Supporting Information

Sequence-Function Space of Radical SAM Cyclophane Synthases Reveal Conserved Active Site Residues that Influence Substrate Specificity

Chin-Soon Phan,[1,2] and Brandon I. Morinaka*,[1]

[1]Department of Pharmacy, National University of Singapore, Singapore 117544, Singapore. [2]Latvian Institute of Organic Synthesis, Aizkraukles street 21, LV-1006 Riga, Latvia.

Correspondence: Brandon I. Morinaka, phambi@nus.edu.sg

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EXPERIMENTAL SECTION

General. Chemicals and reagents were purchased from either Merck or Bio Basic unless otherwise specified. Synthetic genes inserted into expression vectors were obtained from Twist Bioscience. Gene sequences used in this study are listed in Table S1. Trypsin protease was purchased from Merck. Antibiotics (kanamycin and spectinomycin) were purchased from GoldBio. *Escherichia coli* NiCo21(DE3) and DH5α strains purchased from NEB were used for protein expression and plasmid preparation, respectively. Electroporation was carried out using a Bio-Rad MicroPulser. *E. coli* cells were lysed using a Fisherbrand Model 505 Sonic Dismembrator fitted with an FB4208 ¾" probe or FB4220 ½" probe equipped with either an FB4420 ¼" Microtip or FB4418 1/8" Microtip. LC-MS experiments were performed on a Waters Acquity UPLC System coupled to Xevo G1 QToF Mass Spectrometer. Preparative HPLC was carried out on a Shimadzu Nexera Prep System. Sample solutions were concentrated using a centrifugal evaporator (Genevac EZ-2 Elite) or freeze dryer (LaboGene ScanVac CoolSafe). NMR spectra were acquired at 298K using a Bruker 400 MHz Avance Neo Nanobay with Bruker iProbe 5 mm SmartProbe. NMR solvents were purchased from Cambridge Isotope Labs. 1D/2D NMR data were processed and analyzed with Bruker Topspin software. Chemical shift values were referenced using the signals of residual sodium trimethylsilylpropanesulfonate (δ_H 0.00 ppm; δ_C 0.00 ppm) when using D_2O as NMR solvent and DMSO-d_6 (δ_H 2.50 ppm; δ_C 39.50 ppm).

Bioinformatic analysis to search for triceptide precursors. Our previous bioinformatic mapping of the sequence-function space for the 3-residue cyclophane forming enzymes (3-CyFEs) has constructed a sequence similarity network (SSN, Figure 1) and has identified a total of 34 putative precursor sequences associated with C09 (Figure 2).[1] The SSN for triceptide precursors (extracted from C09) using RODEO[2] were constructed using Enzyme Function Initiative−Enzyme Similarity Tool (EFI-EST)[3] and visualized in Cytoscape 3.5.1.

Transformation of plasmids into *E. coli* NiCo21 (DE3). Plasmids containing precursor and rSAM enzyme genes were obtained from Twist Bioscience or Gene Universal and dissolved in MilliQ grade water to a final concentration of 5-10 ng/μL. 70 μL of *E. coli* NiCo21(DE3) electrocompetent cells were transformed in a 2 mm electroporation cuvette with either 1 μL of plasmid DNA containing the precursor gene for the precursor-only expression or 1 μL of plasmid DNA containing the precursor gene + 1 μL of plasmid DNA containing the rSAM enzyme gene for the coexpression of the precursor and the rSAM enzyme. The transformed cells were then grown overnight at 37 °C on lysogeny broth (LB) agar supplemented with appropriate antibiotics at a final concentration of 50 μg/mL.

Protein expression and purification of NHis$_6$-precursor. A colony from the transformation above was picked up by a toothpick and added to 10 mL Terrific Broth (TB) medium supplemented with appropriate antibiotics in a 50 mL falcon tube. The 10 mL culture was grown overnight at 37 °C and shaken at 250 rpm. The overnight culture was used to inoculate either 250 mL of antibiotic-supplemented TB media in a 500 mL Ultra Yield™ flask or 1 L of antibiotic-supplemented TB medium in a 2.5 L Ultra Yield™ flask in a 1:100 (v:v) ratio. The cells were then grown at 37 °C, 250 rpm until $OD_{600\,nm}$ reached 1.6-3.0. The culture was then placed on ice water for 30 min and protein expression was induced by addition of IPTG at a 0.8-1 mM final concentration for precursor only and precursor + rSAM coexpression. After induction, the culture was shaken at 16 °C, 250 rpm for 16 h. The cells were collected by centrifugation at 4000 rpm for 15 min. For precursor only and precursor and rSAM coexpression, either denaturing lysis buffer (100 mM NaH_2PO_4, 10 mM Tris, 6.5 M guanidine hydrochloride or 9 M Urea, 10 mM imidazole, pH 8) or NPI-10 buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) was added to cell pellets in a ratio of 3:1 (v:w). The cell pellets were reconstituted and lysed by sonication with a ¾" inch solid probe (10 sec on and 10 sec off for 30 cycles at 25% amplitude). After sonication, the cell debris was removed by centrifugation at 15,000 rpm for 15 min. HisPur Ni-NTA resin (0.7 mL) was added to ~15-20 mL of supernatant in a 50 mL falcon tube and gently shaken for 1 h to allow binding of the precursor peptide to the Ni-NTA resin. Peptide-bound Ni-NTA resin was then washed with denaturing lysis buffer (2 x 1 mL for 0.7 mL resin if this buffer was used to resuspend the cell pellet), NPI-20 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8, 5 x 1 mL for 0.7 mL resin) and eluted with NPI-250 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8, 2.5 mL for 0.7 mL resin). Elution fractions were desalted into 50 mM Tris buffer (pH 8.0) using PD Minitrap G-10 columns, and then digested with 1 mg/mL trypsin (1:100, trypsin/eluant v:v) at 37 °C for 16 h.

Mobile phases used for LC-MS methods. A: MeCN + 0.1% formic acid (FA); B: MeCN:Isopropanol (1:1, v:v) + 0.5% FA; C: H_2O + 0.1% FA; D: H_2O + 0.5% FA, E: MeCN:Isopropanol (1:1 v:v) + 0.1% FA.

LC-MS conditions. Data acquisition was performed using MassLynx 4.1 (Waters) at the following conditions.

Figures 3, 4, S14-S15, S25-S32 and S35. LC: column = Phenomenex Kinetex XB-C18, 5 μm, 150 x 4.6 mm; mobile phase/gradient = solvent A: H_2O (0.1% formic acid, FA), solvent B: CH_3CN (0.1% FA), isocratic 4% B for 2 min, followed by a linear gradient to 60% B over 10 min; flow rate = 0.5 mL/min; column temp. = 50 °C.

Figure S33. LC: column = Phenomenex Kinetex XB-C8, 5 μm, 150 x 4.6 mm; mobile phase/gradient = solvent A: H_2O (0.1% FA), solvent B: mixture of CH_3CN:IPA (1:1 v:v and 0.1% FA), isocratic 4% B for 2 min, followed by a linear gradient to 60% B over 10 min; flow rate = 0.5 mL/min; column temp. = 50 °C.

MS: polarity = positive; capillary voltage = +0.6 kV; cone voltage = 30.0 V; source temperature = 120 °C; desolvation temperature = 600 °C; cone gas flow = 30 L/h; desolvation gas flow = 800 L/h; collision energy = 4 V; mass range = *m/z* 250–2000; scan duration = 0.5 s; interscan delay = 0.025 s; data acquisition = continuum mode; Lockspray (Leucine enkephalin); scan duration = 1.0 s; interval = 10 scans.

MS/MS: polarity = positive; scan duration = 1.0 s; inter-scan delay = 0.025 s; data acquisition = continuum mode; mass range and collision energy are specified in the respective figure legends.

Purification of products 1 and S1a. The concentrated trypsin digests were dissolved in deionized water and subjected to preparative reversed phase HPLC using a Phenomenex Kinetex XB-C18 column (5 µm, 100 x 30 mm). Fractions containing the product of interest were combined and concentrated using a centrifugal evaporator followed by lyophilization.

Product **1**. The purification was carried out using a gradient condition: mobile phase = 10-23% CH_3CN (0.1% FA) in H_2O (0.1% FA) at 0-20 min, flow rate = 15 mL/min, λ = 220 nm. Product **1** with a yield of 0.45 mg/L.

Product **S1a**. The purification was carried out using an isocratic condition: mobile phase = 16% CH_3CN (0.1% FA) in H_2O (0.1% FA) at 0-20 min, flow rate = 15 mL/min, λ = 220 nm. Product **2a** with a yield of 0.34 mg/L.

The trypsin digest prepared from 8 L of culture was subjected to preparative reversed phase HPLC to give ~3 mg of the product **S1a**. The FTK macrocycle of **S1a** was determined by 2D NMR which is also assigned in a similar manner as HTK motif (Figure S25 and Table S9). COSY/TOCSY correlation at the doublet signals of Phe1-H2 (δ_H 6.79, d, $^3J_{HH}$ = 7.0 Hz) to Phe1-H3 (δ_H 6.98) and Phe1-H5 (δ_H 7.06, d, $^3J_{HH}$ = 7.0 Hz) to Phe1-H6 (δ_H 7.19) supported a para-substituted Phe and existence of planar chirality. This was further confirmed by NOESY correlations (Figures S23-S24).

For NMR analysis, the dried materials of products **1** and **S1a** were dissolved in DMSO-d_6, moreover product **1** also measured in D_2O.

Advanced Marfey's analysis. 100 µg of **1** was hydrolyzed in 6 M HCl (1 mL) at 110 °C for 18 h. The hydrolysate was concentrated using a centrifugal evaporator and reconstituted in water (100 µL), followed by addition of 1 M $NaHCO_3$ (40 µL) and 1% w/v L-FDVA in acetone (200 µL). The mixture was incubated at 42°C for 1 h and quenched with 2 M HCl (20 µL). Both L-amino acids and L-*allo*-Thr standards were derivatized in the same manner using L- and D-FDVA. The reaction mixtures were diluted with CH_3CN/H_2O (1:1 v/v) and analyzed by LC-MS using negative ion mode. Retention times of the derivatized samples and standards are summarized in Table S8 with detailed LC conditions.

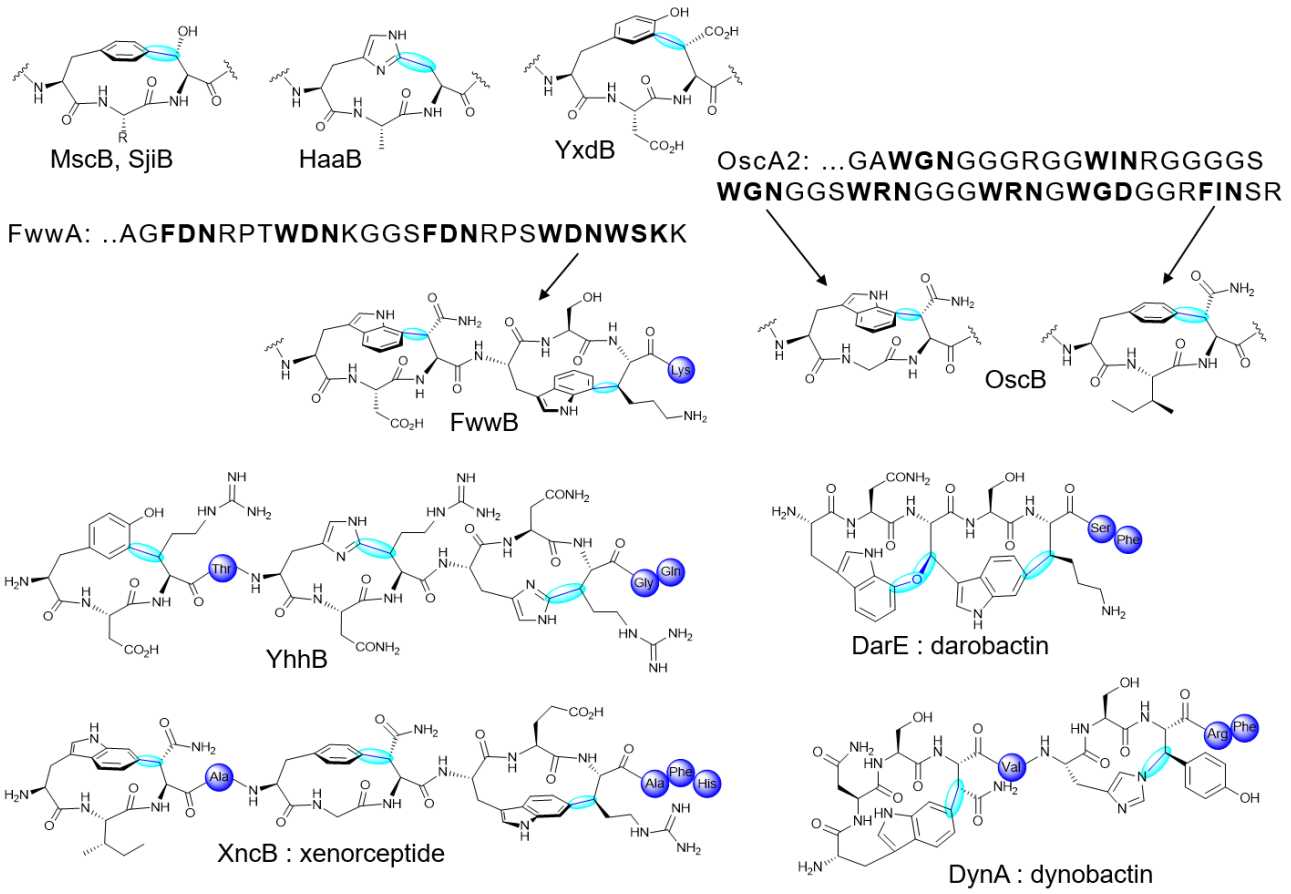

Figure S1. Characterized cyclophane synthases and structures of modified products.

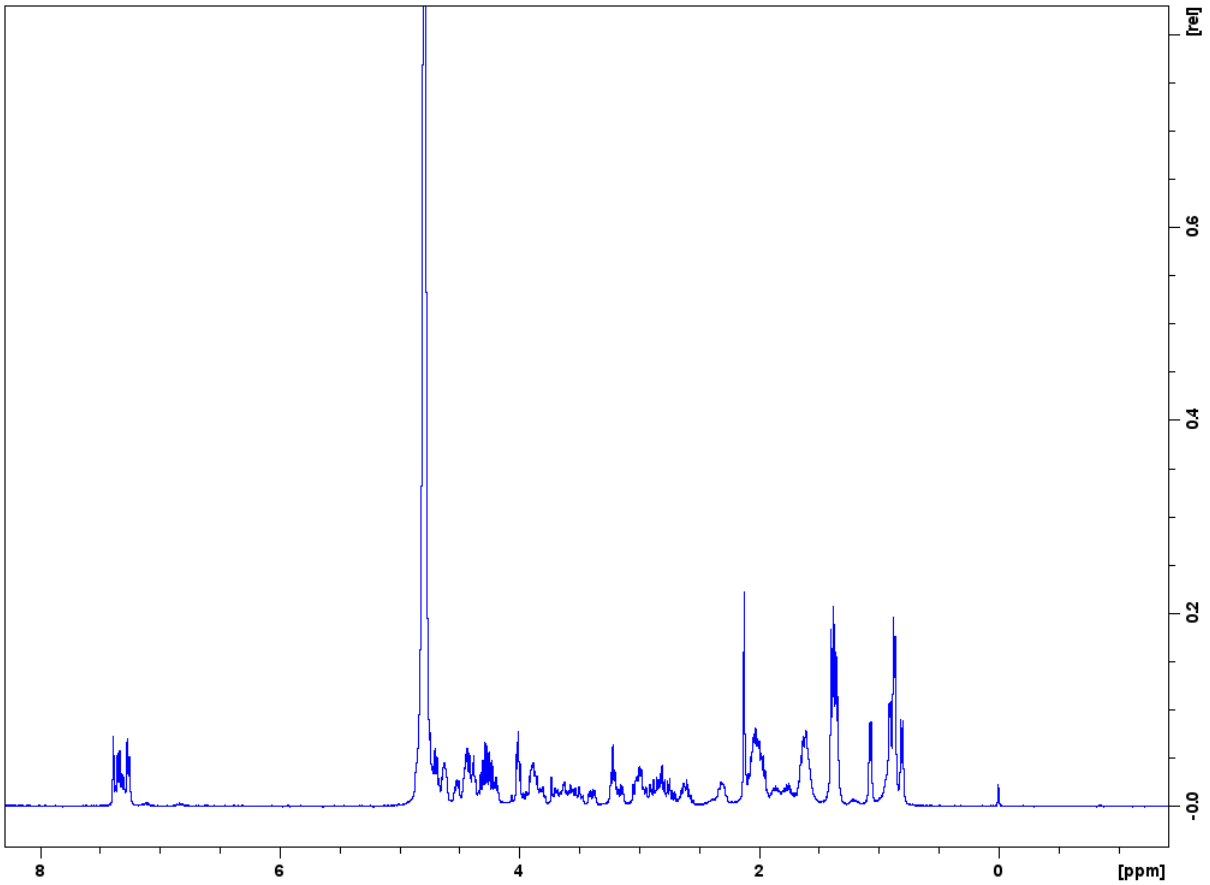

Figure S2. ^{1}H NMR (400 MHz) spectrum of **1** in D_2O

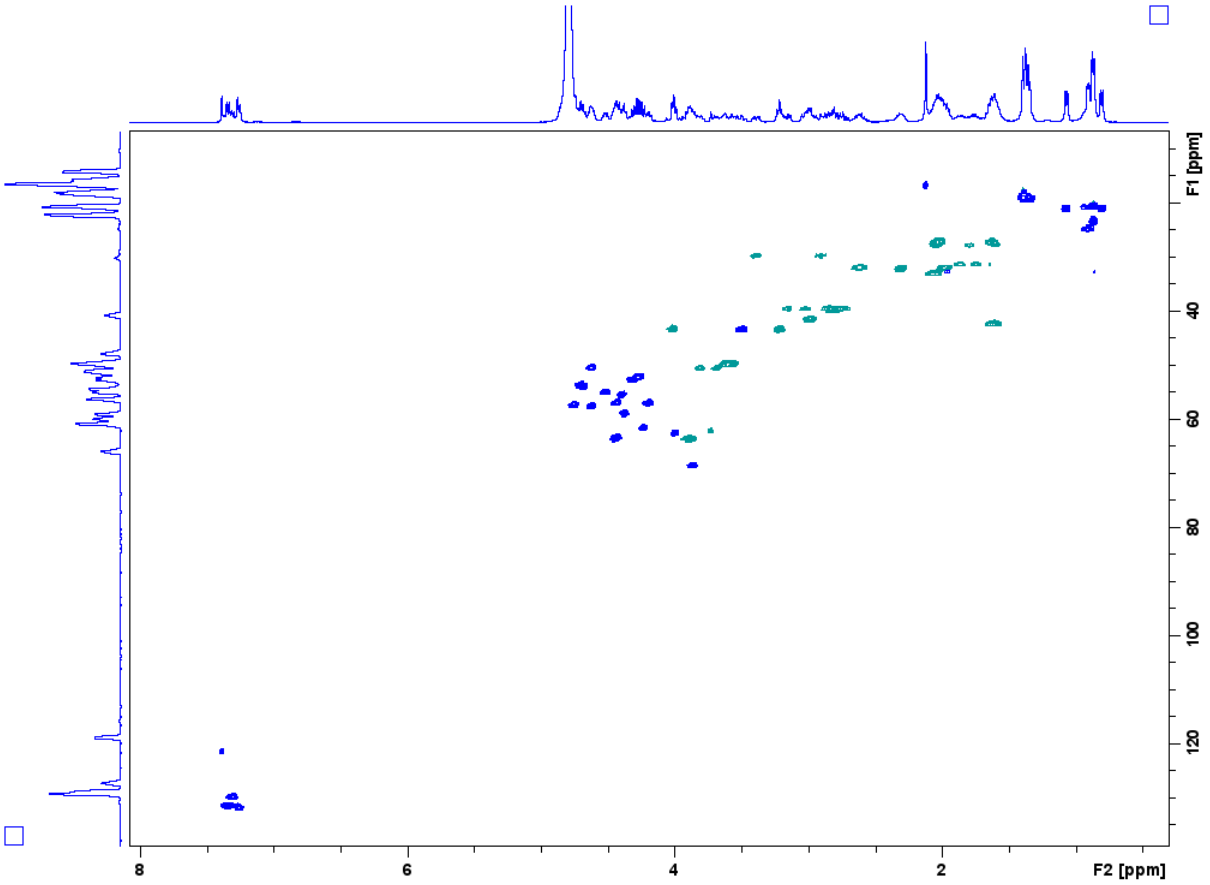

Figure S3. HSQC spectrum (400 MHz) of **1** in D_2O

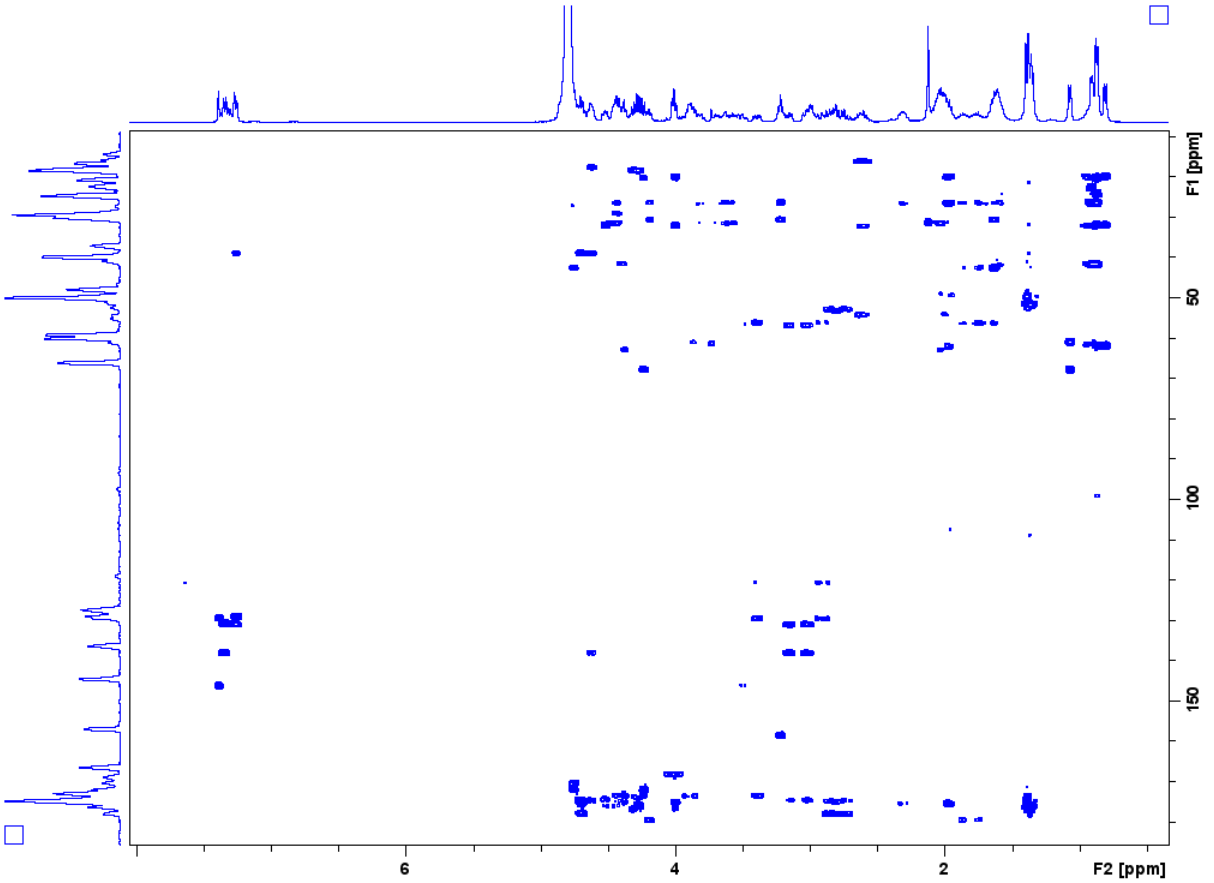

Figure S4. HMBC spectrum (400 MHz) of **1** in D_2O

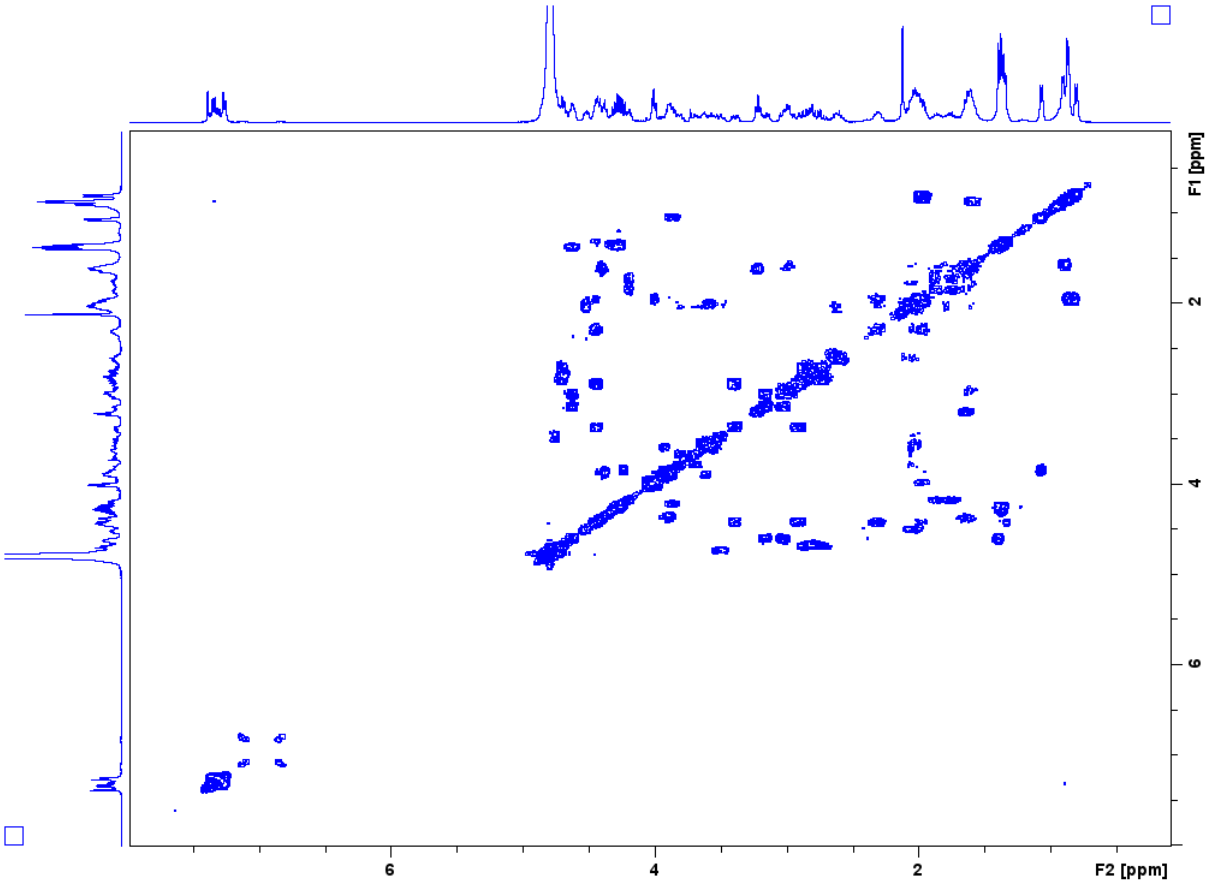

Figure S5. COSY spectrum (400 MHz) of **1** in D_2O

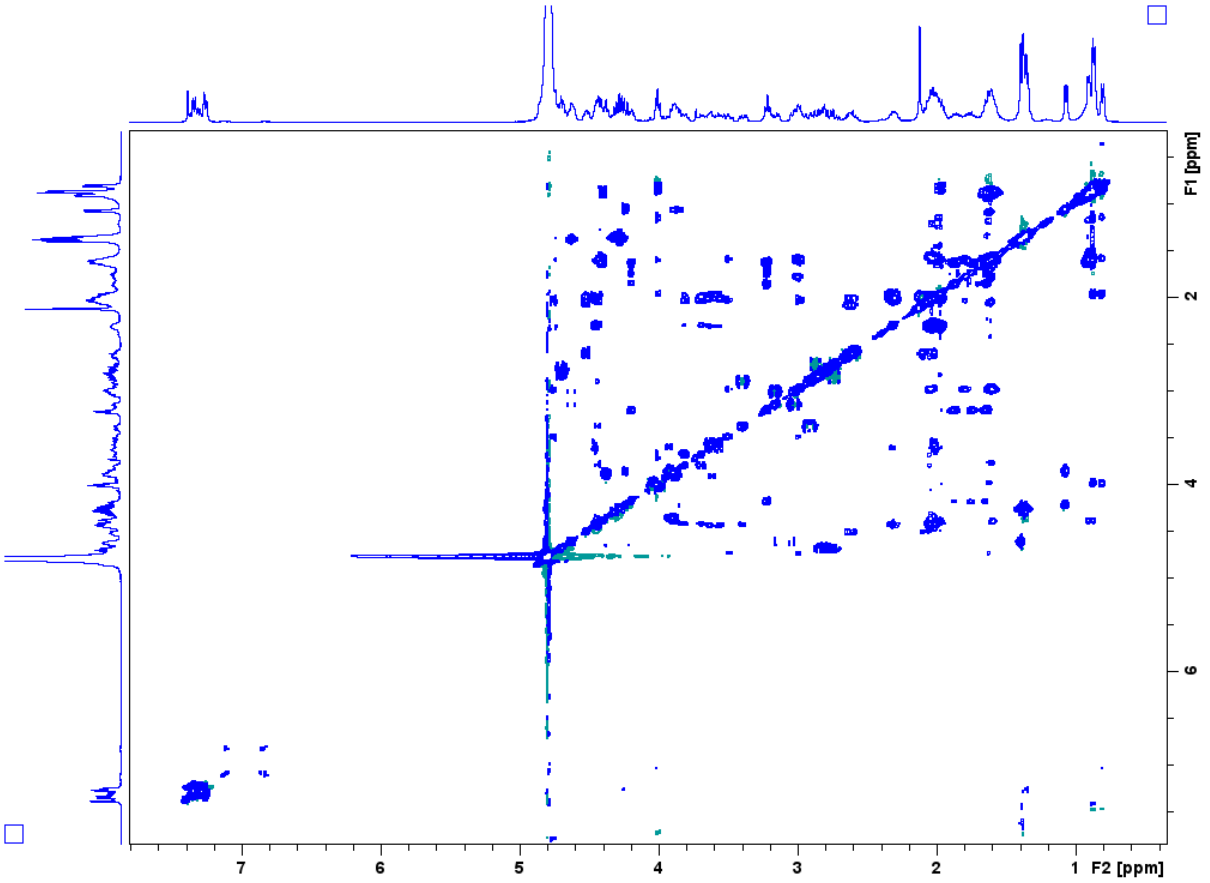

Figure S6. TOCSY spectrum (400 MHz) of **1** in D_2O

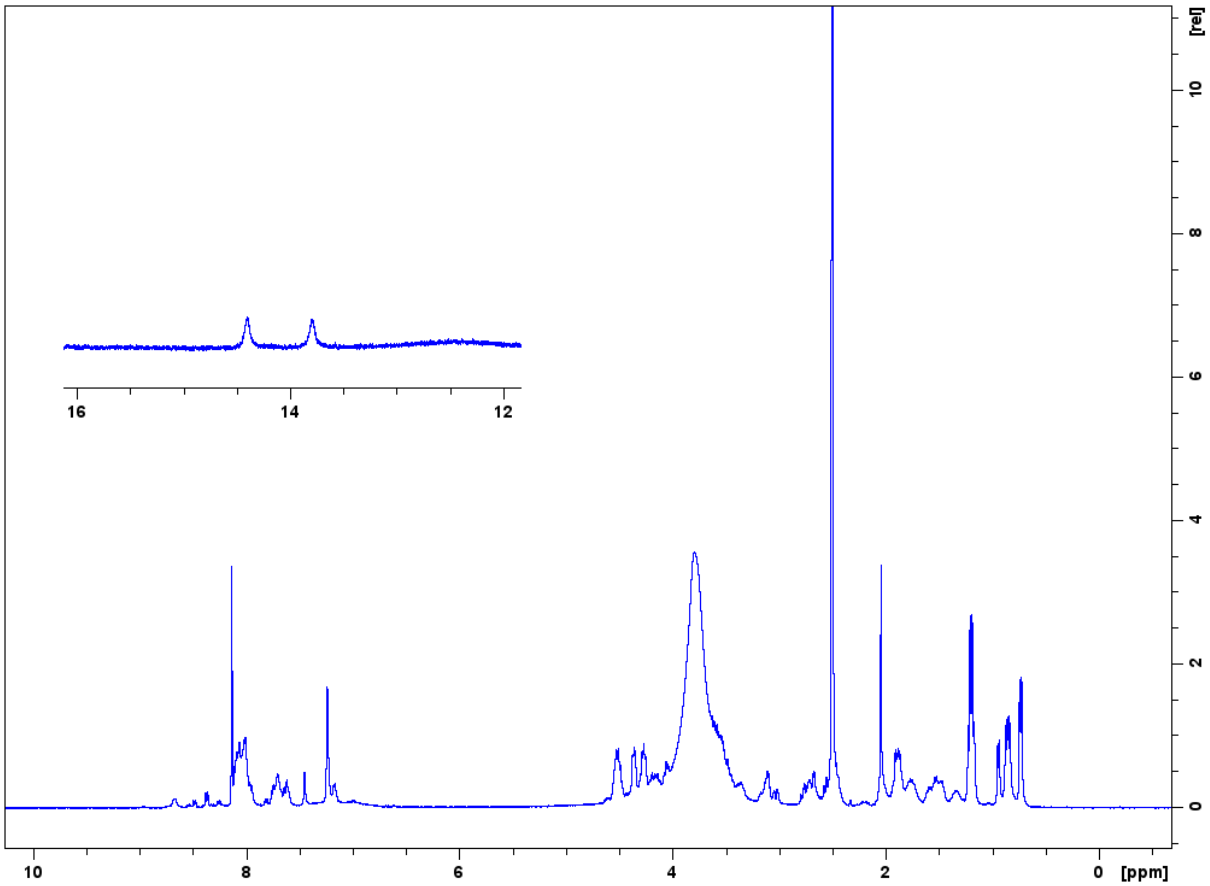

Figure S7. ^{1}H NMR (400 MHz) spectrum of **1** in DMSO-d_6 with 0.15% TFA-*d*

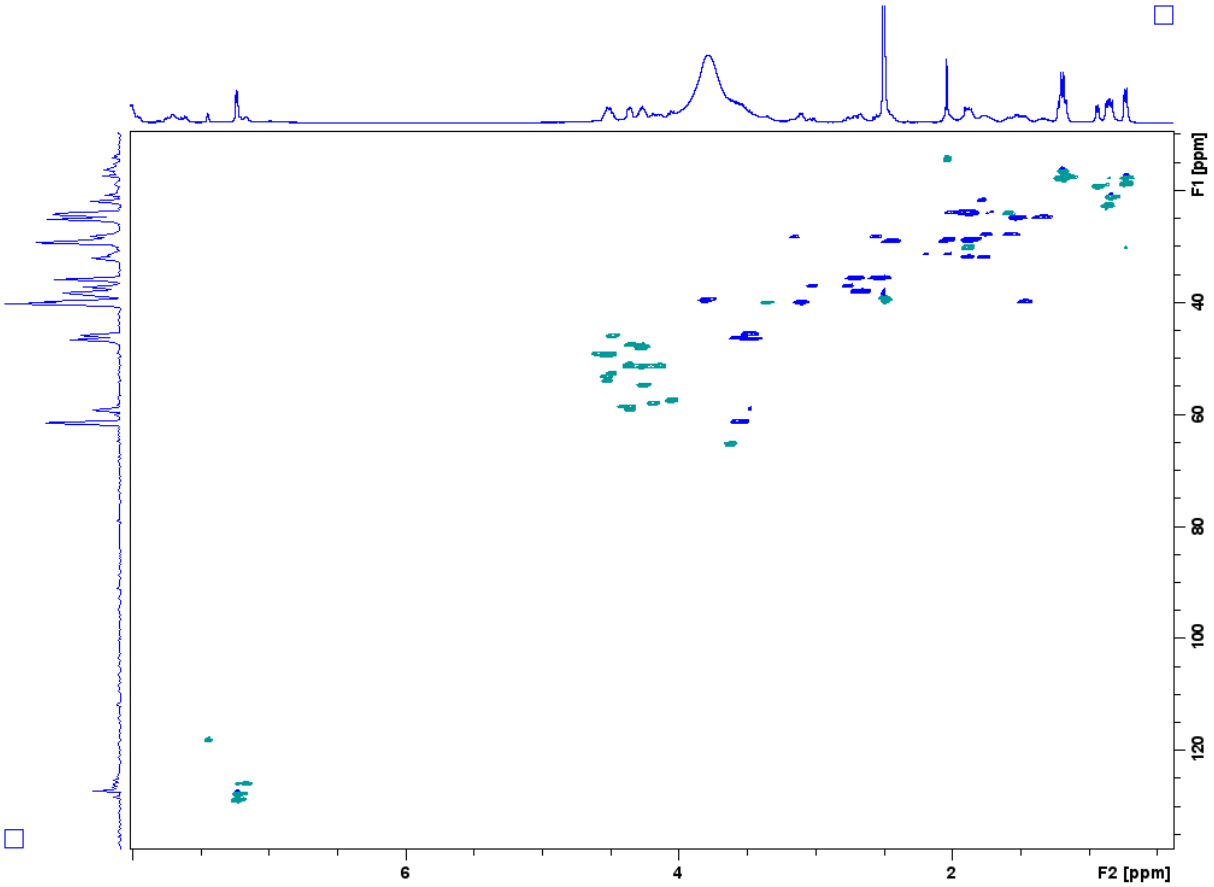

Figure S8. HSQC spectrum (400 MHz) of **1** in DMSO-d_6 with 0.15% TFA-*d*

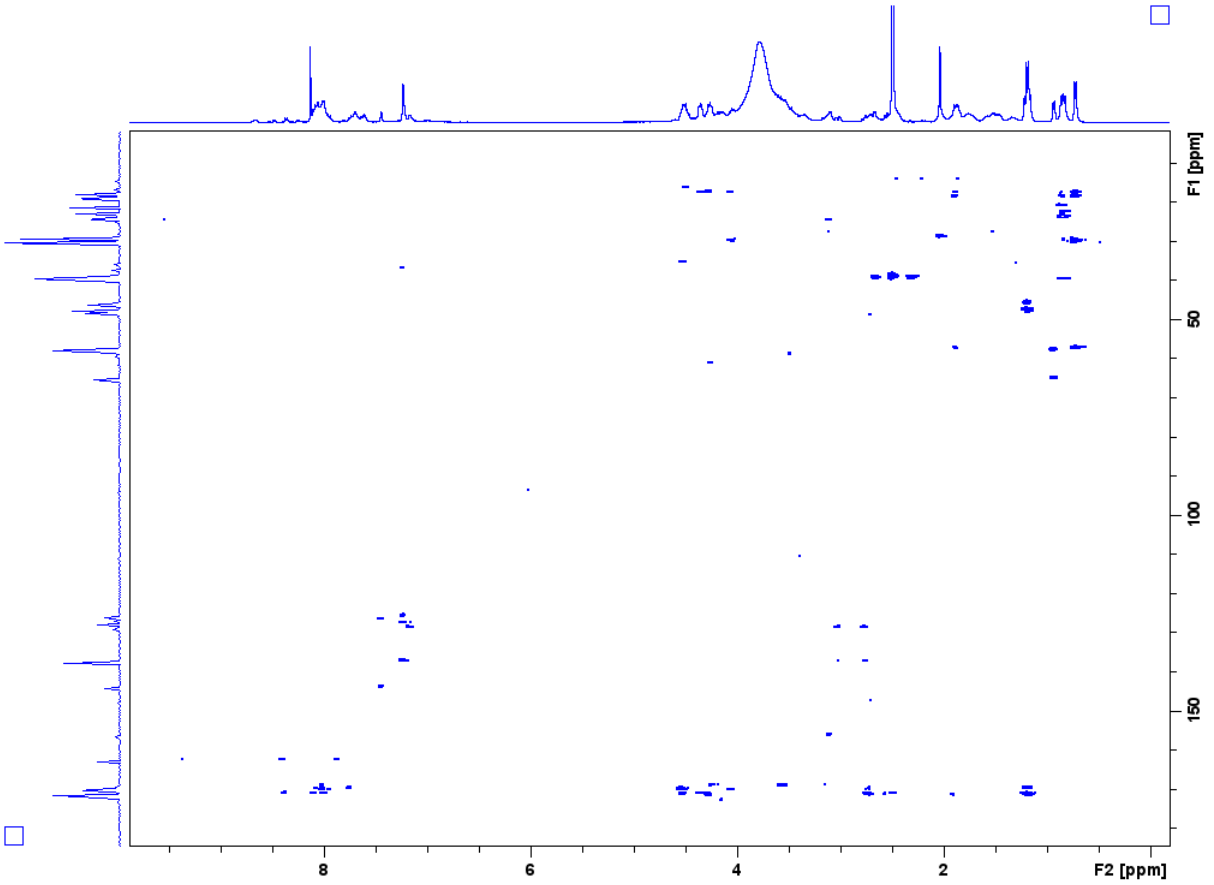

Figure S9. HMBC spectrum (400 MHz) of **1** in DMSO-d_6 with 0.15% TFA-*d*

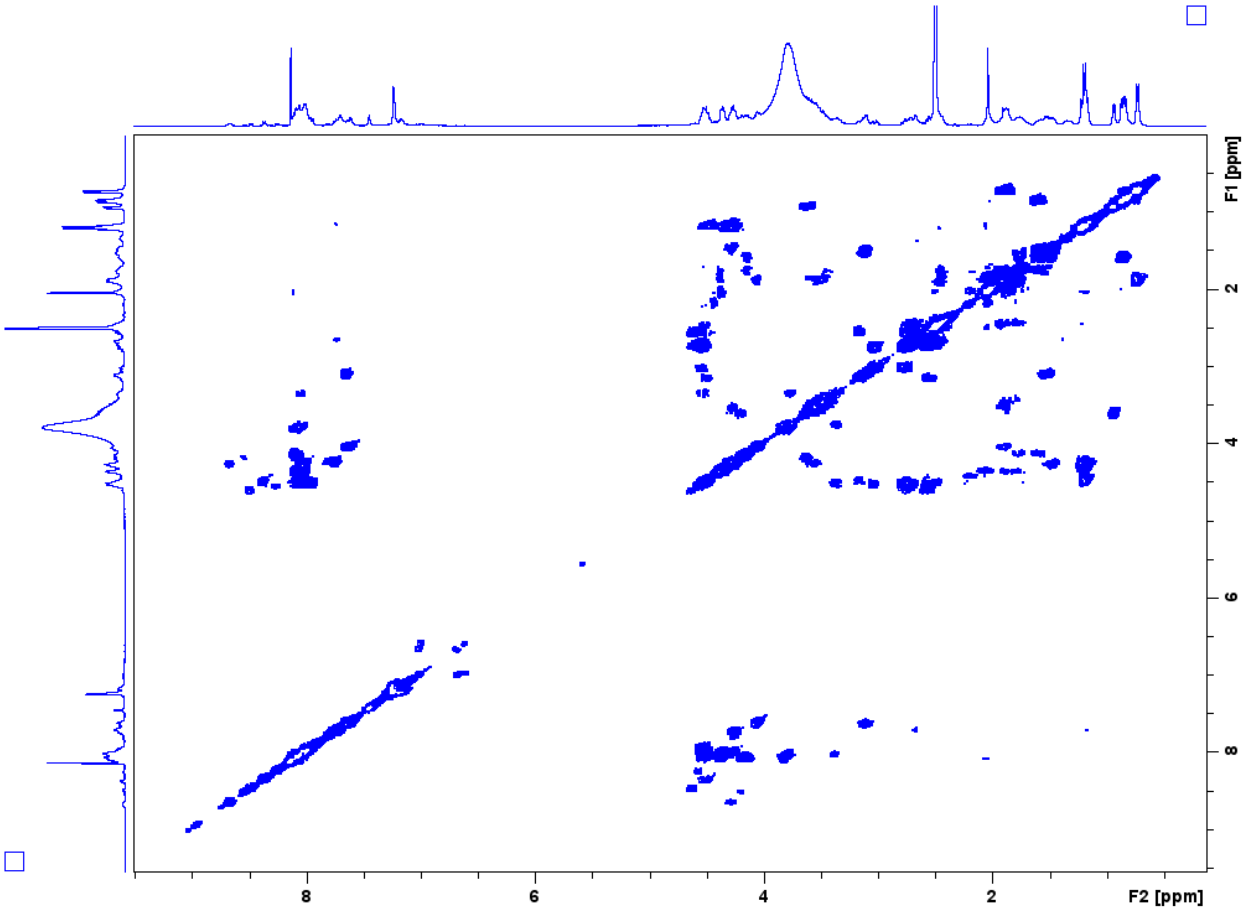

Figure S10. COSY spectrum (400 MHz) of **1** in DMSO-d_6 with 0.15% TFA-*d*

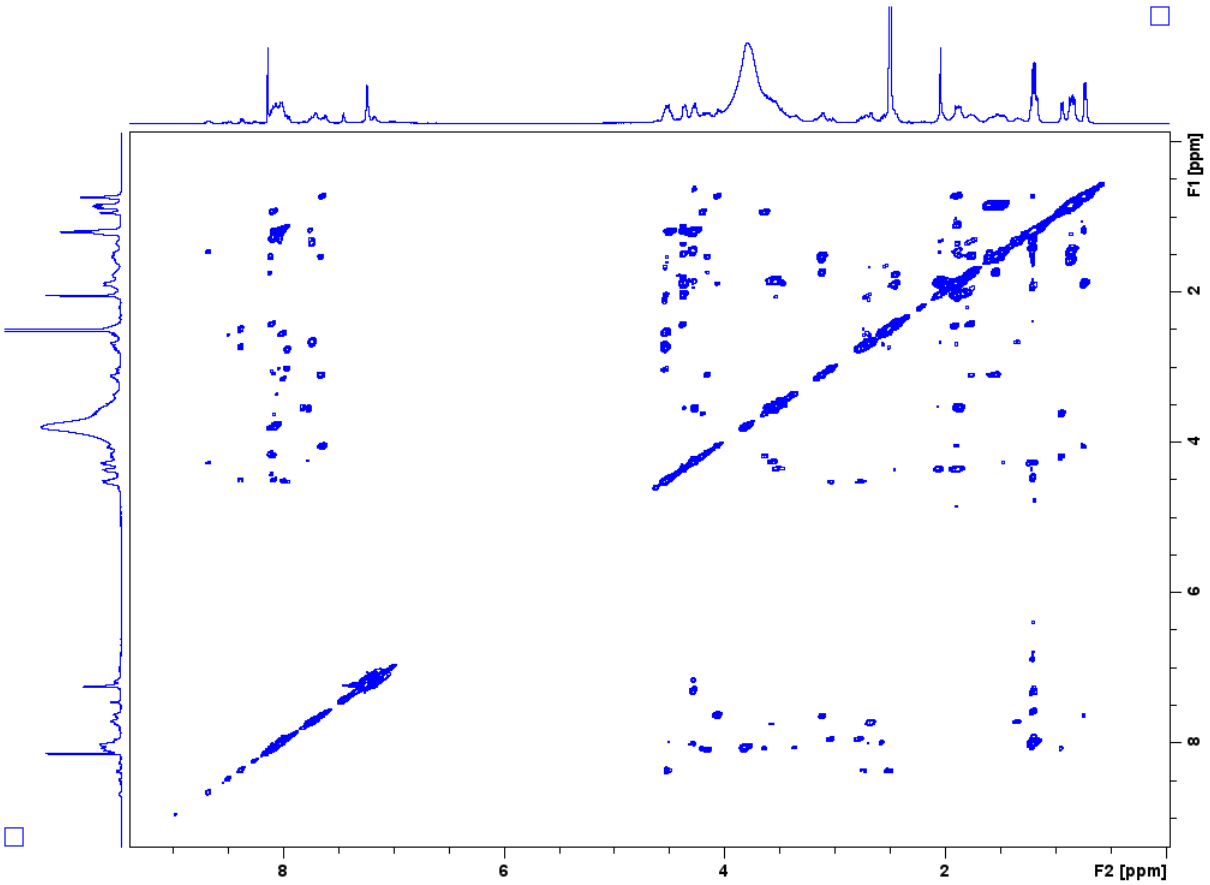

Figure S11. TOCSY spectrum (400 MHz) of **1** in DMSO-d_6 with 0.15% TFA-*d*

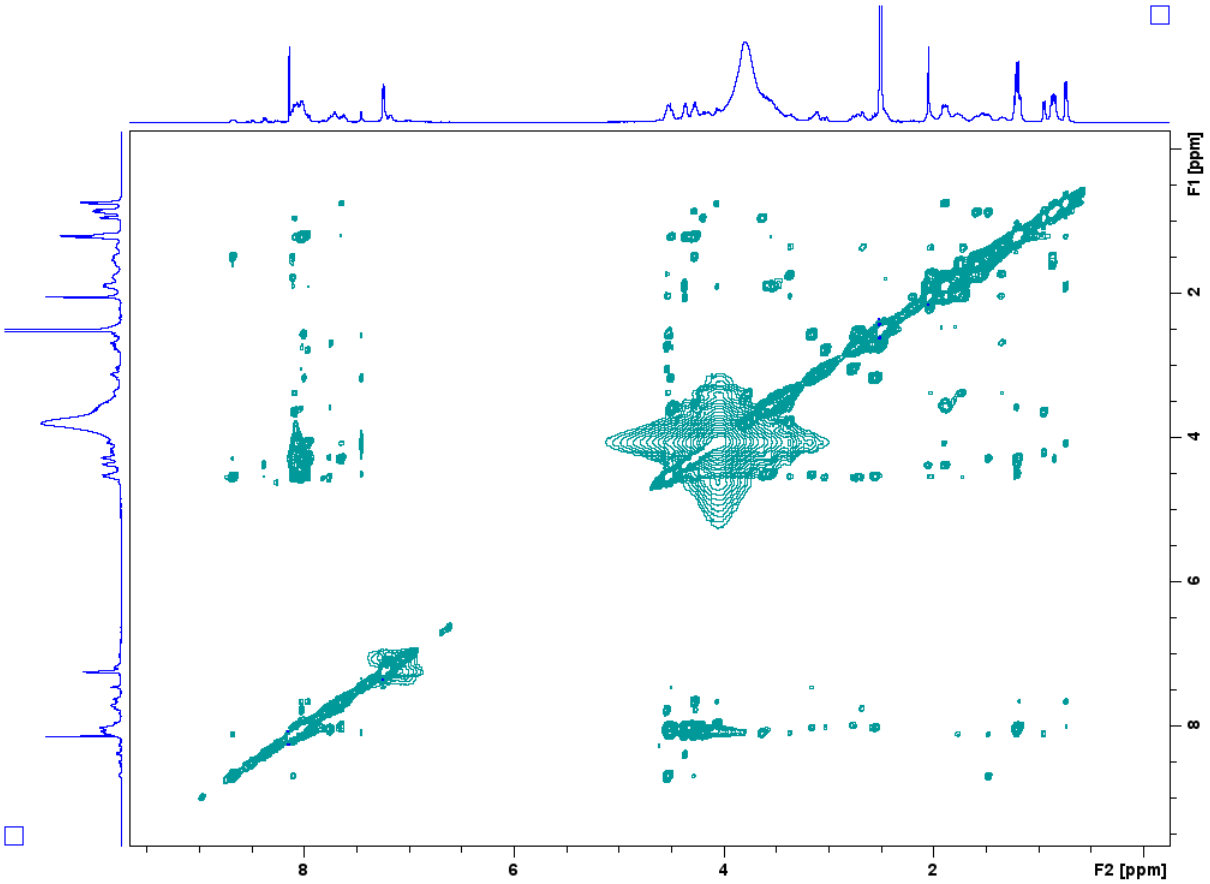

Figure S12. NOESY spectrum (400 MHz) of **1** in DMSO-d_6 with 0.15% TFA-*d*

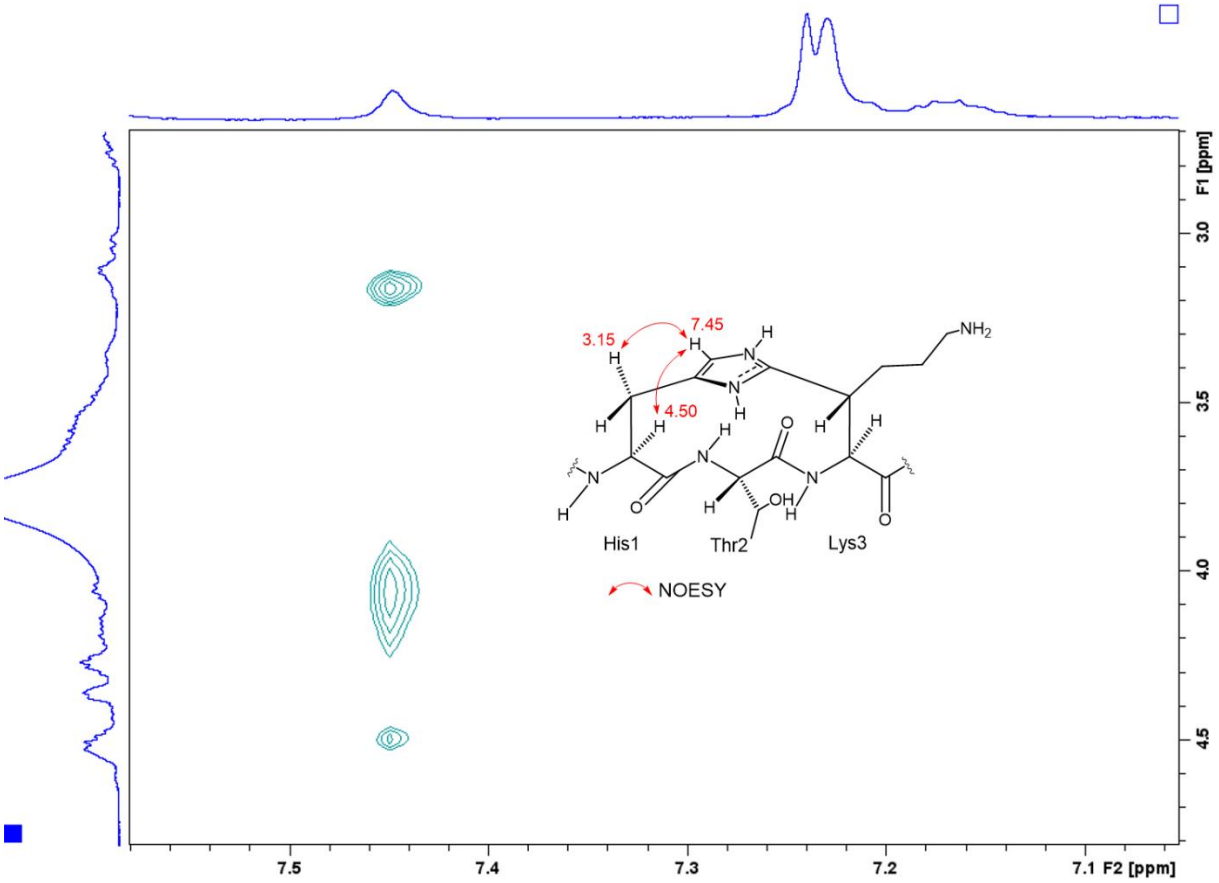

Figure S13. NOESY spectrum (His1-H5) of **1** in DMSO-d_6 with 0.15% TFA-*d*

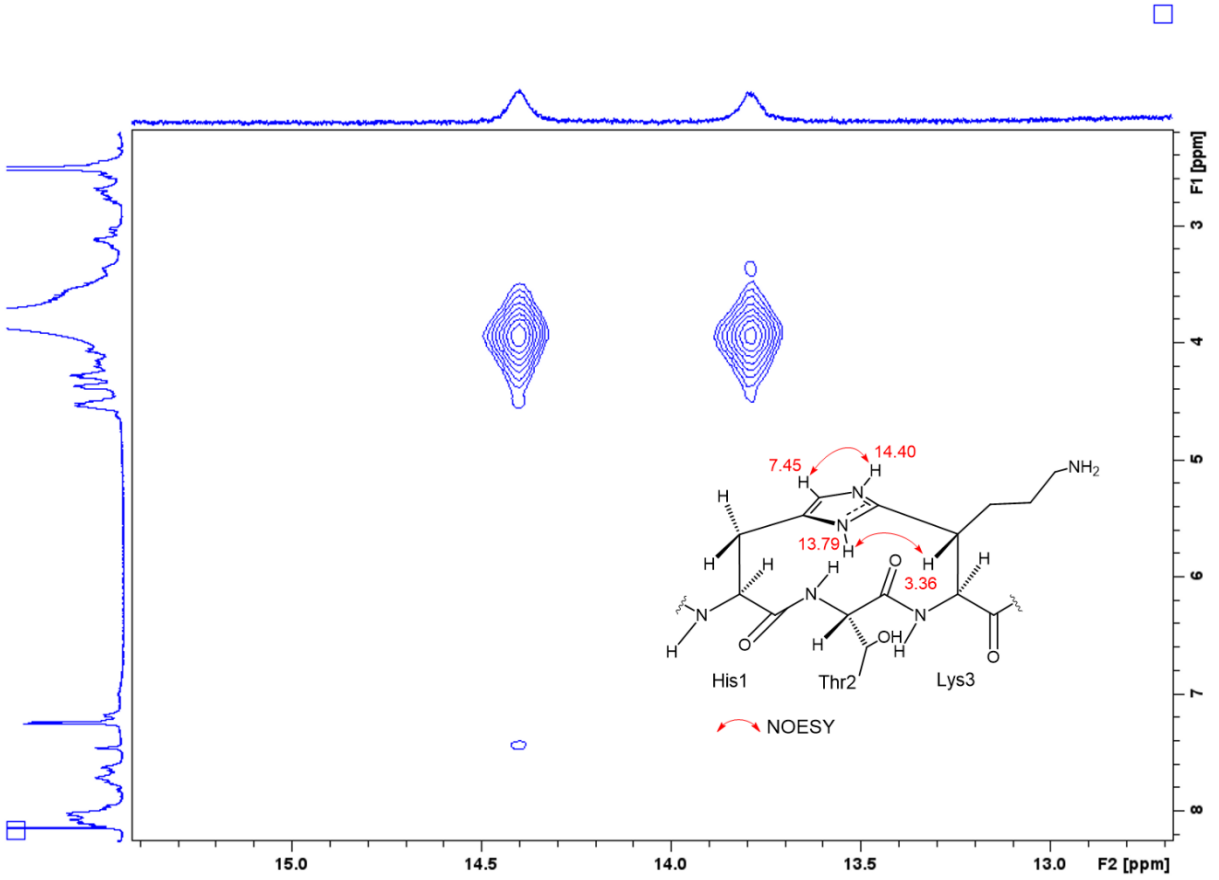

Figure S14. NOESY spectrum (His1-NHτ and NHπ) of **1** in DMSO-d_6 with 0.15% TFA-*d*

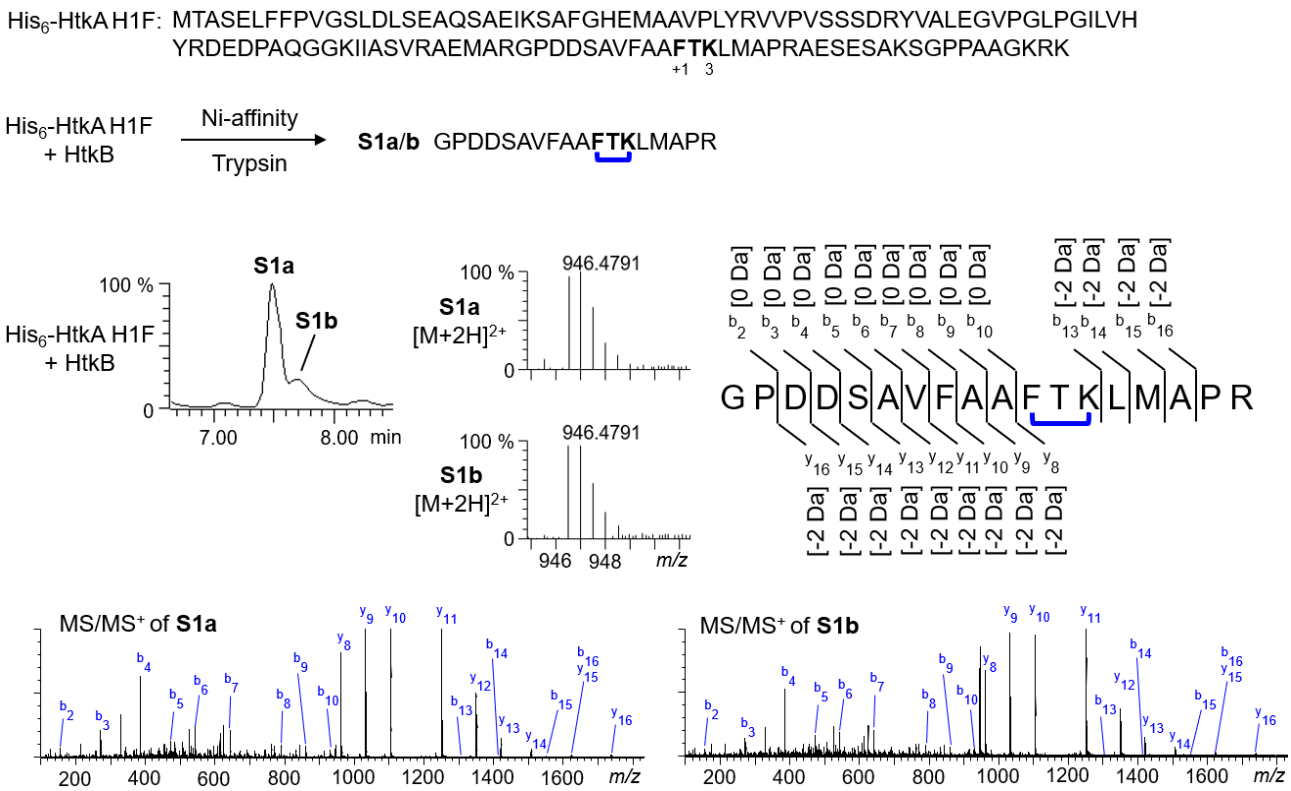

Figure S15. *In vivo* coexpression of His_6-HtkA H1F + HtkB followed by Ni-affinity purification and trypsin digestion yielded fragment **S1a**/**b**. The EIC, MS and MS/MS spectra of **S1a**/**b**.

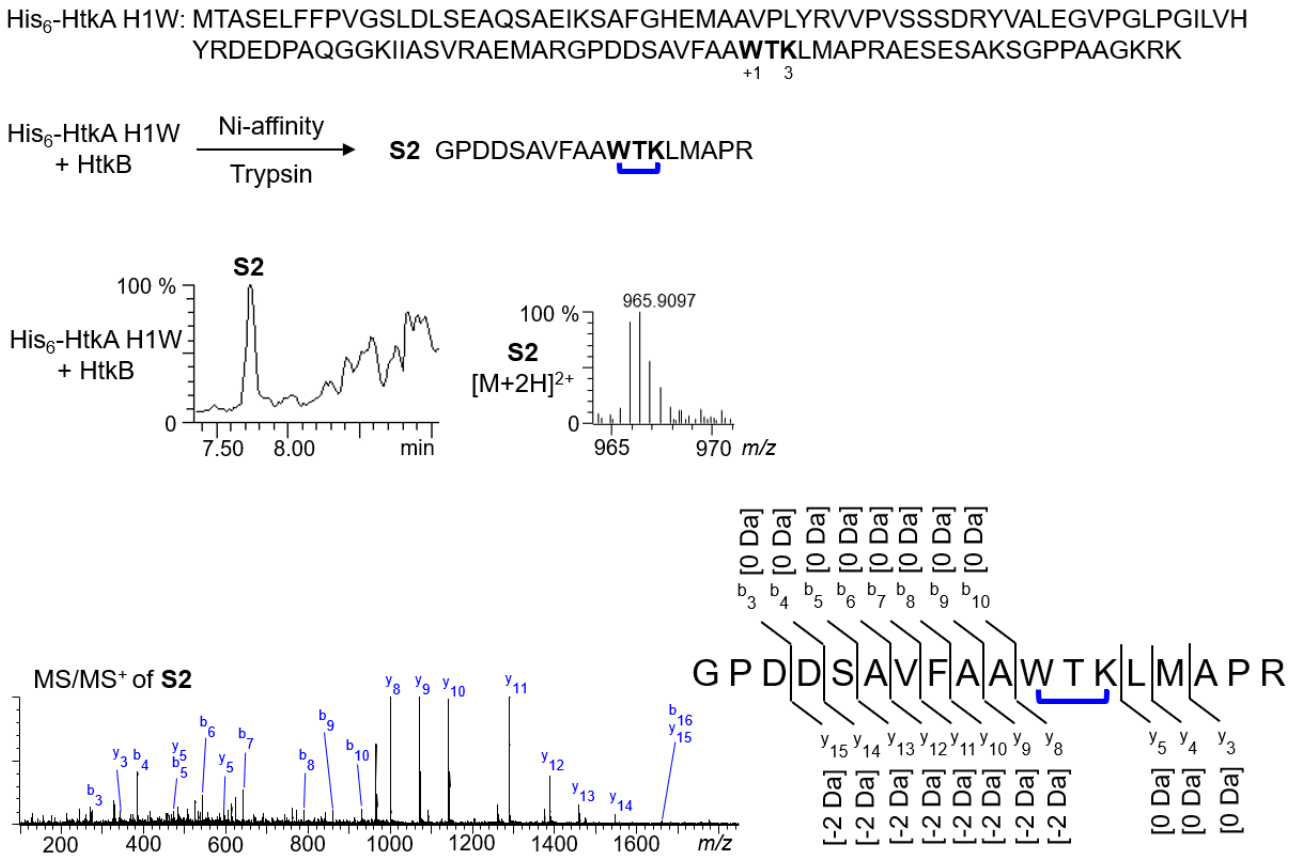

Figure S16. *In vivo* coexpression of His_6-HtkA H1W+ HtkB followed by Ni-affinity purification and trypsin digestion yielded fragment **S2**. The EIC, MS and MS/MS spectra of **S2**.

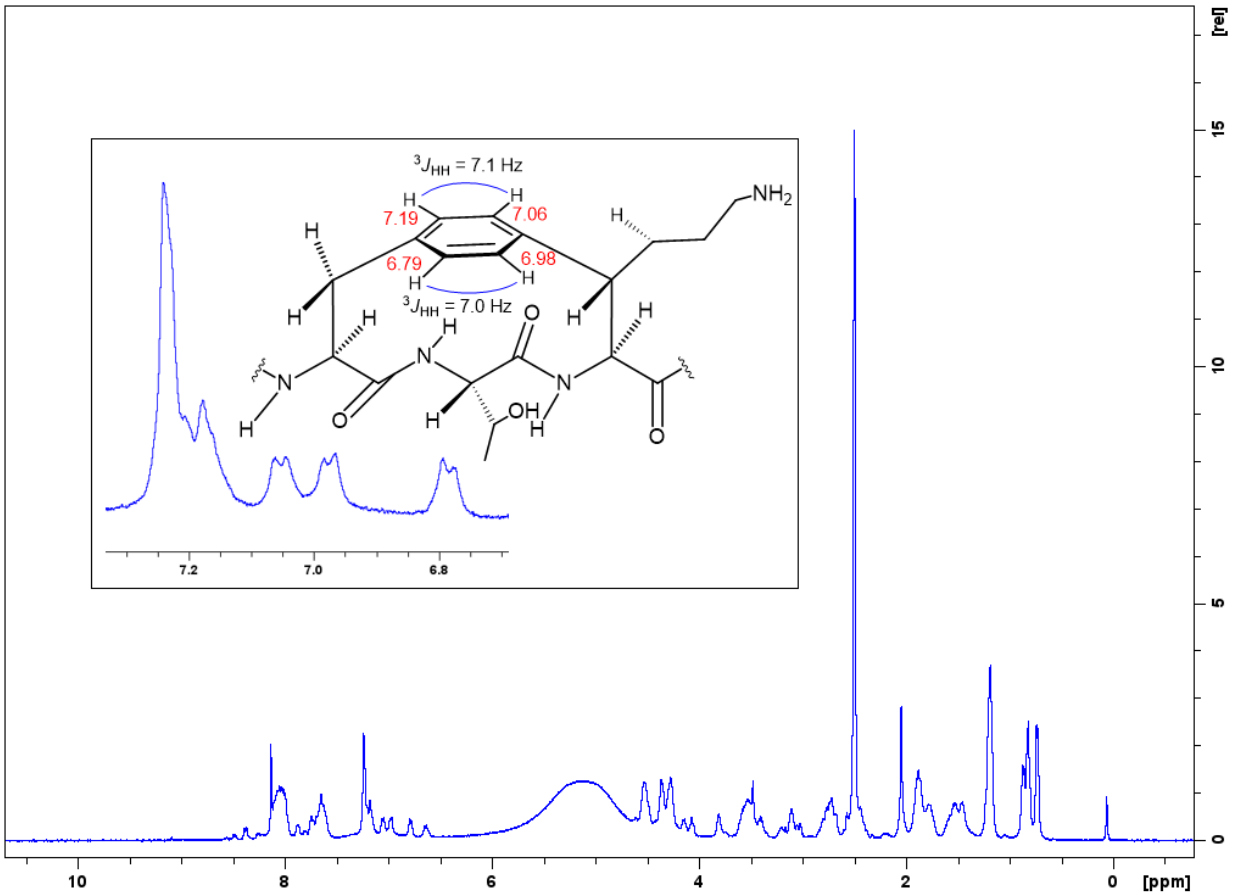

Figure S17. ^{1}H NMR (400 MHz) spectrum of **S1a** in DMSO-d_6 with 0.3% TFA-*d*

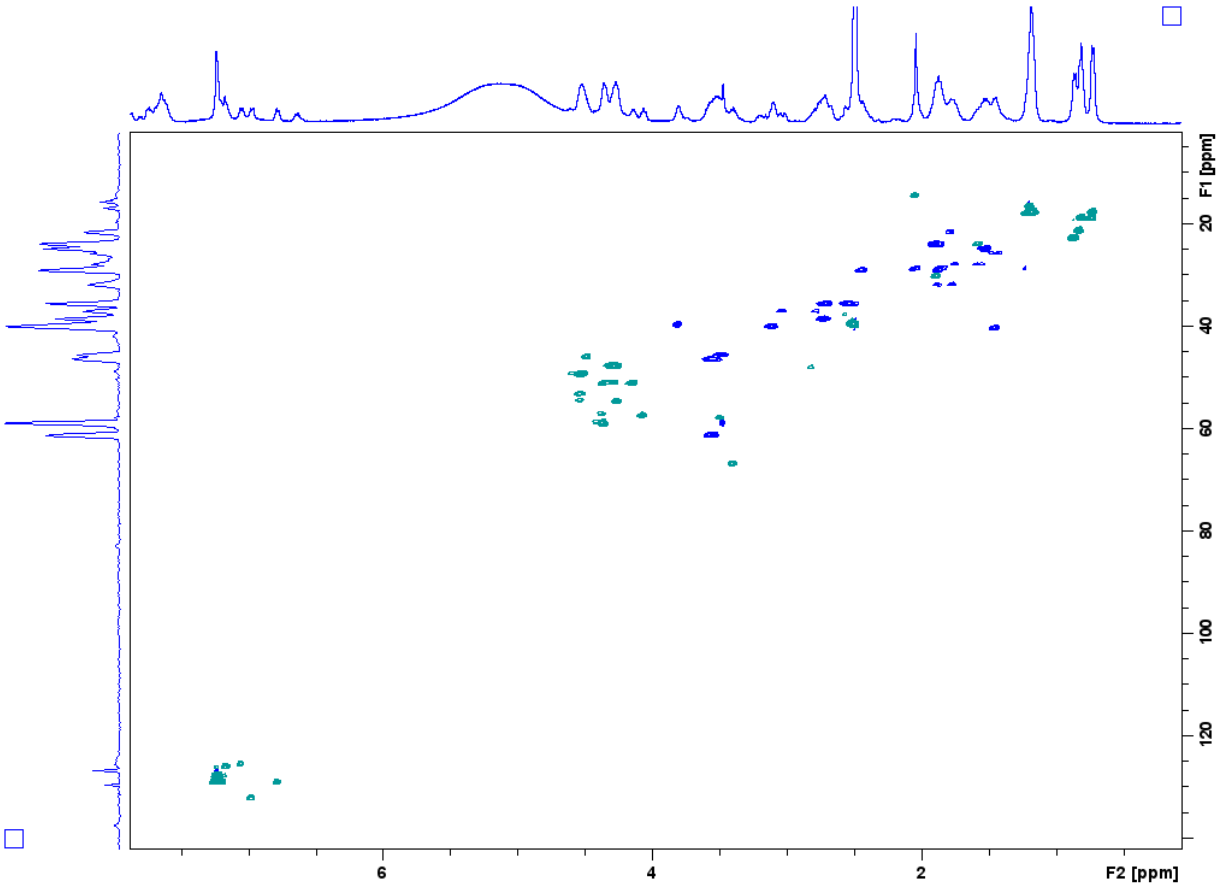

Figure S18. HSQC spectrum (400 MHz) of **S1a** in DMSO-d_6 with 0.3% TFA-*d*

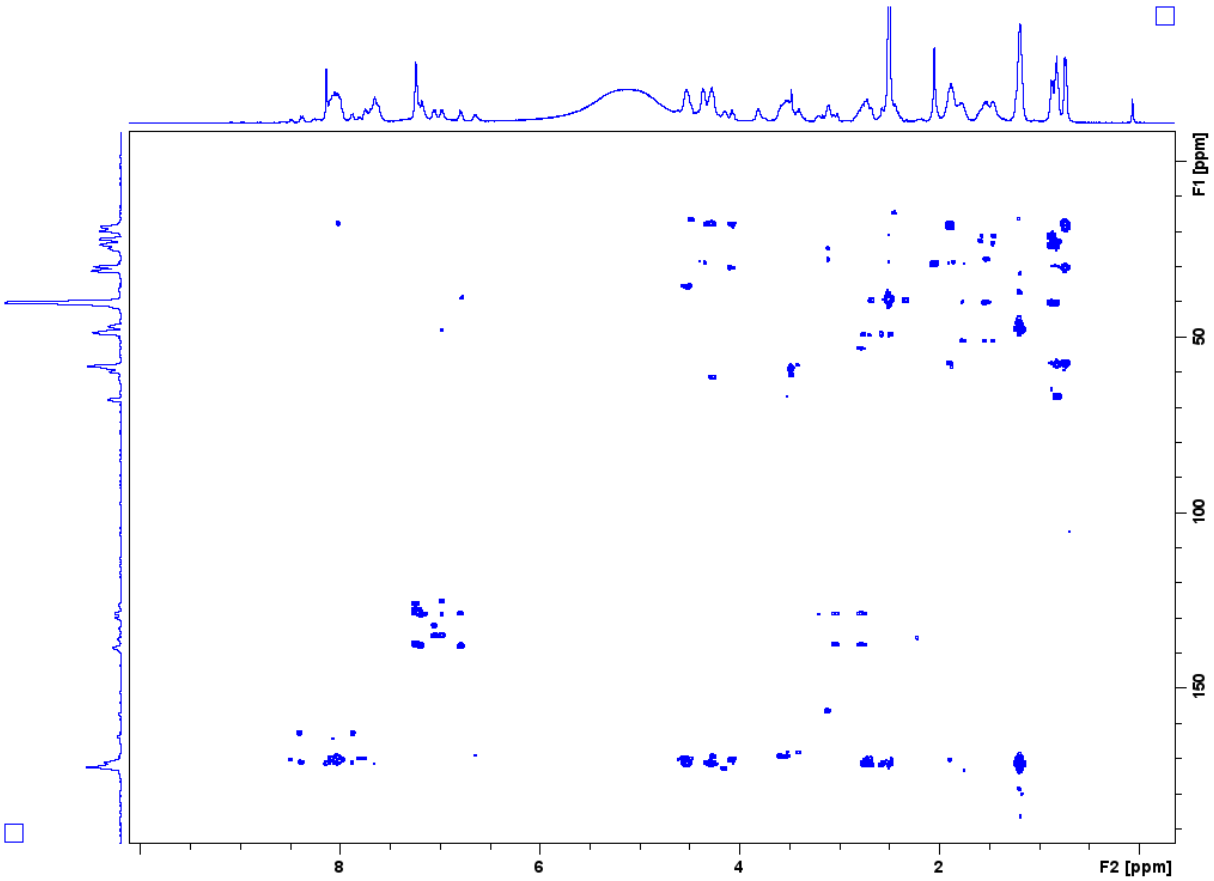

Figure S19. HMBC spectrum (400 MHz) of **S1a** in DMSO-d_6 with 0.3% TFA-*d*

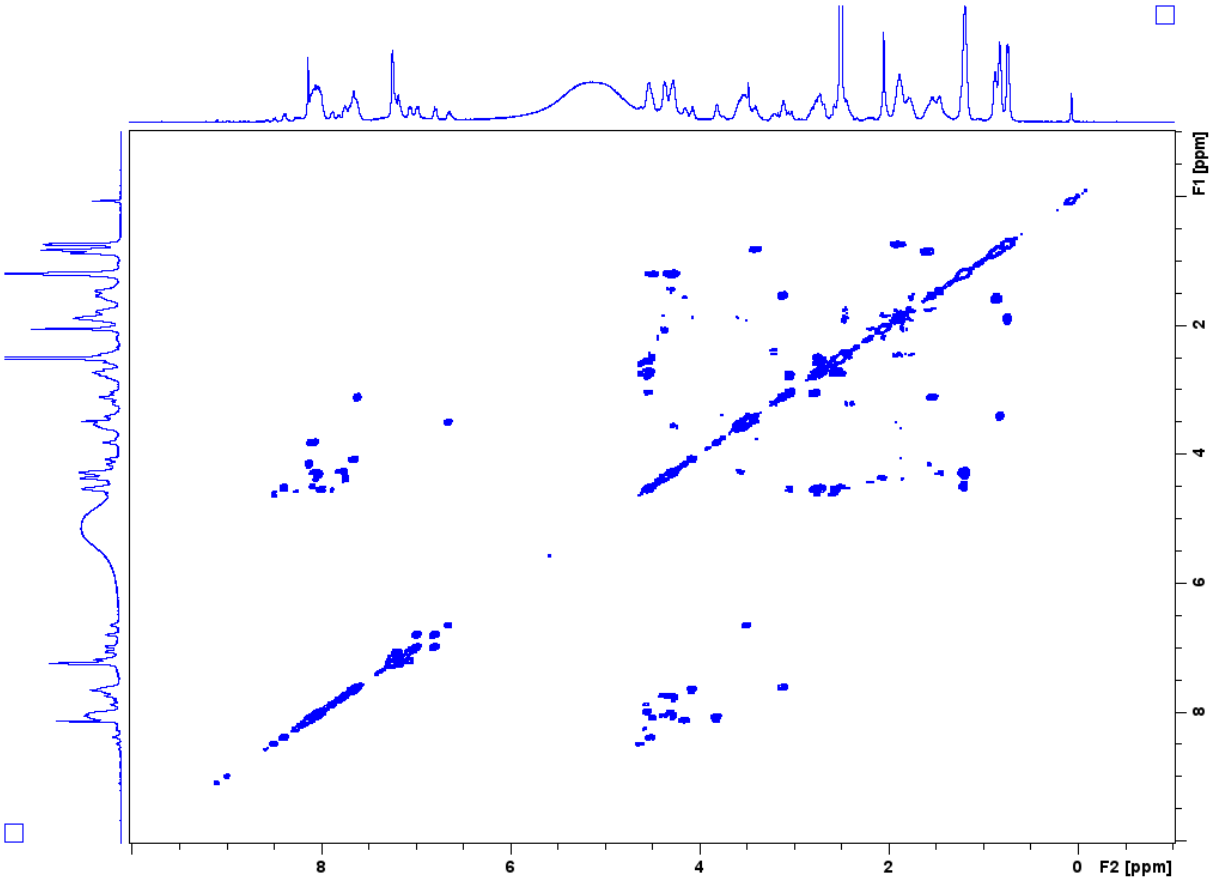

Figure S20. COSY spectrum (400 MHz) of **S1a** in DMSO-d_6 with 0.3% TFA-*d*

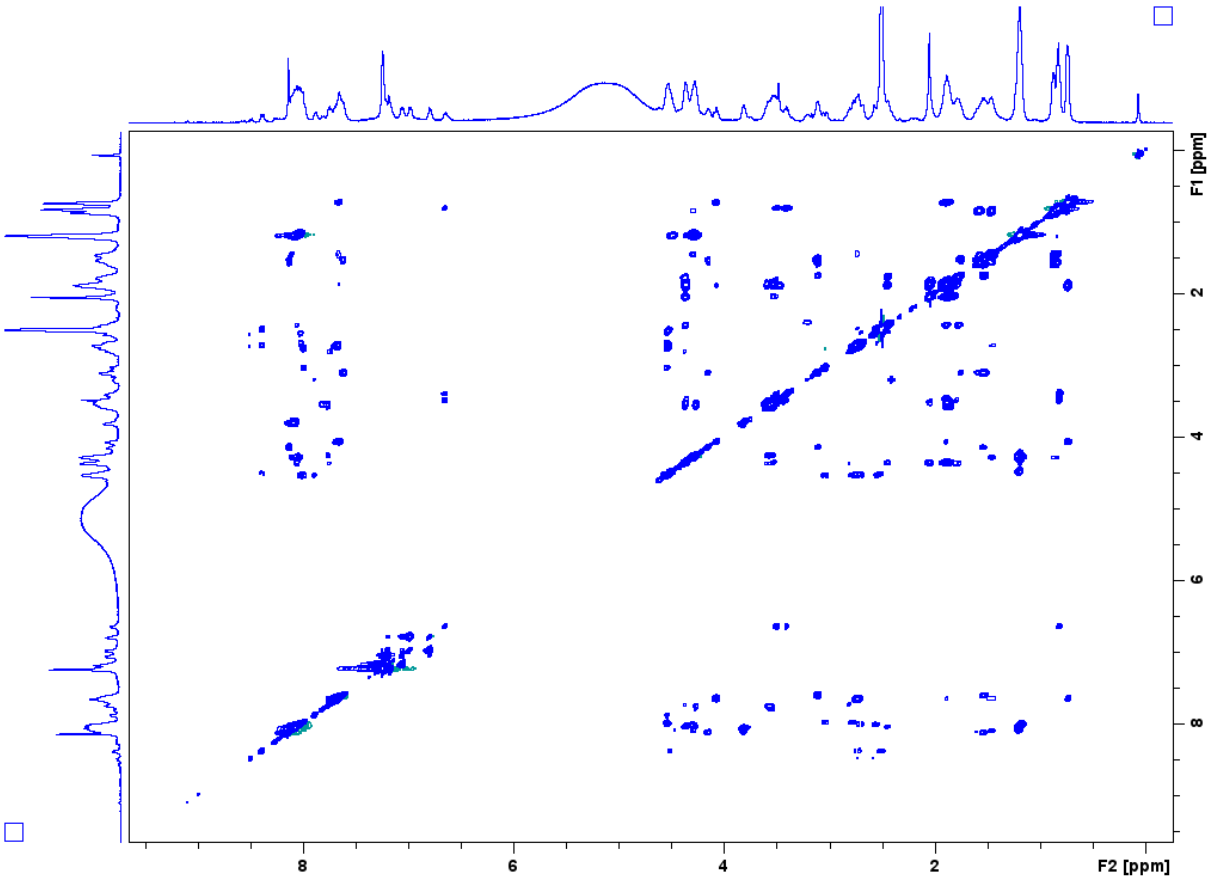

Figure S21. TOCSY spectrum (400 MHz) of **S1a** in DMSO-d_6 with 0.3% TFA-*d*

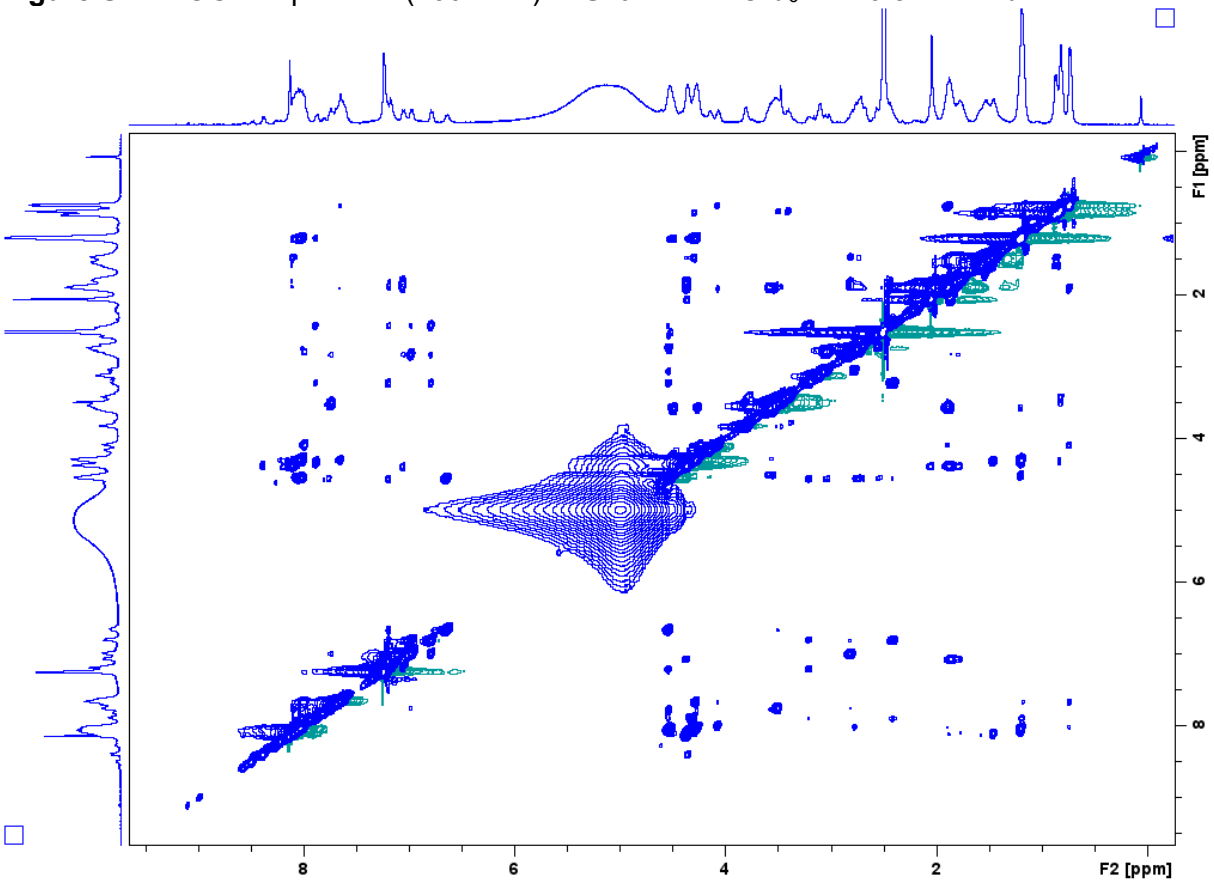

Figure S22. NOESY spectrum (400 MHz) of **S1a** in DMSO-d_6 with 0.3% TFA-*d*

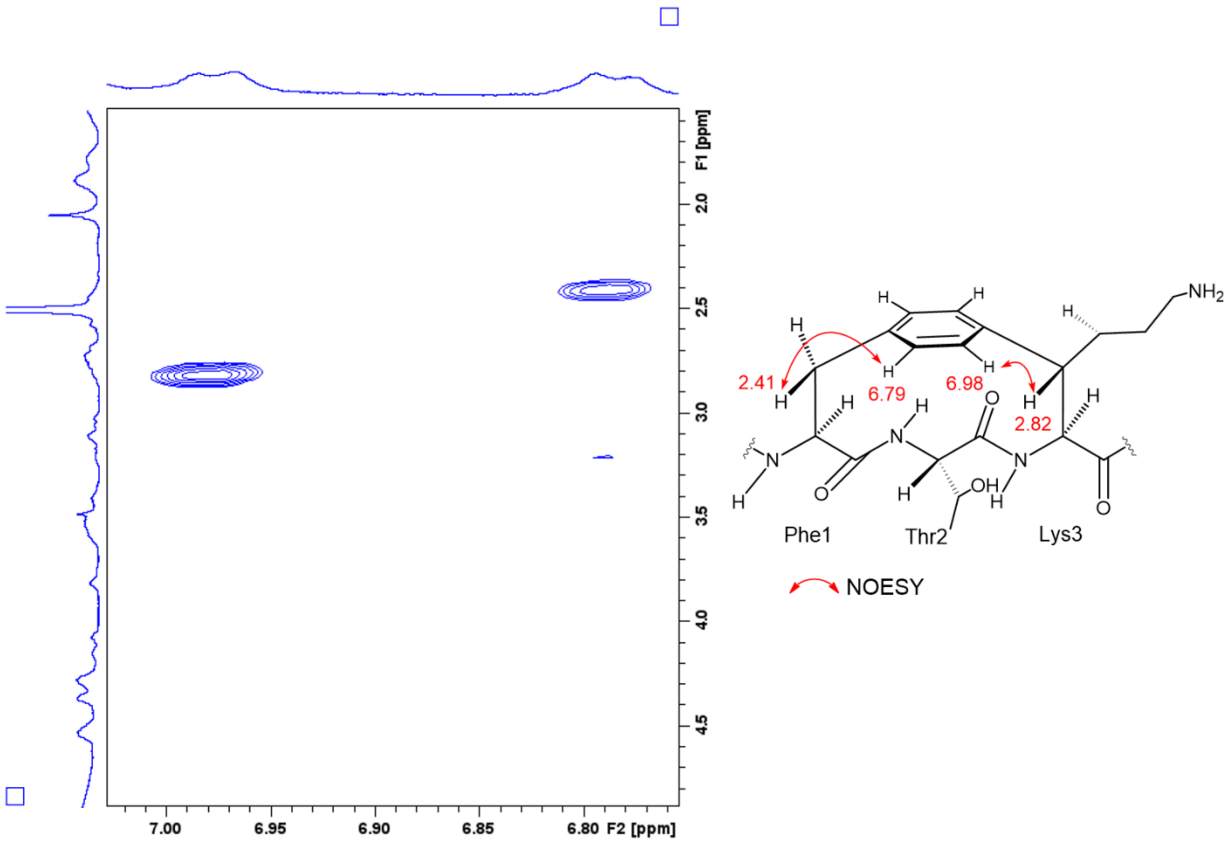

Figure S23. NOESY spectrum (Phe1-H2 and H3) of **S1a** in DMSO-d_6 with 0.3% TFA-*d*

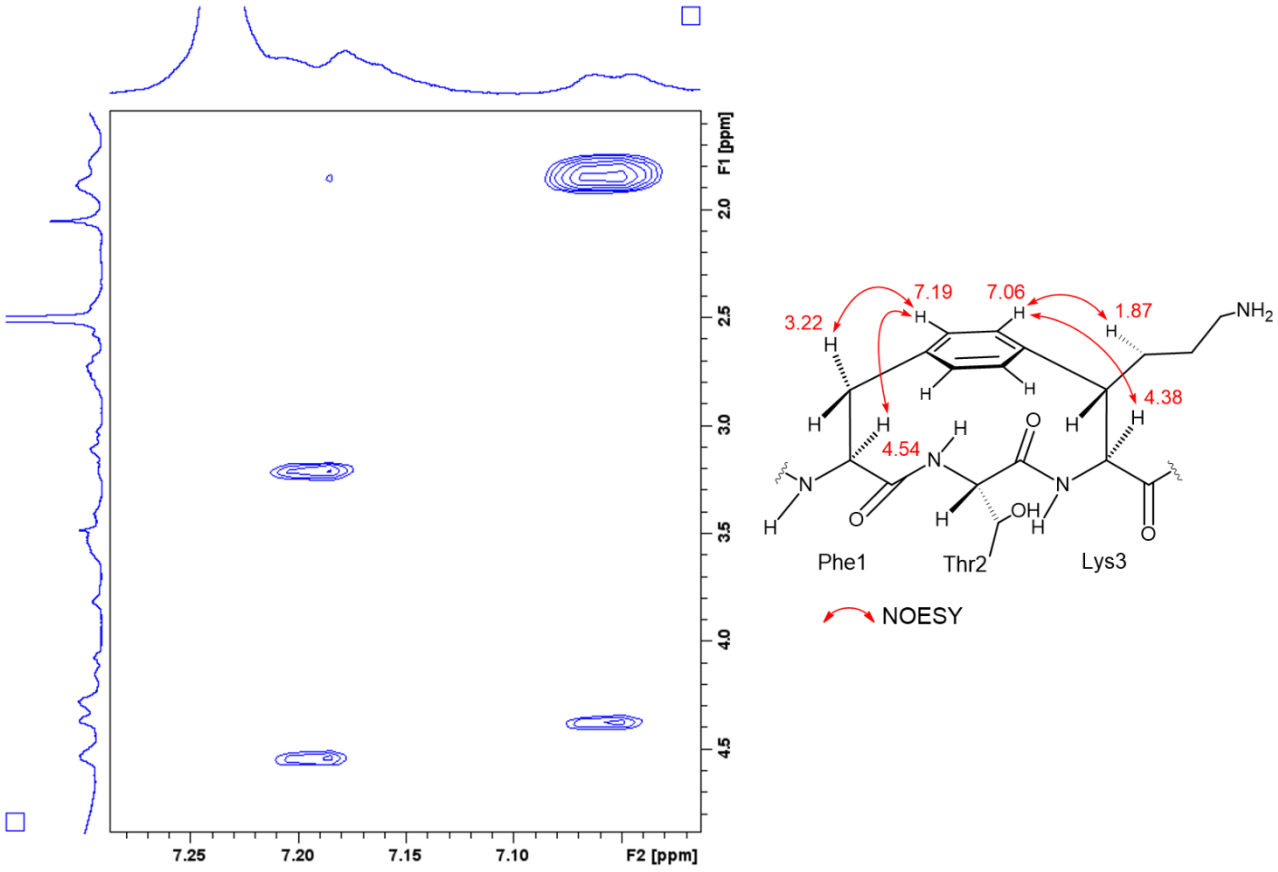

Figure S24. NOESY spectrum (Phe1-H5 and H6) of **S1a** in DMSO-d_6 with 0.3% TFA-*d*

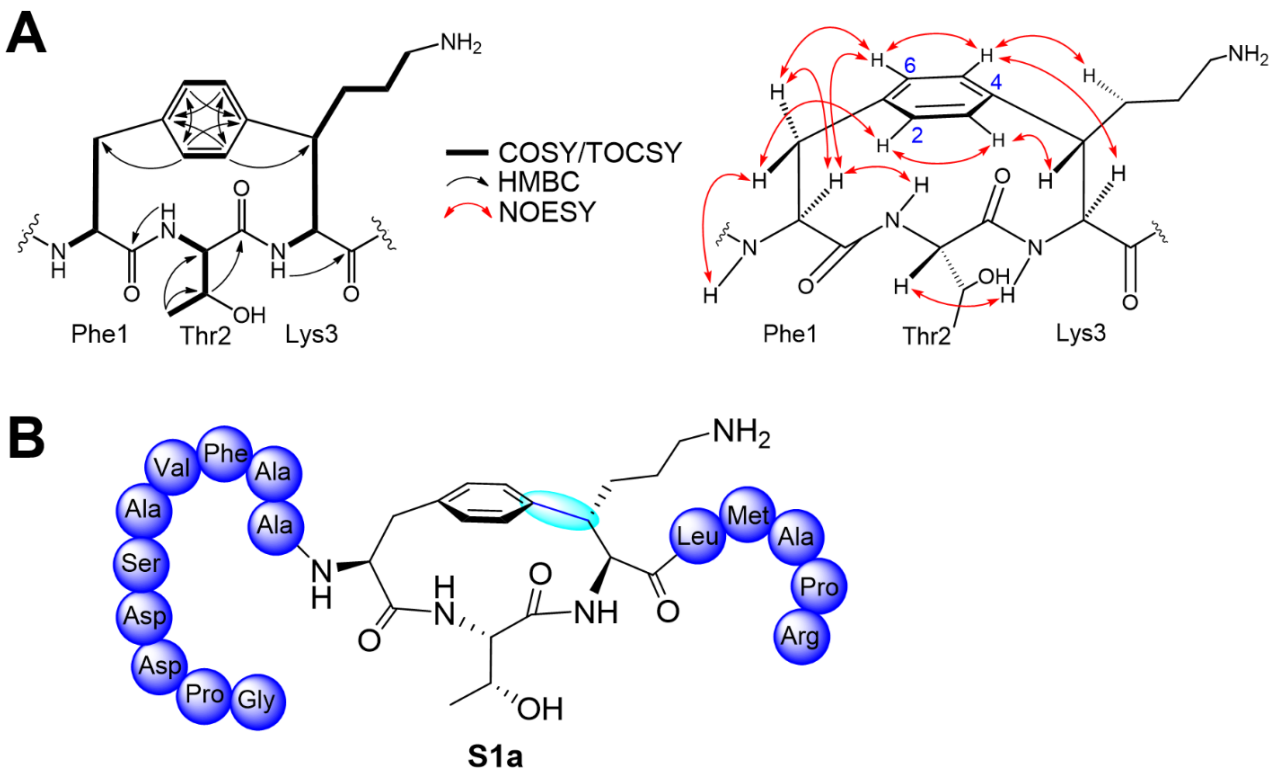

Figure S25. (A) 2D NMR correlations of the FTK macrocycle in fragment **S1a**. (B) Structure of fragment **S1a**.

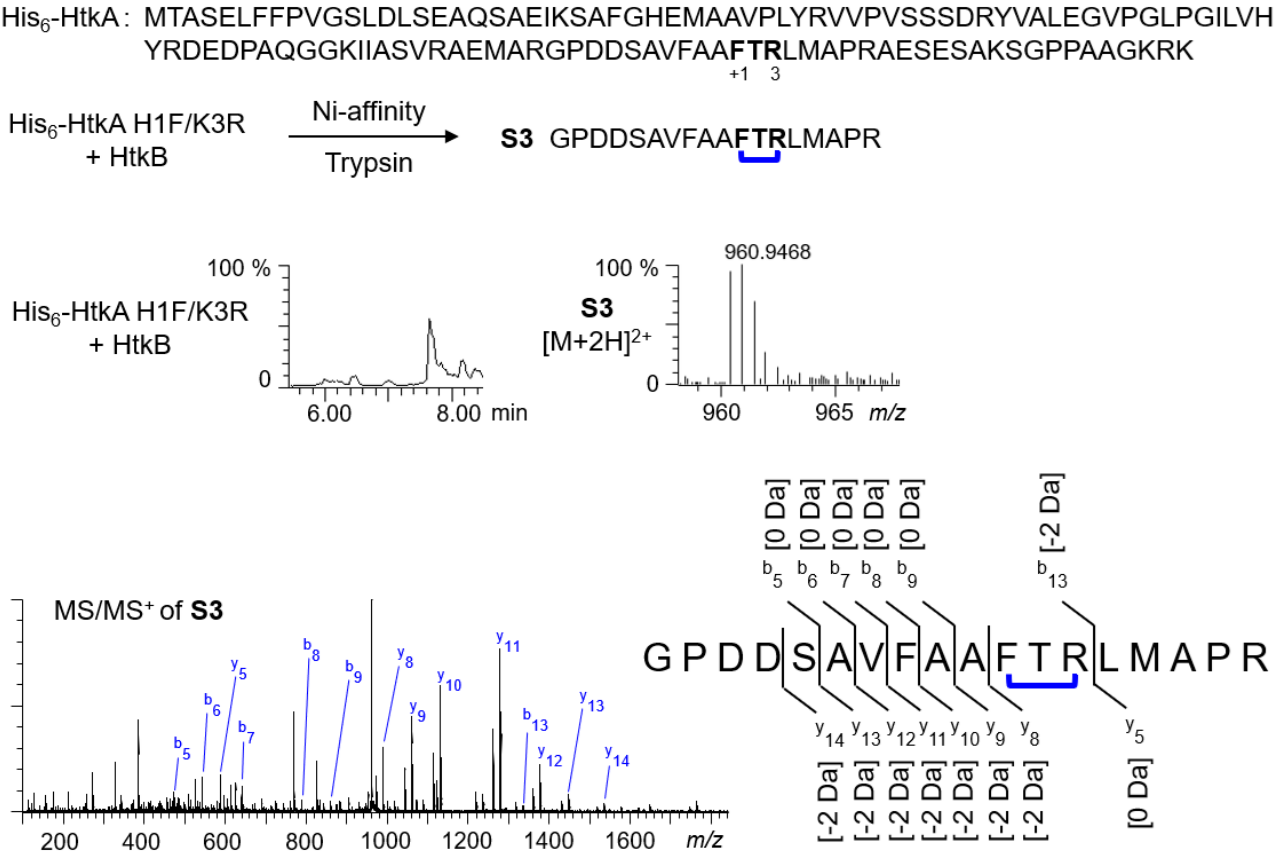

Figure S26. *In vivo* coexpression of His_6-HtkA H1F/K3R + HtkB followed by Ni-affinity purification and trypsin digestion yielded fragment **S3**. The EIC, MS and MS/MS spectra of **S3**.

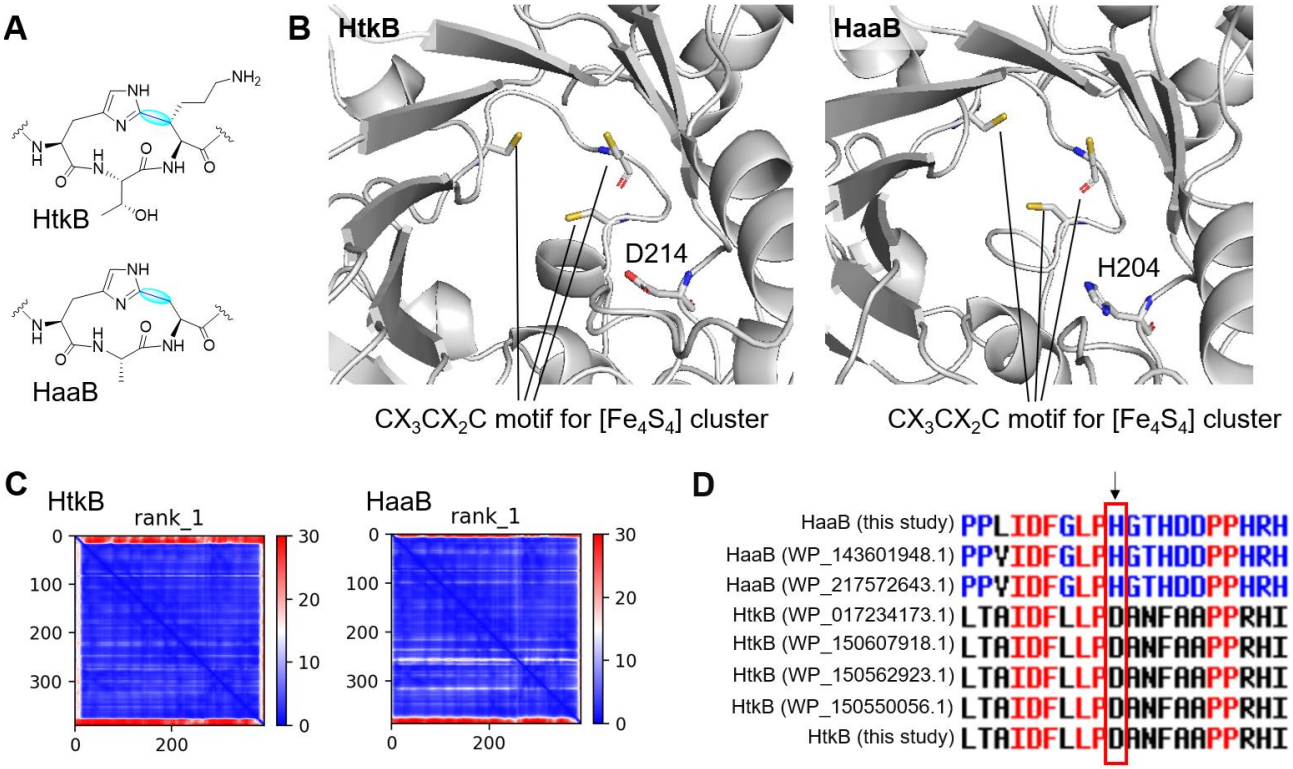

Figure S27. Protein structure and sequence alignment of HtkB and HaaB. (A) HTK and HAA motifs catalyzed by HtkB and HaaB, respectively. (B) Predicted protein structure of HtkB and HaaB by ColabFold[5] showed D214 in HtkB and H204 in HaaB are sticking inward to the CX_3CX_2C motif. (C) AlphaFold2 confidence measure of PAE for modeling of HtkB and HaaB. (D) Primary sequences alignment identified D214 in HtkB is aligned to H204 in HaaB.

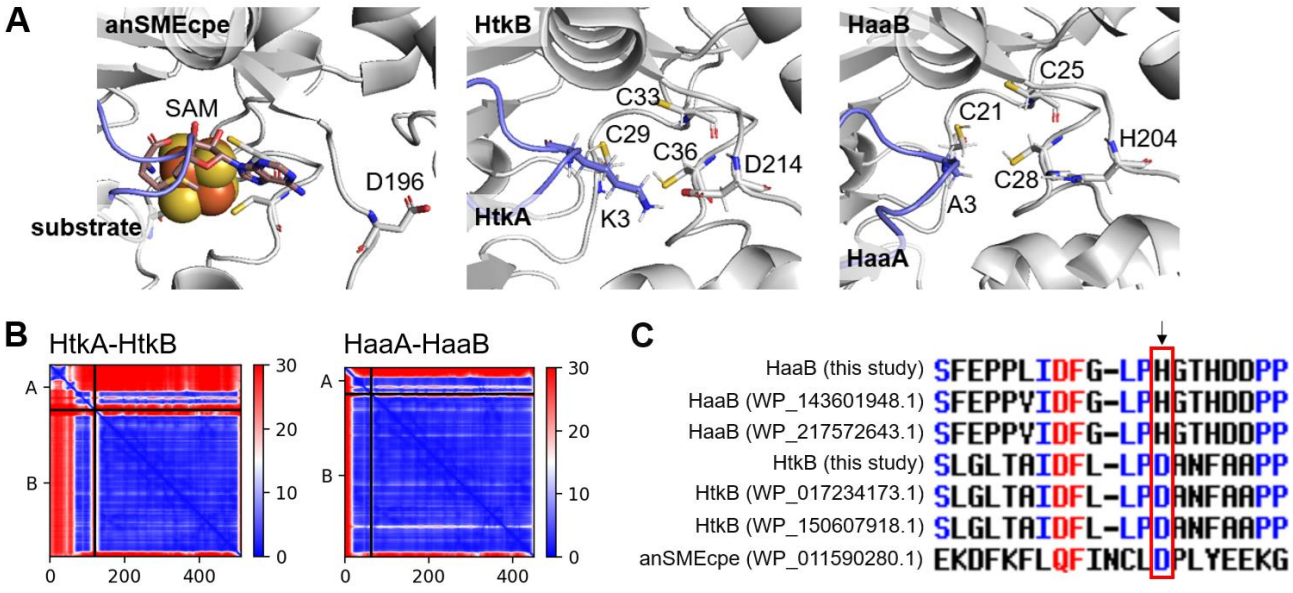

Figure S28. Protein structure of enzyme-substrate complex. (A) Predicted protein complex structures of HtkA-HtkB and HaaA-HaaB by ColabFold[5] showed Asp214 in HtkB and His204 in HaaB are sticking towards to the X3 position of substrates. Structure for anSMEcpe (PDB: 4K39). (B) AlphaFold2 confidence measure of PAE for modeling the complexes of HtkA-HtkB and HaaA-HaaB. (D) Primary sequences alignment identified D214 in HtkB and H204 in HaaB is aligned to D196 in anSMEcpe.

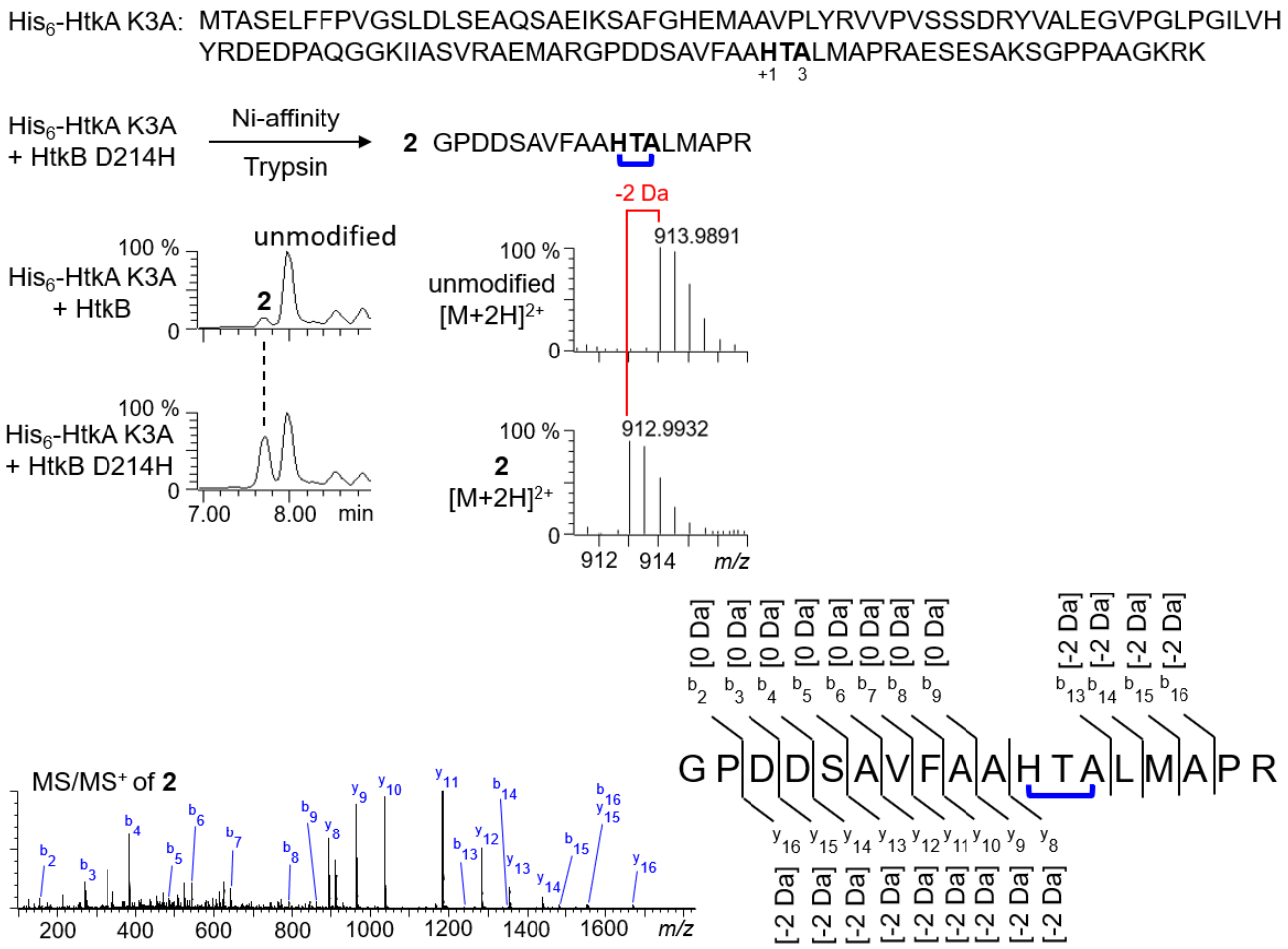

Figure S29. *In vivo* coexpression of His_6-HtkA K3A + HtkB D214H followed by Ni-affinity purification and trypsin digestion yielded fragment **2**. The EIC, MS and MS/MS spectra of **2**.

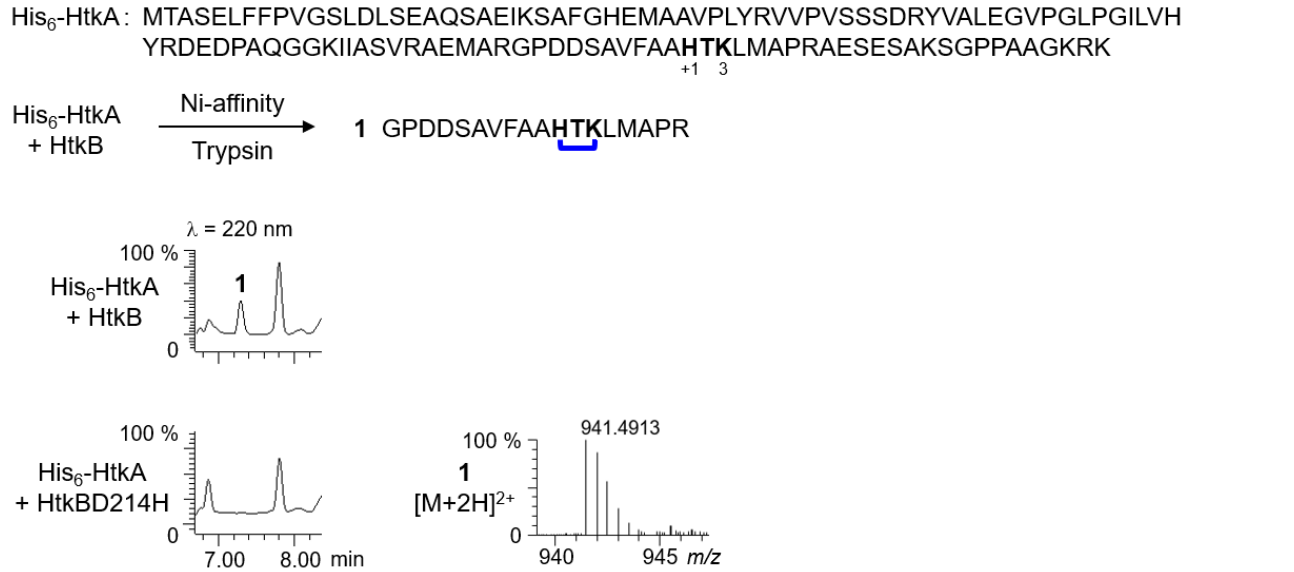

Figure S30. *In vivo* coexpression of His_6-HtkA + HtkB D214H followed by Ni-affinity purification and trypsin digestion yielded fragment **1**. The UV 220 nm chromatogram and MS spectra of **1**.

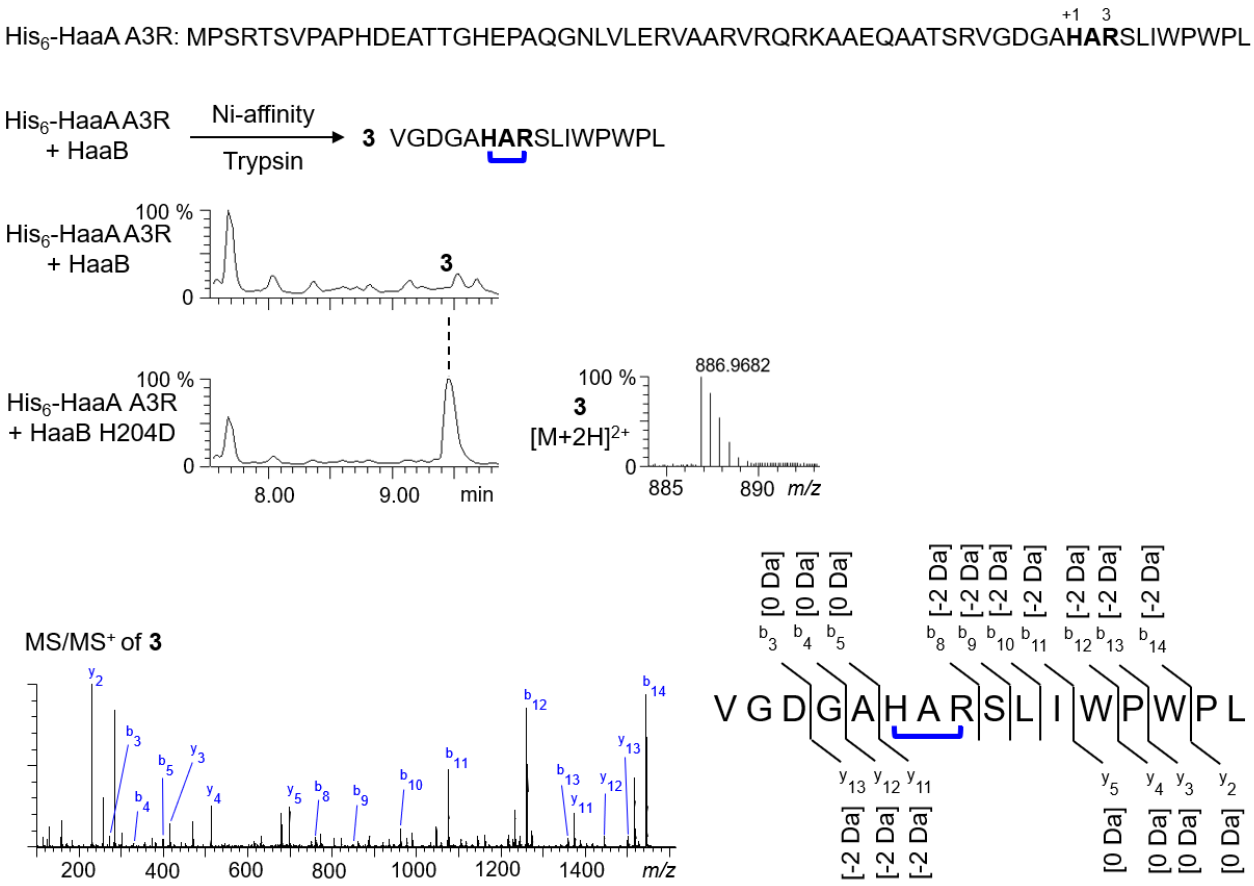

Figure S31. *In vivo* coexpression of His_6-HaaA A3R + HaaB H204D followed by Ni-affinity purification and trypsin digestion yielded fragment **3**. The EIC, MS and MS/MS spectra of **3**.

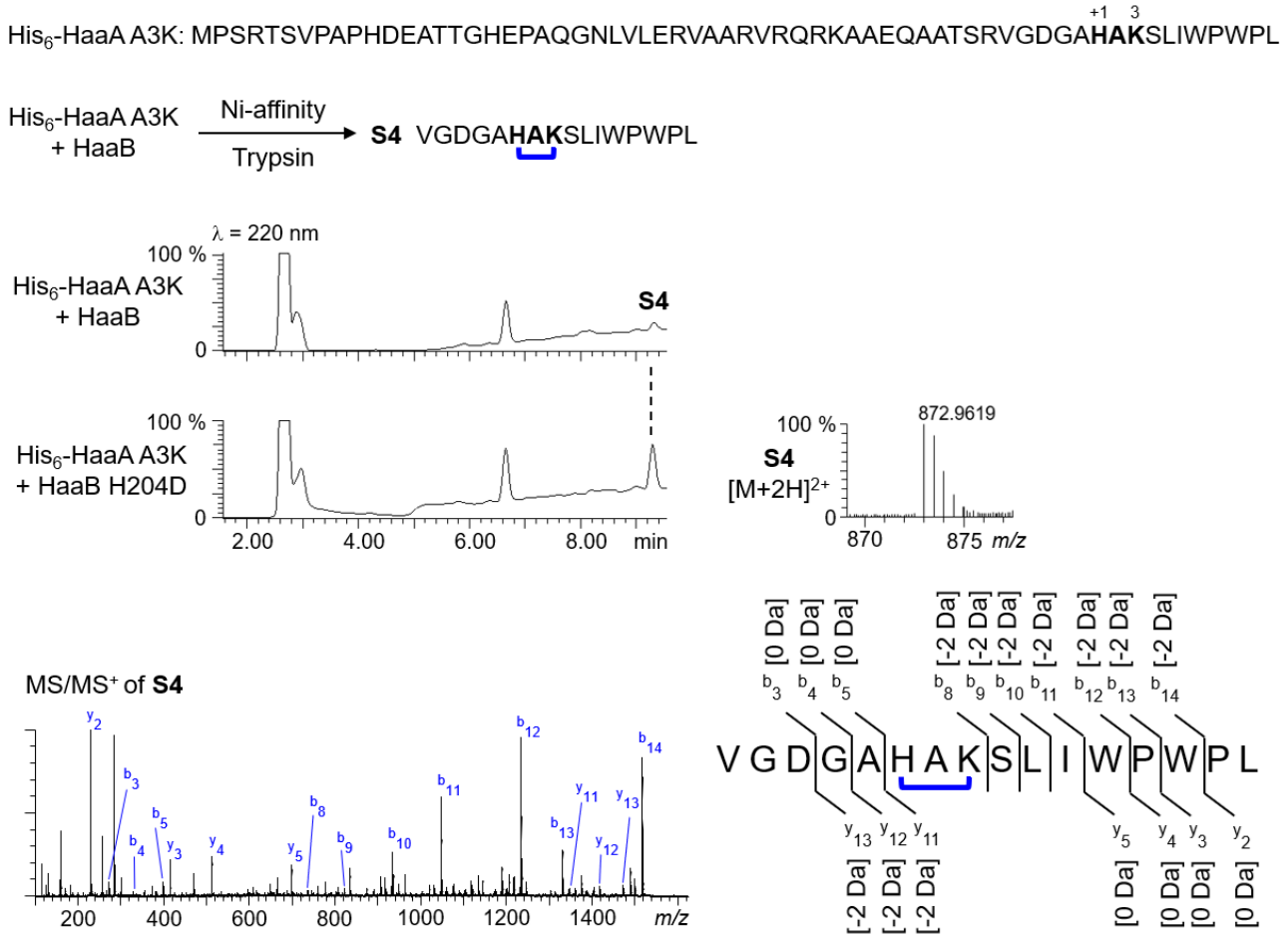

Figure S32. *In vivo* coexpression of His_6-HaaA A3K + HaaB H204D followed by Ni-affinity purification and trypsin digestion yielded fragment **S4**. The UV 220 nm chromatogram, MS and MS/MS spectra of **S4**.

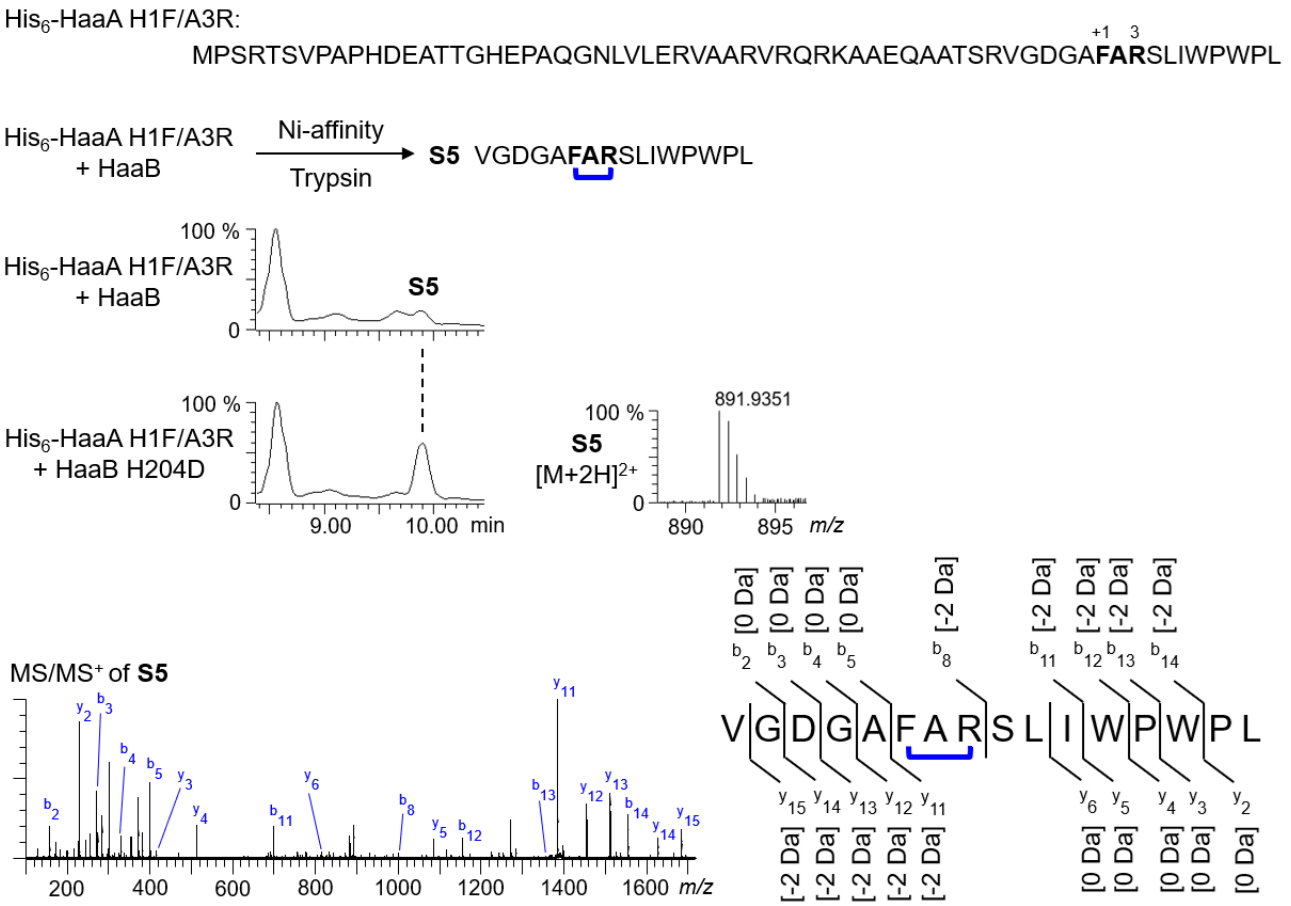

Figure S33. *In vivo* coexpression of His_6-HaaA H1F/A3R + HaaB H204D followed by Ni-affinity purification and trypsin digestion yielded fragment **S5**. The EIC, MS and MS/MS spectra of **S5**.

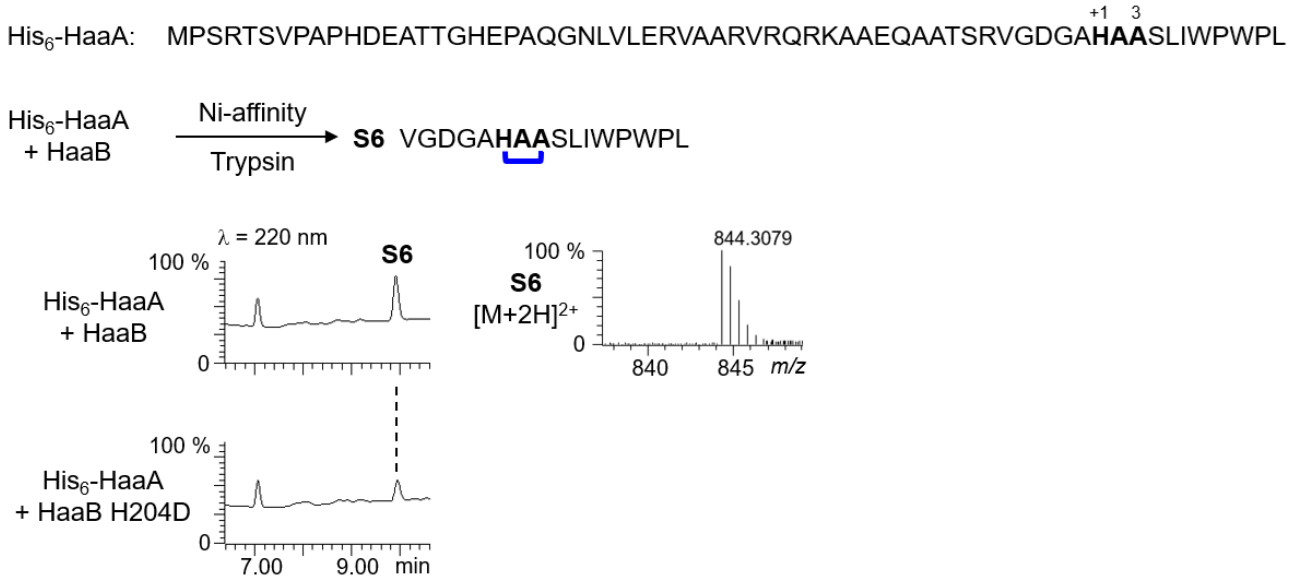

Figure S34. *In vivo* coexpression of His_6-HaaA + HaaB H204D followed by Ni-affinity purification and trypsin digestion yielded fragment **S6**. The UV 220 nm chromatogram and MS spectrum of **S6**.

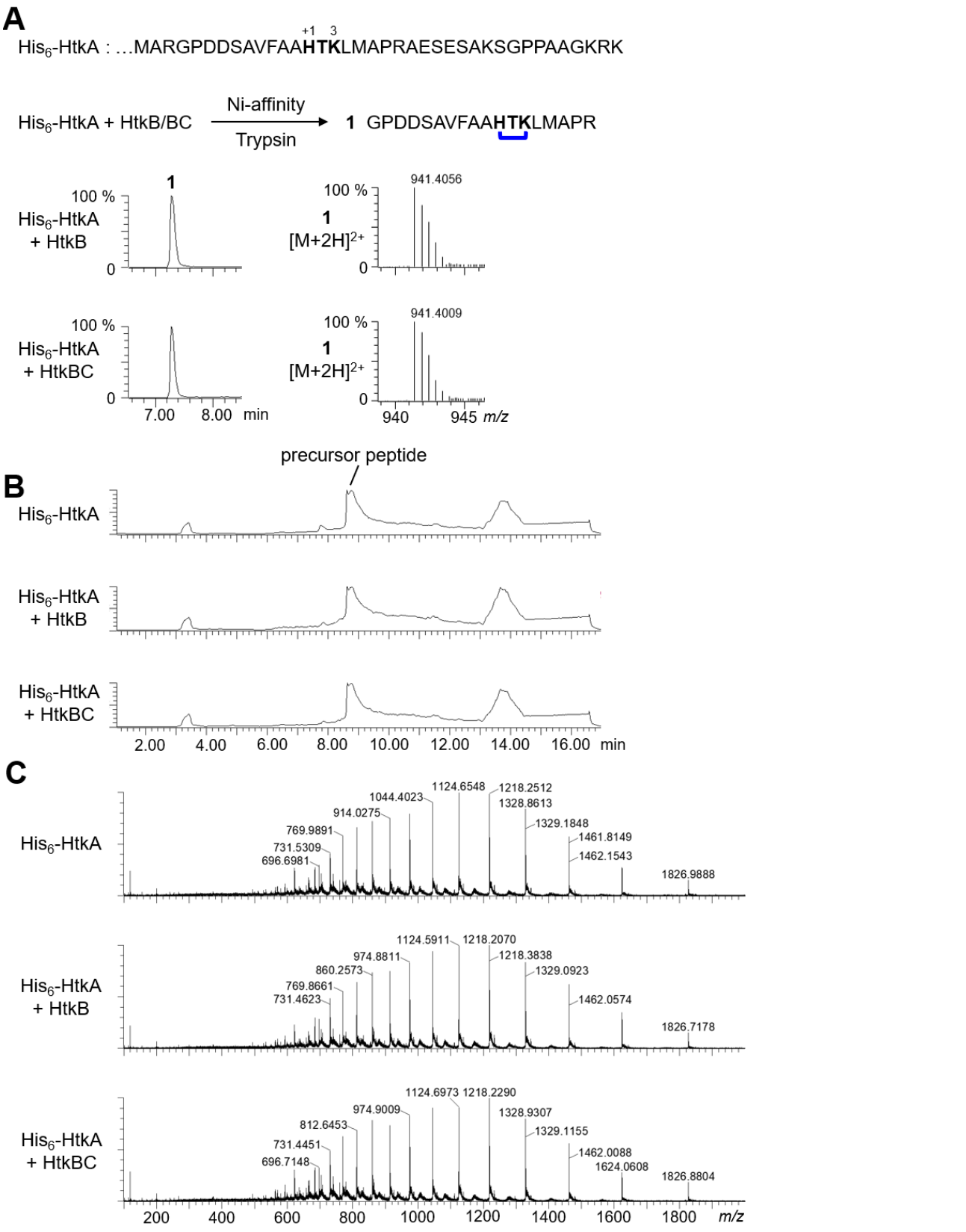

Figure S35. Functional study of HtkC. (A) *In vivo* coexpression of His_6-HtkA + HtkB and His_6-HtkA + HtkBC followed by Ni-affinity purification and trypsin digestion yielded fragment **1**. The MS spectra and EIC of **1**. (B) *In vivo* coexpression of His_6-HtkA, His_6-HtkA + HtkB and His_6-HtkA + HtkBC followed by Ni-affinity purification yielded full-length precursor peptides. TIC of full-length precursor peptides. (C) MS spectra of full-length precursor peptides.

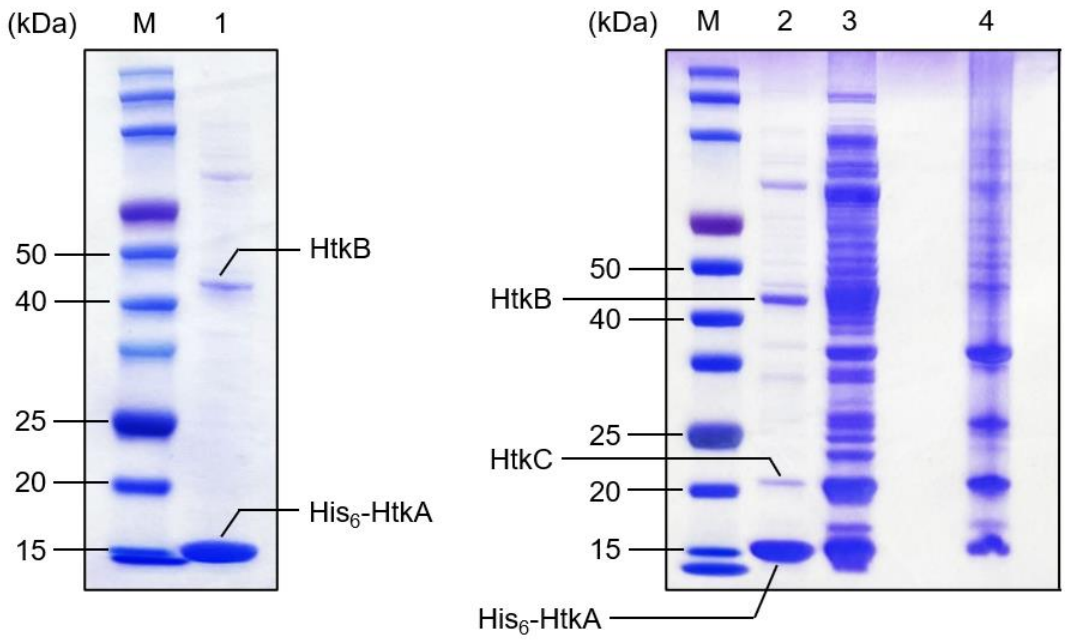

Figure S36. SDS-PAGE of recombinant HtkA/B/C. M: molecular weight marker. 1: *in vivo* coexpression of His_6-HtkA + HtkB in *E. coli* NiCo21 (DE3) and HtkB is pulled down with His_6-HtkA via Ni-affinity purification. 2: *in vivo* coexpression of His_6-HtkA + HtkB + HtkC in *E. coli* NiCo21 (DE3) and both HtkB/C are pulled down with His_6-HtkA via Ni-affinity purification. 3: supernatant from 2. 4: precipitate from 2. Theoretical molecular weight of recombinant His_6-HtkA, HtkB and HtkC are 14.7, 43.3 and 21.4 kDa, respectively.

Table S1. Gene sequences used in this study.

Gene	Vector (Restriction Sites)	Insert Sequence[a]
HtkA[b] WP_130026180	pET28a(+) (NdeI_XhoI)	ACAGCATCAGAGTTATTCTTCCCTGTTGGGTCTCTGGATTT ATCAGAAGCACAGTCCGCGGAAATTAAGAGTGCATTTGGC CACGAAATGGCAGCGGTCCCTCTGTACCGTGTTGTACCAG TCAGTAGTTCCGATAGATATGTCGCATTAGAAGGTGTTCCA GGCCTGCCAGGGATCTTAGTACATTATCGAGATGAGGACC CAGCGCAGGGCGGTAAAATTATAGCCTCAGTGCGAGCTGA GATGGCTCGAGGGCCTGATGATAGTGCAGTGTTTGCTGCG CATACAAAATTGATGGCGCCCCGTGCGGAATCGGAAAGCG CAAAGTCCGGTCCACCCGCAGCTGGGAAACGTAAATAA
HtkB[b] WP_130026179	pCDFDuet-1 (NdeI_XhoI) pET28a(+) (NdeI_XhoI)	CTTTCCTCTCCTGGTGCCACAGCCCACGGTAATGCCAATG CCCATGGTGACCTTCTGGTTATGGTAAAAGTTGCAGAAAG ATGTAACATAGATTGCTCGTACTGTTATATGTACCATGGGGT AGACCAAGGATGGAGAGAAAGACCAAAACGCTTGCATGC CTCTCATCTGGACGCTTTGGTTACACGTCTTGTAGAGCAC CGGAGCGCATACCCAACCGCAAGAATGACTCTTGAAGTCC ATGGCGGCGAACCCTTATTACTGGGAAAGAGCCATACAGA ACGATTCTTTAGTAATTTACGCCGCAGATTGCCCGCATCAG ATTTGGCTATAATAACACAATCGAATGGTGTATTAATTGATGA CGATTGGCTCGACATATATGCTCGCTATGATGCTTCATTAG GAATTAGTTGTGACGGTCCGCCAGATATGCACGATCGCCA CCGTTACGATTTTGCACGGAAGGGAACCGGAGAACGGGT TGAACAGGCTATATTACGGTGCCTGGCACACCGTGCGAGC CAACGTGTATTTAACGGAGTACTTGCTGTTATTGACCCAAA CCACGATCCCGTCCGTATGCTTGACTATTTCCGCAGTCTTG GTTTAACCGCTATAGATTTTCTTCTGCCAGACGCAAACTTT GCGGCTCCCCCGCGGCATATACAAGCGTATACCCATGCGC GTTTACTTTCATTTCTTTGCCGCGGCTTTGACGCTTGGCTG GCTATTGATGACCCTGGCTTTTCGGTTCGTATATTTGAGAC CTTTATCAGAGGAGTTCTGGGCCGACGTAGCGAGCTGGAC GCATTCGGCGGCGCCTTGGCACCAATTGTTGTAGTTGAAA GTGACGGTTCCTACCGTCTTTTAGATGTTTTAGCTATATGTG AAGAAGGGGCCACACATACAGGTTTTAATTTAACCTCACAT CCTCTCCAGGCGTGTTCTGACTTTGCCCGGACACGATATC CGGAACCGCATGCAACCTGTTTGCAGTGTGAAGCGTTCG CCGCTTGTGGAGGAGGCTATGTTGCACATCGCTTTGATGG ACGTTCGTATGATAATCCTTCATTCTACTGCCATGCTTTGCT TGGGTTCTACCGTCATGTGCGTGCACGTGTTCAAGGAGTC ACCCCACCAATTAGCCTTACAGCCAATGCCAAAGGTTAA
HtkC[b] WP_130026181.1	pACYCDuet-1 (NcoI(-G)/EcoRI)	CACGACAGTATAATTGTGACCGACGATTTCTACCGTGATCC TGCCGACGTGCGCGACTTCGCACTGAGACAGACCTTTGC CGTGAAAGGCAATTATCCAGGGGCCCGGACGGAGGCATT TGTCCATGACGGCGTCACTTCTGCCATCCAGGATATTGTCA AATCTCCGATAACCTACTGGCCTGTGGACACCTACAATGGT GCATTTCAGTATTCGGTTCAGCGTGACATAACTTGGGTCCA TGCAGACCATACGACGGTTTGGTCCGGTGTTGTTTACTTAA CACCGGATGCGCCCCCGACGGCCGGTACTGCTTTTTTCC AACATATTGAAACAGGATTTGATCTCTACCCAGATGATGATG ATCAGCGCCAACGTTGTGACGCTGATGCAGCGGCGTGGG AGCGTTGGGAGATGACGGATCGCATCGCAAACCGATTTAA

		TCGTCTTATCCTGTTCCGTGGTCGTCGTTTTCACGCTAGTC AGGGTCATTTTGGTGATTGCAAGGAGAACGGGCGTTTGTT TCAGACGTTCTTTTTTAATACTCAGTATTAA
HtkA K3A	pET28a(+) (NdeI_XhoI)	ACAGCATCAGAGTTATTCTTCCCTGTTGGGTCTCTGGATTT ATCAGAAGCACAGTCCGCGGAAATTAAGAGTGCATTTGGC CACGAAATGGCAGCGGTCCCTCTGTACCGTGTTGTACCAG TCAGTAGTTCCGATAGATATGTCGCATTAGAAGGTGTTCCA GGCCTGCCAGGGATCTTAGTACATTATCGAGATGAGGACC CAGCGCAGGGCGGTAAAATTATAGCCTCAGTGCGAGCTGA GATGGCTCGAGGGCCTGATGATAGTGCAGTGTTTGCTGCG ttcACAAAATTGATGGCGCCCCGTGCGGAATCGGAAAGCGC AAAGTCCGGTCCACCCGCAGCTGGGAAACGTAAATAA
HtkA H1F	pET28a(+) (NdeI_XhoI)	ACAGCATCAGAGTTATTCTTCCCTGTTGGGTCTCTGGATTT ATCAGAAGCACAGTCCGCGGAAATTAAGAGTGCATTTGGC CACGAAATGGCAGCGGTCCCTCTGTACCGTGTTGTACCAG TCAGTAGTTCCGATAGATATGTCGCATTAGAAGGTGTTCCA GGCCTGCCAGGGATCTTAGTACATTATCGAGATGAGGACC CAGCGCAGGGCGGTAAAATTATAGCCTCAGTGCGAGCTGA GATGGCTCGAGGGCCTGATGATAGTGCAGTGTTTGCTGCG tggACAAAATTGATGGCGCCCCGTGCGGAATCGGAAAGCG CAAAGTCCGGTCCACCCGCAGCTGGGAAACGTAAATAA
HtkA H1W	pET28a(+) (NdeI_XhoI)	ACAGCATCAGAGTTATTCTTCCCTGTTGGGTCTCTGGATTT ATCAGAAGCACAGTCCGCGGAAATTAAGAGTGCATTTGGC CACGAAATGGCAGCGGTCCCTCTGTACCGTGTTGTACCAG TCAGTAGTTCCGATAGATATGTCGCATTAGAAGGTGTTCCA GGCCTGCCAGGGATCTTAGTACATTATCGAGATGAGGACC CAGCGCAGGGCGGTAAAATTATAGCCTCAGTGCGAGCTGA GATGGCTCGAGGGCCTGATGATAGTGCAGTGTTTGCTGCG CATACAgctTTGATGGCGCCCCGTGCGGAATCGGAAAGCGC AAAGTCCGGTCCACCCGCAGCTGGGAAACGTAAATAA
HtkB D214H	pCDFDuet-1 (NdeI_XhoI)	CTTTCCTCTCCTGGTGCCACAGCCCACGGTAATGCCAATG CCCATGGTGACCTTCTGGTTATGGTAAAAGTTGCAGAAAG ATGTAACATAGATTGCTCGTACTGTTATATGTACCATGGGGT AGACCAAGGATGGAGAGAAAGACCAAAACGCTTGCATGC CTCTCATCTGGACGCTTTGGTTACACGTCTTGTAGAGCAC CGGAGCGCATACCCAACCGCAAGAATGACTCTTGAAGTCC ATGGCGGCGAACCCTTATTACTGGGAAAGAGCCATACAGA ACGATTCTTTAGTAATTTACGCCGCAGATTGCCCGCATCAG ATTTGGCTATAATAACACAATCGAATGGTGTATTAATTGATGA CGATTGGCTCGACATATATGCTCGCTATGATGCTTCATTAG GAATTAGTTGTGACGGTCCGCCAGATATGCACGATCGCCA CCGTTACGATTTTGCACGGAAGGGAACCGGAGAACGGGT TGAACAGGCTATATTACGGTGCCTGGCACACCGTGCGAGC CAACGTGTATTTAACGGAGTACTTGCTGTTATTGACCCAAA CCACGATCCCGTCCGTATGCTTGACTATTTCCGCAGTCTTG GTTTAACCGCTATAGATTTTCTTCTGCCAcatGCAAACTTTG CGGCTCCCCCGCGGCATATACAAGCGTATACCCATGCGCG TTTACTTTCATTTCTTTGCCGCGGCTTTGACGCTTGGCTGG CTATTGATGACCCTGGCTTTTCGGTTCGTATATTTGAGACC TTTATCAGAGGAGTTCTGGGCCGACGTAGCGAGCTGGAC GCATTCGGCGGCGCCTTGGCACCAATTGTTGTAGTTGAAA GTGACGGTTCCTACCGTCTTTTAGATGTTTTAGCTATATGTG

		AAGAAGGGGCCACACATACAGGTTTTAATTTAACCTCACAT CCTCTCCAGGCGTGTTCTGACTTTGCCCGGACACGATATC CGGAACCGCATGCAACCTGTTTGCAGTGTGAAGCGTTCG CCGCTTGTGGAGGAGGCTATGTTGCACATCGCTTTGATGG ACGTTCGTATGATAATCCTTCATTCTACTGCCATGCTTTGCT TGGGTTCTACCGTCATGTGCGTGCACGTGTTCAAGGAGTC ACCCCACCAATTAGCCTTACAGCCAATGCCAAAGGTTAA
HaaA[b] WP_133931327.1	pET28a(+) (NdeI_XhoI)	CCGTCTCGTACCTCTGTTCCGGCGCCGCACGACGAAGCG ACCACCGGTCACGAACCGGCGCAGGGTAACCTGGTTCTG GAACGTGTTGCGGCGCGTGTTCGTCAGCGTAAAGCGGCG GAACAGGCGGCGACCTCTCGTGTTGGTGACGGTGCGCAC GCGGCGTCTCTGATCTGGCCGTGGCCGCTGTAATGAAATT ACTTAGAAATGTAGCTAGATTTTTACCCACGCACCTAATTAG TCCACGTTTGGTTTTTAGAACCCGATGATCTTCAAAACATTA TAAGATTCTCAACTTGCTTT
HaaB[b] WP_133931326.1	pCDFDuet-1 (NdeI_XhoI)	ACGCTTCTGGAAGAGATTGCGCCGTTTCGTACTTTTATTCT TAAAGTGGCGAATCGTTGTAATATTGATTGCGATTACTGTTT CGTGTTTAATTCTAAAGATCAAACCGCACGTCGTCTTCCGG CGCGCATGAGTCTGGATGTGGCACGCGCAGCGGCACGCC GCATTGGTGATCATGTTACGGCACATGGGTTACCAGCGGT TCATGTGGTCTTGCACGGCGGCGAGCCGTTACTGGCGGG ATTACGTCACATGGCTAATTTACTGGATACGATCCGTGATC GTATTCCCGATGATGCAGAACTGCGTTTTGAGTTGCAAACA AATGCGACACTGCTGAGCGAGGCCTGGTTGGATTTATTTG AACGCTATGAAGTTGCGGTTGGTGTATCCATTGATGGGCC CCCAGCTGCCAACGATTTACATCGTTTGACCCATGCGGGT CGCAGTTCTGCAGCAAGCACAGTTCGTGGTATTGAGTTGC TTCGTAGCCGTCCGCATTTGTTTGCTGGCTTACTGGCTGTA GTAGATCTTGCAAATGATCCTGTAGAAGTACATGATTATTTG GCGAGCTTTGAACCCCCTCTGATTGATTTTGGTCTCCCGC ATGGTACGCACGATGATCCACCACATCGTCATGATCCCGG GGTTCCGGAATATGGGAAGTGGATGTCTCGTGTTTATGATG CGTGGCTGGCTCGTCCGGAATATCAACATTCTGTCCGTAT GTTAGAAGATATTGTTGCCCTGTCTAGCGGGGTTCGCGGG AGCGTCGAAACCTTGGGCCTCGCTCCGCCCACCTCAGTT GTAATTGAAAGCGATGGTACGATTGAAGGTGTCGATACATT GCGCAGTGTGGAAGAAGGTGCGAGCTGGCTTGGCTTGGA TGTTTTCCATCAATCGTTTGATGAGGCTTTGCGCCACTCCA AATTGACCCATCGCCAATATGGTAAAGCAAGCCTGGCTGAA CAATGTCAATCGTGTCCTCTTGTCGATGTATGTGGAGGAG GATACCTGCCGCACCGTTTCTCGGCGGATCGTGGTTATCG TAATCCGAGTGTTTATTGTGCCGATCTTGAATATCTGATTCG TCATGTACAAGGGTCACTTCGCCAACATGGTTGGGATGATA CGCACGCCTCTACGACGCCACCGCCCTAA
HaaA A3R	pET28a(+) (NdeI_XhoI)	CCGTCTCGTACCTCTGTTCCGGCGCCGCACGACGAAGCG ACCACCGGTCACGAACCGGCGCAGGGTAACCTGGTTCTG GAACGTGTTGCGGCGCGTGTTCGTCAGCGTAAAGCGGCG GAACAGGCGGCGACCTCTCGTGTTGGTGACGGTGCGGCA GCGcgtTCTCTGATCTGGCCGTGGCCGCTGTAATGAAATTA CTTAGAAATGTAGCTAGATTTTTACCCACGCACCTAATTAGT CCACGTTTGGTTTTTAGAACCCGATGATCTTCAAAACATTAT AAGATTCTCAACTTGCTTT
HaaA	pET28a(+)	CCGTCTCGTACCTCTGTTCCGGCGCCGCACGACGAAGCG

H1F/A3R	(NdeI_XhoI)	ACCACCGGTCACGAACCGGCGCAGGGTAACCTGGTTCTG GAACGTGTTGCGGCGCGTGTTCGTCAGCGTAAAGCGGCG GAACAGGCGGCGACCTCTCGTGTTGGTGACGGTGCGttcG CGcgtTCTCTGATCTGGCCGTGGCCGCTGTAATGAAATTACT TAGAAATGTAGCTAGATTTTTACCCACGCACCTAATTAGTCC ACGTTTGGTTTTTAGAACCCGATGATCTTCAAAACATTATAA GATTCTCAACTTGCTTT
HaaA A3K	pET28a(+) (NdeI_XhoI)	CCGTCTCGTACCTCTGTTCCGGCGCCGCACGACGAAGCG ACCACCGGTCACGAACCGGCGCAGGGTAACCTGGTTCTG GAACGTGTTGCGGCGCGTGTTCGTCAGCGTAAAGCGGCG GAACAGGCGGCGACCTCTCGTGTTGGTGACGGTGCGCAC GCGaaaTCTCTGATCTGGCCGTGGCCGCTGTAATGAAATTA CTTAGAAATGTAGCTAGATTTTTACCCACGCACCTAATTAGT CCACGTTTGGTTTTTAGAACCCGATGATCTTCAAAACATTAT AAGATTCTCAACTTGCTTT
HaaB H204D	pCDFDuet-1 (NdeI_XhoI)	ACGCTTCTGGAAGAGATTGCGCCGTTTCGTACTTTTATTCT TAAAGTGGCGAATCGTTGTAATATTGATTGCGATTACTGTTT CGTGTTTAATTCTAAAGATCAAACCGCACGTCGTCTTCCGG CGCGCATGAGTCTGGATGTGGCACGCGCAGCGGCACGCC GCATTGGTGATCATGTTACGGCACATGGGTTACCAGCGGT TCATGTGGTCTTGCACGGCGGCGAGCCGTTACTGGCGGG ATTACGTCACATGGCTAATTTACTGGATACGATCCGTGATC GTATTCCCGATGATGCAGAACTGCGTTTTGAGTTGCAAACA AATGCGACACTGCTGAGCGAGGCCTGGTTGGATTTATTTG AACGCTATGAAGTTGCGGTTGGTGTATCCATTGATGGGCC CCCAGCTGCCAACGATTTACATCGTTTGACCCATGCGGGT CGCAGTTCTGCAGCAAGCACAGTTCGTGGTATTGAGTTGC TTCGTAGCCGTCCGCATTTGTTTGCTGGCTTACTGGCTGTA GTAGATCTTGCAAATGATCCTGTAGAAGTACATGATTATTTG GCGAGCTTTGAACCCCCTCTGATTGATTTTGGTCTCCCGga tGGTACGCACGATGATCCACCACATCGTCATGATCCCGGG GTTCCGGAATATGGGAAGTGGATGTCTCGTGTTTATGATGC GTGGCTGGCTCGTCCGGAATATCAACATTCTGTCCGTATGT TAGAAGATATTGTTGCCCTGTCTAGCGGGGTTCGCGGGAG CGTCGAAACCTTGGGCCTCGCTCCGCCCACCTCAGTTGTA ATTGAAAGCGATGGTACGATTGAAGGTGTCGATACATTGCG CAGTGTGGAAGAAGGTGCGAGCTGGCTTGGCTTGGATGT TTTCCATCAATCGTTTGATGAGGCTTTGCGCCACTCCAAAT TGACCCATCGCCAATATGGTAAAGCAAGCCTGGCTGAACA ATGTCAATCGTGTCCTCTTGTCGATGTATGTGGAGGAGGAT ACCTGCCGCACCGTTTCTCGGCGGATCGTGGTTATCGTAA TCCGAGTGTTTATTGTGCCGATCTTGAATATCTGATTCGTCA TGTACAAGGGTCACTTCGCCAACATGGTTGGGATGATACG CACGCCTCTACGACGCCACCGCCCTAA

[a]Red color letters indicate engineered sequences. [b]Codons were optimized for heterologous expression in *E. coli*.

Table S2. Primers used in this study.

Primer	Sequence[a]
HtkA K3A-F	ttcACAAAATTGATGGCGCC
HtkA K3A-R	CGCAGCAAACACTGCACTAT
HtkA H1F-F	tggACAAAATTGATGGCGCCC
HtkA H1F-R	CGCAGCAAACACTGCACTAT
HtkA H1W-F	gctTTGATGGCGCCCCGT
HtkA H1W-R	TGTATGCGCAGCAAACACTG
HtkB D214H-F	catGCAAACTTTGCGGCTCC
HtkB D214H-R	TGGCAGAAGAAAATCTATAGCGG
HaaA A3R-F	ttcGCGCGTTCTCTGATCTGG
HaaA A3R-R	CGCACCGTCACCAACAC
HaaA A3K-F	aaaTCTCTGATCTGGCCGTGGCC
HaaA A3K-R	CGCGTGCGCACCGTCAC
HaaA H1F-F	ttcGCGcgtTCTCTGATCTGGC
HaaA H1F-F	CGCACCGTCACCAACACG
HaaB H204D-F	gatGGTACGCACGATGATCC
HaaB H204D-R	CGGGAGACCAAAATCAATCA

[a]Small letters indicate engineered sequences.

Table S3. Plasmids constructed in this study.

Plasmids	Description
HtkA K3A-pET28a(+)	Plasmid for expression of HtkA K3A
HtkA H1F-pET28a(+)	Plasmid for expression of HtkA H1F
HtkA H1W-pET28a(+)	Plasmid for expression of HtkA H1W
HtkB D214H-pCDFDuet-1	Plasmid for expression of HtkB D214H
HaaA A3R-pET28a(+)	Plasmid for expression of HaaA A3R
HaaA A3K-pET28a(+)	Plasmid for expression of HaaA A3K
HaaA H1F/A3R-pET28a(+)	Plasmid for expression of HaaA H1F/A3R
HaaB H204D-pCDFDuet-1	Plasmid for expression of HaaB H204D

Table S4. Strains used in this study.

Strains	Description
E. coli HtkA	*E. coli* NiCo (DE3) harboring HtkA-pET28a(+)
E. coli HtkA + HtkB	*E. coli* NiCo (DE3) harboring HtkA-pET28a(+) and HtkB-pCDFDuet-1
E. coli HtkA + HtkB + HtkC	*E. coli* NiCo (DE3) harboring HtkA-pET28a(+), HtkB-pCDFDuet-1 and HtkC-pACYCDuet-1.
E. coli HtkA + HtkB D214H	*E. coli* NiCo (DE3) harboring HtkA-pET28a(+) and HtkB D214H-pCDFDuet-1
E. coli HtkA H1F + HtkB	*E. coli* NiCo (DE3) harboring HtkA H1F-pET28a(+) and HtkB-pCDFDuet-1
E. coli HtkA H1W + HtkB	*E. coli* NiCo (DE3) harboring HtkA H1W-pET28a(+) and HtkB-pCDFDuet-1
E. coli HtkA K3A + HtkB	*E. coli* NiCo (DE3) harboring HtkA K3A-pET28a(+) and HtkB-pCDFDuet-1
E. coli HtkA K3A + HtkB D214H	*E. coli* NiCo (DE3) harboring HtkA K3A-pET28a(+) and HtkB D214H-pCDFDuet-1
E. coli HaaA A3R + HaaB	*E. coli* NiCo (DE3) harboring HaaA A3R-pET28a(+) and HaaB-pCDFDuet-1
E. coli HaaA A3K + HaaB	*E. coli* NiCo (DE3) harboring HaaA A3K-pET28a(+) and HaaB-pCDFDuet-1
E. coli HaaA H1F/A3R + HaaB	*E. coli* NiCo (DE3) harboring HaaA H1F/A3R-pET28a(+) and HaaB-pCDFDuet-1
E. coli HaaA A3R + HaaB H204D	*E. coli* NiCo (DE3) harboring HaaA A3R-pET28a(+) and HaaB H204D-pCDFDuet-1
E. coli HaaA A3K + HaaB H204D	*E. coli* NiCo (DE3) harboring HaaA A3K-pET28a(+) and HaaB H204D-pCDFDuet-1
E. coli HaaA H1F/A3R + HaaB H204D	*E. coli* NiCo (DE3) harboring HaaA H1F/A3R-pET28a(+) and HaaB H204D-pCDFDuet-1
E. coli HaaA + HaaB H204D	*E. coli* NiCo (DE3) harboring HaaA-pET28a(+) and HaaB H204D-pCDFDuet-1

Table S5. Precursor peptides used in this study.

Precursor Peptides	**Amino Acids Sequence**[a]
HtkA	MTASELFFPVGSLDLSEAQSAEIKSAFGHEMAAVPLYRVVPVSS SDRYVALEGVPGLPGILVHYRDEDPAQGGKIIASVRAEMARGPD DSAVFAAHTKLMAPRAESESAKSGPPAAGKRK
HtkA H1F	MTASELFFPVGSLDLSEAQSAEIKSAFGHEMAAVPLYRVVPVSS SDRYVALEGVPGLPGILVHYRDEDPAQGGKIIASVRAEMARGPD DSAVFAAFTKLMAPRAESESAKSGPPAAGKRK
HtkA H1W	MTASELFFPVGSLDLSEAQSAEIKSAFGHEMAAVPLYRVVPVSS SDRYVALEGVPGLPGILVHYRDEDPAQGGKIIASVRAEMARGPD DSAVFAAWTKLMAPRAESESAKSGPPAAGKRK
HtkA K3A	MTASELFFPVGSLDLSEAQSAEIKSAFGHEMAAVPLYRVVPVSS SDRYVALEGVPGLPGILVHYRDEDPAQGGKIIASVRAEMARGPD DSAVFAAHTALMAPRAESESAKSGPPAAGKRK
HaaA	MPSRTSVPAPHDEATTGHEPAQGNLVLERVAARVRQRKAAEQA ATSRVGDGAHAASLIWPWPL
HaaA A3R	MPSRTSVPAPHDEATTGHEPAQGNLVLERVAARVRQRKAAEQA ATSRVGDGAHARSLIWPWPL
HaaA H1F/A3R	MPSRTSVPAPHDEATTGHEPAQGNLVLERVAARVRQRKAAEQA ATSRVGDGAFARSLIWPWPL
HaaA A3K	MPSRTSVPAPHDEATTGHEPAQGNLVLERVAARVRQRKAAEQA ATSRVGDGAHAKSLIWPWPL

[a]Red color residues indicate mutated residues.

Table S6. NMR spectroscopic data (400 MHz, D_2O) of **1**.

Residue	**Position**	**^{1}H**	**^{13}C^a**
Gly(-10)	C=O		169.2
	α	4.01	43.4
Pro(-9)	C=O		176.2
	α	4.45	63.5
	β	2.31	32.1
		1.98	
	γ	2.04	27.3
	δ	3.81	50.5
		3.69	
Asp(-8)	C=O		178.9
	α	4.69	54.0
	β	2.75-2.83	39.7
Asp(-7)	C=O		176.0
	α	4.70	53.7
	β	2.75-2.83	39.7
Ser(-6)	C=O		174.6
	α	4.38	58.9
	β	3.90	63.6
Ala(-5)	C=O		177.8
	α	4.31	52.6
	β	1.36	19.1
Val(-4)	C=O		176.1
	α	4.01	62.5
	β	1.96	32.8
	γ-Me1	0.87	20.6
	γ-Me2	0.80	21.0
Phe(-3)	C=O		175.5
	α	4.63	57.6
	β	3.15	39.6
		3.02	
	1		138.9
	2/6	7.27	131.9
	3/5	7.35	131.5
	4	7.30	129.9
Ala(-2)	C=O		175.3
	α	4.29	52.3
	β	1.39	19.0
Ala(-1)	C=O		176.3
	α	4.63	50.4
	β	1.34	19.0

His(1)	C=O		174.5
	α	4.44	56.9
	β	3.40	29.8
		2.91	
	2		147.1
	4		130.4
	5	7.39	121.5
Thr(2)	C=O		173.0
	α	4.24	61.7
	β	3.87	68.6
	γ	1.07	21.0
Lys(3)	C=O		171.5
	α	4.76	57.3
	β	3.50	43.4
	γ	2.04	27.8
		1.79	
	δ	1.60	27.2
	ε	2.99	41.5
Leu(4)	C=O		176.2
	α	4.40	55.4
	β	1.60	42.3
	γ	1.60	27.1
	δ-Me1	0.87	23.2
	δ-Me2	0.91	24.8
Met(5)	C=O		175.4
	α	4.52	55.0
	β	2.08	33.0
	γ	2.62	31.9
	S-Me	2.12	16.7
Ala(6)	C=O		177.2
	α	4.27	52.1
	β	1.36	19.0
Pro(7)	C=O		176.2
	α	4.45	63.5
	β	2.31	32.1
		1.98	
	γ	2.04	27.3
	δ	3.63	49.7
		3.57	
Arg(8)	C=O		180.6
	α	4.20	57.0
	β	1.86	31.3
		1.75	
	γ	1.63	27.2

	δ	3.22	43.4
	C (guanidine)		159.5

[a]Assigned by HSQC and HMBC.

Table S7. NMR spectroscopic data (400 MHz, DMSO-d_6, 0.15% TFA-*d*) of **1**.

Residue	**Position**	**^{1}H**	**^{13}C[a]**
Gly(-10)	C=O		n.d.[b]
	NH	8.07	
	α	3.81	39.7
Pro(-9)	C=O		n.d.[b]
	α	4.42	58.6
	β	2.18	31.4
		2.03	
	γ	1.78	21.6
	δ	3.57	46.4
		3.43	
Asp(-8)	C=O		171.4
	NH	8.37	
	α	4.53	49.3
	β	2.72	35.6
		2.54	
Asp(-7)	C=O		171.4
	NH	8.01	
	α	4.53	49.3
	β	2.74-271	38.0
Ser(-6)	C=O		170.2
	NH	7.76	
	α	4.25	54.8
	β	3.56	61.3
Ala(-5)	C=O		171.6
	NH	8.02	
	α	4.27	48.0
	β	1.19	17.7
Val(-4)	C=O		170.7
	NH	7.64	
	α	4.06	57.6
	β	1.89	30.2
	γ-Me1	0.73	18.9
	γ-Me2	0.73	17.8
Phe(-3)	C=O		170.5
	NH	7.95	
	α	4.53	53.3

	β	3.03	37.0
		2.76	
	1		137.5
	2/6	7.23	128.9
	3/5	7.23	127.7
	4	7.16	125.9
Ala(-2)	C=O		171.6
	NH	8.02	
	α	4.27	48.0
	β	1.20	17.8
Ala(-1)	C=O		171.6
	NH	8.02	
	α	4.48	46.0
	β	1.20	16.6
His(1)	C=O		169.6
	NH	8.00	
	α	4.50	52.8
	β	3.15	28.3
		2.55	
	2		144.4
	4		126.9
	5	7.45	118.1
	NHτ	14.40	
	NHπ	13.79	
Thr(2)	C=O		170.5
	NH	8.09	
	α	4.19	58.1
	β	3.63	65.3
	γ	0.94	19.3
Lys(3)	C=O		171.8
	NH	8.08	
	α	4.53	54.0
	β	3.36	40.1
	γ	2.02	23.9
		1.73	
	δ	1.88	24.1
	ε	3.48	45.7
Leu(4)	C=O		171.6
	NH	8.02	
	α	4.27	51.5
	β	1.47	39.9
	γ	1.59	24.0
	δ-Me1	0.86	22.7
	δ-Me2	0.84	21.2
Met(5)	C=O		171.5

	NH	8.12	
	α	4.36	51.3
	β	1.89	31.9
		1.77	
	γ	2.45	29.0
	S-Me	2.04	14.4
Ala(6)	C=O		170.3
	NH	8.02	
	α	4.27	48.0
	β	1.19	17.7
Pro(7)	C=O		n.d.[b]
	α	4.42	58.6
	β	2.18	31.4
		2.03	
	γ	1.78	21.6
	δ	3.57	46.4
		3.43	
Arg(8)	C=O		173.3
	NH	8.11	
	α	4.15	51.3
	β	1.52	24.8
		1.34	
	γ	1.74	27.9
		1.57	
	δ	3.11	40.1
	$N^{\delta}H$	7.64	
	C (guanidine)		156.6

[a]Assigned by HSQC and HMBC. [b]Not detected.

Table S8. Advanced Marfey's analysis of product **1**.

Amino acid	**Retention time (min)**		
	L-FDVA-standard	D-FDVA-standard	Hydrolysate of **1**[b]
L-Pro	9.14	10.23	9.14
L-Asp	8.01	8.51	8.01
L-Ser	7.31	7.72	7.31
L-Ala	9.14	10.67	9.14
L-Val	10.71	13.22	10.71
L-Phe	12.03	13.99	12.03
L-Thr	7.45	9.17	7.45
L-*allo*-Thr	8.49	7.74	-
L-Leu	11.78	14.48	11.78
L-Met	10.62	12.59	10.62
L-Arg	4.61	4.20	4.61

[a]Analytical condition: MS polarity = negative; column: Kinetex XB-C18, 2.6 µm, 150 x 4.6 mm; flow rate: 0.50 mL/min; column temperature: 50°C; mobile phase/gradient: 30% H_2O/CH_3CN + 0.1% FA isocratic for 2 min followed by linear gradient to 77% H_2O/CH_3CN + 0.1% FA over 17 min.
[b]Derivatized with L-FDVA.

Table S9. NMR spectroscopic data (400 MHz, DMSO-d_6, 0.3% TFA-*d*) of **S1a**.

Residue	**Position**	**^{1}H**	**^{13}C[a]**
Gly(-10)	C=O		169.6
	NH	8.08	
	α	3.81	39.7
Pro(-9)	C=O		n.d.[b]
	α	4.43	58.7
	β	2.20	31.4
		2.04	
	γ	1.79	21.6
	δ	3.57	46.4
		3.46	
Asp(-8)	C=O		171.8
	NH	8.38	
	α	4.53	49.4
	β	2.74	38.6
		2.50	
Asp(-7)	C=O		171.8
	NH	8.01	
	α	4.53	49.4
	β	2.74	35.6
		2.55	
Ser(-6)	C=O		169.7
	NH	7.78	
	α	4.27	54.8
	β	3.56	61.4
Ala(-5)	C=O		171.7
	NH	8.09	
	α	4.29	47.9
	β	1.19	17.8
Val(-4)	C=O		170.9
	NH	7.64	
	α	4.08	57.4
	β	1.89	30.1
	γ-Me1	0.74	18.9
	γ-Me2	0.74	17.7
Phe(-3)	C=O		169.9
	NH	8.00	
	α	4.54	53.3
	β	3.04	37.1
		2.79	
	1		137.7
	2/6	7.24	129.9
	3/5	7.24	127.7

	4	7.17	126.0
Ala(-2)	C=O		171.7
	NH	8.09	
	α	4.29	47.9
	β	1.19	17.8
Ala(-1)	C=O		171.7
	NH	8.08	
	α	4.49	46.0
	β	1.20	16.6
Phe(1)	C=O		169.4
	NH	7.88	
	α	4.54	54.5
	β	3.22	38.7
		2.41	
	1		135.3
	2	6.79 (d, *J* = 7.0 Hz)	129.1
	3	6.98 (d, *J* = 7.0 Hz)	132.1
	4		138.1
	5	7.06 (d, *J* = 7.1 Hz)	125.4
	6	7.19 (d, *J* = 7.1 Hz)	128.8
Thr(2)	C=O		168.6
	NH	6.65	
	α	3.50	58.0
	β	3.41	67.0
	γ	0.82	18.8
Lys(3)	C=O		170.3
	NH	7.74	
	α	4.38	57.1
	β	2.82	48.1
	γ	2.05	29.0
		1.87	
	δ	1.45	25.8
	ε	3.49	45.6
Leu(4)	C=O		171.5
	NH	8.02	
	α	4.30	51.0
	β	1.47	40.4
	γ	1.59	23.9
	δ-Me1	0.88	22.7
	δ-Me2	0.83	21.3
Met(5)	C=O		173.6
	NH	8.05	
	α	4.36	51.2
	β	1.89	31.9
		1.77	

	γ	2.46	29.0
	S-Me	2.05	14.4
Ala(6)	C=O		170.4
	NH	8.09	
	α	4.29	47.9
	β	1.19	17.8
Pro(7)	C=O		171.6
	α	4.43	58.7
	β	2.20	31.4
		2.04	
	γ	1.79	21.6
	δ	3.57	46.4
		3.46	
Arg(8)	C=O		173.4
	NH	8.12	
	α	4.15	51.2
	β	1.53	24.8
	γ	1.75	27.9
		1.58	
	δ	3.11	40.0
	$N^{\delta}H$	7.61	
	C (guanidine)		156.6

[a]Assigned by HSQC and HMBC. [b]Not detected.

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