1 M SDS was added, and the solutions were incubated for 1 h at room temperature (ca. 22 °C) prior to data acquisition.

Protease Susceptibility Assays. To determine the relative susceptibility of HD5-CD, $HD5_{ox}$, and $HD5_{red}$ to proteolysis by trypsin (Affymetrix), α-chymotrypsin (Worthington), and proteinase K (Worthington), 150-μL solutions of peptide (80 μM) were prepared at pH 8.0 (100 mM Tris-HCl, 20 mM $CaCl_2$). A 25-μL aliquot was removed for the no enzyme control. The assays were initiated by addition of protease (1 mg/mL) to afford final enzyme concentrations of 25 or μg/mL or 10 μg/mL. The reactions were gently mixed with a pipet, and incubated at room temperature. A 25-μL aliquot of each reaction was removed at t = 2, 5, 10, 30, and 60 min and quenched by addition of 5% TFA (10 μL). The samples were vortexed immediately, stored on ice, centrifuged (13,000 rpm x 10 min, 4 °C) to remove any precipitate, and analyzed by analytical HPLC (10–60% B over 30 min, 1 mL/min). For double-digest assays, 100-μL solutions of HD5-CD and $HD5_{ox}$ were prepared (140 μM and 280 μM, respectively; 1 mg/mL) at pH 6.5 (100 mM Tris-HCl, 1 mM $CaCl_2$), and α-chymotrypsin and trypsin were added from 1 mg/mL

stock solutions to achieve 10 or 25 μg/mL of each protease.[S5] The double digests were incubated at 37 °C for 60 h, quenched by addition of 5% TFA (aqueous), and prepared for analytical HPLC as described above.

Peptide Reduction Assays. The reduction of HD5-CD and $HD5_{ox}$ to $HD5_{red}$ by TCEP was examined at pH 8.2 (75 mM HEPES). The solutions (160 μL) contained 20 μM peptide and 0-150 mM TCEP. The solution pH was monitored after each TCEP addition and adjusted to 8.2 by drop-wise addition of 0.4 M $NaHCO_3$. Following a 10-min incubation at room temperature, the solutions were acidified by addition of 10 μL of 5% TFA (aq), centrifuged (13 000 rpm x 10 min, 4 °C), and analyzed by HPLC (10–60% B over 30 min, 1 mL/min).

Midpoint Potential Determination. The midpoint potential (E_m) of HD5-CD was determined by following an established procedure.[S6] In brief, buffers with known reduction potentials spanning the −260 to −360 mV range were prepared at pH 7.0 (75 mM HEPES) by using the reduced (DTT_{red}) and oxidized (DTT_{ox}) forms of dithiothreitol (DTT). HD5-CD (25 μM, 100 μL) was incubated in these buffers with gentle rocking for 48 h, and the resulting mixtures were acidified by addition of 5% aqueous TFA (10 μL) and analyzed by HPLC (10–60% B over 30 min, 1 mL/min; HD5-CD, 12.9 min retention time; $HD5_{red}$, 19.9 min retention time). The integrated HPLC peak areas were used to determine %HD5-CD at each redox potential and the reported E_m value is the potential where the solution contained 50% HD5-CD as determined from a plot of %HD5-CD versus redox potential. The same experiment was performed over a 72 h period and the same product ratios were obtained.

Sedimentation Velocity Experiments. SV experiments were performed with a Beckman XL-I Analytical Ultracentrifuge using an An-50 Ti rotor as previously described.[S2] The rotor housed sample cells with quartz (absorption optics at 280 nm) windows and conventional double-sector charcoal-filled epon centerpieces. The samples were centrifuged at 42,000 rpm and 20 °C for 14 h. SEDNTERP[S7] was employed to calculate the buffer viscosity (η), buffer density (ρ), and protein partial specific volume ($\bar{v}$) values at 20 °C based on database values (http://www.jphilo.mailway.com). Sedimentation coefficients were obtained by fitting the

sedimentation velocity data with the program DCDT+.[S8,S9] The apparent sedimentation coefficient distribution, $g(s^*)$, was generated from 20-26 scans with a peak broadening limit of 40 kDa (Tables S5,S6).

All SV window assemblies were loaded with 410 μL of buffer reference and 400 μL of peptide sample. Starting from a lyophilized peptide, a concentrated stock solution of each peptide was prepared from a solution of 10 mM sodium phosphate (pH adjusted to 2.0 – 8.0) that was filtered through a 0.45-μm membrane. Aliquots of the peptide stock solutions were diluted to 400 μL to provide the desired concentrations prior to loading the AUC sample cells. To determine the effect of peptide concentration on sedimentation, HD5-CD samples with concentrations of 40, 60, 90, 120 μM were prepared at pH 7.0 in 10 mM sodium phosphate buffer. To evaluate the consequences of pH on the sedimentation, samples of HD5-CD (20 μM) were prepared in 10 mM sodium phosphate adjusted to pH 2.0, 4.0, 6.0, 7.0, and 8.0.

To estimate the sedimentation coefficients for HD5-CD, hydrodynamic modeling computations were performed with HYDROPRO[S10] using the NMR solution structure reported in this work (PDB: 2MIT), the NMR solution structure of the $HD5_{ox}$ monomer (PDB: 2LXZ),[S2] and the X-ray crystal structure of the non-covalent $HD5_{ox}$ dimer (PDB: 1ZMP).[S11] All HYDROPRO calculations used the buffer density (ρ) and buffer viscosity (η) values for water at 20 °C, and a partial specific volume ($\bar{v}$) of 0.7094 mL g^{-1} for $HD5_{ox}$ (Table S7).

Sedimentation Equilibrium Experiments. The Beckman XL-I Analytical Ultracentrifuge equipped with an An-50 Ti rotor was employed for all sedimentation equilibrium (SE) experiments. The absorption wavelength of 280 nm was used for optical detection at 20 °C. Samples (400 μL) of HD5-CD were prepared in 10 mM sodium phosphate buffer at pH 7.0 (28, 46, 62 and 92 μM) as described above. Equilibrium profiles were established at rotor speeds of 25,000, 36,000, and 42,000 rpm based on sedimentation coefficients of ~1.2 S obtained from the SV experiments.[S12] Upon confirmation of equilibrium establishment using WinMatch (http://www.biotech.uconn.edu/auf/?i=aufftp), 10 scans with 5 replicates were recorded.

SEDNTERP was employed to calculate the buffer viscosity (η), buffer density (ρ), and protein partial specific volume ($\bar{\nu}$) values at 20 °C. Initial SE data analysis was performed by linear regression fitting of $\ln(c_r)$ and r^2 using the Lamm equation (eq. S1)

$$\frac{\ln(c_r)}{r^2} = \frac{M(1-\bar{\nu}\rho)\omega^2}{2RT} \qquad \text{(eq. S1)}$$

which describes the movement of a particle in a centrifugal field where c_r is the concentration of the species at a certain radius (mg mL^{-1}), r is the distance from the center of the rotor (cm), M is molecular weight (kDa), ρ is the buffer density (g mL^{-1}), $\bar{\nu}$ is partial specific volume (mL g^{-1}), ω is the angular velocity (rad s^{-1}), R is the universal gas constant (8.314 $JK^{-1}mol^{-1}$), and T is the temperature (293 K).[S13] This data treatment provides a first approximation of self-association mode (mono- versus poly-dispersity) and molecular weight ($MW_{apparent}$).

Molecular weights were determined by global fitting of the multi-speed equilibrium data across all loading concentrations using the program SEDPHAT.[S14] The Species Analysis model and Single Species of an Interacting System model, both with mass conservation, were employed for data analysis with the bottom of the sample sector assigned as a floating parameter. To ensure the least squares curve-fitting procedure reached the global minimum, the alternate methods of Simplex, Marquardt-Levenberg, and simulated annealing were employed and the global reduced chi-square value was monitored. The critical chi-square values for each fitting process were obtained and Monte-Carlo analysis (95% confidence level) was also performed to provide the observed non-linear regression fits (Table S9).

Solution NMR Sample Preparation. Preliminary data acquisition was performed on a 625-μM sample of [*U*-^{15}N]-HD5-CD (^{15}N-HD5-CD) that was dissolved in 90:10 H_2O/D_2O and adjusted to pH 4.0 by addition of 5% TFA. The NMR sample was prepared immediately after the peptide was HPLC purified and lyophilized to dryness. A 540-μM sample of [*U*-^{13}C,^{15}N]-HD5-CD (^{13}C-^{15}N-HD5-CD) was prepared in the same manner for collection of relaxation, triple resonance, and X-edited NOESY experiments. For the acquisition of the 2D ^{13}C-edited/^{15}N,^{13}C-

filtered HSQC-NOESY experiment, an anaerobic solution containing a 1:1 molar ratio of unlabeled-$HD5_{red}$ and [*U*-^{13}C,^{15}N]-$HD5_{red}$ was prepared at pH 7.4 in 75 mM HEPES buffer (Chelex-treated) containing 12 mM GSH and 0.48 mM GSSG for preparative-scale oxidative folding (*vide supra*). Following HPLC purification (10–45% B over 25 min), lyophilization, and dissolution in 90:10 H_2O/D_2O adjusted to pH 4.0 with 5% TFA, a 638-μM sample of [^{12}C,^{14}N/*U*-^{13}C,^{15}N]-HD5-CD was obtained. The product contains a mixture of unlabeled, mix-labeled, and globally-labeled HD5-CD and the final peptide concentration corresponds to the total peptide concentration in the sample.

Solution NMR Spectroscopic Studies. ^{15}N-R_1, ^{15}N-R_2, and {^1H}-^{15}N NOE experiments were collected on a 900 MHz NMR spectrometer housed in the MIT Francis Bitter Magnet Laboratory (FBML). [^{15}N-^1H]-TRACT experiments were performed on a 500 MHz Bruker Avance spectrometer equipped with a cryogenic probe housed at Harvard Medical School. Sequence-specific assignments were derived from a suite of standard triple-resonance experiments (HNCO, HNCA, HNCACO, HNCACB, HCCONH, and CCONH) and an HCCH-TOCSY experiment collected using non-uniform sampling. The sampling schedule was created based on the Poisson Gap sampling method[S15] and missing data points were reconstructed by using the istHMS algorithm.[S16] The 3D HCCH-TOCSY and 2D X-edited NOESY experiments were collected with 40 ms and 200 ms mixing times, respectively. Spectral data were processed by using NMRPipe[S17] and analyzed in CARA[S18] and XEASY.[S19]

NMR Solution Structure Calculations and Refinement. A semi-automated approach was applied for chemical shift assignment and initial NOE assignment using the PINE-NMR server[S20] and the NOEASSIGN module of CYANA 3.0.[S21] Backbone dihedral constraints were generated using the TALOS+ module.[S22] The initial structure was refined manually to eliminate constraint violations. During the initial iterative structural calculations, explicit disulfide bonds were omitted and χ_1 angles were also excluded. X-PLOR was used for further refinement in which physical force field terms and explicit water solvent molecules were added to the experimental constraints. Table 2 lists the statistics for PROCHECK-NMR validation of the final

20 conformers.[S23] These coordinates are deposited in the Protein Data Bank (code: 2MIT). The UCSF Chimera[S24] package and PyMol[S25] were employed for final graphical presentation.

Covalent disulfide bonding restraints for Cys^{3}—Cys^{31}, Cys^{5}—$Cys^{20'}$, $Cys^{5'}$—Cys^{20}, and Cys^{10}—Cys^{30} were included in the final structure calculations. The Cys^{10}—Cys^{30} bonds were confirmed from the 2D X-edited NOESY spectra as was previously done for $HD5_{ox}$.[S2] Inter-cysteine NOEs from both the ^{13}C-edited/^{15}N,^{13}C-filtered HSQC-NOESY (Figure S12) and the 3D X-edited NOESY experiments confirmed the intermolecular disulfide bonding pattern for Cys^{5}—$Cys^{20'}$ and $Cys^{5'}$—Cys^{20}. In combination with NOEs from the 2D X-edited NOESY experiments, the observed $\chi 1$ angles aided assignment of the intramolecular Cys^{3}—Cys^{31} bonds and completed the full assignment of the disulfide array within HD5-CD.

Antimicrobial Activity Assays Using a Micro-Drop Colony Forming Unit Method. Frozen stocks (-80 °C) of bacterial strains were streaked onto TSB-dextrose agar plates and incubated overnight at 37 °C for all strains except *Bacillus cereus* which was grown at 30 °C. Overnight cultures from single colonies of bacteria (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213, *B. cereus* ATCC 14579, *Klebsiella pneumoniae* ATCC 13883, *Acinetobacter baumannii* ATCC 17961, *A. baumannii* ATCC 17978*, Listeria monocytogenes* ATCC 19115, *Pseudomonas aeruginosa* PAO1) and *Candida albicans* ATCC SC5314 were grown with shaking according to the conditions detailed in Table S2. Each overnight culture was prepared by inoculating 5 mL of media housed in a sterile 14-mL polypropylene culture tube (VWR) with the desired bacterial or fungal strain. Each overnight culture was diluted 1:100 in 6 mL of fresh media in sterile 14-mL polypropylene culture tubes, and grown to mid-log phase (OD_{600} 0.5–0.6) with shaking. Each culture was then centrifuged (3500 rpm x 10 min, 4 °C) to pellet the bacterial cells. The supernatant was discarded and the cell pellet was resuspended in 5 mL of 1.1x AMA buffer (11 mM sodium phosphate buffer supplemented with 1.1% TSB minus dextrose (Becton Dickinson), pH 7.4; this solution provides 10 mM sodium phosphate buffer supplemented with 1% TSB for the AMA). The cell suspension was centrifuged again (3500 rpm x 10 min, 4°C) and the supernatant

discarded. The pellet was resuspended in 4 mL of AMA buffer and further diluted with AMA buffer to obtain an OD_{600} of 0.5 for *E. coli*, *K. pneumoniae*, *L. monocytogenes*, *A. baumannii*, *P. aeruginosa, and C. albicans*, and an OD_{600} of 0.6 for *B. cereus* and *S. aureus*. The bacterial suspensions were further diluted in AMA buffer according to the AMA dilution factors listed in Table S2 and used immediately.

Antimicrobial activity assays were performed in sterile polystyrene 96-well plates (Costar). Each well contained 10 μL of 10x sterile-filtered peptide stock (i.e., $HD5_{ox}$ or HD5-CD prepared in Milli-Q water) or a no-peptide control. A 90-μL aliquot of the diluted bacterial culture was added to each well. The plate was covered and sealed with parafilm, and incubated for 1 h with shaking (37°C, 150 rpm for all strains except *B. cereus*, which was incubated at 30°C, 150 rpm). Immediately after the 1-h incubation, a 20-μL aliquot was taken from each well and diluted with 180 μL of AMA buffer (10^{-1} dilution) in another sterile polystyrene 96-well plate. The resulting solution was pipetted up and down ten times for mixing. The solution was further diluted serially from 10^{-2} to 10^{-4} by removing 20 μL from the dilution and adding it to 180 μL of AMA buffer. A 5-μL aliquot of each dilution and an undiluted sample were spotted onto pre-warmed TSA minus dextrose (Becton Dickinson) agar plates (VWR). The spots were allowed to dry, agar side up, for at least 30 min prior to being inverted and incubated (37°C for all strains except *B. cereus*, which was incubated at 30°C) for 13-15 h. The number of colony forming units (CFU) obtained for each species and peptide treatment was determined by colony counting. All assays were plated in triplicate in three independent trials. The resulting averages and standard deviations are reported.

Table S1. Molecular Weights and Extinction Coefficients

Peptide	Sequence[a]	MW (Da)[b]	ε_{278} ($M^{-1}cm^{-1}$)[c]
$HD5_{red}$	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR	3588.2	2800
$HD5_{ox}$	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR	3582.1	3181
HD5-CD	(ATCY**C**RTGRCATRESLSGV**C**EISGRLYRLCCR)$_2$	7164.2	6362

[a] Cysteine residues involved in intermolecular disulfides are underlined and in bold. [b] Molecular weights were calculated by using PROTEIN CALCULATOR v3.3 available at http://www.scripps.edu/~cdputnam/protcalc.html. [c] Extinction coefficients (278 nm) were calculated by using PROTEIN CALCULATOR v3.3.

Table S2. Strains and Growth Conditions

Strain	Source	Culture conditions[a]	AMA dilution factor[b]
E. coli 25922	ATCC	37 °C, TSB-dextrose	1:200
A. baumannii 17978	ATCC	37 °C, TSB-dextrose	1:200
A. baumannii 17961	ATCC	37 °C, TSB-dextrose	1:200
K. pneumoniae 13883	ATCC	37 °C, TSB-dextrose	1:100
P. aeruginosa PAO1	Katharina Ribbeck, MIT	37 °C, TSB-dextrose	1:100
B. cereus 14579	ATCC	30 °C, TSB-dextrose	1:25
S. aureus 29213	ATCC	37 °C, TSB-dextrose	1:100
S. aureus 25923	ATCC	37 °C, TSB-dextrose	1:100
L. monocytogenes 19115	ATCC	37 °C, BHI	1:500
C. albicans SC5314	Susan Lindquist, Whitehead Institute	37 °C, YPD	1:20

[a] TSB-dextrose and BHI were obtained from Becton Dickinson (BD). YPD was prepared in-house. [b] The AMA dilution factor indicates the dilution of each mid-log phase culture employed in the antimicrobial activity assays.

Table S3. ESI-MS of Major HPLC Peaks Observed in Crude HD5 from SPPS

Peak	Retention Time (min)[a]	Obs. *m/z* $[M+H]^+$	Calc. *m/z* $[M+H]^+$	Fragment
1	15.6	1237.68	1237.51	ISGRLYRLCR
2	19.5	1825.02	1824.95	LSGVCEISGRLYRLCR
3	20.8	3585.67	3585.70	$HD5_{red}$

[a] Retention times were obtained by using analytical HPLC gradient of 10–60% B over 30 min at 1 mL/min.

Table S4. Characterization of Purified Peptides

Peptide	HPLC Retention Time (min)[a]	Calculated Mass (Da)[b]	Observed Mass (Da)[b]	Sedimentation Coefficient (S)[c]	Free Thiol Content[d]
$HD5_{red}$	20.1	3588.2	3588.0	0.6[e]	6.24±0.47
$HD5_{ox}$	15.2	3582.2	3582.5	1.8[f]	0.23±0.20
HD5-CD	13.1	7164.4	7164.1	1.2[f]	0.55±0.39

[a] Analytical HPLC using a gradient of 10–60% B over 30 min at 1 mL/min. [b] Analyzed using Agilent Technologies Quantitative Analysis 2009 software version B.03.02. [c] Determined by fitting –dc/dt data unless otherwise noted. [d] Determined by using the DTDP assay. [e] Theoretical value derived from hydrodynamic modeling with HYDROPRO. [f] Average S-value from concentration range of 30-437 μM $HD5_{ox}^{S2}$ and 40-120 μM HD5-CD in 10 mM sodium phosphate buffer, pH 7.0.

Table S5. Sedimentation Velocity of HD5-CD at Different Concentrations[a]

[HD5-CD] (μM)	D value (F)	S value (S)	MW (kDa)
40	12.99	1.279	8.22
60	13.47	1.257	7.67
90	13.82	1.235	7.46
120	16.07	1.196	6.21

[a] All data were analyzed by fitting –dc/dt data with the end of each cell set to 6.90 cm, using 12-22 scans with ~30-40 kDa peak broadening, and fitting from 0.15-3.5 S. All samples were prepared at pH 7.0 in 10 mM sodium phosphate buffer, 20 °C. Sedimentation coefficients are $s_{20,w}$ values, adjusted with $\bar{\nu}$ = 0.7094 mL/g at 20 °C, solvent density (ρ) of 0.99967 g/mL, and a solvent viscosity (η) of 1.0061 cP. Viscosity units are in centipoise (cP) (1 Poise = 1 g · cm^{-1} · s^{-1}). Sedimentation coefficients are in Svedbergs (1 Svedberg = 100 fs = 1 x 10^{-13} s). Diffusion coefficients correspond to the best-fit molecular mass in Fick units (1 Fick = 1 x 10^{-7} cm^2/s).

Table S6. Sedimentation Velocity of HD5-CD at Different pH Values[a]

[HD5-CD] (μM)	D value (F)	S value (S)	MW (kDa)	pH
20	15.10	1.286	7.11	2.0
20	14.23	1.211	7.11	4.0
20	13.27	1.272	8.00	6.0
20	13.55	1.236	7.34	7.0
20	13.74	1.234	7.50	8.0

[a] All data were analyzed by fitting –dc/dt data with the end of each cell set to 6.90 cm, using 12-22 scans with ~30-40 kDa peak broadening, and fitting from 0.15-3.5 S. All samples were prepared at in 10 mM sodium phosphate, 20 °C, and the indicated pH was achieved by addition of 1 M HCl. Sedimentation coefficients are $s_{20,w}$ values, adjusted with $\bar{\nu}$ = 0.7094 mL/g at 20 °C, solvent density (ρ) of 0.99967 g/mL, and a solvent viscosity (η) of 1.0061 cP. Viscosity units are in centipoise (cP) (1 Poise = 1 g · cm^{-1} · s^{-1}). Sedimentation coefficients are in Svedbergs (1 Svedberg = 100 fs = 1 x 10^{-13} s). Diffusion coefficients correspond to the best-fit molecular mass in Fick units (1 Fick = 1 x 10^{-7} cm^2/s).

Table S7. Sedimentation Coefficient Calculations using HYDROPRO

Species (PDB ID)	$s_{T,B}$ (S)[a]	$s_{20,w}$ (S)	Partial Specific Volume (mL/g)[b]
$HD5_{red}$ monomer[c,d]	0.5836	0.5867	0.7093
$HD5_{red}$ monomer[c,e]	0.503	0.5064	0.7093
$HD5_{ox}$ monomer (1ZMP)	0.659	0.662	0.7094
$HD5_{ox}$ monomer (2LXZ)	0.712	0.726	0.7094
$HD5_{ox}$ dimer (1ZMP)	1.149	1.156	0.7094
HD5-CD (2MIT)	1.114	1.083	0.7094
$HD5_{ox}$ tetramer (1ZMP)	1.718	1.727	0.7094
$HD5_{ox}$ tetramer (4E83)	1.713	1.722	0.7094

[a] Modeled using $\bar{v}$ at 20 °C, solvent density (ρ) of 0.99967 g/mL, and a solvent viscosity (η) of 1.0061 cP unless otherwise noted. Viscosity units are in centipoise (cP) (1 Poise = 1 g · cm^{-1} · s^{-1}). Sedimentation coefficients are in Svedbergs (1 Svedberg = 100 fs = 1 x 10^{-13} s). [b] Monomer, dimer, or tetramer $\bar{v}$ values for $HD5_{ox}$ and HD5-CD are predicted to be the same value with SEDNTERP. [c] Structures generated from CYANA calculation of primary sequence. [d] Molten globule form. [e] Extended coil form.

Table S8. Initial HD5-CD Sedimentation Equilibrium Analysis: ln(c) versus r^2

Concentration (μM) [a]	$MW_{apparent}$ (Da)	Coefficient of Determination (R^2) [b]
15	7443	0.9815
19	8071	0.9835
31	8456	0.9965
32	8103	0.9949
37	7364	0.9952
41	7376	0.9960
47	8381	0.9981
50	8085	0.9984
68	7319	0.9990
73	7376	0.9989

[a] Samples prepared in 10 mM sodium phosphate buffer, pH 7.0 [b] Linear regression fitting of equilibrium data at 42,000 rpm.

Table S9. SEDPHAT Molecular Weight Fitting of Sedimentation Equilibrium Data for HD5-CD[a]

Sample[b]	MW_{calc} (Da)	95% Confidence Interval[c]	Standard Deviation[c]	Global Reduced Chi-Square Value	Critical Chi-Square Value[d]
HD5-CD (Cell 2)	6394	5405 – 7317 Da	± 490 Da	0.4776	0.5367
HD5-CD (Cell 3)	7145	6198 – 8072 Da	± 487 Da	0.4985	0.5715
HD5-CD (Cell 4)	7038	6206 – 7894 Da	± 445 Da	0.4677	0.5214
HD5-CD (Cell 5)	7254	6640 – 7902 Da	± 334 Da	0.4894	0.5434
HD5-CD (Cell 6)	7294	6458 – 8211 Da	± 821 Da	0.4927	0.5646
HD5-CD (Global Fit)	6958	6598 – 7330 Da	± 176 Da	0.4663	0.4896

[a] Fitting used $\bar{\nu}$ of 0.7094 mL/g at 20 °C and a solvent density (ρ) of 0.99967 g/mL. [b] All samples were prepared at pH 7.0 in 10 mM sodium phosphate buffer. [c] Calculated from 1000 iterations of Monte-Carlo analysis. [d] Calculated at a 95% confidence level.

Table S10. HPLC and Mass Spectrometry of ^{15}N- and ^{13}C,^{15}N-Labeled Peptides

Peptide	Calculated Mass (Da)[a]	Observed Mass (Da)	Retention Time (min)[b]
[*U*-^{15}N]-HD5-CD	7263.50	7263.51	13.2
[*U*-^{13}C,^{15}N]-HD5-CD	7549.38	7549.37	13.2
[^{12}C,^{14}N/*U*-^{13}C,^{15}N]-HD5-CD[c]	7356.79	7356.82	13.2

[a] Molecular weights were calculated by using PROTEIN CALCULATOR v3.3 available at http://www.scripps.edu/~cdputnam/protcalc.html. [b] HPLC retention times were determined using a gradient of 10–60% B over 30 min at 1 mL/min. [c] This samples also included unlabeled and uniformly-labeled HD5-CD (see Experimental Section).

Table S11. Statistics for 20 Lowest Energy Conformers of HD5-CD pH 4.0 at 298 K

Experimental constraints		
Distance constraints		
Long ($\|i - j\| > 5$)		380
Medium ($\|i - j\| < 4$)		116
Sequential ($\|i - j\| = 1$)		232
Intraresidue $\|i = j\|$		270
Intermolecular		107
Total		1105
Dihedral angle constraints (ϕ, ψ, and χ_1)		
ϕ		28
ψ		26
χ_1		28
Total		82
Average atomic RMSD to the mean structure (Å)		
All Residues		
Backbone (C^{α}, C′, N)		0.46 ± 0.07
Heavy atoms		0.94 ± 0.15
Ordered Residues [a]		
Backbone (C^{α}, C′, N)		0.29 ± 0.06
Heavy atoms		0.64 ± 0.16
Deviations from idealized covalent geometry		
Bond lengths	RMSD (Å)	0.020
Torsion angle violations	RMSD (°)	1.50
Lennard-Jones energy [b] (kJ mol^{-1})		-1284 ± 38.1
Constraint violations [c, d]		
NOE distance	Number > 0.5 Å	0 ± 0
NOE distance	RMSD (Å)	0.0219 ± 0.0011
Torsion angle violations	Number > 5°	0 ± 0
Torsion angle violations	RMSD (°)	0.9636 ± 0.2300
Ramachandran statistics (% of all residues)		
Most favored		84.4
Additionally allowed		9.8
Generously allowed		3.4
Disallowed		2.3
Ramachandran statistics (% of ordered residues) [d]		
Most favored		99.1
Additionally allowed		0.9
Generously allowed		0.0
Disallowed		0.0

[a] Ordered residues defined as 2-10, 16-32, 2'-10', and 16'-32'. [b] Nonbonded energy was calculated in XPLOR-NIH. [c] The largest NOE violation in the ensemble of structures was 0.67 Å. [d] The largest ϕ,ψ angle violation in the ensemble of structures was 6.3°.

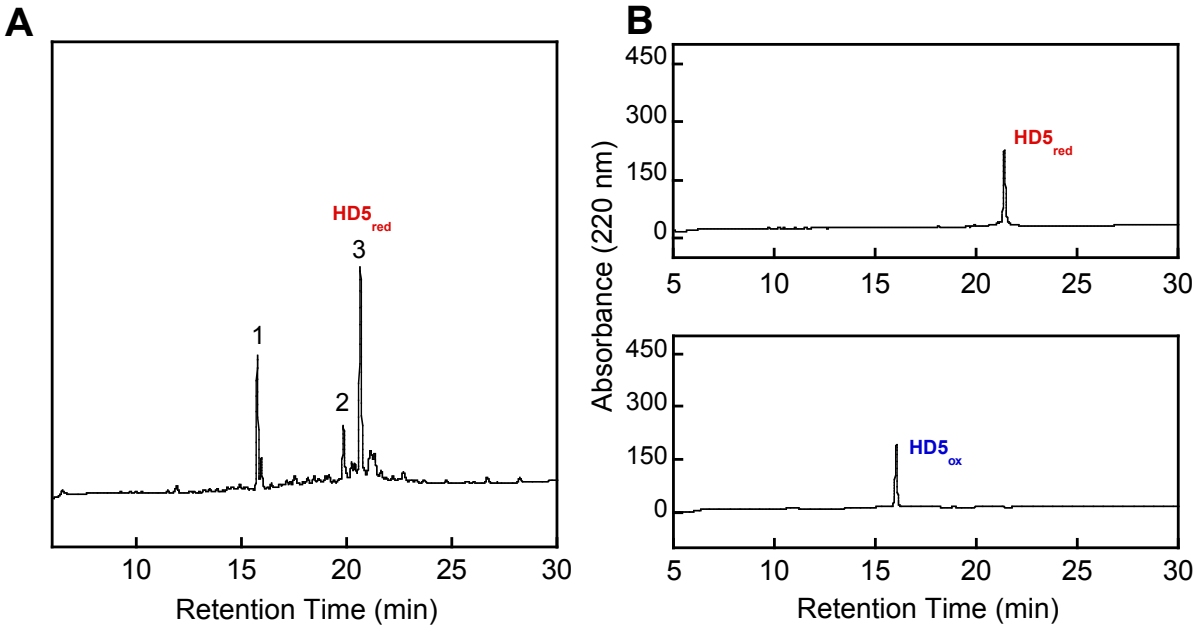

Figure S1. Fmoc solid-phase peptide synthesis of HD5. (A) Example HPLC trace from the crude reaction. LC/MS analysis for peaks 1-3 is provided in Table S3. (B) Purified $HD5_{red}$ and $HD5_{ox}$ obtained from the solid-phase peptide synthesis. All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min.

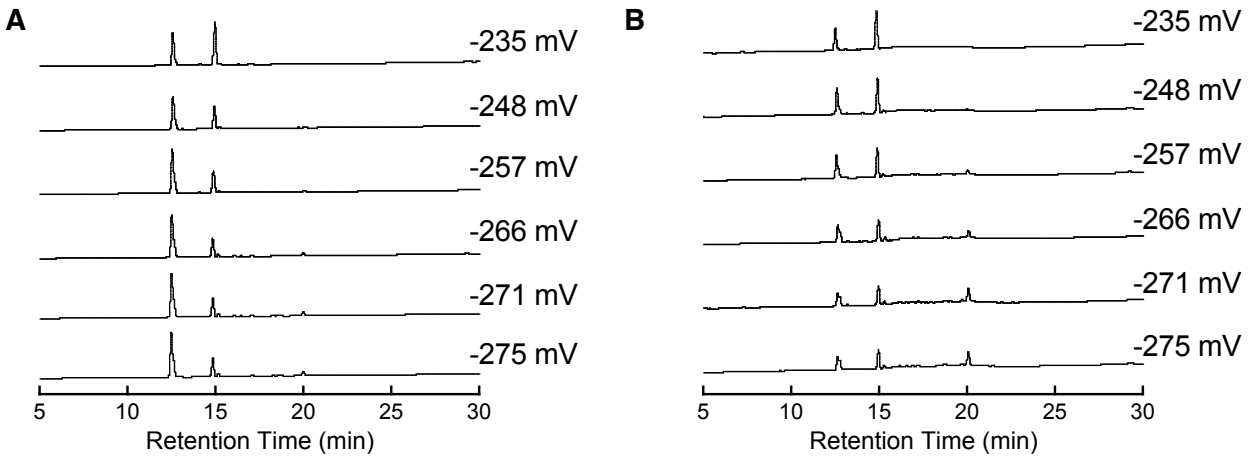

Figure S2. Analytical HPLC traces showing HD5-CD formation from (A) 25 μM $HD5_{ox}$ and (B) 25 μM $HD5_{red}$ following incubation in redox buffers containing GSH/GSSG (75 mM HEPES, pH 7.0, rt, anaerobic). All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min.

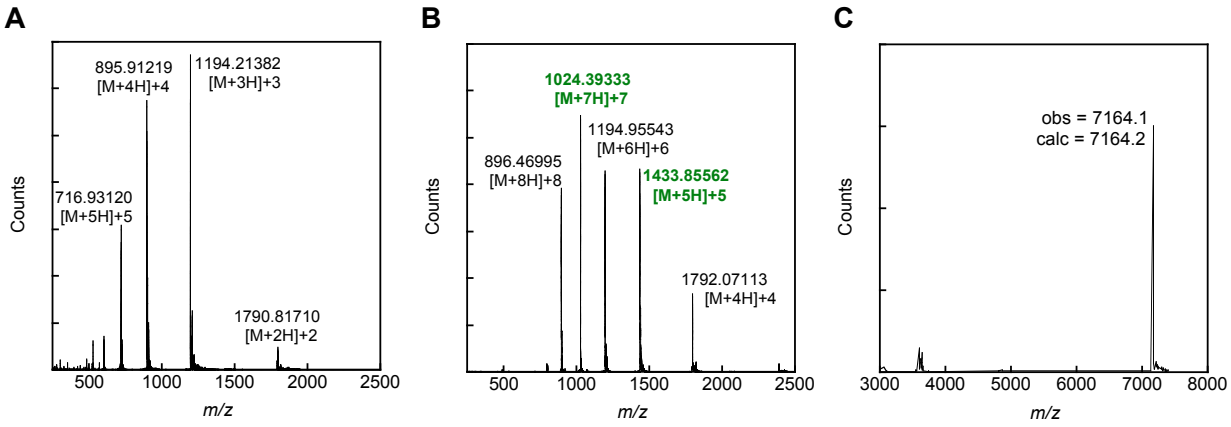

Figure S3. LC/MS analysis of HD5-CD. (A) LC/MS ionization pattern for $HD5_{ox}$ presented for comparison. (B) LC/MS ionization pattern for HD5-CD. The unique molecular ions of HD5-CD are labeled in green. (C) The deconvoluted molecular mass for HD5-CD.

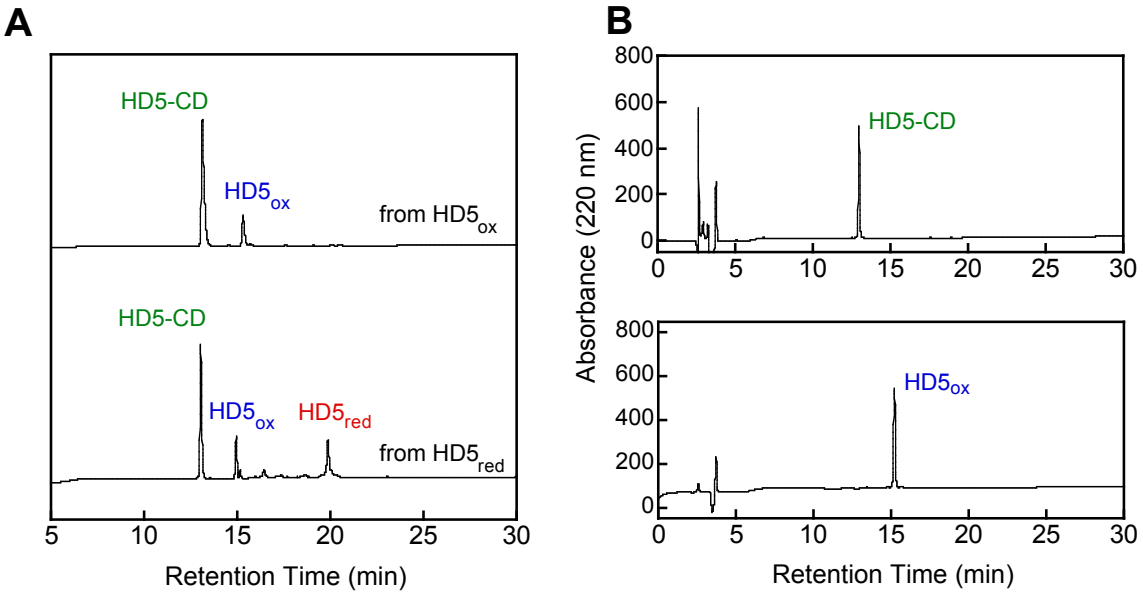

Figure S4. Preparative-scale formation and isolation of HD5-CD. (A) Analytical HPLC traces for HD5-CD formation from 50 μM $HD5_{red}$ (75 mM HEPES, pH 7.4 containing 12.0 mM GSH and 0.48 mM GSSG; t = 48 h, anaerobic) and 50 μM $HD5_{ox}$ (75 mM HEPES, pH 7.4 containing 12.0 mM GSH and 0.12 mM GSSG; t = 48 h, anaerobic). (B) Representative analytical HPLC traces of purified HD5-CD and $HD5_{ox}$. All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min.

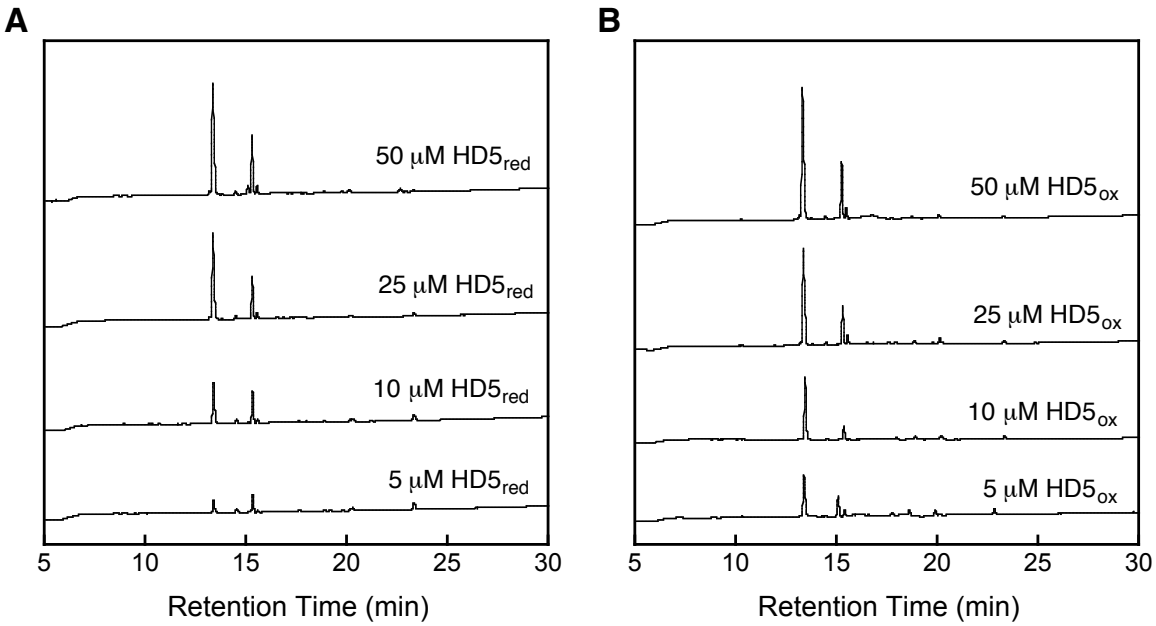

Figure S5. HD5-CD formation over a range (5 – 50 μM) of initial peptide concentrations. (A) Starting from $HD5_{red}$ (75 mM HEPES, pH 7.4 containing 12.0 mM GSH and 0.48 mM GSSG; t = 24 h, anaerobic). (B) Starting from varying concentrations of $HD5_{ox}$ (75 mM HEPES, pH 7.4 containing 12.0 mM GSH and 0.12 mM GSSG; t = 24 h, anaerobic). All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min.

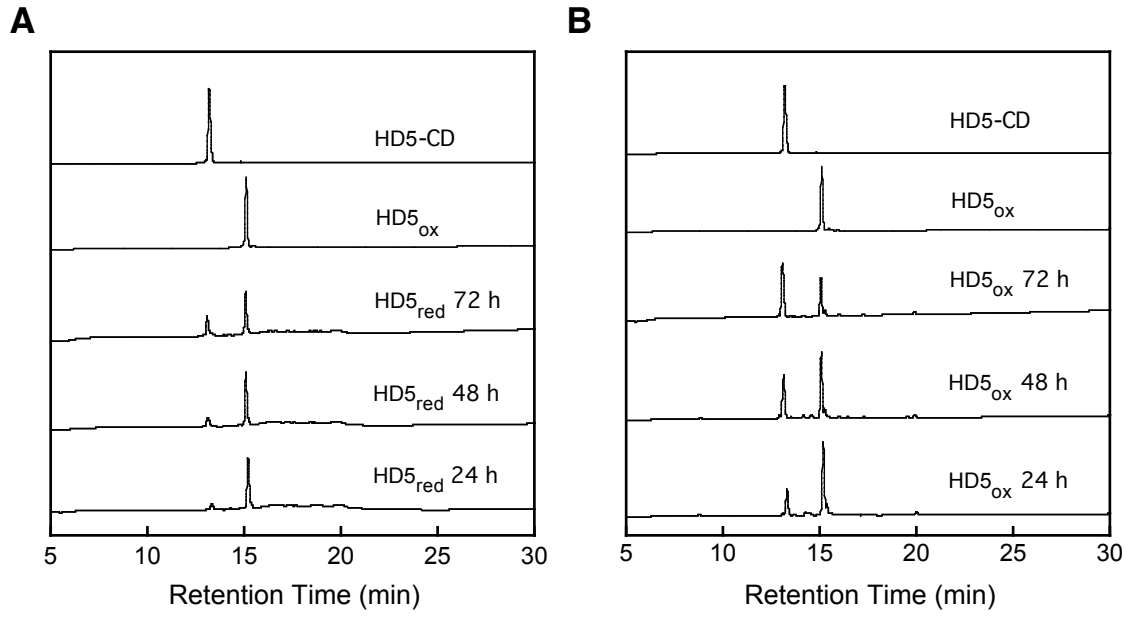

Figure S6. HD5-CD formation under aerobic conditions and in the presence of GSH/GSSG. (A) Starting from 25 μM $HD5_{red}$ (75 mM HEPES, pH 7.4 containing 12.0 mM GSH and 0.48 mM GSSG). (B) Starting from 25 μM $HD5_{ox}$ (75 mM HEPES, pH 7.4 containing 12.0 mM GSH and 0.12 mM GSSG). $HD5_{ox}$ and HD5-CD standards are included for reference. The incubations were performed on the benchtop and the buffers were not deoxygenated prior to the experiment. All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min.

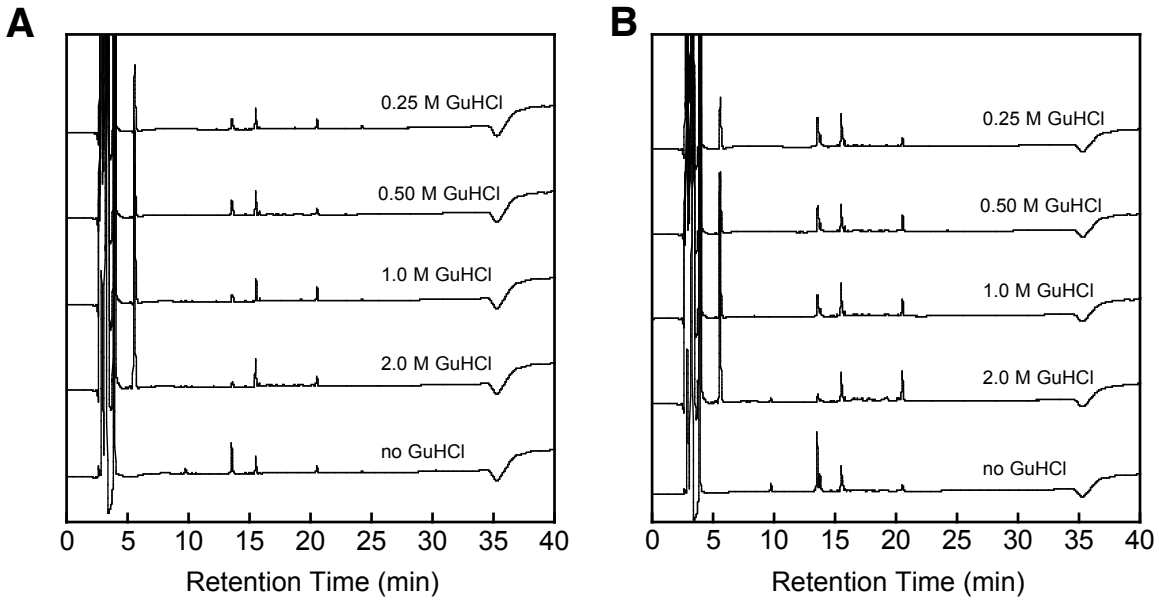

Figure S7. Formation of HD5-CD is attenuated in buffers containing GuHCl. (A) Formation of HD5-CD from 50 μM $HD5_{red}$ (75 mM HEPES, pH 7.4 containing 12.0 mM GSH and 0.48 mM GSSG; t = 24 h). (B) Formation of HD5-CD from 50 μM $HD5_{ox}$ (75 mM HEPES, pH 7.4 containing 12.0 mM GSH and 0.12 mM GSSG; t = 24 h). All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min.

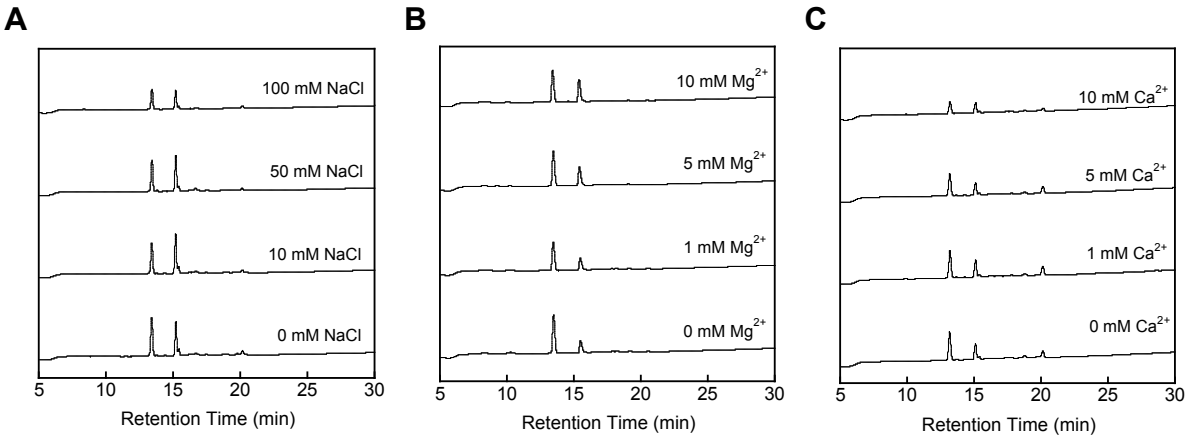

Figure S8. Formation of HD5-CD from 50 μM $HD5_{ox}$ in the presence of cations (75 mM HEPES, pH 7.4 containing 12.0 mM GSH and 0.12 mM GSSG; t = 24 h). (A) In the presence of 0 – 100 mM NaCl. (B) In the presence of 0 – 10 mM $MgSO_4$. (C) In the presence of 0 – 10 mM $CaCl_2$. All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min.

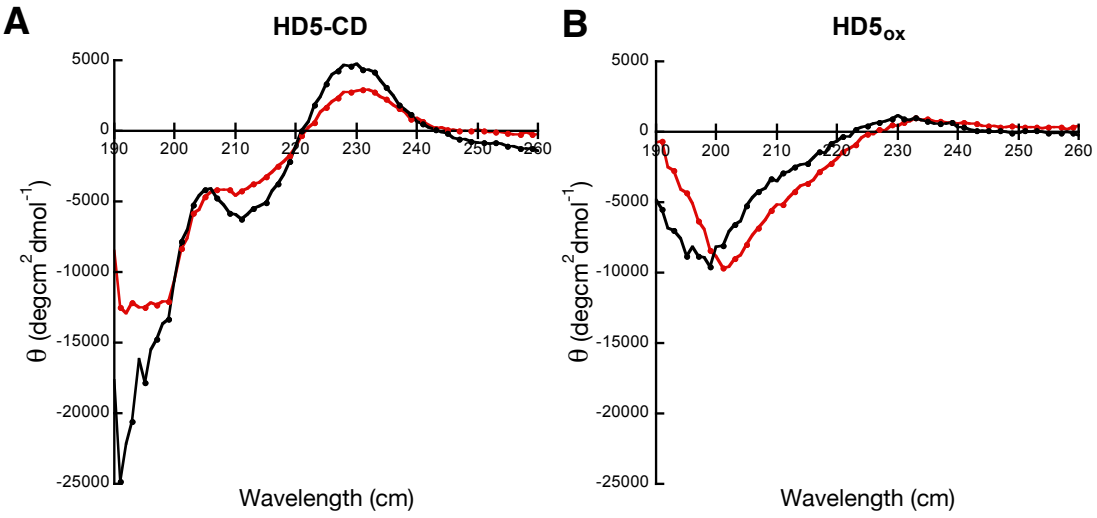

Figure S9. CD spectra of HD5-CD and $HD5_{ox}$. (A) HD5-CD (20 μM) in the absence (black) and presence (red) of 10 mM SDS. (B) $HD5_{ox}$ (20 μM) in the absence (black) and presence (red) of 10 mM SDS. Samples were prepared at pH 7.0 in 10 mM sodium phosphate buffer.

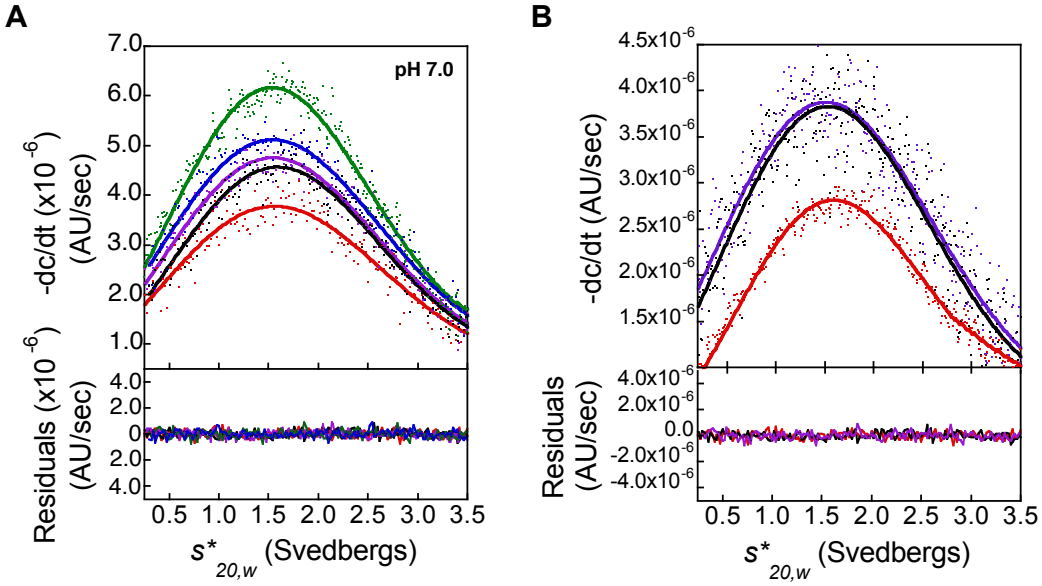

Figure S10. Sedimentation velocity of HD5-CD. (A) The HD5-CD concentration was varied from 40 – 120 μM (10 mM sodium phosphate buffer, pH 7.0). (B) The HD5-CD concentration was 20 μM and pH was adjusted to 2.0 (purple), 4.0 (red), and 6.0 (black). In both data sets, the dots are the –dc/dt data measured using UV absorbance at 280 nm and the lines are the single Gaussian fits. These data yielded a sedimentation coefficient of ca. 1.2 S for all peptide concentrations, which supports a single sedimenting species of ca. 7.4 kDa (Tables S5,S6).

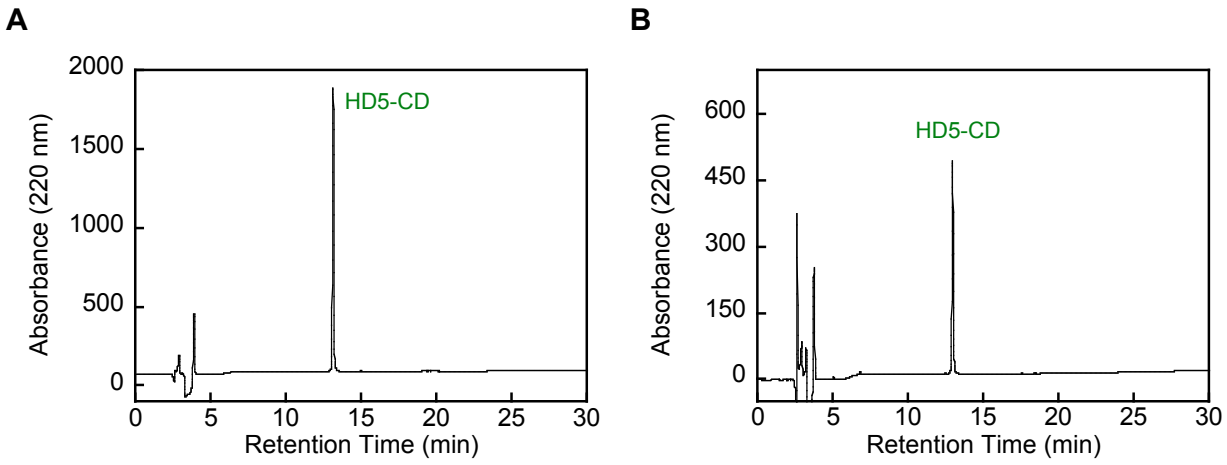

Figure 11. Analytical HPLC traces of purified isotopically-labeled HD5-CD. (A) Mixed $^{12}C/^{13}C$-labeled [^{12}C,^{14}N/*U*-^{13}C,^{15}N]-HD5-CD dissolved in 5% aqueous TFA. (B) [*U*-^{13}C,^{15}N]-HD5-CD dissolved in 5% aqueous TFA . All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min.

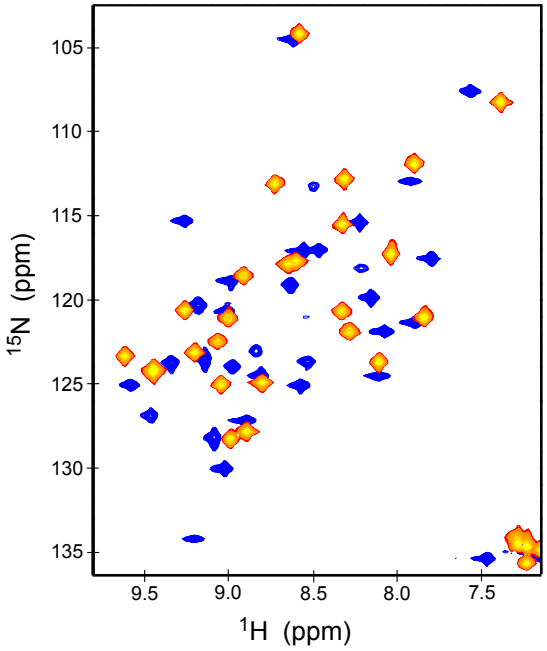

Figure S12. Overlay of the ^{1}H–^{15}N HSQC spectra of ^{15}N-$HD5_{ox}$ (blue peaks, 460 μM) and ^{15}N-HD5-CD (yellow-orange peaks, 625 μM). Both samples were prepared in 90:10 H_2O/D_2O adjusted to pH 4.0.

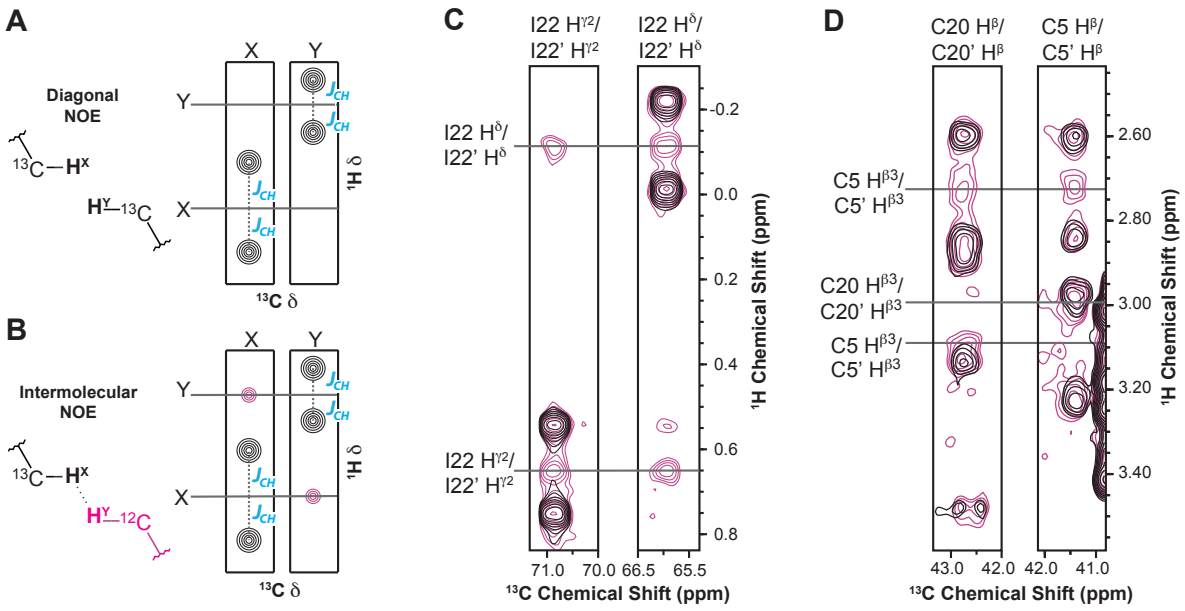

Figure S13. Identification of the intermolecular disulfide bonds. The 2D ^{13}C-edited/^{15}N,^{13}C-filtered HSQC-NOESY spectrum identifies intermolecular disulfide bond between Cys^{5} and Cys$^{20'}$ and supports molecular symmetry. (A) Diagonal NOEs, originating from ^{1}H directly bonded to ^{13}C, will produce two resonances (black) separated from the chemical shift value by the J_{CH} constant (light blue). (B) Intermolecular NOEs (magenta), resulting from ^{1}H directly bonded to ^{12}C, will be distinct from diagonal NOEs (black) because they produce only a single resonance at the chemical shift. (C) Overlay of strips from 2D ^{13}C-edited/^{15}N,^{13}C-filtered HSQC-NOESY spectra collected with (magenta), and without (black), a NOE mixing period. Ile^{22} Hγ2 and Hδ produce NOEs to their symmetric mates (Ile$^{22'}$ Hγ2 and Hδ) on the opposite protomer. (D) Overlay of strips from 2D ^{13}C-edited/^{15}N,^{13}C-filtered HSQC-NOESY spectra collected with (magenta), and without (black), a NOE mixing period discern intermolecular disulfide bridges between Cys^{5}—Cys$^{20'}$ and Cys$^{5'}$—Cys^{20}.

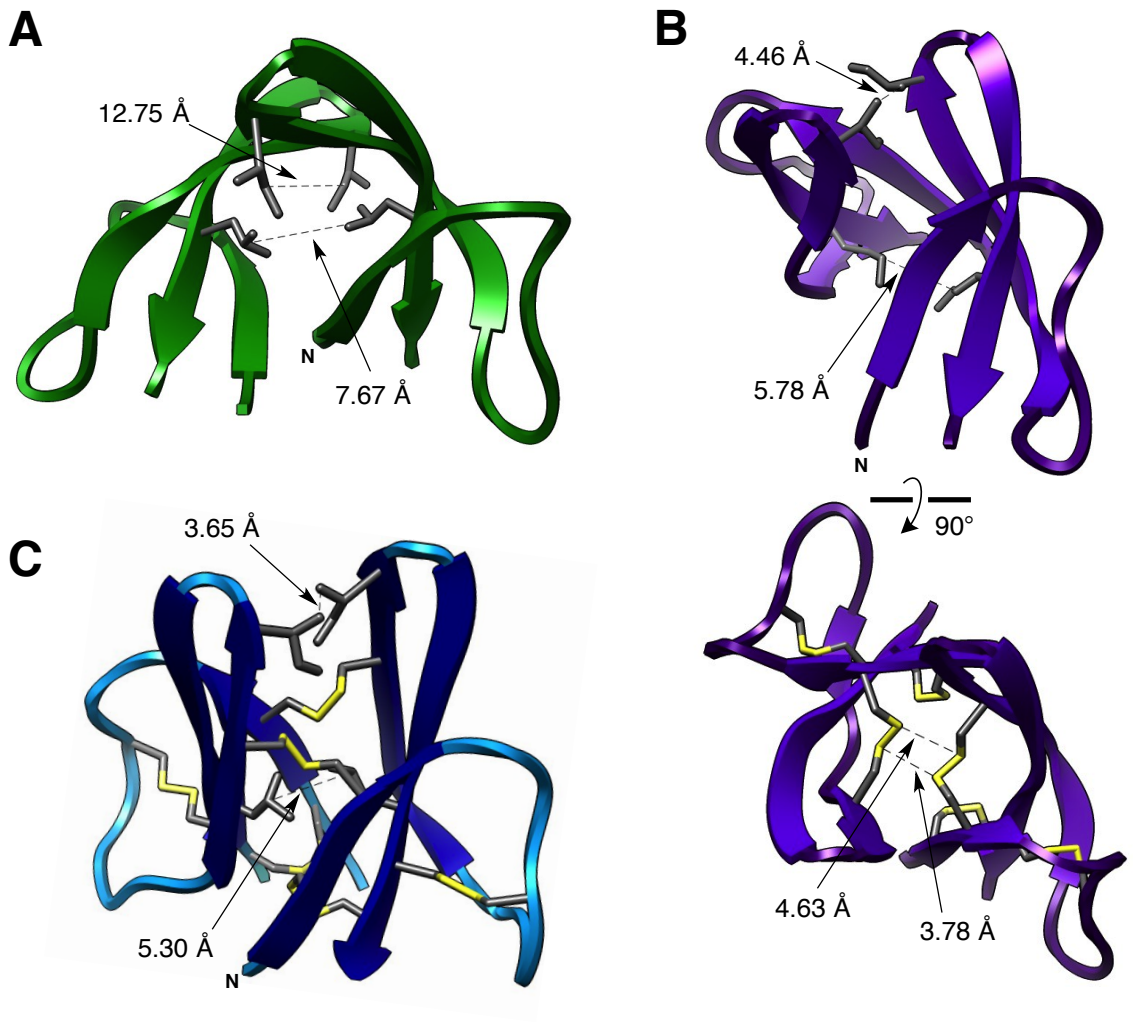

Figure S14. The alternative non-covalent binding modes of $HD5_{ox}$ suggest orientations between monomers that facilitate intermolecular disulfide bond formation. (A) The $HD5_{ox}$ dimeric crystal structure (PDB ID 1ZMP)[S11] is arranged with the aliphatic side chains Ile^{22} and Leu^{29} separated by long distances (Ile^{22} Cγ to $Ile^{22'}$ Cγ = 12.75 Å and Leu^{29} Cγ to $Leu^{29'}$ Cγ = 7.67 Å). (B, top) Alternatively, when the Leu^{29} side chain is mutated to *n*Leu (PDB ID 4E83)[S26], the $HD5_{ox}$ non-covalent dimer is arranged with the aliphatic side chains Ile^{22} and $nLeu^{29}$ at shorter distances (Ile^{22} Cγ to $Ile^{22'}$ Cγ = 4.46 Å and $nLeu^{29}$ Cγ to $nLeu^{29'}$ Cγ = 5.78 Å). (B, bottom) The binding mode of the $nLeu^{29}$ $HD5_{ox}$ mutant brings the Cys^5—Cys^{20} intramolecular disulfide in close proximity to the $Cys^{5'}$—$Cys^{20'}$ bond (Cys^5 S to $Cys^{20'}$ S = 4.63 Å and $Cys^{5'}$ S to Cys^{20} S = 3.78 Å). The alternative crystal packing observed for these two structures indicates that multiple modes of non-covalent dimerization are possible. (C) The covalent dimer, HD5-CD (PDB ID 2MIT, hydrogen atoms omitted for clarity), orients the Ile^{22} and Leu^{29} aliphatic side chains close together (Ile^{22} Cγ to $Ile^{22'}$ Cγ = 3.65 Å and Leu^{29} Cγ to $Leu^{29'}$ Cγ = 5.30 Å).

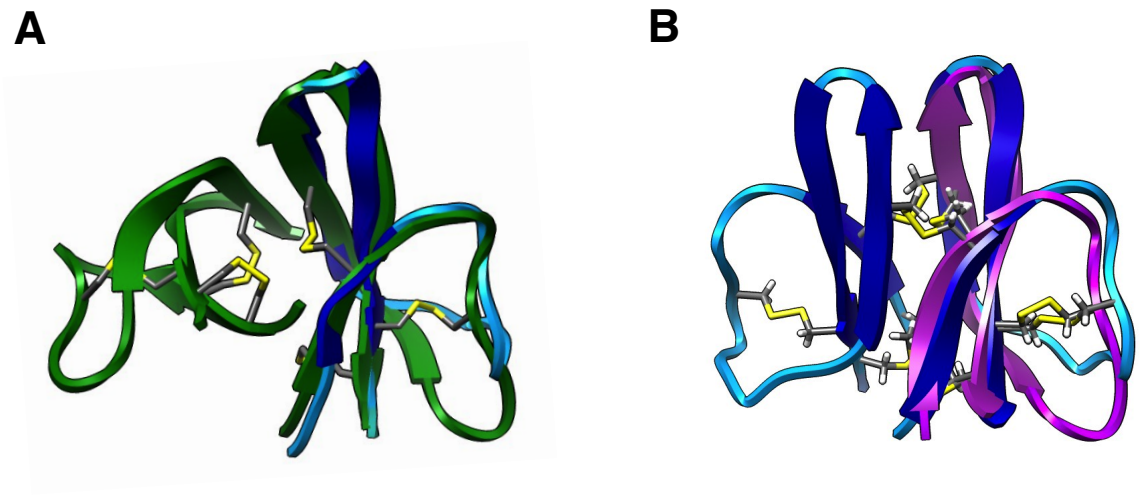

Figure S15. Overlay of the HD5-CD solution structure with structures of $HD5_{ox}$. Each monomer of HD5-CD exhibits tertiary structure that is similar to that of $HD5_{ox}$. (A) A protomer from the HD5-CD structure (blue, PDB ID 2MIT) overlays with a protomer from the $HD5_{ox}$ dimeric crystal structure (green with disulfides shown, PDB ID 1ZMP)[S11] with a Cα RMSD = 1.36 Å. (B) The tertiary structure of $HD5_{ox}$ determined by solution NMR (magenta with disulfides shown, PDB ID 2LXZ)[S2] aligns with a protomer of the HD5-CD (blue with disulfides shown, PDB ID 2MIT) structure with a Cα RMSD = 1.46 Å.

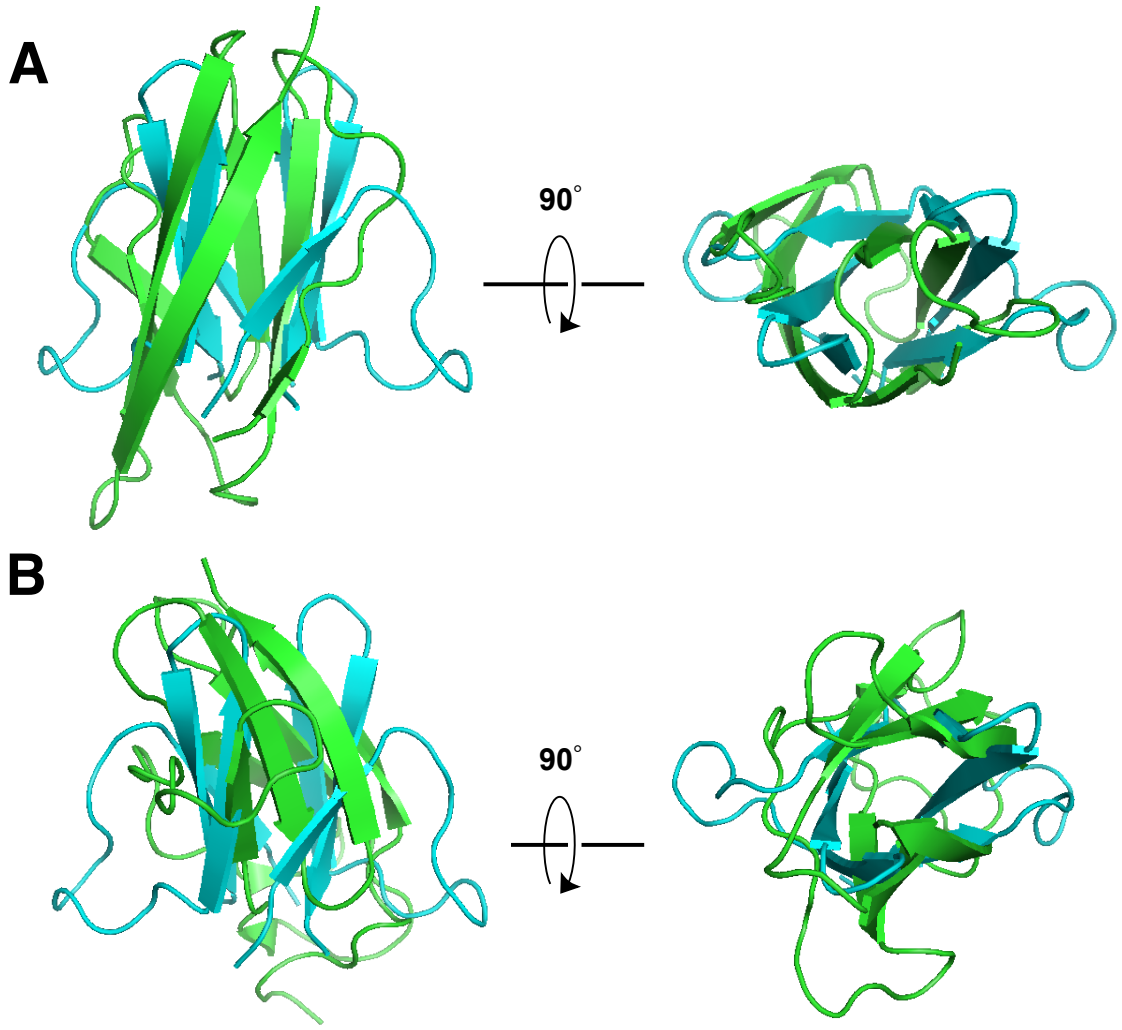

Figure S16. HD5-CD exhibits a unique fold. Following structural alignment using the PDBefold server,[S27] the HD5-CD β-barrel-like core is found to most closely match proteins classified as β-sandwich structures. (A) HD5-CD (light blue) aligns with the PKD domain from the glycoprotein polycystine-1 (green, PDB ID 1B4R)[S28] with a Cα RMSD = 3.09 Å. (B) HD5-CD (light blue) aligns with domain III of an envelope protein of Langat flavivirus (green, PDB ID 1Z66)[S29] with a Cα RMSD = 3.10 Å.

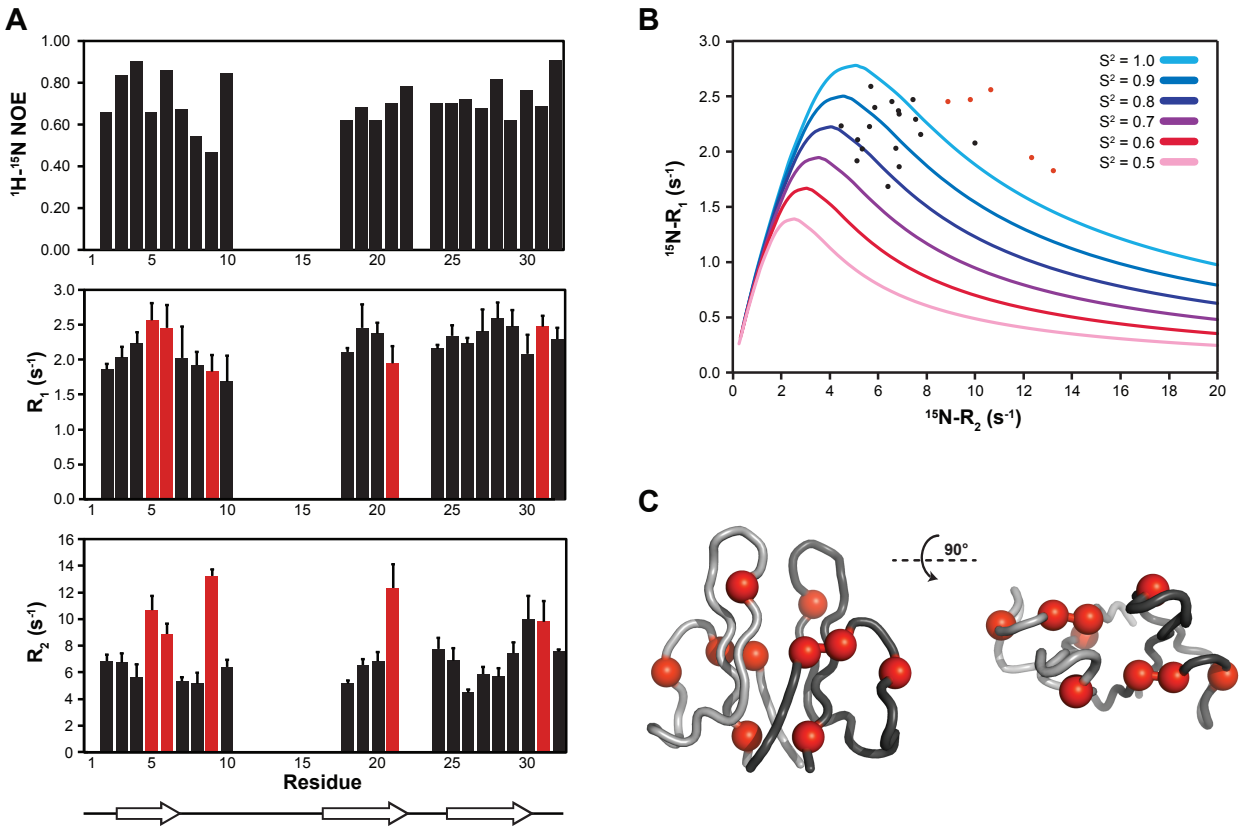

Figure S17. The ^{15}N backbone dynamics of HD5-CD are consistent with a rigid covalent dimer. (A) The intensity of ^{1}H-^{15}N heteronuclear NOEs reflect constrained motion on the picosecond-nanosecond timescale and a rigid structure. The ^{15}N-R_1 and ^{15}N-R_2 relaxation parameters are relatively constant across the entire molecule. Residues 11-16 appear to be in intermediate exchange, indicating microsecond-millisecond motions, and do not possess observable resonances. Ser^{17} and Ser^{23} are omitted due to peak overlap and the data from Ile^{22} could not be fit. (B) The combined R_1/R_2 relaxation parameters of residues highlighted in red were inconsistent with $S^2 \leq 1.0$. The R_1/R_2 values of HD5-CD (dots) were compared with R_1/R_2 values generated assuming various S^2 order parameters using Lipari-Szabo model-free analysis. (C) Residues possessing R_1/R_2 values outside the bounds of $S^2 = 1.0$ are highlighted red and indicated on the HD5-CD structure (PDB ID 2MIT).

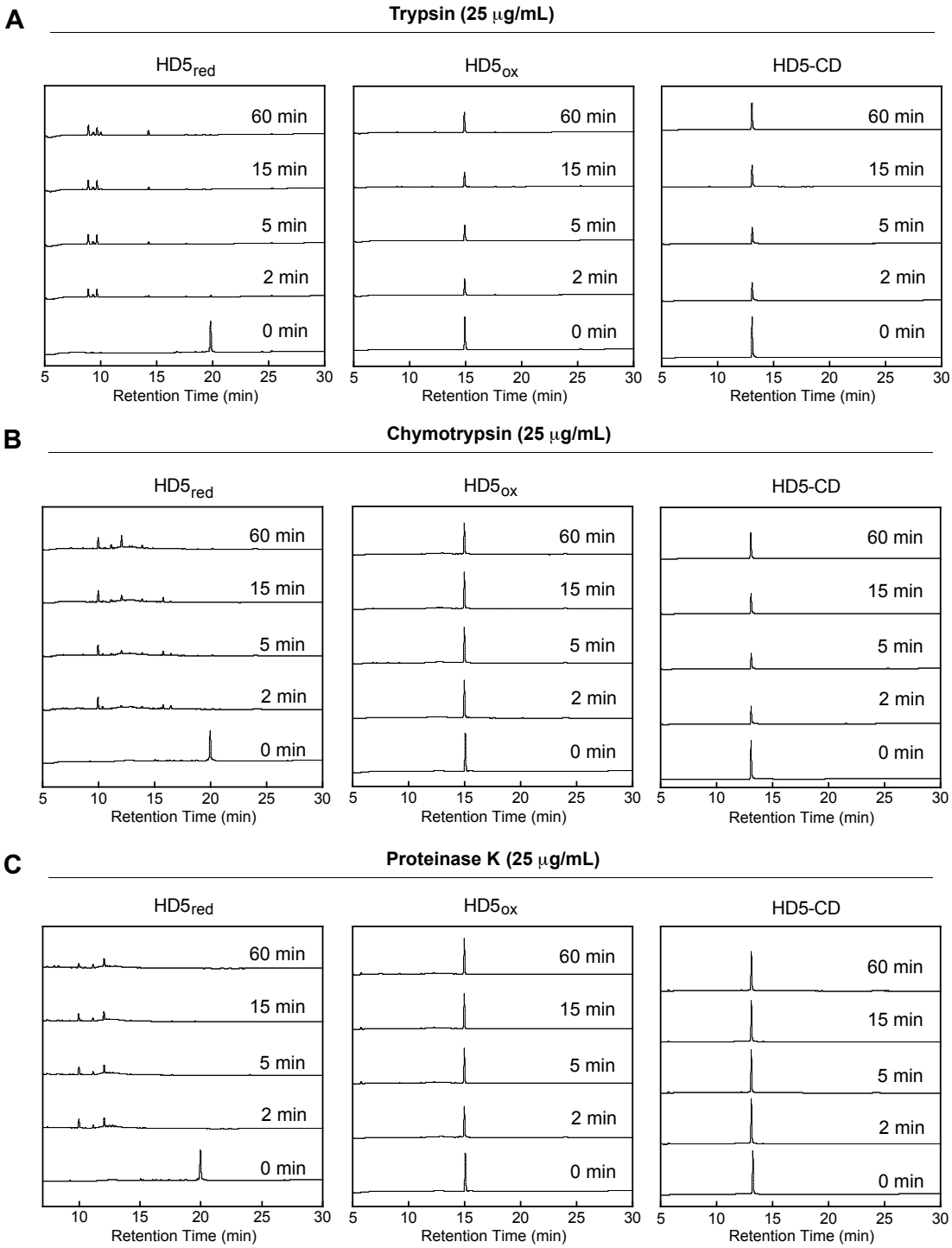

Figure S18. Protease degradation assays monitored by HPLC (100 mM Tris-HCl, 20 mM $CaCl_2$, pH 8.0) show that HD5-CD is resistant to proteolysis. (A) Treatment of peptides (80 μM, 100 μL) with trypsin. (B) Treatment of peptides (80 μM, 100 μL) with chymotrypsin. (C) Treatment of peptides (80 μM, 100 μL) with proteinase K. $HD5_{red}$ was employed as a positive control. All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min.

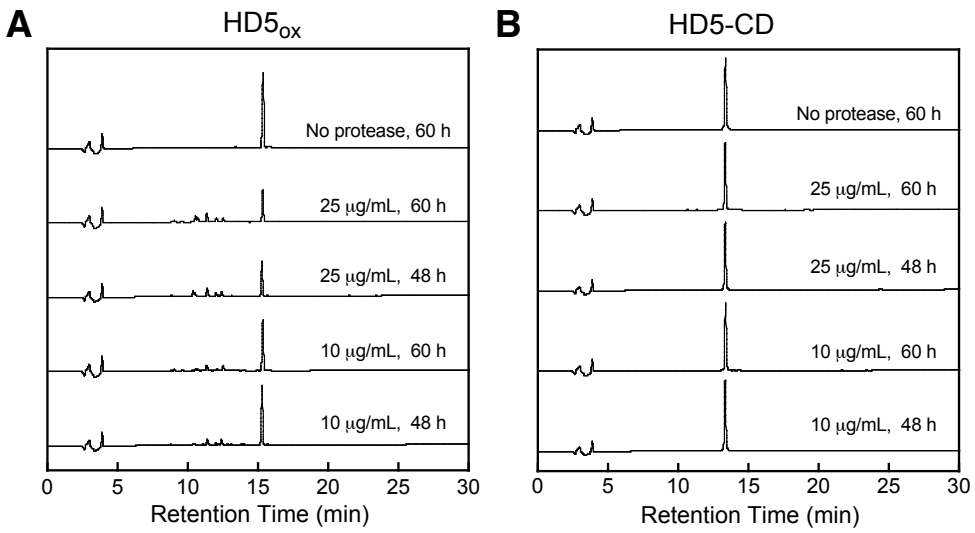

Figure S19. Double-digest protease degradation assays monitored by HPLC (100 mM Tris-HCl, 1 mM $CaCl_2$, pH 6.5). (A) Treatment of $HD5_{ox}$ with a protease mixture containing 1:1(w/w) trypsin and chymotrypsin (10 or 25 μg/mL) affords proteolysis at 37 °C.[S5] (B) Treatment of HD5-CD with the same protease mixtures affords no proteolysis at 37 °C. In each panel, "No protease" indicates a no-protease control and confirms the stability of the peptide in the absence of enzyme (t = 60 h, 37 °C). All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min.

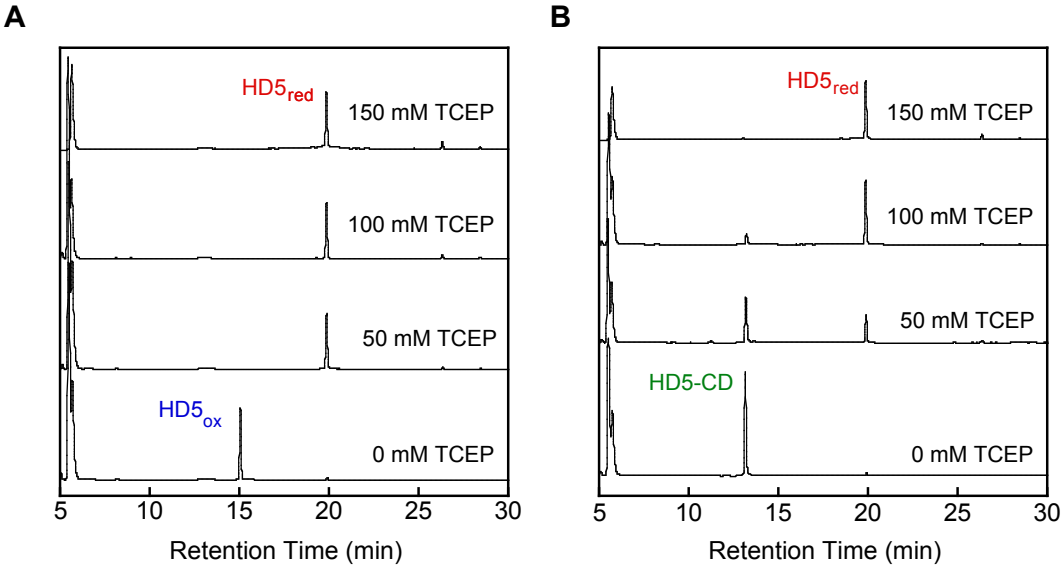

Figure S20. Peptide reduction assays employing TCEP at pH 8.2. (A) Reduction of $HD5_{ox}$ (15.1 min) to $HD5_{red}$ (19.9 min) following a 10-min incubation with TCEP. (B) Reduction of HD5-CD to $HD5_{red}$ following a 10-min incubation with TCEP. Reduction of $HD5_{ox}$ is more facile than for HD5-CD. All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min.

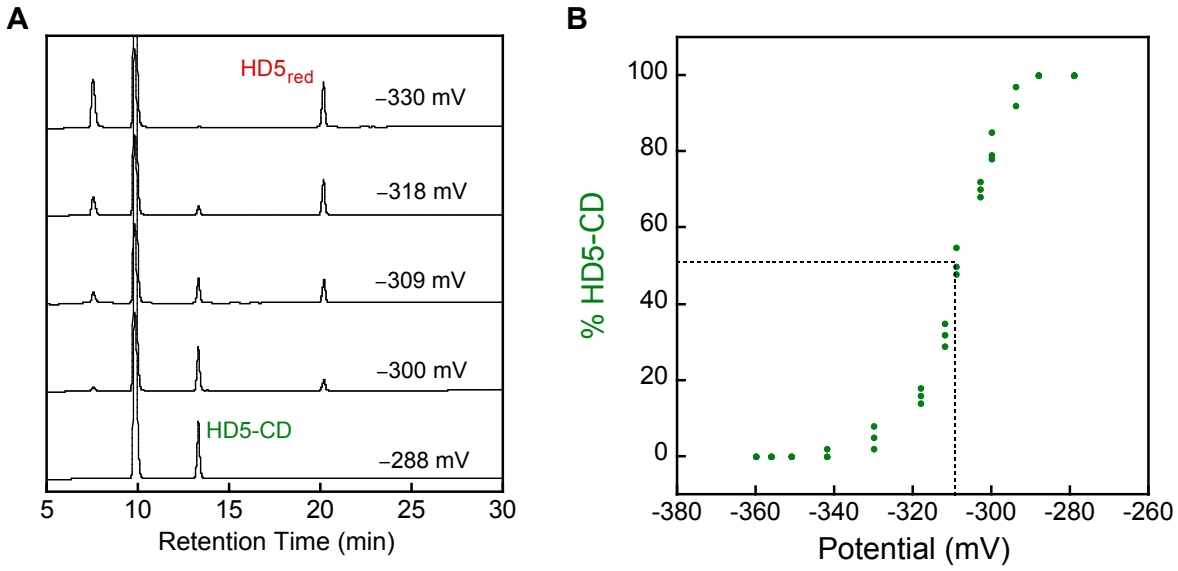

Figure S21. Midpoint potential determination of HD5-CD (75 mM HEPES, pH 7.0, t = 48 h). (A) Analytical HPLC traces of HD5-CD after incubation and equilibrium establishment in redox buffers. All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min. Peaks at ca. 7 and 10 min are DTT and DTT_{ox}, respectively. (B) The observed HD5-CD midpoint potential of –309 mV was determined by graphical analysis and indicates that HD5-CD is less susceptible to reduction than $HD5_{ox}$ (E_m = -257 mV).[S6]

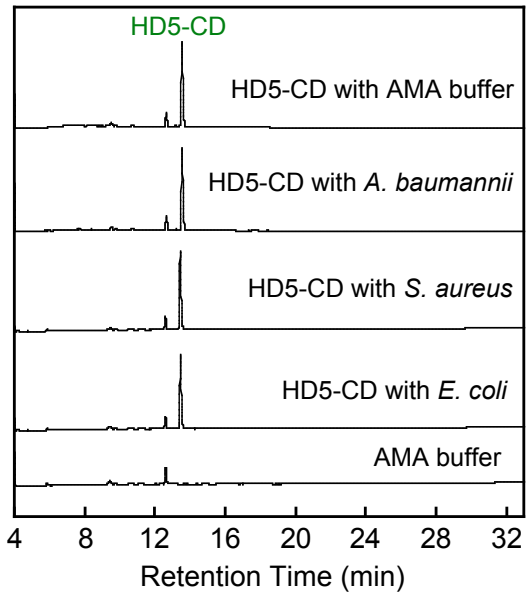

Figure S22. Stability of HD5-CD under the antimicrobial activity assay conditions and in the presence of select bacterial species monitored by HPLC. The peak at 12.4 min is from the AMA buffer. There is no change in the HD5-CD peak following incubation in the AMA buffer for 1 h at 37 °C in the absence or presence of bacteria. All chromatograms (220 nm absorbance) were obtained using a solvent gradient of 10–60% B over 30 min at 1 mL/min.

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