Design, synthesis and protein-targeting properties of thioether-linked hydrogen bond surrogate helices

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Supplementary Information

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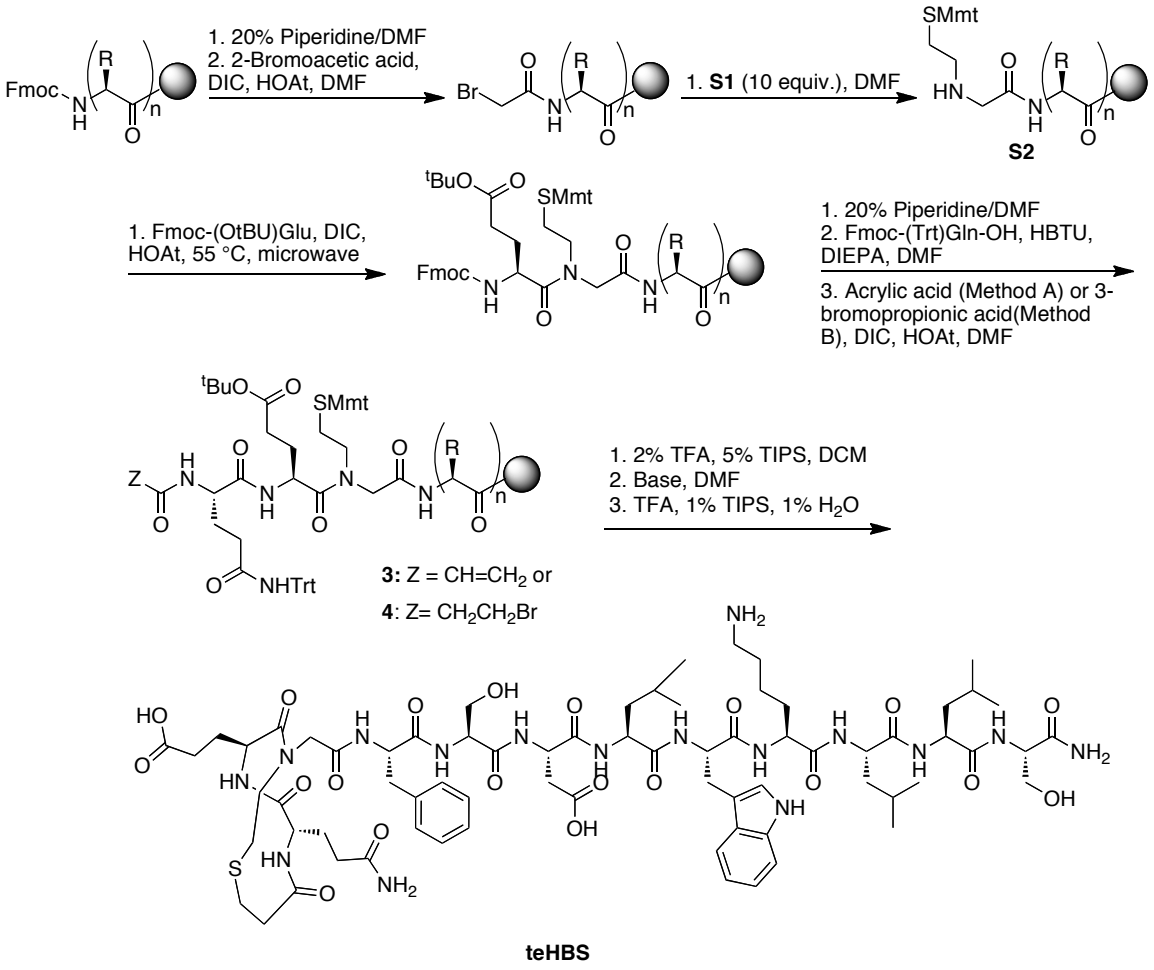
General. Commercial-grade reagents and solvents were used without further purification except as indicated. All Fmoc amino acids, peptide synthesis reagents, and Rink Amide MBHA resin were obtained from Novabiochem (San Diego, USA). All other reagents were obtained from Sigma-Aldrich (St. Louis, USA). Reversed-phase HPLC experiments were conducted with 4.6 x 150 mm (analytical scale) or 21.4 x 150 mm (preparative scale) Waters C_{18} Sunfire columns using a Beckman Coulter HPLC equipped with a System Gold 168 Diode array detector. The typical flow rates for analytical and preparative HPLC were 1 mL/min and 8 mL/min, respectively. In all cases, 0.1% aqueous trifluoroacetic acid and acetonitrile buffers were used. Proton and carbon NMR spectra of monomers were obtained on a Bruker AVANCE 400 MHz spectrometer. Proton NMR spectra of HBS peptides were recorded on a Bruker AVANCE 500 MHz spectrometer. High-resolution mass spectra (HRMS) were obtained on a LC/MSD TOF (Agilent Technologies). LCMS data was obtained on an Agilent 1100 series LC/MSD (XCT) electrospray trap. MS/MS sequencing was performed using a Bruker Daltronics Ultraflex MALDI TOF/TOF mass spectrometer. Ms/ms spectra were analyzed using Bruker Daltronics flexAnalysis software, version 2.4.

Synthesis of teHBS 1



Scheme S1. Synthetic scheme for the preparation of teHBS **1**.

Knorr amide resin (0.69 mmol/g; 362 mg, 0.25 mmol) was swelled in DMF (5 mL) for 10 min prior to Fmoc group removal by treatment with 3 mL of 20% piperidine in NMP (5 min and then 20 min). The resin was then washed with DMF (3 x 5mL), DCM (3 x 5mL) and DMF (3 x 5mL). The free amine was treated with pre-activated Fmoc-Ser(OtBU)-OH, which was prepared from Fmoc-Ser(OtBu)-OH (409 mg, 1.25 mmol), HBTU (474 mg, 1.25 mmol) and *N*,*N*-diisopropylethylamine (218 μL, 1.25 mmol) in DMF (3 mL).

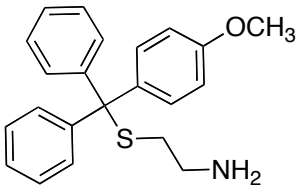
After 2 h of shaking, the resin was washed with DMF (3 x 5mL), DCM (3 x 5mL) and DMF (3 x 5mL). The Fmoc group was removed from the Fmoc-Ser(OtBu) functionalized resin using 20% piperidine in NMP (5 min and then 20 min) and the above procedure repeated for additional amino acid residues.

For inclusion of the ethane-2-thiol group, free amine was treated with pre-activated 2-bromoacetic acid, which was prepared from 2-bromoacetic acid (347 mg, 2.5 mmol), DIC (391 μL, 2.5 mmol) and HOAt (170 mg, 1.25 mmol) in DMF (3 mL). After 1 h of shaking, the resin was washed with DMF (3 x 5mL), DCM (3 x 5mL) and DMF (3 x 5mL). The bromoacetyl group was treated with *S*-(4-methoxytrityl)-2-aminoethanethiol (873 mg, 2.5 mmol) dissolved in DMF(3 mL). After 30 min of shaking, the resin was washed with DMF (3 x 5mL), DCM (3 x 5mL) and DMF (3 x 5mL). Chloranil test was used to monitor the reaction progress.

The secondary amine **S2** (scheme S1) was treated with pre-activated Fmoc-Glu(OtBu)-OH and heated to 55 °C for 60 min under microwave conditions. Pre-activated Fmoc-Glu(OtBu)-OH was prepared from Fmoc-Glu(OtBu)-OH (532 mg, 1.25 mmol), DIC (196 μL, 1.25 mmol) and HOAt (85 mg, 0.63 mmol) in DMF (3 mL). The reaction was monitored using a chloranil test. The subsequent Fmoc-Gln(Trt) residue was incorporated using the method outlined above for coupling of Fmoc-Ser(OtBu)-OH to resin. After removal of the Fmoc group, the free amine was treated with pre-activated acrylic acid for **3**, which was prepared from acrylic acid (86 μL, 1.25 mmol), DIC (196 μL, 1.25 mmol) and HOAt (85 mg, 0.63 mmol) in DMF (3 mL). After 1 h of shaking, the resin was

washed with DMF (3 x 5 mL), DCM (3 x 5 mL) and DMF (3 x 5 mL). 3-Bromopropionic acid (191 mg, 1. 25 mmol) was used in the place of acrylic acid for synthesis of **4**.

_S_-(4-Methoxytrityl)-2-aminoethanethiol (S1)[1]



Cysteamine hydrochloride (1.75 g, 16.2 mmol) and 4-methoxytrityl chloride (5 g, 16.2 mmol) were dissolved in a mixture of DMF(25 mL) and dichloromethane (25 mL) and stirred at room temperature under an atmosphere of argon for 1 h. The reaction mixture was concentrated *in vacuo* and diluted with water (150 mL) before extraction with diethyl ether (3 x 50 mL). The organic layers were combined, washed with brine (100 mL), dried over anhydrous magnesium sulfate and evaporated to dryness to afford a colorless oil (5.5 g, 15.7 mmol, 97%). ^{1}H NMR (400 MHz; $CDCl_3$) d 2.26 (2H, t, *J* 6.6 Hz), 2.53 (2H, t, *J* 6.6 Hz), 3.70 (3H, s), 6.72 (4H, m), 7.11 (1H, m), 7.16-7.25 (7H, m), 7.30-7.34 (2H, m). ^{13}C NMR (100 MHz; $CDCl_3$) d 36.24, 41.09, 55.23, 65.62, 113.11, 126.56, 127.85, 129.41, 130.69, 137.30, 145.53, 158.06.

Selective *S*-4-methoxytrityl deprotection and base catalyzed cyclization. Resin (0.25 mmol) was swelled in DCM (3 mL) before treatment with 2% TFA and 5% TIPS in DCM (5 mL). After shaking for 15 min, the resin was washed with DCM (3 x 5 mL). This deprotection procedure was repeated until no yellow color persisted in the reaction solvent (3 x 15 min). The resin was then washed with DMF (3 x 5mL), DCM (3 x 5mL) and DMF (3 x 5 mL). An Ellman test was used to confirm the presence of free thiol. [2, 3]

Synthesis of teHBS 1 *via* Method A. The free thiol functionalized resin, **3**, was swelled in DMF (3 mL) before addition of appropriate base and the reaction was monitored using an Ellman test (see Table 1 for reaction conditions). Reactions were carried out at 25 °C.

Table S1. On resin cyclization conditions for Michael addition reaction with **3**.

Base	Equivalents	Reaction Time (h)	Ellman Test for Thiols
Triethylamine	5	16	Weak positive
Diisopropylethylamine	5	16	Weak positive
n-Butylamine	5	16	Negative
DBU	5	12	Negative

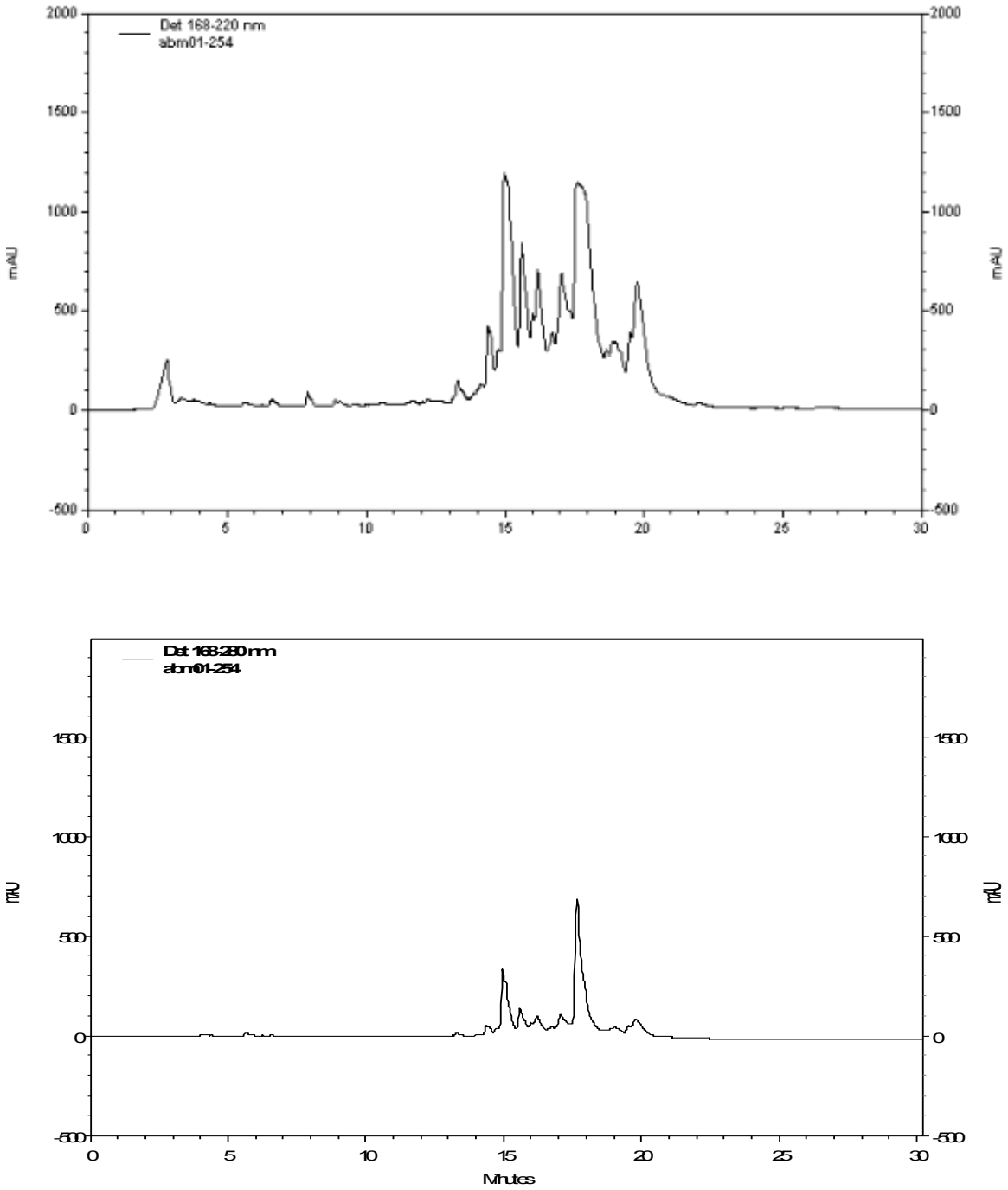


Figure S1. Crude analytical HPLC traces at 220 and 280 nm for reaction of **3** with DBU. 10% B to 60% B in 30 min; A: 0.1% aqueous TFA, B: acetonitrile; flow rate: 1 mL/min.

Synthesis of teHBS *via* Method B. Free thiol functionalized resin, **4**, was swelled in DMF (3 mL) before addition of appropriate base and the reaction was monitored using an Ellman test (see Table 1 for reaction conditions). Reactions were carried out at 25 °C.

Table S2. On resin cyclization reaction conditions for substitution reaction with **4**.

Base	Equivalents	Reaction Time (h)	Ellman Test for Thiols
Triethylamine	5	30 min	Weak positive
N,N-Diisopropylethylamine	5	30 min	Weak positive
n-Butylamine	5	2 h	Negative
DBU	5	10 min	Negative

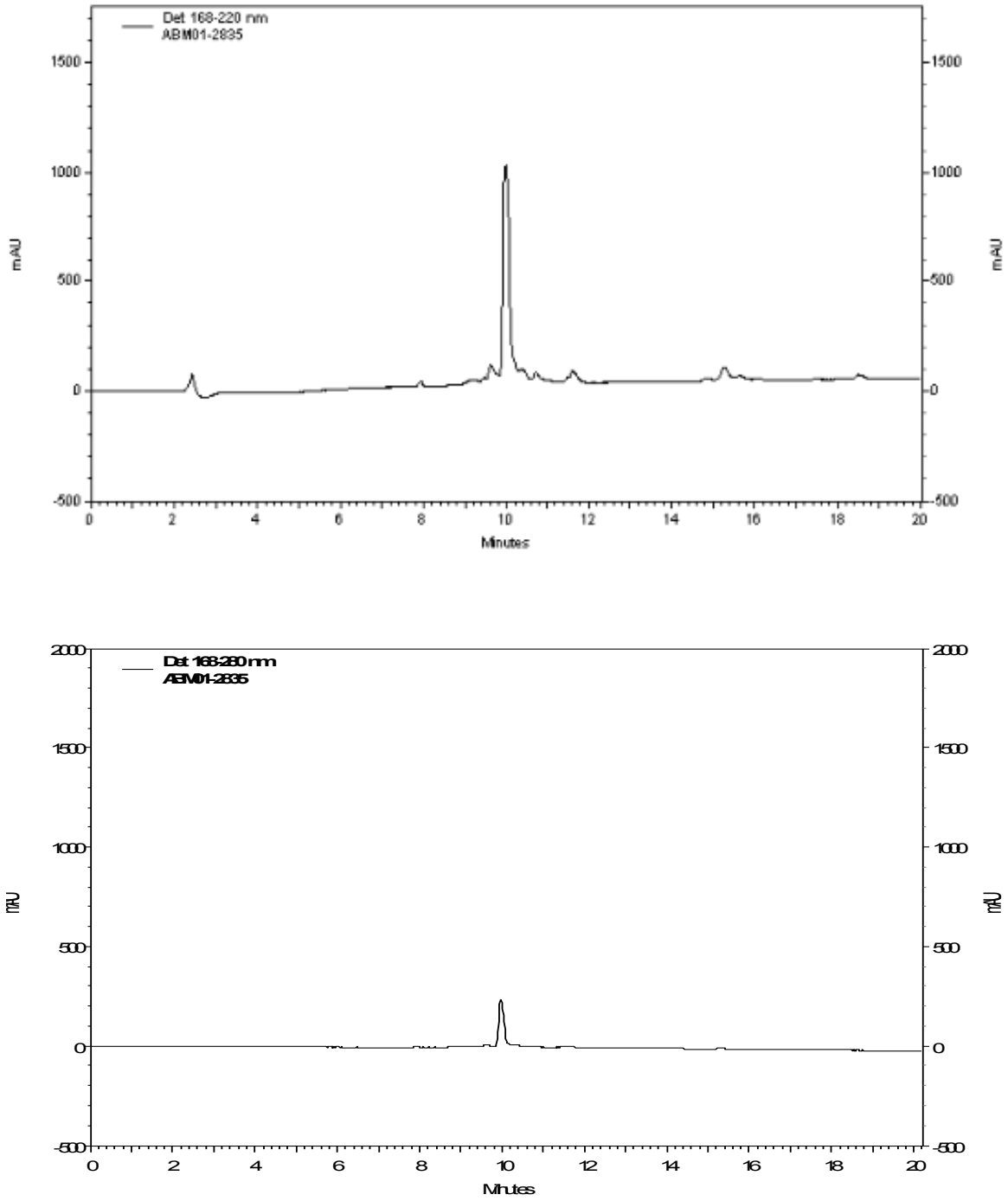
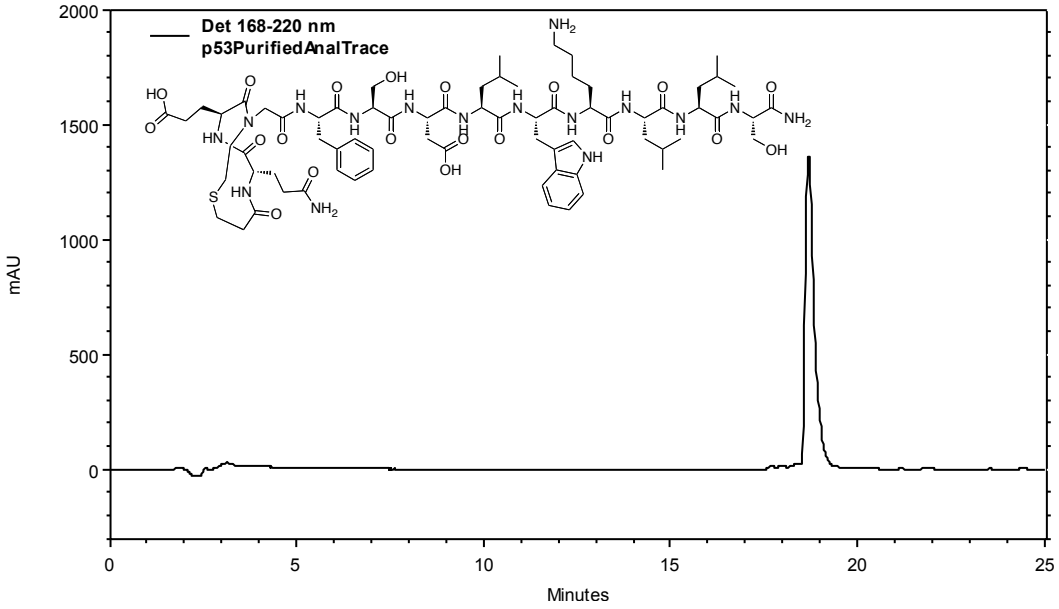


Figure S2. Crude analytical HPLC traces at 220 and 280 nm after reaction of **4** with DBU. 10% B to 60% B in 20 min; A: 0.1% aqueous TFA, B: acetonitrile; flow rate: 1 mL/min.



5% B to 95% B in 25 min; A: 0.1% aqueous TFA, B: acetonitrile; flow rate: 1 mL/min.

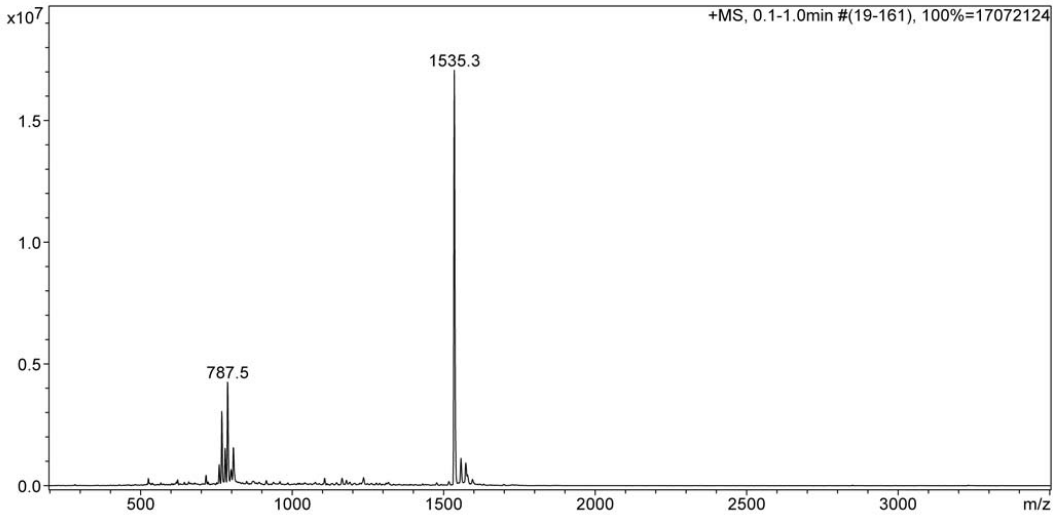


Figure S3. Analytical HPLC trace and mass spectrum after semi-preparative HPLC purification of teHBS **1**. Exact mass calc'd $[M+H]^+$ (m/z): 1535.7; found: 1535.3.

Description of Mdm2 Binding Studies

Protein Expression and Purification. Competent BL21 DE3 pLySS E. coli cells were transformed by heat shocking the bacteria at 42° C for 30 sec in media containing a pET-14B vector containing a His_6-tagged $Mdm2_{25-117}$ fusion protein. Cells were grown on ampicillin containing agar plates (50 mg/mL), and a single culture was used to inoculate a 100 mL overnight culture of LB media containing ampicillin (50 mg/mL) at 37 °C. 500 mL of terrific broth (4L flask) was seeded with 25 mL of overnight culture and incubated at 30 °C for 5 hours (UV abs =1, 600 nm) before induction of protein expression with 0.4 mM IPTG. The flask was incubated at 30 °C for an additional 4.5 hours. The cells were harvested by centrifugation at 3700 g for 45 minutes and the supernatant was discarded. The cells were resuspended in 50 mL of binding buffer (5mM NaH_2PO_4, 30 mM NaCl, 0.5 mM imidazole, and 0.2 mM BME, Roche® protease inhibitor cocktail, pH 7.9), and lysed by sonication in ice (8 x 15 seconds pulses over 30 minutes). The cells were again centrifuged at 3700 g for 40 minutes at 4 °C, and the resulting supernatant containing the desired Mdm2 fusion protein was allowed to bind nickel beads with shaking at 4 °C for 2 hours. Protein was eluted form the beads with elution buffer (5 mM NaH_2PO_4, 30 mM NaCl, 25 mM imidazole and 0.2mM BME). Protein was concentrated using an Amicon®Ultra centifuge filter (3 kD cut-off) and characterized by SDS-PAGE analysis.

Description of protein binding studies. The relative affinity of peptides for N-terminal His_6-tagged $Mdm2_{25-117}$ was determined using a fluorescence polarization based competitive binding assay with fluorescein labeled p53 peptide **fl-p53**. The polarization experiments were performed with a DTX 880 Multimode Detector (Beckman) at 25° C,

with excitation and emission wavelengths at 485 and 525 nm, respectively. All samples were prepared in 96 well plates in dialysis buffer with 0.1% pluronic F-68 (Sigma). The binding affinity (K_D) values reported for each peptide are the averages of 3 individual experiments, and were determined by fitting the experimental data to a sigmoidal dose-response nonlinear regression model on GraphPad Prism 4.0. The concentration of the Mdm2 protein was determined by UV absorbance at 280 nm.

Prior to the competition experiments, the affinity of the **fl-p53** for $Mdm2_{25\text{-}117}$ was determined by monitoring polarization of the fluorescent probe upon binding $Mdm2_{25\text{-}117}$ (Figure S4). Addition of an increasing concentration (0 nm to 4 ìM) of $Mdm2_{25\text{-}117}$ protein to a 15 nM solution of **fl-p53** in $Mdm2_{25\text{-}117}$ dialysis buffer afforded the saturation binding curve (Figure 4). The IC_{50} value obtained from this binding curve was fit into equation (**1**) to calculate the dissociation constant (K_{D1}) for the p53/Mdm2 complex.[4]

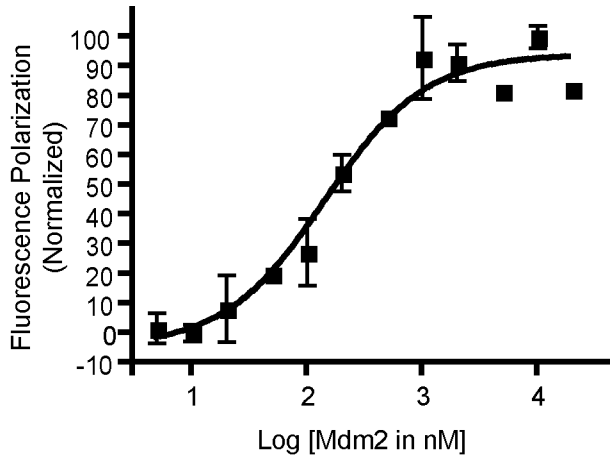


Figure S4. Saturation binding of $Mdm2_{25\text{-}117}$ with **fl-p53**.

$$K_{D1} = (R_T*(1-F_{SB}) + L_{ST}*F_{SB}^2)/F_{SB}-L_{ST} \qquad (1)$$

where:

R_T = Total concentration of Mdm2 protein

L_{ST} = total concentration of p53 fluorescent peptide

F_{SB} = Fraction of bound p53 fluorescent peptide

The K_{D1} of **fl-p53** was determined to be 129 ± 38 nM. For competition experiments, appropriate concentrations of the teHBS or HBS (10 nm to 100 ìM) were added to a solution of 300 nM Mdm2 and 15 nM FluP53. The resulting mixtures were incubated at 25° C for 60 minutes before measuring the degree of dissociation of **fl-p53** by polarization. The IC_{50} was fit into equation (**2)** to calculate the K_{D2} value of teHBS **1** and HBS **2**.[4]

$$K_{D2} = K_{D1}*F_{SB}*((L_T/(L_{ST}*F_{SB}^2 - (K_{D1} + L_{ST} + R_T)*F_{SB} + R_T)) - 1/(1 - F_{SB})) \quad (\mathbf{2})$$

where:

K_{D1} = K_D of fluorescent probe fl-p53

R_T = Total concentration of Mdm2 protein

L_{ST} = total concentration of p53 fluorescent peptide

F_{SB} = Fraction of bound p53 fluorescent peptide

L_T = total concentration of HBS peptide

CD Spectroscopy. CD spectra were recorded on AVIV 202SF CD spectrometer equipped with a temperature controller using 1 mm length cells and a scan speed of 5 nm/min. The spectra were averaged over 10 scans with the baseline subtracted from analogous conditions as that for the samples. The samples were prepared in phosphate buffered saline (13.7 mM NaCl, 1 mM phosphate, 0.27 mM KCl, pH 7.4), containing 10% trifluoroethanol, with the final peptide concentration of 50 μM. The concentrations of unfolded peptides were determined by the UV absorption of tyrosine residue at 275 nm in 6.0 M guanidinium hydrochloride aqueous solution. The helix content of each peptide was determined from the mean residue CD at 222 nm, $[\theta]_{222}$ (deg cm^2 $dmol^{-1}$) corrected for the number of amino acids. Percent helicity was calculated from the ratio $[\theta]_{222}/[\theta]_{max}$, where $[\theta]_{max} = (-44000 + 250T)(1 - k/n) = -25{,}170$ for k = 4.0 and n = 12, the number of amino acid residues in the peptide.[5-7]

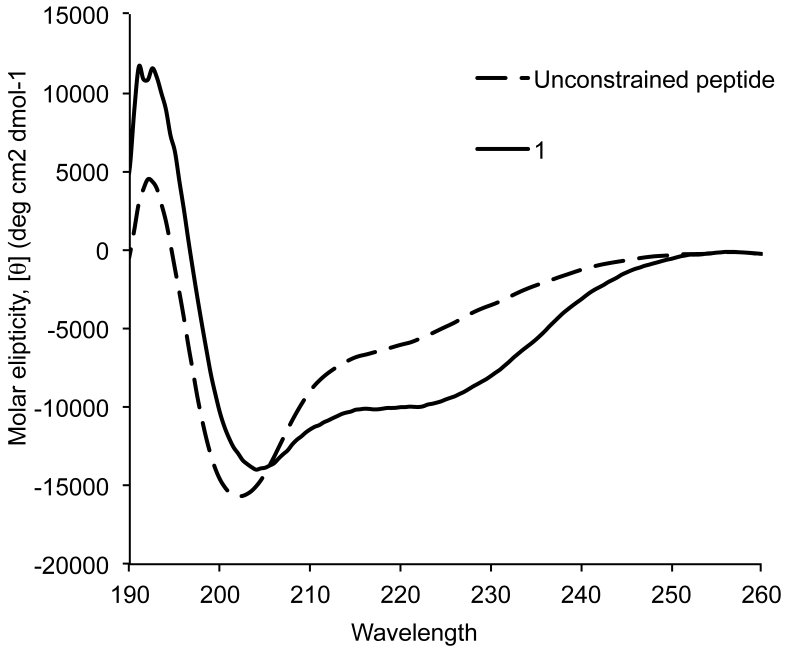


Figure S5. CD Spectrum of **1** and unconstrained peptide derivative (AcQEGFSDLWKLLS-NH_2) in 1 mM phosphate buffered saline, pH 7.4.

2D NMR Spectroscopy.

All experiments were carried out on a Bruker AVANCE 500 MHz spectrometer at 25 °C. Samples of 1 were prepared by dissolving 2 mg of peptide in 400 μL phosphate buffered saline (13.7 mM NaCl, 1 mM phosphate, 0.27 mM KCl, pH 3.5) and 100 μL TFE-d3. The pH of the solution was adjusted to 3.5 by adding 1 M HCl. The 1D proton spectra or 2D TOCSY spectra (when overlapping is severe) were employed to read the chemical shifts of the amide protons. Solvent suppression was achieved with a 3919 Watergate pulse sequence. All 2D spectra were recorded by collecting 4092 complex data points in the t2 domain by averaging 64 scans and 128 increments in the t1 domain with States-TPPI mode. All TOCSY experiments are performed with a mixing time of 80 ms on 6000 Hz spin lock frequency, and all NOESY with the mixing time of 300 ms. The data were processed and analyzed using Bruker TOPSPIN program. The original free induction decays (FIDs) were zero-filled to give a final matrix of 2048 by 2048 real data points. A 90° sine-square window function was applied in both dimensions.

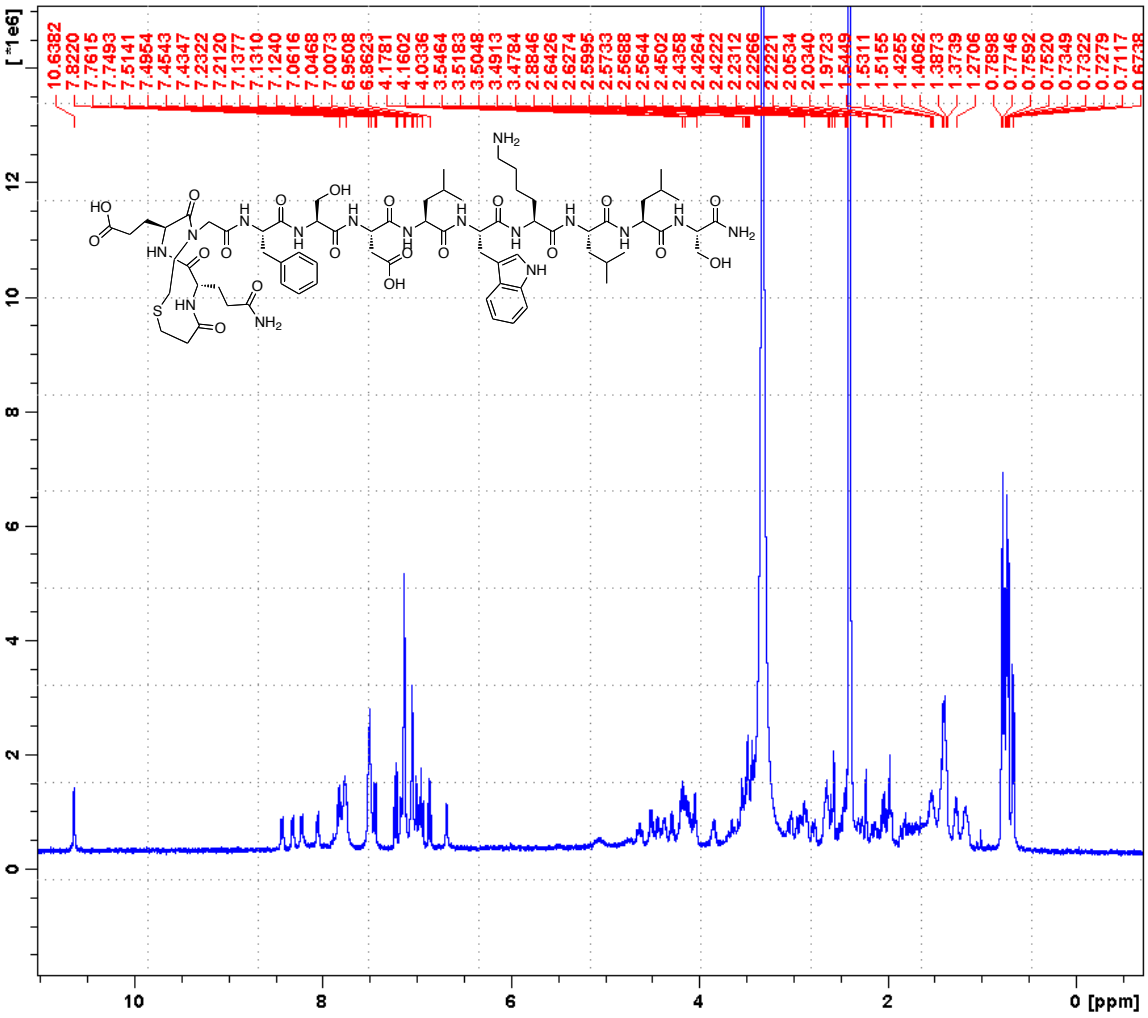


Figure S6. ^{1}H NMR spectrum of teHBS **1** in DMSO-d_6.

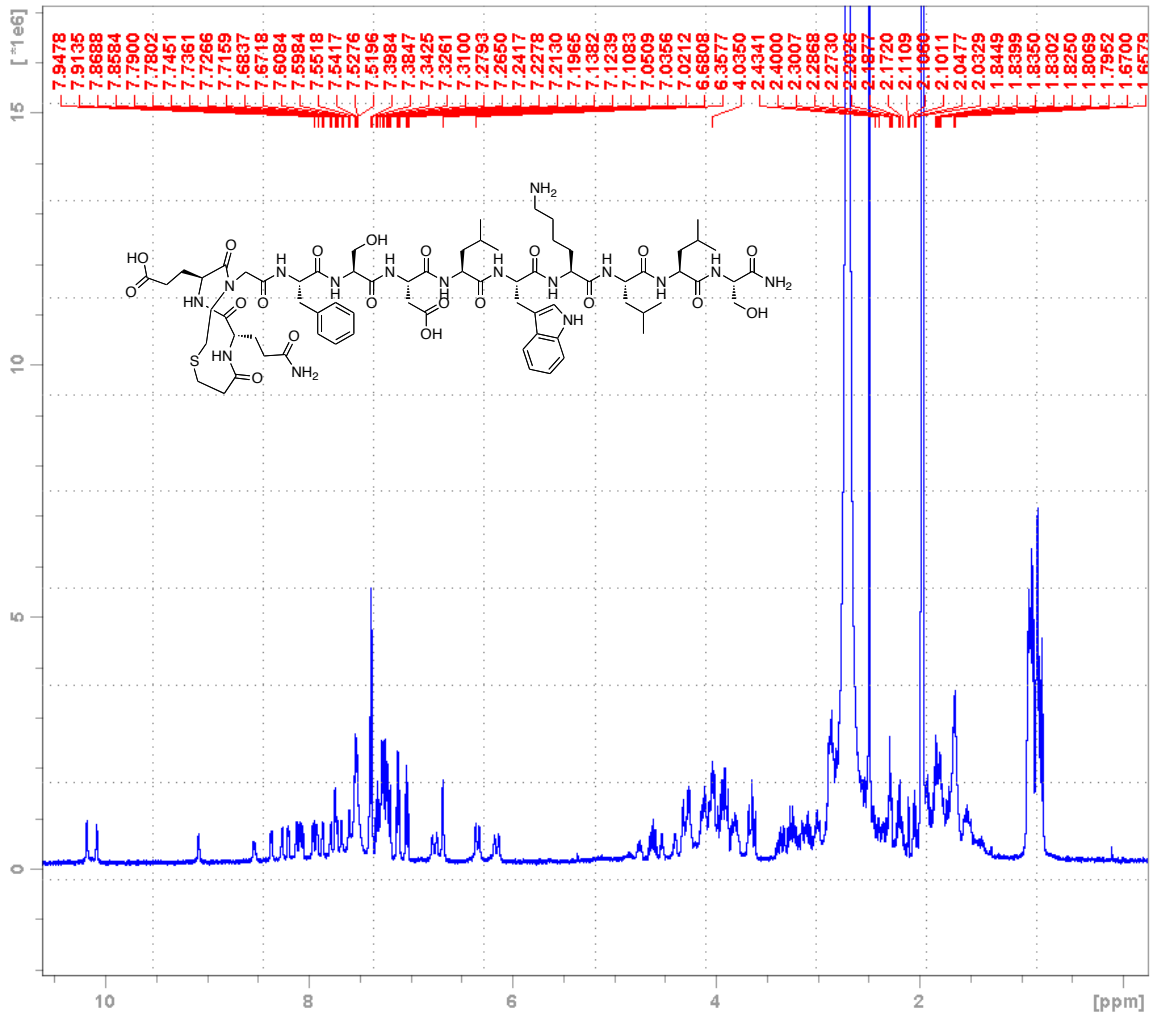


Figure S7. ^{1}H NMR spectrum of teHBS **1** in ACN-d_3/5% DMSO-d_6.

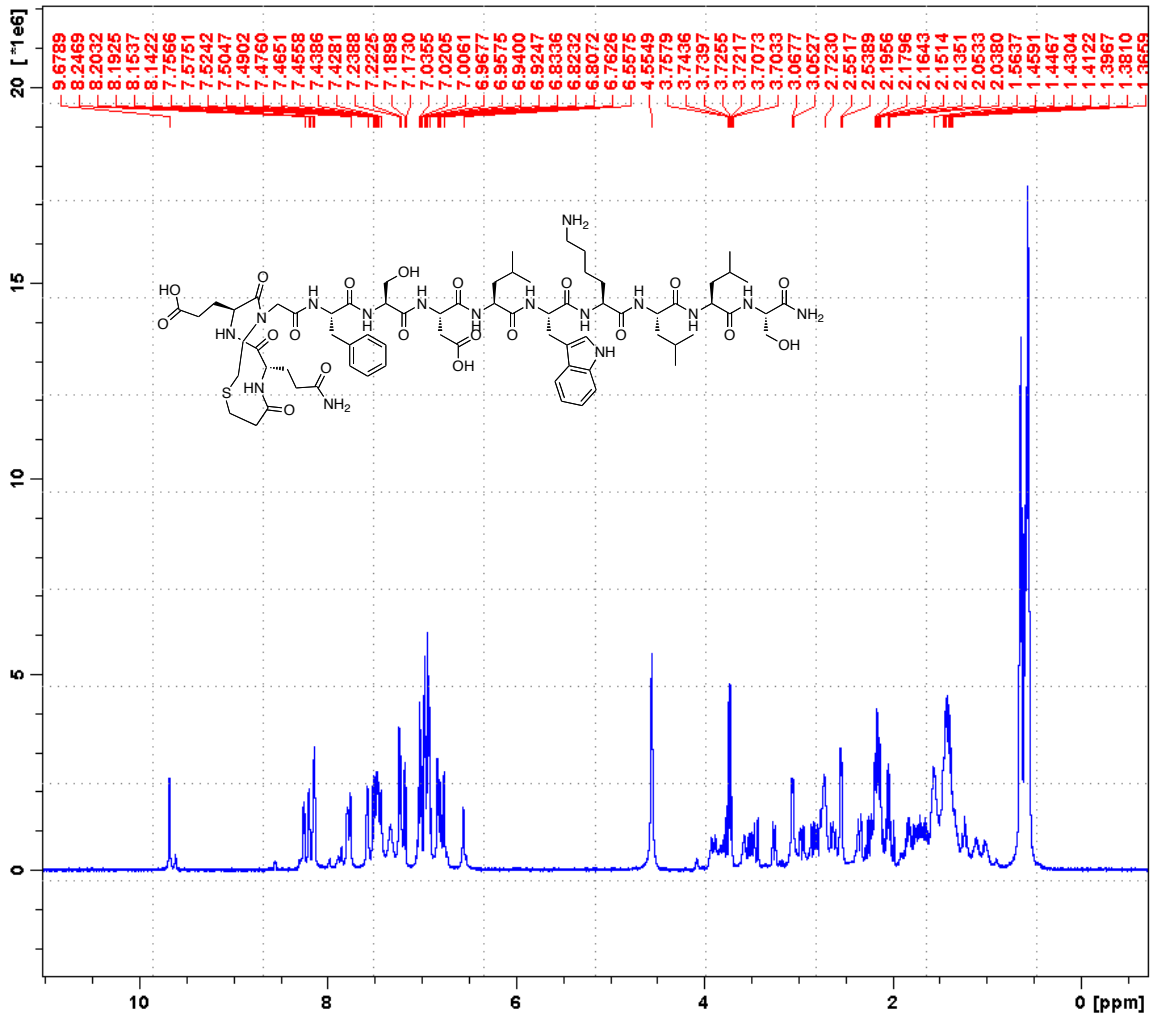


Figure S8. ^{1}H NMR spectrum of teHBS **1** in 20% trifluoroethanol-d_3 and PBS.

2-D NMR spectra of teHBS 1.

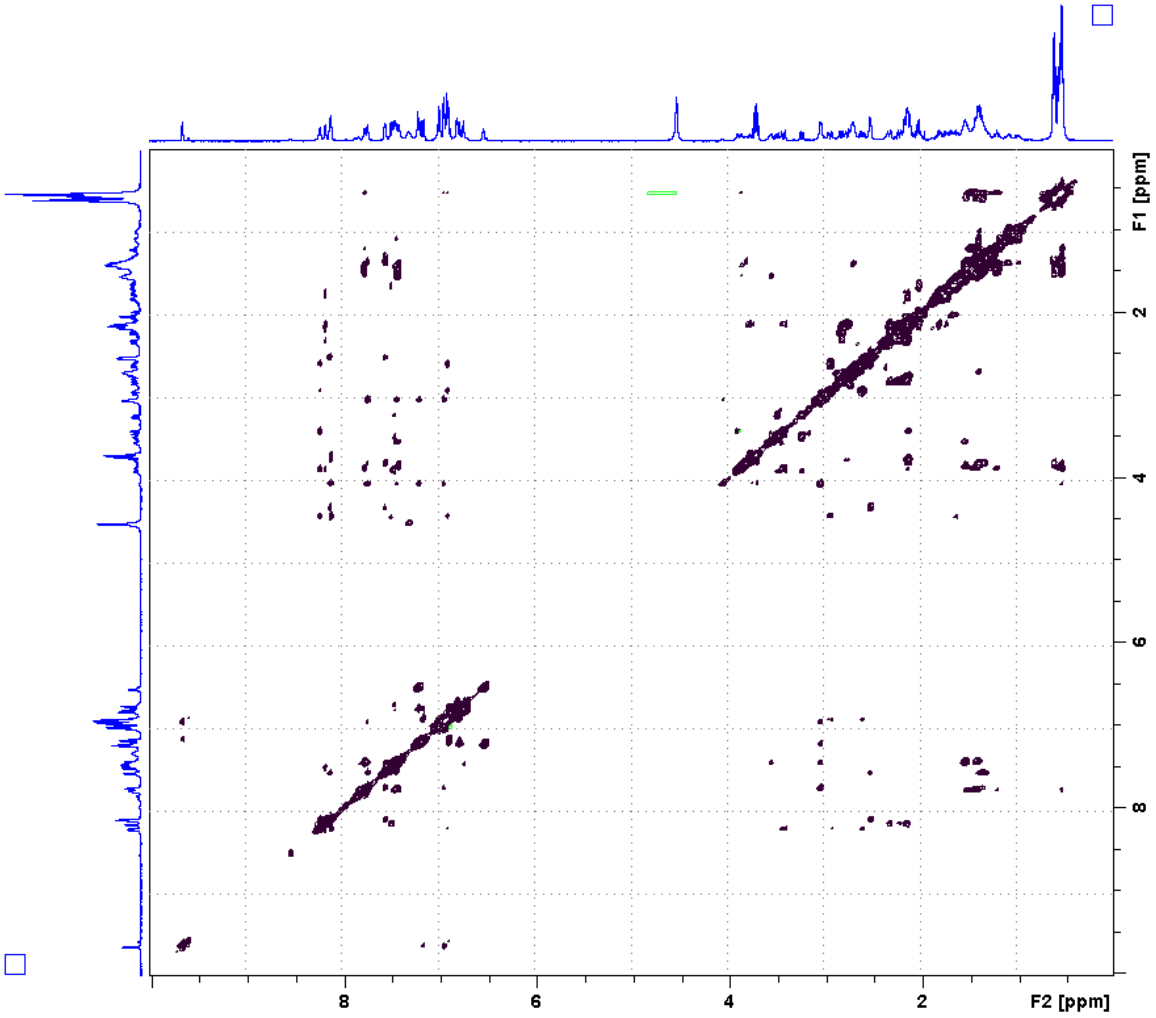


Figure S9. NOESY NMR spectrum of teHBS **1** in PBS (pH 3.5) and 20% trifluoroethanol-d_3.

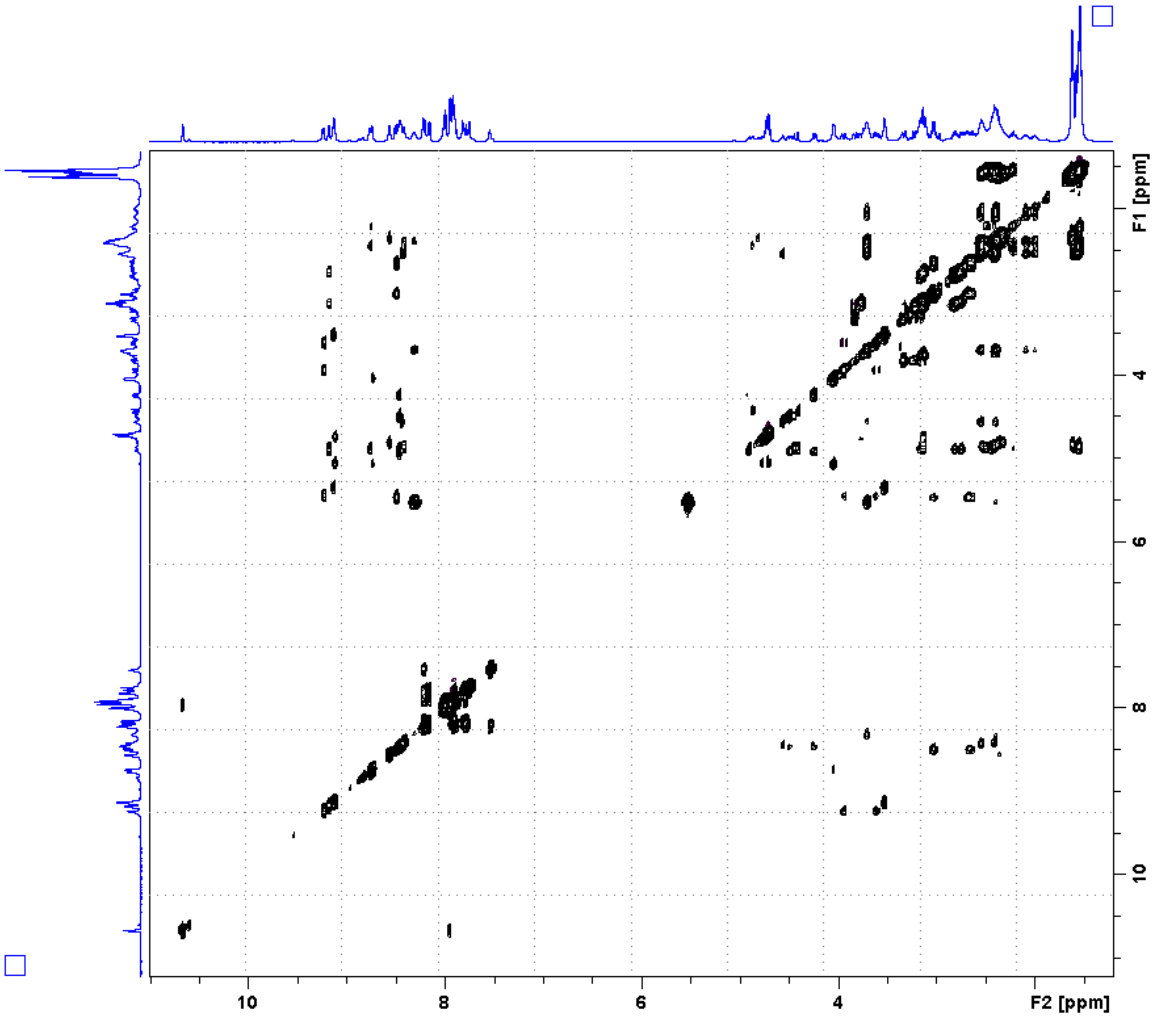


Figure S10. TOCSY NMR spectrum of teHBS **1** in PBS (pH 3.5) and 20% trifluoroethanol-d_3.

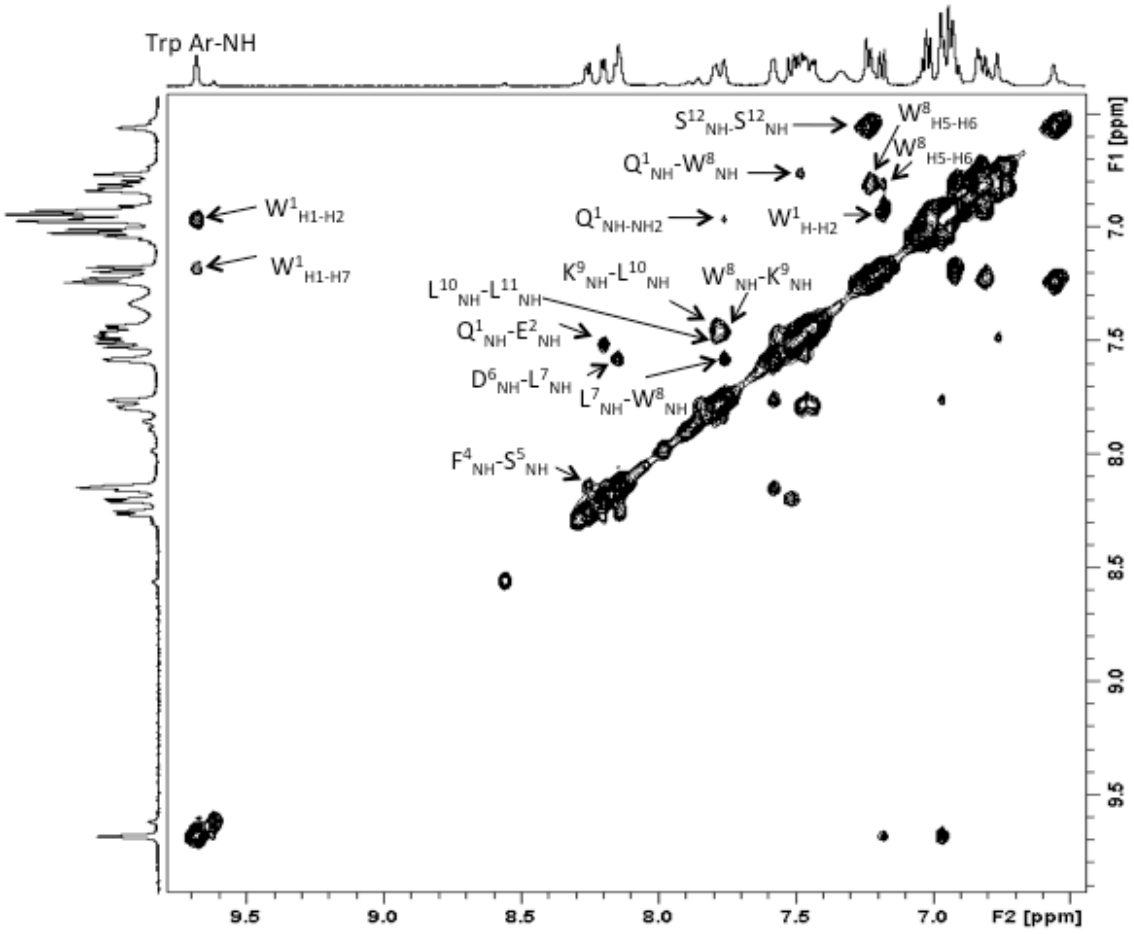


Figure S11. NH region of NOESY spectrum of teHBS **1** in PBS and 20% trifluoroethanol-d_3.

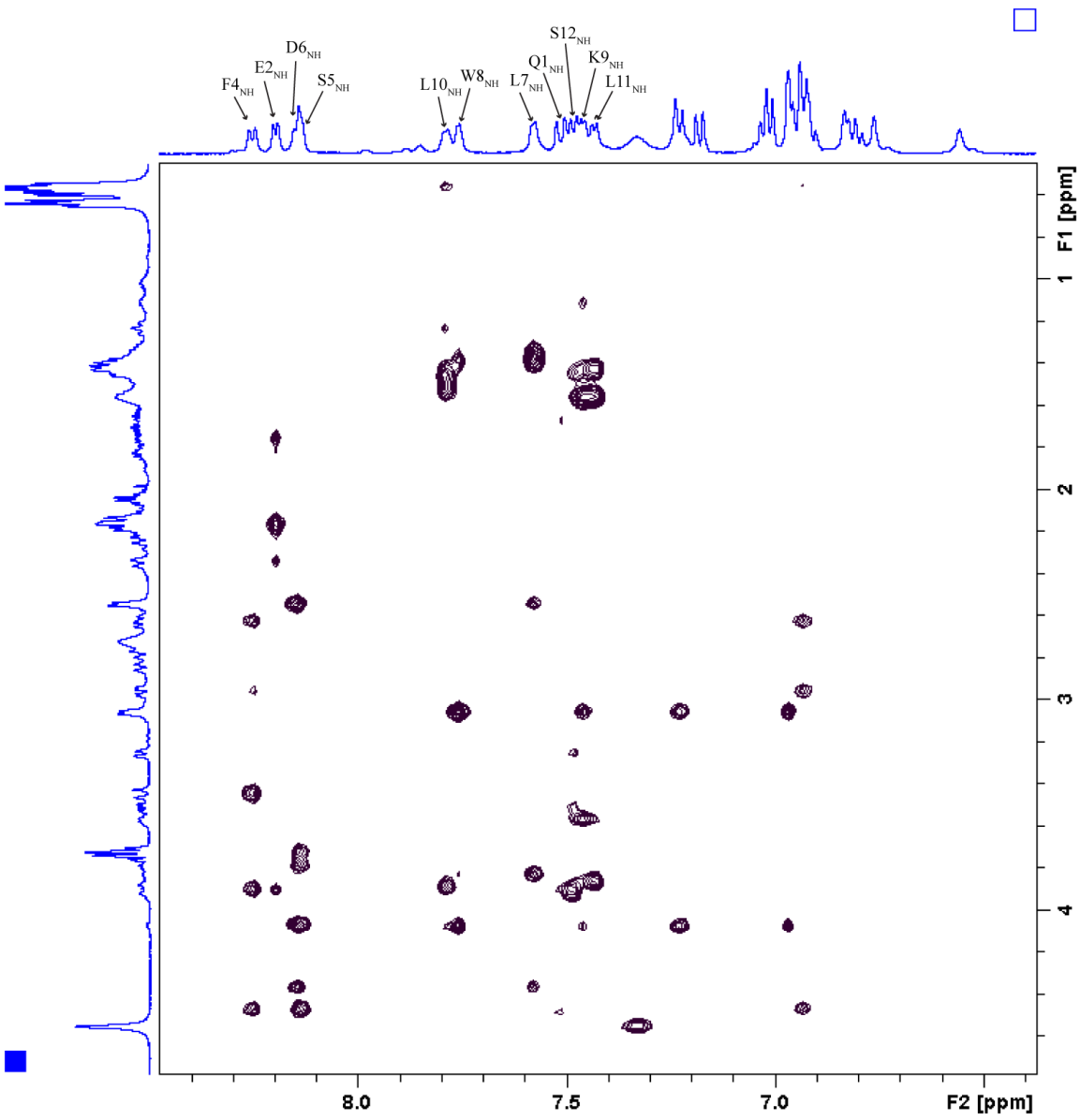


Figure S12. NH-αCH region of 2D NOESY spectrum for teHBS **1** in PBS and 20% trifluoroethanol-d_3.

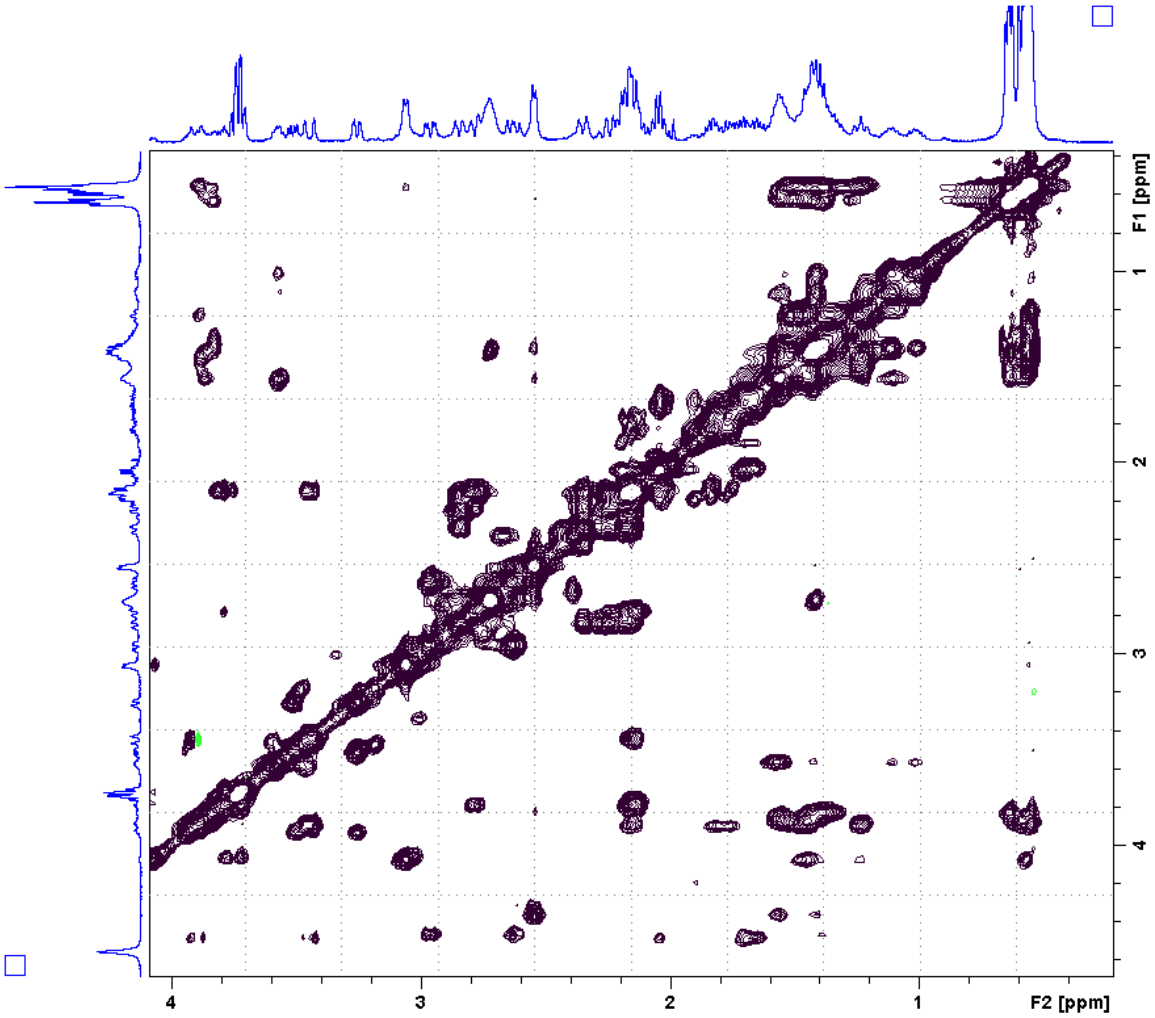


Figure S13. Aliphatic region of 2D NOESY spectrum for teHBS **1** in PBS and 20% trifluoroethanol-d_3.

Table S3. $_1$H NMR assignments and chemical shifts (δ, ppm) for teHBS **1 in** PBS and 20% trifluoroethanol-d_3.

Residue	NH	Hα	Hβ	Hγ	Hδ	Hε
Q^1	7.51	4.50	1.67	2.05	-	
E^2	7.22	3.91	1.79	2.17	-	
G^3	-	3.95, 3.47	-	-	-	
F^4	8.25	4.47	2.97, 2.65	-	-	
S^5	8.14	4.08	3.76	-	-	
D^6	8.15	4.37	2.53	-	-	
L^7	7.58	3.81	1.35	1.25	0.53	
W^8	7.76	4.10	3.07	-	-	
K^9	7.46	3.55	1.53	1.38	1.06, 1.00	2.70
L^{10}	7.78	3.91	1.47	1.24	0.54	
L^{11}	7.43	3.89	1.45	1.22	0.59	
S^{12}	7.48	3.95	3.52, 3.26	-	-	

Table S4. $^3J_{NHCH\alpha}$ coupling constants and calculated ϕ angles.

	Q_1	E_2	G_3	F_4	S_5[c]	D_6[c]	L_7	W_8	K_9	L_{10}	L_{11}	S_{12}
$^3J_{NH\alpha CH}$[a]	9.75	5.15	N/A	6.95	6.45	4.85	3.70	3.20	4.35	4.00	5.15	7.20
ϕ (deg)[b]	-120	-68	N/A	-81	-78	-65	-55	-56	-61	-58	-68	-84

[a] J values are in Hz; [b]Calculated using the Karplus equation; [c] Coupling constants were derived from 1D ^{1}H NMR spectra acquired at 313 K due to overlapping resonances at 298 K.

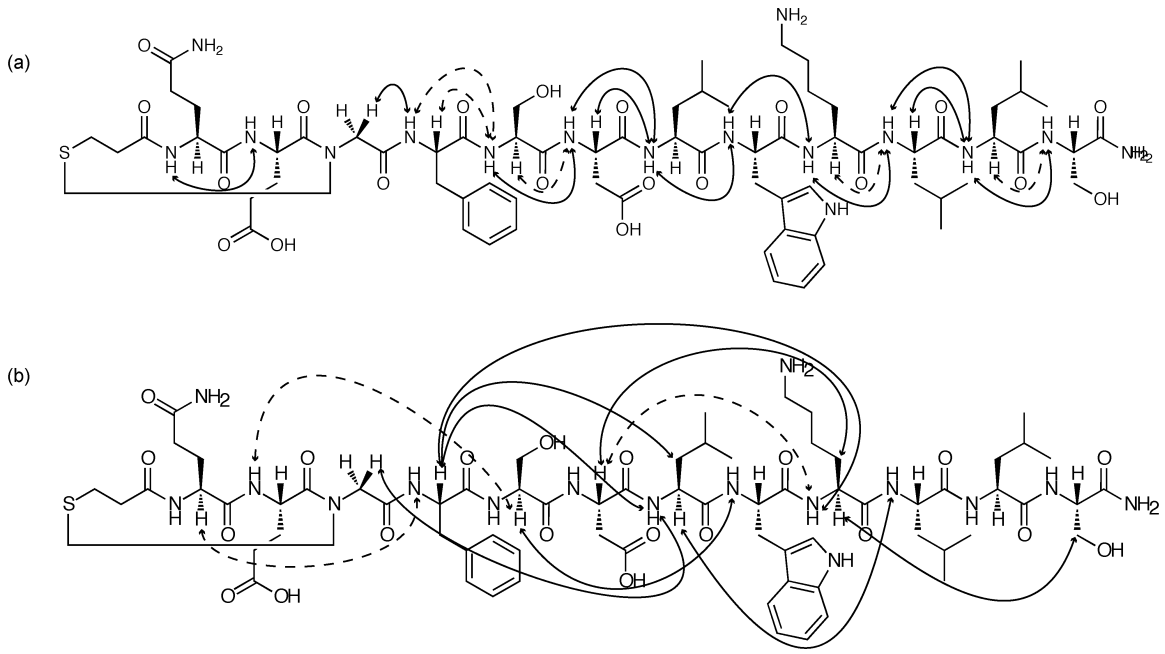


Figure S14. Short-range and (b) medium-range NOE's observed for **1**. Dashed lines indicate NOEs that could not be assigned definitively because of overlapping resonaces.

Ms/MS sequence analysis of teHBS 1.

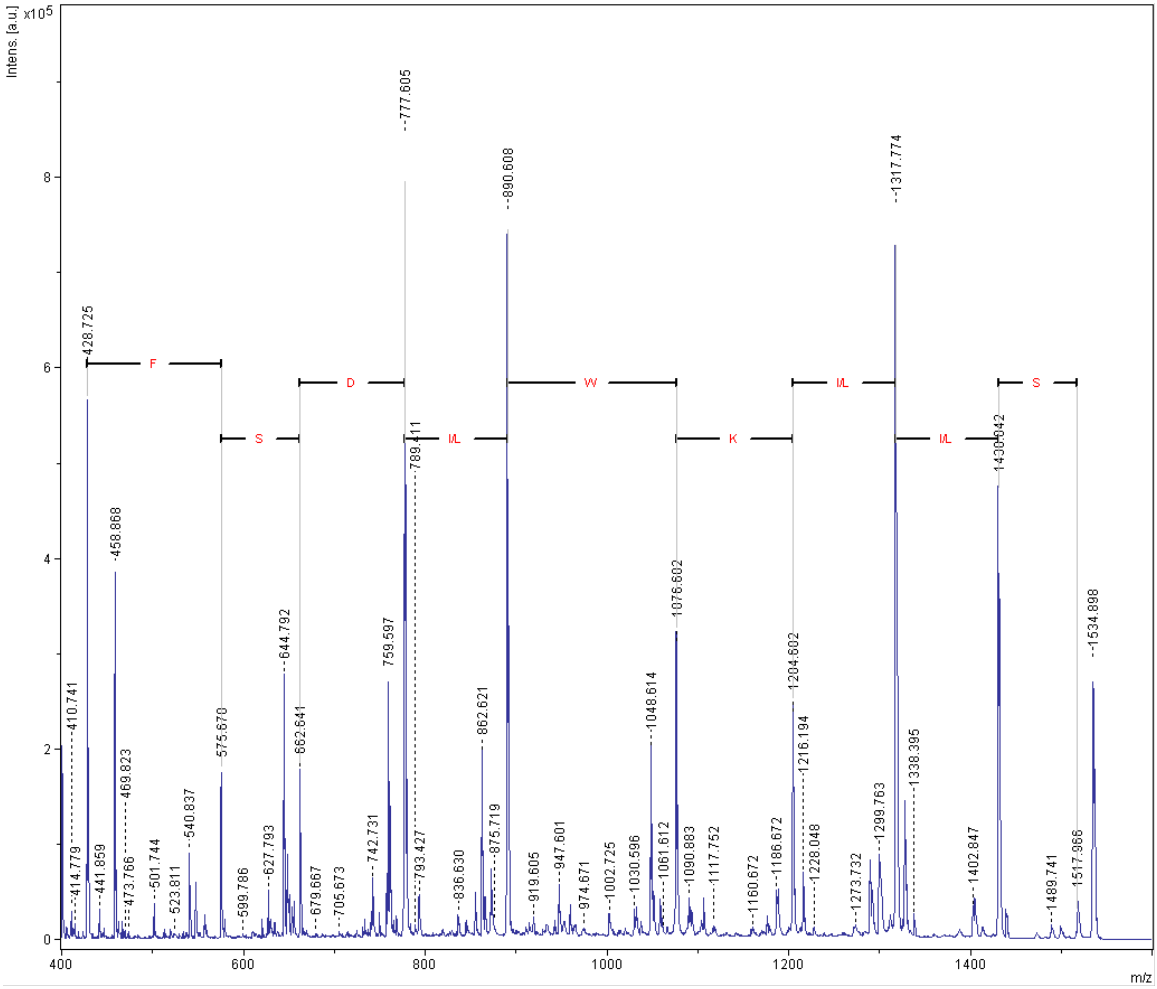


Figure S15. Labeled B ions from MS/MS sequencing of teHBS **1**.

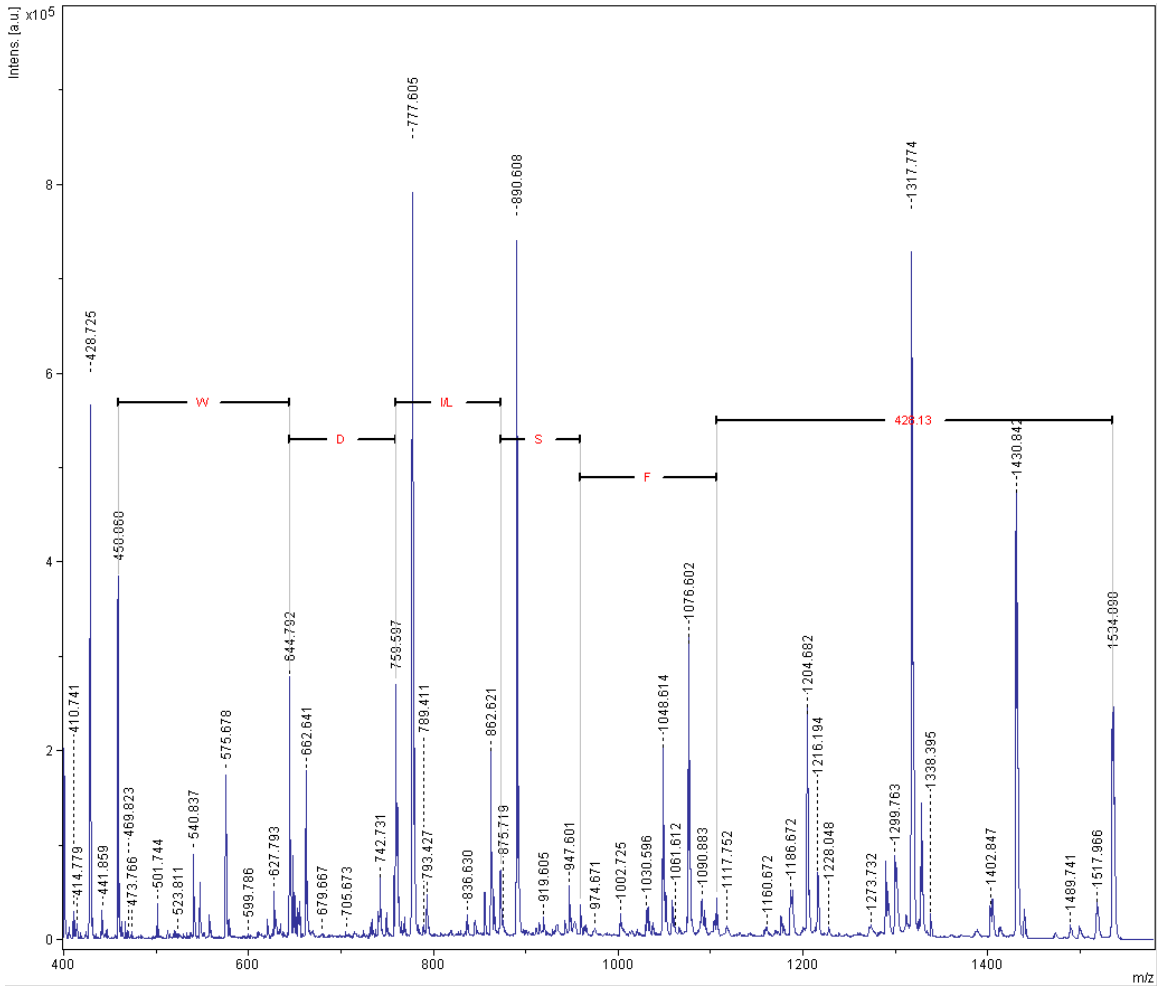


Figure S16. Labeled Y ions from MS/MS sequencing of teHBS **1**.

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