Materials and Methods

Amine Synthesis

Trityl- and methoxytrityl-protected cysteamine were prepared as described previously.[1]

Peptide Synthesis and Purification

Linear peptides were synthesized manually and/or using a Gyros Protein Technologies Prelude® X automated peptide synthesizer by standard Fmoc peptide synthesis with Rink amide resin (0.8 mmol/g). For manual synthesis, Fmoc-protected amino acids were activated for 30 minutes with 1-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIC) in dimethylformamide (DMF). For automated synthesis, (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and diisopropylethylamine (DIEA) were used for peptide activation. Fmoc deprotection was performed using 20% (v/v) piperidine in N-methyl-2-pyrrolidone (NMP) for all syntheses. The HP7 peptide, which contains a free carboxylic acid at the C-terminus, was synthesized on 2-chlorotrityl resin. To generate cystine disulfide macrocyclic peptides, partially purified cysteine-containing peptides were dissolved in 20% dimethyl sulfoxide (DMSO) and 80 mM ammonium carbonate in 5% (v/v) acetic acid to a final concentration of approximately 1 mM and reacted for 2 hours.

HBS peptides were synthesized as previously described except for the placement of the C-terminal HBS functional group.[2-4] For all HBS syntheses, 5 eq. of bromoacetic acid was activated with HOBt and DIC in DMF for 30 minutes and added to Fmoc-deprotected Rink Amide resin (loading = 0.2-0.25 mmol/g) for 2 hours. After washing with dichloromethane (DCM) and DMF, 5 eq. of the appropriate amine (allylamine, Mmt-cysteamine, and Trt-cysteamine for olefin, thioether, and disulfide HBS peptides, respectively) and 15 eq. of DIEA were added for 2 hours to install the first HBS functional group. Secondary amine coupling was performed overnight using 5 eq. of Fmoc-amino acid pre-activated with HOAt and DIC in DMF. The remaining amino acids and N-terminal carboxylic acids (4-pentenoic acid, 3-bromopropionic acid, and dithiodiglycolic acid for olefin, thioether, and disulfide HBS peptides, respectively) were coupled using standard Fmoc peptide synthesis procedures.

Once both HBS functional groups were installed, appropriate ring-closing protocols were followed as described previously.[2-4] For olefin HBS ring closure, the peptide was dried overnight and added to a microwave tube. Hoveyda-Grubbs II catalyst (20 mol%) and dry 1,2-dichloroethane (2 mL/mmol) were added sequentially with 5-10 minutes of nitrogen purging between additions. The reaction vessel was irradiated in a microwave (120°C, 10 minutes). For thioether HBS ring closure, the Mmt protecting group was removed by incubating 6-8x with 1% trifluoroacetic acid (TFA) and 5% triisopropylsilane (TIPS) in DCM for 10 minutes per incubation. After washing, 5 equivalents of 1,8-diazabicyclo(5.4.0)undec-7-ene in DMF was added for 20 minutes for macrocycle formation. On-resin ring closure for olefin and thioether HBS peptides was confirmed by microcleavage. Olefin and thioether HBS peptides were globally deprotected and cleaved from resin after ring closure by incubation with reagent K (82.5% TFA, 5% water, 5% phenol, 5% thioanisole, and 2.5% 1,2-ethanedithiol) for 2 hours, rotary evaporation of TFA, and peptide precipitation with diethyl ether. Disulfide ring closure was performed after a single round of HPLC purification by dissolving peptide in 20% dimethyl sulfoxide (DMSO) and 80 mM ammonium carbonate in 5% (v/v) acetic acid to a final concentration of approximately 1 mM and reacted for 2 hours.

All peptides were purified by reverse-phase HPLC using preparative-scale C_{18} columns. Purity was confirmed by analytical HPLC and MALDI mass spectrometry (Figure S8, S9).

Circular Dichroism (CD) Spectroscopy

Peptide concentration for all spectroscopy measurements was calculated using A_{280} measurements on a Nanodrop2000 and extinction coefficients calculated using ExPASy ProtParam.[5] Circular dichroism spectra were acquired using a Jasco J-1500 CD spectrometer at a concentration of 30 μM in 10 mM potassium fluoride (pH 7.4) in a 0.1 cm pathlength cell. Data for disulfide HBS reduction (with and without 10 equivalents of tris(2-carboxyethyl)phosphine (TCEP)) were collected as above in 10 mM potassium phosphate pH 7.4.

Nuclear Magnetic Resonance (NMR) Spectroscopy

Experiments were performed on a Bruker AVANCE III-600 MHz NMR spectrometer. HP7Δ-HBS was dissolved in 20 mM sodium phosphate buffer pH 6 to a final concentration of 1 mM. Proton, TOCSY, and NOESY spectra were acquired using Watergate solvent suppression. TOCSY and NOESY mixing times were 60 ms and 150 ms, respectively.[6] Spectral data were processed using the Bruker TOPSIN program. Resonance assignments, $^3J_{NHC\alpha H}$ coupling constants, and calculated φ angles are reported in Table S1. NOE cross-peaks and distance constraints are reported in Table S2.

Molecular Modeling

A starting structure for the olefin HP7 HBS peptide was constructed using the HP7 structure (PDB 2EVQ) in Maestro. Spectral overlap in the 1D NMR spectrum prohibited calculation of all $^3J_{NHC\alpha H}$ coupling constants using the Pardi parameterized Karplus equation,[7] so phi angles were calculated using method #1 of Wishart *et al.*[8] Distance constraints

were assigned to NOE cross-peaks using a simple scheme: strong – 2.5 ± 1.0 Å, medium – 3.0 ± 1.0 Å, and weak – 4.0 ± 1.0 Å. A total of 9 dihedral and 60 NOE parameters were used to constrain a Monte Carlo conformational search in the Macromodel 2015 program using the OPLS_2005 force field and mixed torsional and low-mode sampling. The 20 lowest energy structures show high convergence.

Protease Resistance Assay

Peptides were initially dissolved in distilled water. Equal volumes of peptide (250 pmol) and 200 µg/mL proteinase K stock dissolved in 50 mM Tris pH 8.0, 1 mM $CaCl_2$ were mixed at 25°C to a final volume of 20 µL. After incubation, 4 µL of 15% trichloroacetic acid (TCA) was added to quench the reaction. Samples were centrifuged at 10,000 rpm at 4°C for 10 minutes to remove precipitate. Supernatants were transferred to vials and analyzed by reverse-phase HPLC using an analytical C_{18} column. The fraction of peptide remaining was calculated by comparing integrated peaks corresponding to the original peptide with the integrated peak of the original peptide.

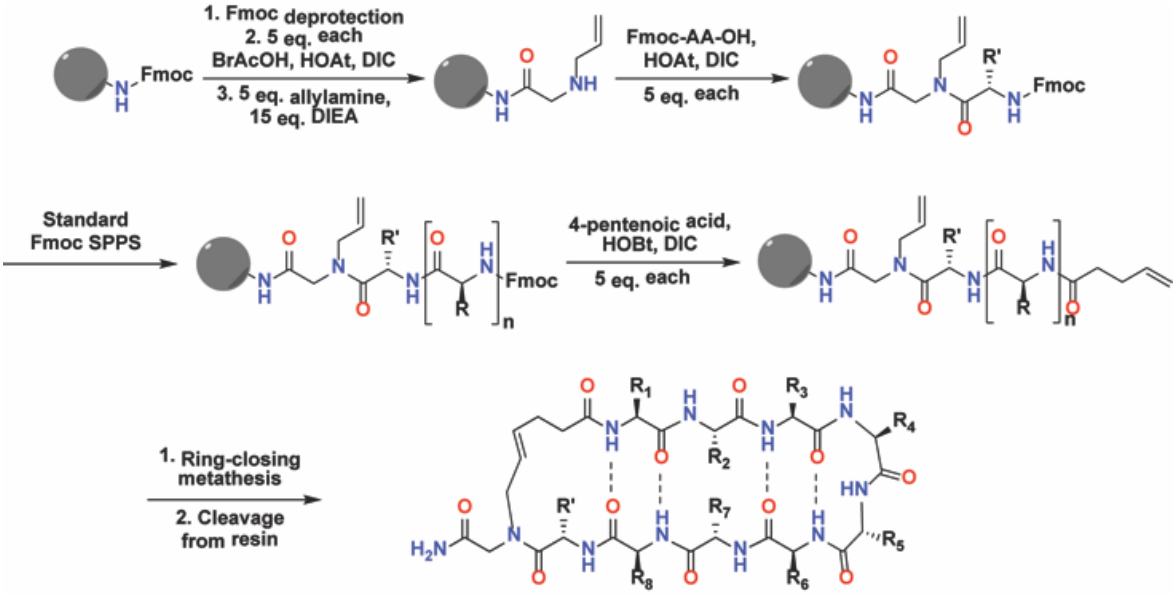


Figure S1. Olefin β-hairpin HBS synthesis

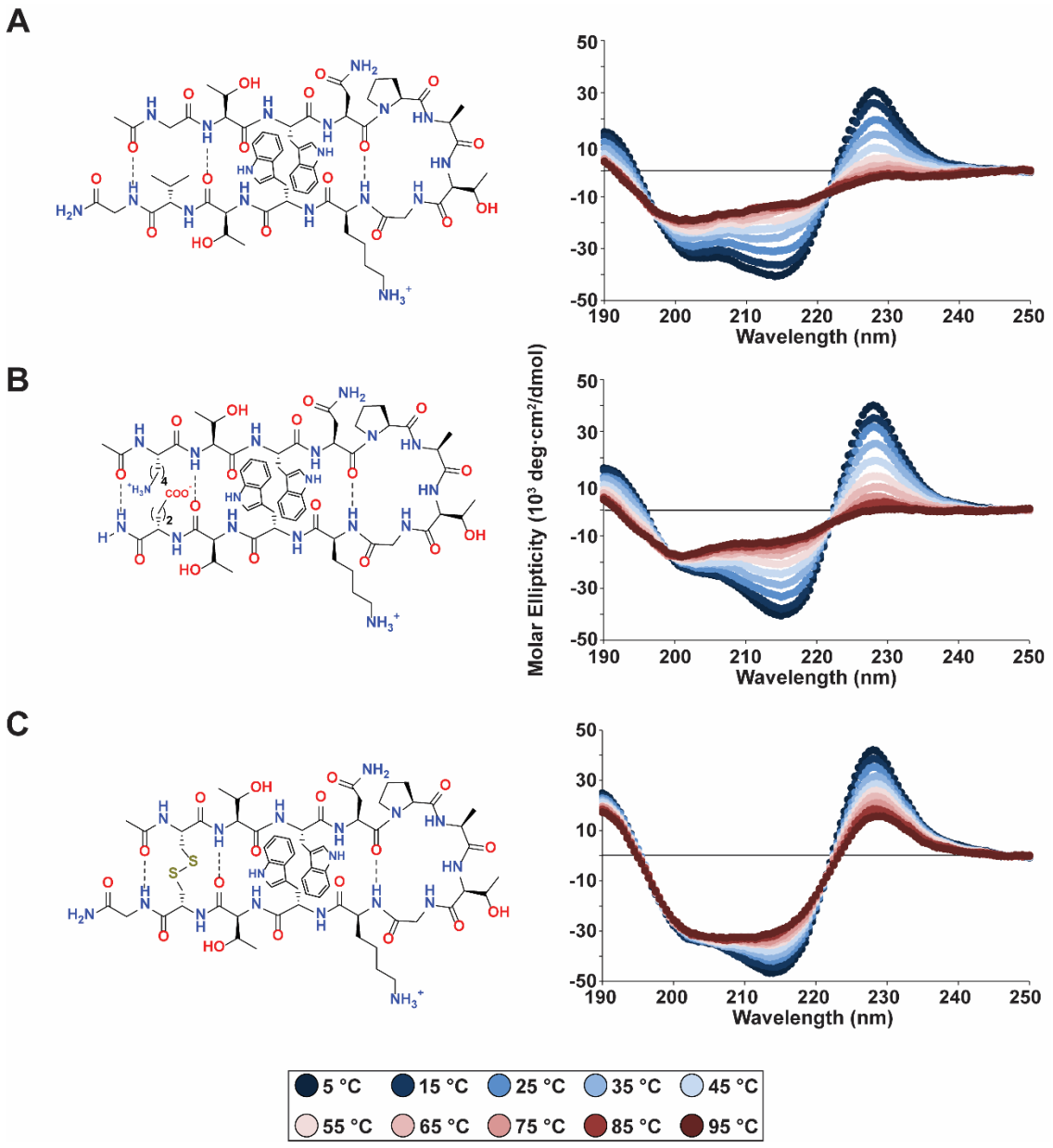


Figure S2. Temperature-dependent circular dichroism spectra for three HP7 control peptides. (A) Structure and CD spectra for HP7Δ-GVG (Ac-GTWNPATGKWTVG-$CONH_2$), which has the same C-terminal VG sequence as HP7Δ-HBS and Ac-G at the N-terminus to mimic the N-terminal 4-pentenoic acid of HP7Δ-HBS. Comparison to HP7Δ (Fig. S3B) suggests that the covalent HBS linker, and not the N- and C-terminal extensions of HP7Δ-HBS, improve structural stability. (B) Structure and CD spectra for HP7-Hbond (Ac-KTWNPATGKWTE-$CONH_2$), which has terminal amides in place of the terminal salt bridge of HP7. The reduced stability relative to HP7 (Fig. S3A) or HP7Δ-HBS (Fig. S3C) demonstrates that the covalent HBS linker is more stabilizing than a cross-strand hydrogen bond. (C) Structure and CD spectra for HP7Δ-dsCys (Ac-CTWNPATGKWTCG-$CONH_2$), which has a cystine disulfide bond between the N- and C-termini. This peptide serves as a model for a fully folded β-hairpin and shows similar stability to HP7Δ-HBS. All spectra were collected from 5-95°C at 10°C intervals and are overlaid using the color scheme above.

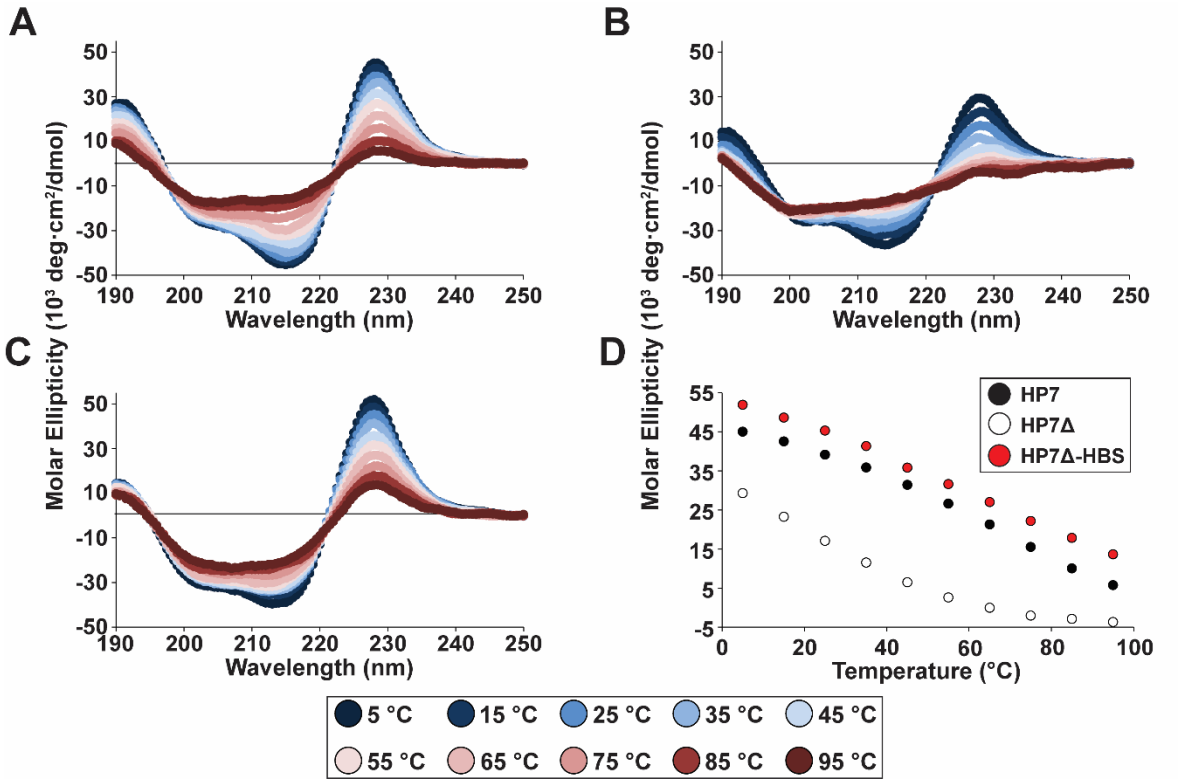


Figure S3. Temperature-dependent circular dichroism spectra for HP7, HP7Δ, and HP7Δ-HBS. Circular dichroism spectra for HP7 (A), HP7Δ (B), and HP7Δ-HBS (C), respectively, were collected from 5-95°C at 10°C intervals and are overlaid using the color scheme above. (D) Molar ellipticity at 228 nm is plotted as a function of temperature.

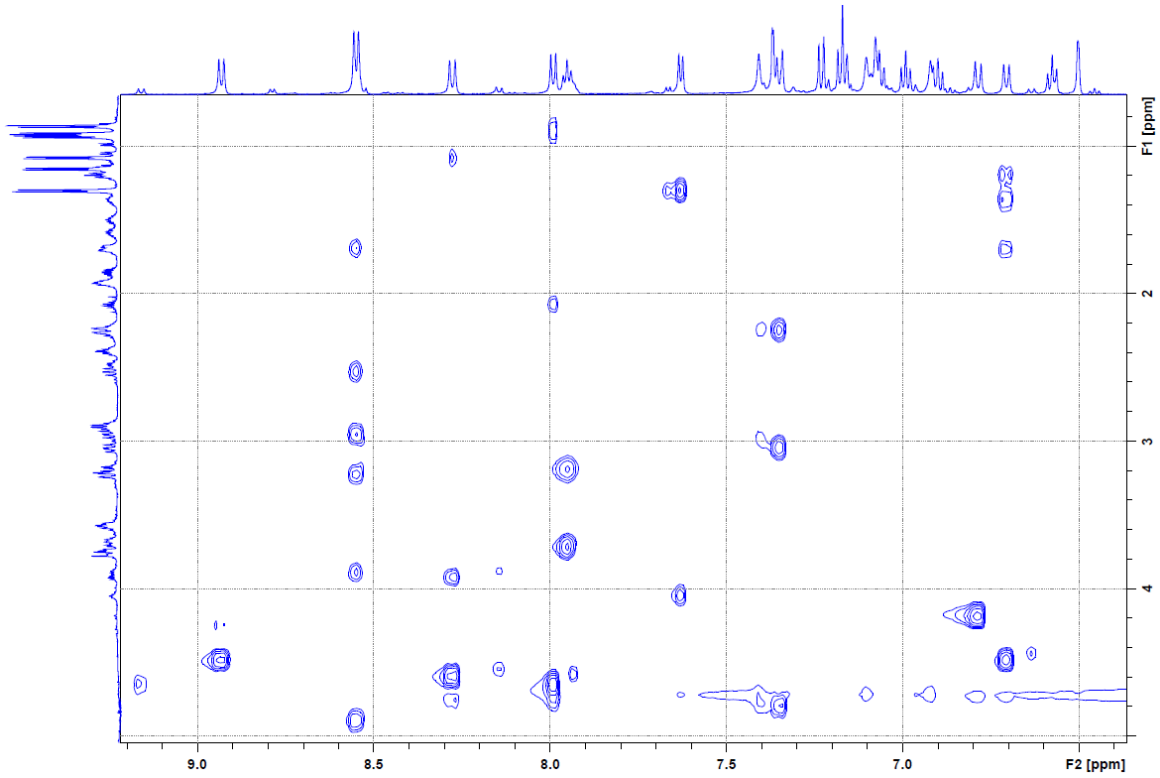


Figure S4. NMR fingerprint region for HP7Δ-HBS

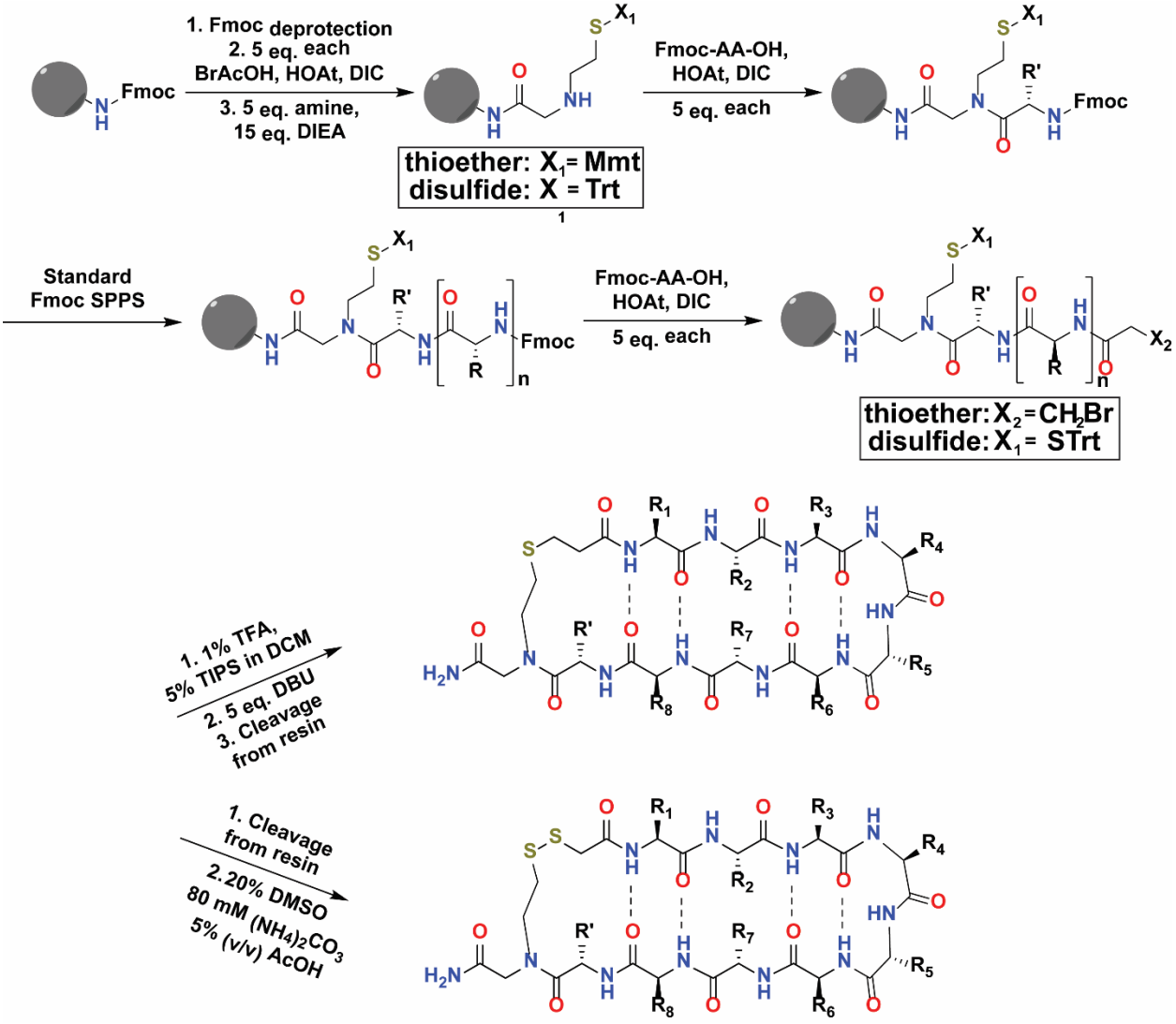


Figure S5. Thioether and disulfide β-hairpin HBS synthesis (for thioether HBS synthesis, X_1 = 4-methoxytrityl (Mmt) and X_2 = CH_2Br; for disulfide HBS synthesis - X_1 = trityl (Trt) and X_2 = S-Trt).

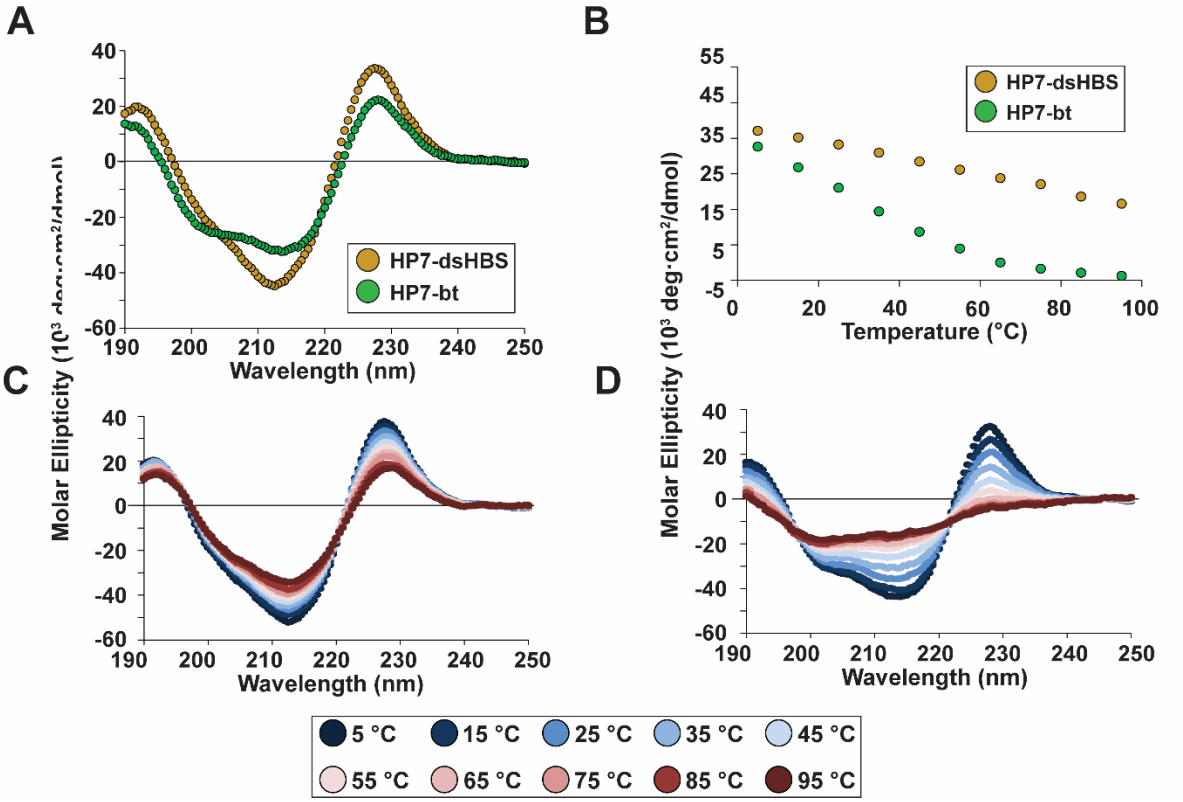


Figure S6. Temperature-dependent circular dichroism spectra for HP7Δ-dsHBS and HP7Δ-bt. (A) Circular dichroism spectra for HP7Δ-dsHBS (yellow) and HP7Δ-bt (green). (B) Molar ellipticity at 228 nm is plotted as a function of temperature. (C, D) Circular dichroism spectra for HP7Δ-dsHBS (C) and HP7Δ-bt (D) were collected from 5-95°C at 10°C intervals and are overlaid using the color scheme above. In panel C, molar ellipticity at 228 nm is plotted as a function of temperature.

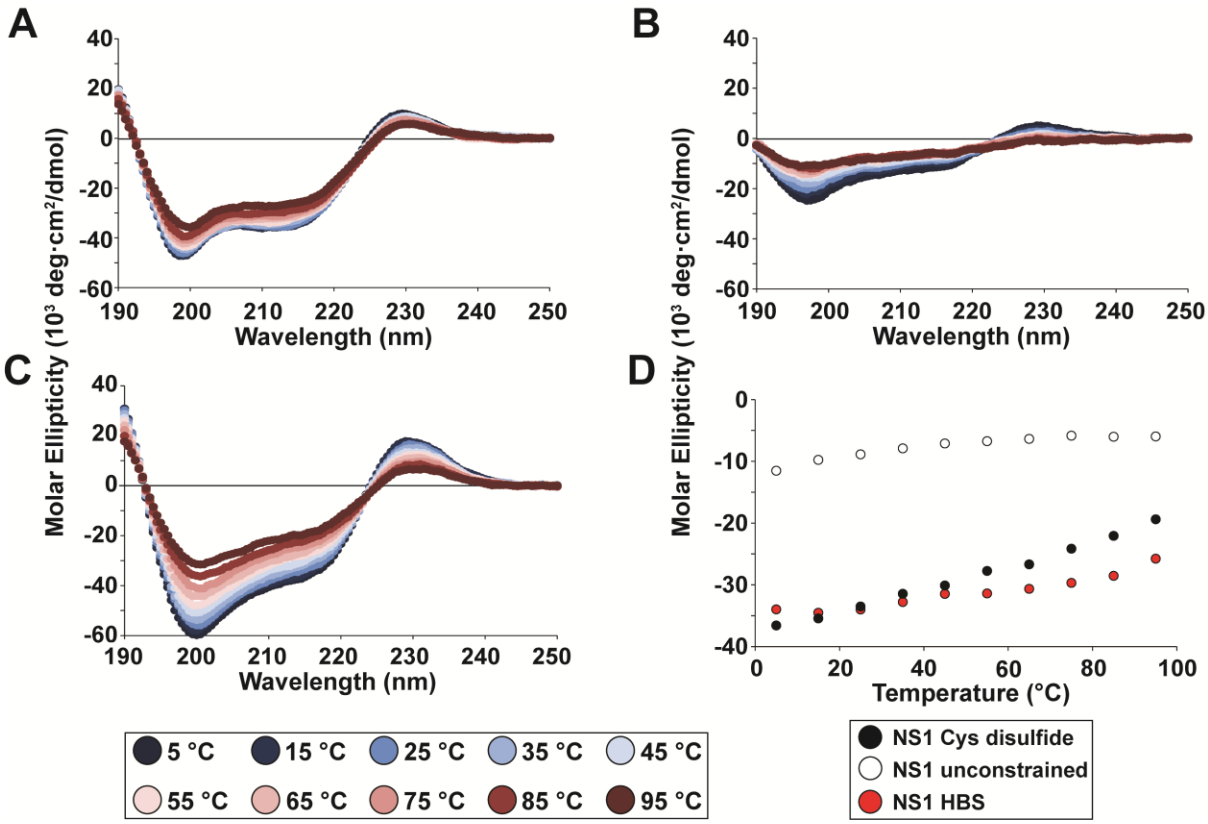
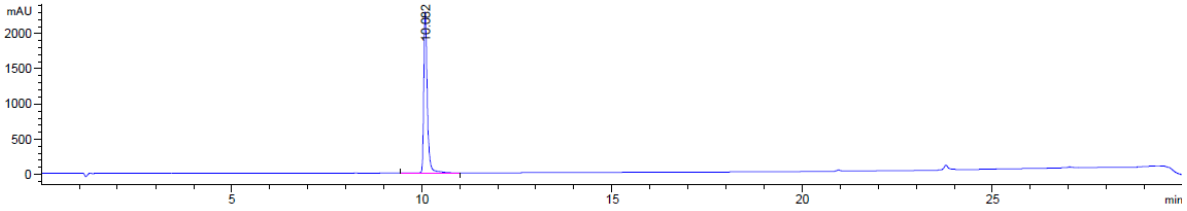
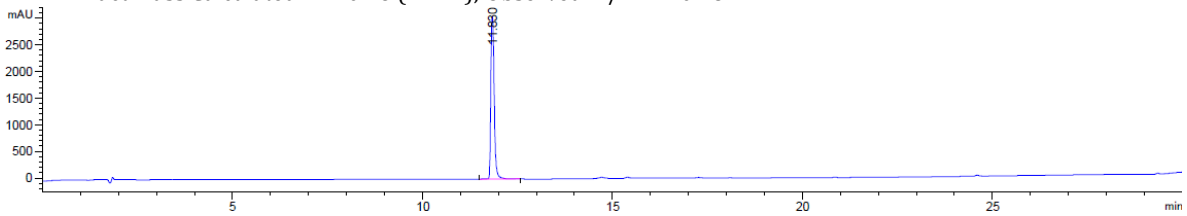


Figure S7. Temperature-dependent CD spectra for NS1-derived peptides. Temperature-dependent circular dichroism spectra for NS1 HBS (A), NS1 unconstrained (B), and NS1 Cys disulfide (C) (sequence: Ac-CGWRGQVYYCG-$CONH_2$), respectively, were collected from 5-95°C at 10°C intervals and are overlaid using the color scheme above. (D) Molar ellipticity at 215 nm is plotted as a function of temperature.

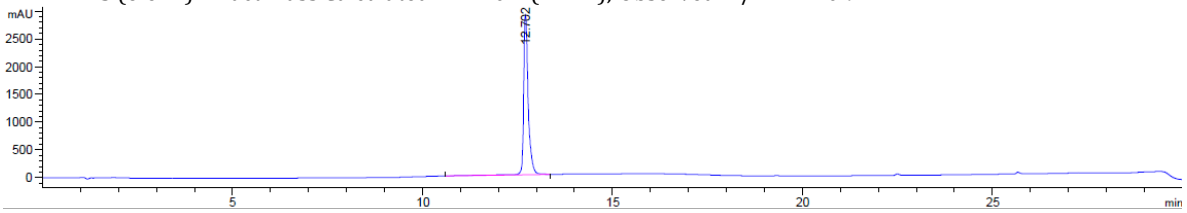
HP7: Exact Mass Calculated – 1418.7 ($M+H^+$); Observed m/z – 1418.9



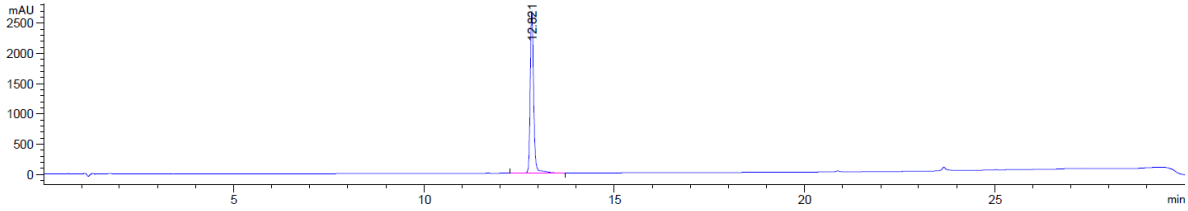
HP7Δ: Exact Mass Calculated – 1202.6 ($M+H^+$); Observed m/z – 1202.8



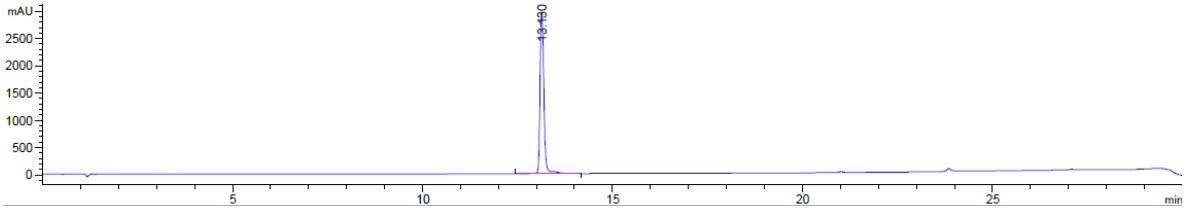
HP7Δ-HBS (olefin): Exact Mass Calculated – 1410.7 ($M+H^+$); Observed m/z – 1410.9



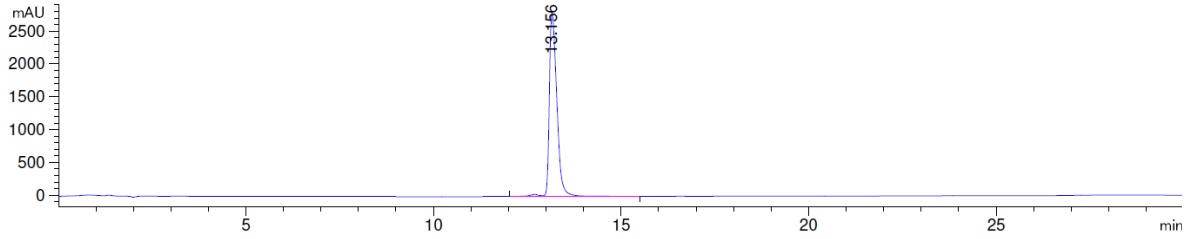
HP7Δ-teHBS: Exact Mass Calculated – 1430.7 ($M+H^+$); Observed m/z – 1430.8



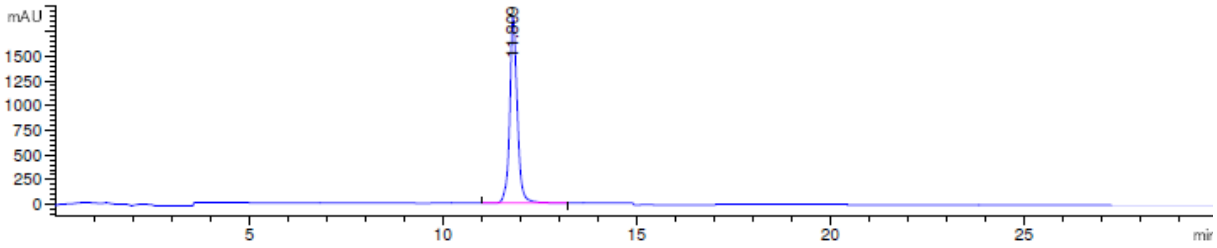
HP7Δ-dsHBS: Exact Mass Calculated – 1448.6 ($M+H^+$); Observed m/z – 1446.8



HP7Δ-GVG: Exact Mass Calculated – 1415.7 ($M+H^+$); Observed m/z – 1415.5



HP7-Hbond: Exact Mass Calculated – 1460.2 ($M+H^+$); Observed m/z – 1460.7



HP7Δ-dsCys: Exact Mass Calculated – 1463.6 ($M+H^+$); Observed m/z – 1464.2

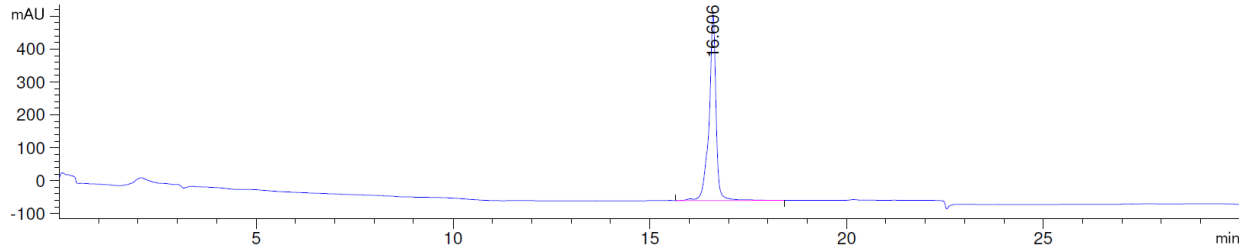
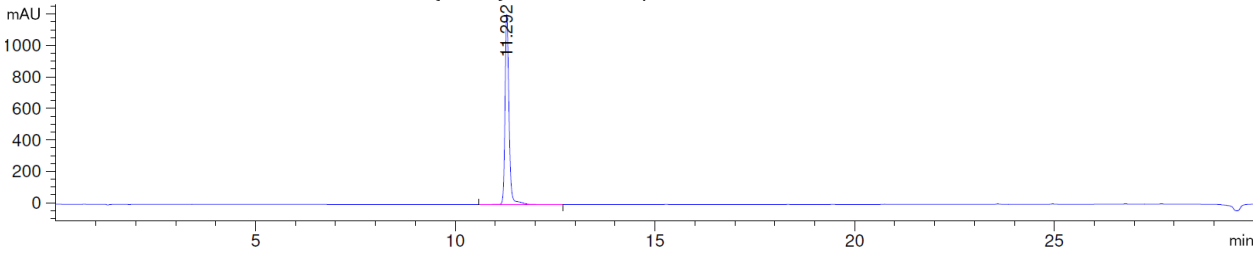
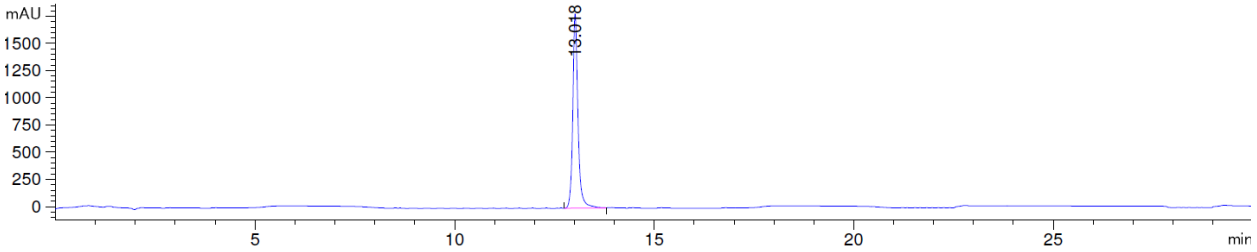


Figure S8. HP7 peptide analytical HPLC chromatograms were collected at 220 nm using a gradient of 5 to 95% acetonitrile over 30 minutes on a XTerra RP18 3.5 µm 2.1 x 150 mm column (Part No. 186000410) at a flow rate of 400 µL/min. Observed m/z values were obtained using a Bruker Maldi-TOF TOF UltrafleXtreme MS spectrometer.

NS1 HBS: Exact Mass Calculated – 1297.6 ($M+H^+$); Observed m/z – 1297.9



NS1 unconstrained: Exact Mass Calculated – 1282.6 ($M+H^+$); Observed m/z – 1282.6



NS1 Cys disulfide: Exact Mass Calculated – 1415.7 ($M+H^+$); Observed m/z – 1415.5

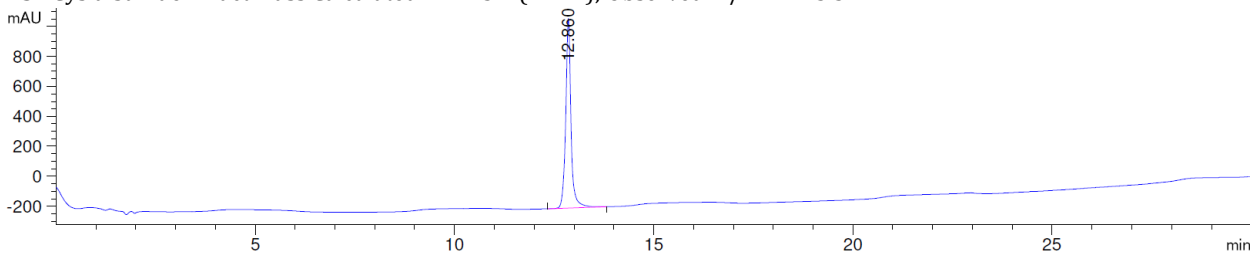


Figure S9. NS1 peptide analytical HPLC chromatograms were collected at 220 nm using a gradient of 5 to 95% acetonitrile over 30 minutes on a XTerra RP18 3.5 µm 2.1 x 150 mm column (Part No. 186000410) at a flow rate of 400 µL/min. Observed m/z values were obtained using a Bruker Maldi-TOF TOF UltrafleXtreme MS spectrometer.

Table S1. NMR assignments

Residue	HN	Hα	Hβ, Hβ'	Other	$^3J_{NHC\alpha H}$ (φ)[a]
4-pentenoic acid-1	n/a	2.401	2.245, 2.497	γ - 5.591 (J = 15.72); δ - 5.678 (J = 15.66); ε,ε' 3.766, 4.363	n/a
Thr2	8.358	4.620	3.929	1.094	9.318 (-106.6)
Trp3	8.639	3.903	2.535, 1.697	ε1 9.888; δ1 6.513; ε3 5.815; ζ3 6.587; η2 6.917; ζ2 7.240	6.618 (-79.1)
Asn4	7.378	4.803	3.060, 2.260	γNH2 7.14	9.318 (-106.6)
Pro5	n/a	3.690	2.305, 2.260	γ,γ' 1.872, 1.929; δ,δ' 3.590, 3.566	n/a
Ala6	7.666	4.066	1.314	n/a	8.118 (-91.9)
Thr7	6.797	4.208	4.060	γ 0.926	4.818 (-65.1)
Gly8	7.982	3.205, 3.724	n/a	n/a	n/a
Lys9	6.704	4.509	1.708, 1.377	γ 1.215; δ,δ'1.513, 1.603; ε 2.907; ζNH3 Exchange	9.618 (-113.8)
Trp10	8.620	4.920	3.253, 2.971	ε1 10.110; δ1 7.384; ε3 7.181; ζ3 7.009; η2 7.078; ζ2 7.186	9.318 (-106.6)
Thr11	8.991	4.505	4.264	γ 1.169	9.318 (-106.6)
Val12	8.077	4.678	2.095	γ,γ' 0.879, 0.952	9.018 (-102.0)
Gly13	n/a	3.786, 4.290	n/a	C-terminal amide: 6.967, 7.481	n/a

[a] Estimated from TOCSY spectra using protocol #1 from Wishart *et al.*[8]

Table S2. NOE cross-peaks and corresponding distance constraints.

Proton 1 (chemical shift)	Proton 2 (chemical shift)	Strength (distance constraint)
Trp10 Hδ1 (7.384)	Trp10 Hβ' (2.971)	Strong (2.5 ± 1.0 Å)
Trp10 Hδ1 (7.384)	Trp10 Hβ (3.253)	Strong (2.5 ± 1.0 Å)
Trp10 Hε3 (7.181)	Trp10 Hβ' (2.971)	Medium (3.0 ± 1.0 Å)
Trp10 Hε3 (7.181)	Trp10 Hβ (3.253)	Strong (2.5 ± 1.0 Å)
Trp10 Hε3 (7.181)	Trp10 Hα (4.920)	Strong (2.5 ± 1.0 Å)
Trp3 Hδ1 (6.513)	Trp3 Hβ' (1.697)	Strong (2.5 ± 1.0 Å)
Trp3 Hδ1 (6.513)	Trp3 Hβ (2.535)	Strong (2.5 ± 1.0 Å)
Trp3 Hδ1 (6.513)	Trp3 Hα (3.903)	Medium (3.0 ± 1.0 Å)
Trp3 Hε3 (7.181)	Trp3 Hβ' (1.697)	Strong (2.5 ± 1.0 Å)
Trp3 Hε3 (7.181)	Trp3 Hβ (2.535)	Strong (2.5 ± 1.0 Å)
Trp3 Hε3 (7.181)	Trp3 Hα (3.903)	Strong (2.5 ± 1.0 Å)
4-pent1 Hδ (5.678)	4-pent1 Hβ (2.245)	Medium (3.0 ± 1.0 Å)
4-pent1 Hδ (5.678)	4-pent1 Hα (2.401)	Weak (4.0 ± 1.0 Å)
4-pent1 Hγ (5.591)	4-pent1 Hα (2.401)	Medium (3.0 ± 1.0 Å)
4-pent1 Hγ (5.591)	4-pent1 Hε (3.766)	Medium (3.0 ± 1.0 Å)
4-pent1 Hγ (5.591)	4-pent1 Hε' (4.363)	Medium (3.0 ± 1.0 Å)
Thr2 HN (8.358)	Thr2 Hβ (3.929)	Medium (3.0 ± 1.0 Å)
Thr7 HN (6.797)	Thr7 Hγ (0.926)	Medium (3.0 ± 1.0 Å)
Thr7 HN (6.797)	Thr7 Hβ (4.060)	Medium (3.0 ± 1.0 Å)
Trp10 HN (8.620)	Trp10 Hβ' (2.971)	Medium (3.0 ± 1.0 Å)
Asn4 HN (7.378)	Asn Hβ' (2.260)	Medium (3.0 ± 1.0 Å)
Asn4 HN (7.378)	Asn Hβ (3.060)	Medium (3.0 ± 1.0 Å)
Ala6 HN (7.666)	Ala Hβ (1.314)	Medium (3.0 ± 1.0 Å)
Lys9 HN (6.704)	Lys9 Hγ (1.215)	Medium (3.0 ± 1.0 Å)
Lys9 HN (6.704)	Lys9 Hβ (1.377)	Medium (3.0 ± 1.0 Å)
Val12 HN (8.077)	Val12 Hγ (0.879)	Medium (3.0 ± 1.0 Å)
Val12 HN (8.077)	Val12 Hβ (2.095)	Medium (3.0 ± 1.0 Å)
Thr7 Hα (4.207)	Thr7 Hγ (0.926)	Strong (2.5 ± 1.0 Å)

Trp10 Hδ1 (7.384)	Trp3 Hε3 (5.815)	Medium (3.0 ± 1.0 Å)
Trp10 Hδ1 (7.384)	Trp3 Hζ3 (6.587)	Strong (2.5 ± 1.0 Å)
Trp3 Hζ2 (7.240)	Gly8 Hα (3.205)	Strong (2.5 ± 1.0 Å)
Trp3 Hζ2 (7.240)	Gly8 Hα' (3.724)	Weak (4.0 ± 1.0 Å)
Trp10 Hε3 (7.181)	Trp3 Hβ' (1.697)	Medium (3.0 ± 1.0 Å)
Trp10 Hε3 (7.181)	4-pent1 Hα (2.401)	Medium (3.0 ± 1.0 Å)
Trp10 Hε3 (7.181)	4-pent1 Hβ' (2.497)	Medium (3.0 ± 1.0 Å)
Trp10 Hε3 (7.181)	Trp3 Hα (3.903)	Medium (3.0 ± 1.0 Å)
Trp10 Hε3 (7.181)	Trp3 Hε3 (5.815)	Weak (4.0 ± 1.0 Å)
Trp10 Hζ3 (7.009)	Trp3 Hβ' (1.697)	Weak (4.0 ± 1.0 Å)
Trp3 Hη2 (6.917)	Gly8 Hα (3.205)	Strong (2.5 ± 1.0 Å)
Trp3 Hδ1 (6.513)	Pro5 Hγ' (1.929)	Medium (3.0 ± 1.0 Å)
Trp3 Hδ1 (6.513)	Pro5 Hβ' (2.260)	Weak (4.0 ± 1.0 Å)
Trp3 Hδ1 (6.513)	Pro5 Hδ (3.590)	Medium (3.0 ± 1.0 Å)
Trp3 Hε3 (5.815)	Trp10 Hα (4.920)	Weak (4.0 ± 1.0 Å)
Trp10 Hα (4.920)	Trp3 Hα (3.903)	Strong (2.5 ± 1.0 Å)
Asn4 Hα (4.803)	Pro5 Hδ (3.590)	Strong (2.5 ± 1.0 Å)
Thr2 Hα (4.620)	Trp3 Hα (3.903)	Medium (3.0 ± 1.0 Å)
Thr7 Hβ (4.060)	Ala6 Hβ (1.314)	Strong (2.5 ± 1.0 Å)
Asn Hβ' (2.260)	Thr11 Hγ (1.169)	Weak (4.0 ± 1.0 Å)
Thr2 HN (8.358)	4-pent1 Hβ (2.245)	Weak (4.0 ± 1.0 Å)
Thr2 HN (8.358)	4-pent1 Hβ' (2.497)	Strong (2.5 ± 1.0 Å)
Thr7 HN (6.797)	Ala6 Hβ (1.314)	Medium (3.0 ± 1.0 Å)
Thr7 HN (6.797)	Asn4 Hβ (3.060)	Medium (3.0 ± 1.0 Å)
Trp3 HN (8.639)	Thr2 Hα (4.620)	Strong (2.5 ± 1.0 Å)
Trp10 HN (8.620)	Trp3 Hβ (2.535)	Medium (3.0 ± 1.0 Å)
Trp10 HN (8.620)	Trp3 Hβ' (1.697)	Medium (3.0 ± 1.0 Å)
Asn4 HN (7.378)	Trp3 Hα (3.903)	Strong (2.5 ± 1.0 Å)
Ala6 HN (7.666)	Pro5 Hα (3.690)	Weak (4.0 ± 1.0 Å)
Lys9 HN (6.704)	Asn4 Hβ (3.060)	Medium (3.0 ± 1.0 Å)
Lys9 HN (6.704)	Gly8 Hα (3.205)	Medium (3.0 ± 1.0 Å)
Lys9 HN (6.704)	Gly8 Hα' (3.724)	Weak (4.0 ± 1.0 Å)
Val12 HN (8.077)	Thr11 Hβ (4.264)	Strong (2.5 ± 1.0 Å)

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