Supporting information

N-terminal Cysteine Mediated Backbone-Side Chain Cyclization for Chemically Enhanced Phage Display

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I. Supplementary Figures

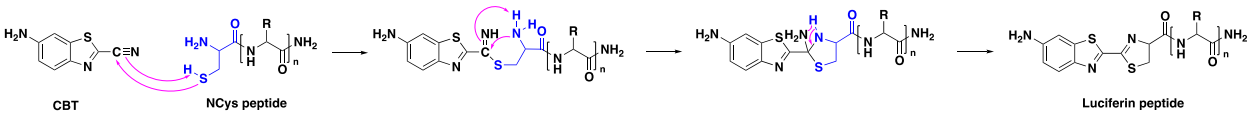


Figure S1. Proposed reaction mechanism between CBT and NCys peptide.

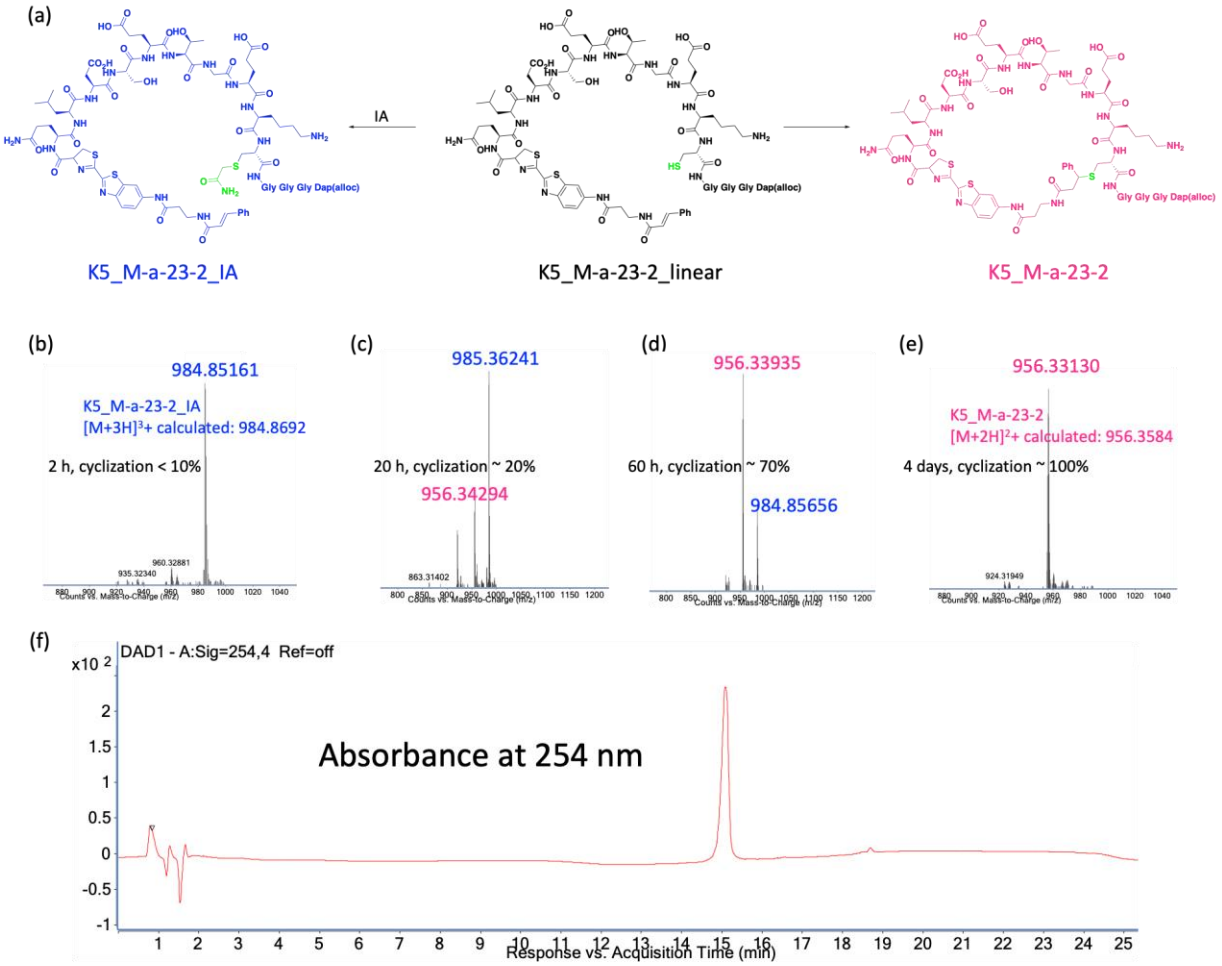


Figure S2. Peptide cyclization of **K5_M-a-23-2** showing slow kinetics. (a) Illustration of the linear peptide **K5_M-a-23-2** for cyclization or alkylation by **IA**. (b-e) Mass-spec analysis of the peptide cyclization at varied time point. An aliquot of 10 µL of the **K5_M-a-23-2**_linear solution was withdrawn at 2 h, 20 h, 60 h and 4 days respectively and treated with **IA** (1 mM) at room temperature for 2 h before subjecting to LC-MS. The data show complete cyclization was achieved after 4 days. (f) LC trace of the HPLC purified cyclic peptide **K5_M-a-23-2**.

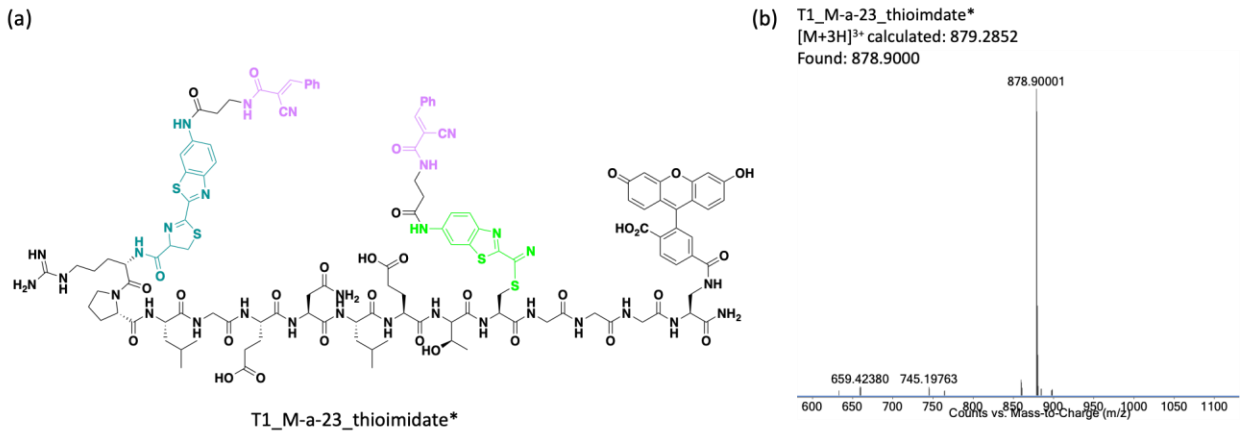


Figure S3. Double **M-a-23** modification of peptide **T1** yielding a thioimidate product (Peak * in Figure 2A of the main text). (a) Chemical structure of the **T1_M-a-23_thioimidate***. (b) Mass of **T1_M-a-23_thioimidate***, $[M+3H]^{3+}$ calculated: 879.2852, found: 878.9000. This thioimidate side product can be cleaved by free cysteine to allow peptide cyclization.

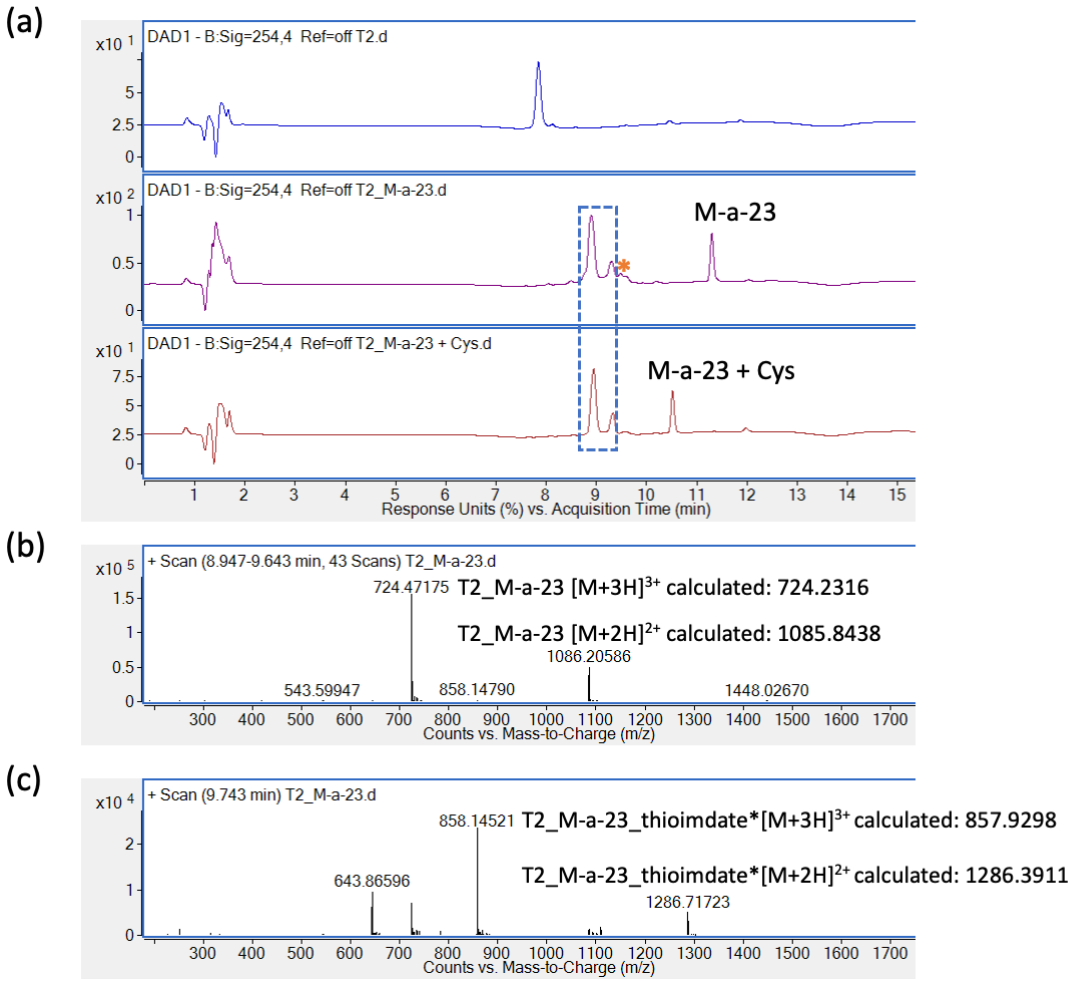


Figure S4. LC-MS analysis of peptide cyclization. Chromatograms show absorbance at 254 nm. (a) Reaction of peptide **T2** (CTWGGTGHNHCGGGDap(Fam), 40 µM) with **M-a-23** (100 µM, 2.5 eq) at rt for 2 h, followed by addition of Cys (250 µM) for 2 h. * A second **M-a-23** molecule reacted with internal cysteine to give a thioimidate product, which could be reversed by the addition of free cysteine. (b) Mass of **T2_M-a-23**, $[M+2H]^{2+}$ calculated: 1085.8438, found: 1086.2059; $[M+3H]^{3+}$ calculated: 724.2316, found: 724.4716. (c) Mass of **T2_M-a-23_thioimidate***, $[M+2H]^{2+}$ calculated: 1286.3911, found: 1286.7172; $[M+3H]^{3+}$ calculated: 857.9298, found: 858.1452.

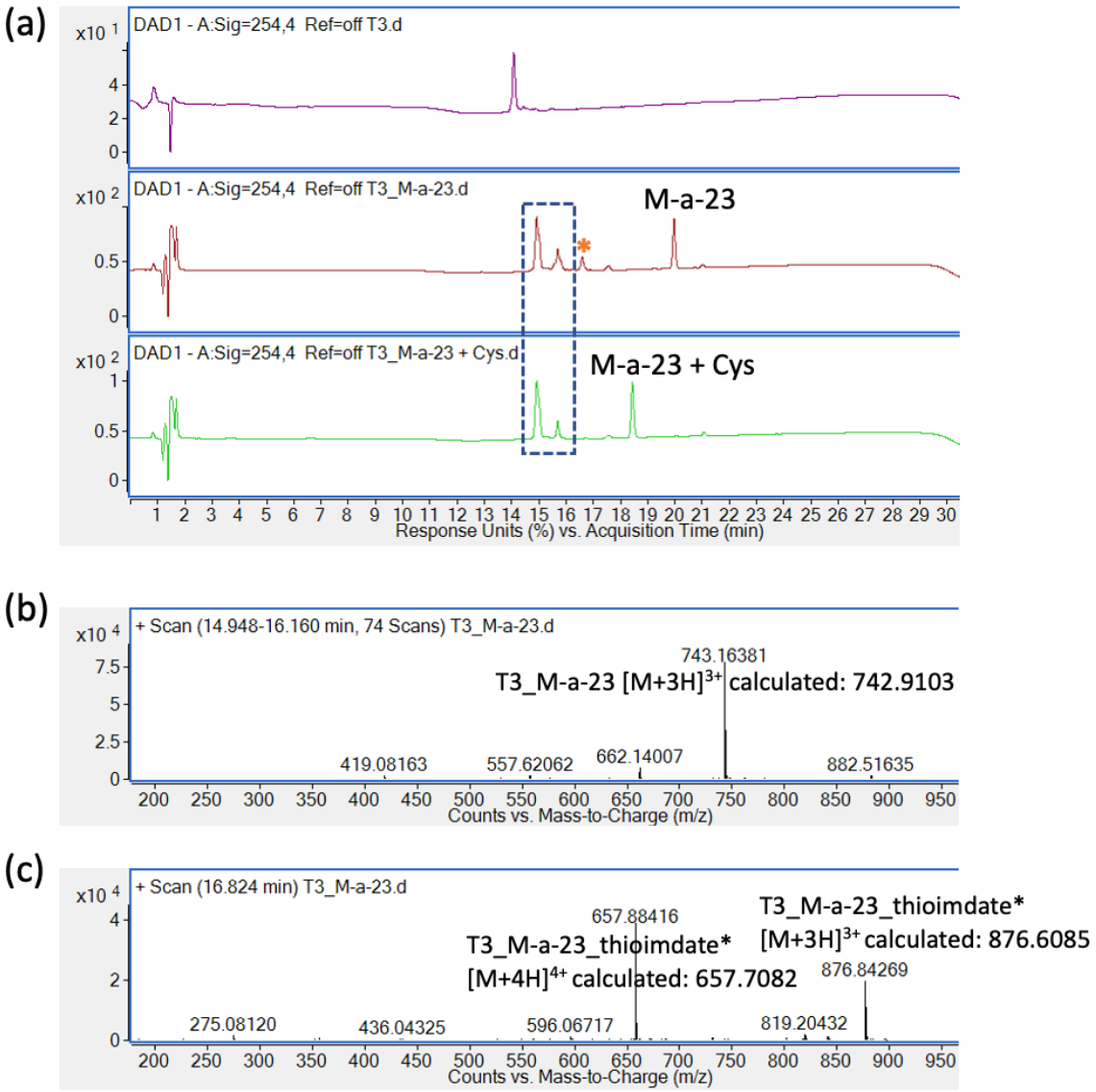


Figure S5. LC-MS analysis of peptide cyclization. Chromatograms show absorbance at 254 nm. (a) Reaction of peptide **T3** (CSTKYHDTGNCGGGDap(Fam), 40 µM) with **M-a-23** (100 µM, 2.5 eq) at rt for 2 h, followed by addition of Cys (250 µM) for 2 h. * A second **M-a-23** molecule reacted with internal cysteine to give a thioimidate product, which could be reversed by the addition of free cysteine. (b) Mass of **T3_M-a-23**, $[M+3H]^{3+}$ calculated: 742.9103, found: 743.1638. (c) Mass of **T3_M-a-23_thioimidate***, $[M+3H]^{3+}$ calculated: 876.6085, found: 876.8427; $[M+4H]^{4+}$ calculated: 657.7082, found: 657.8842.

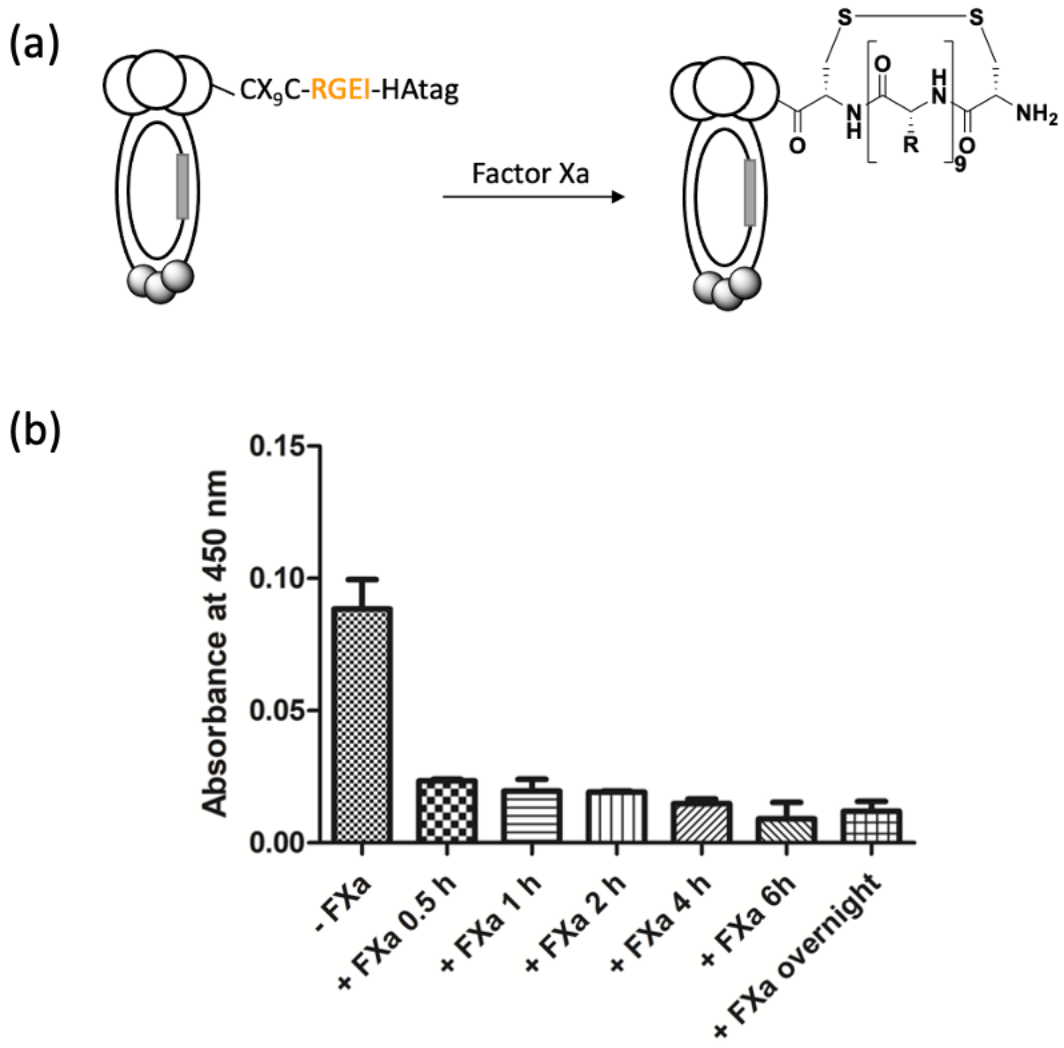


Figure S6. Validating Factor Xa cleavage of the CX_9C phage library. (a) Schematic illustration of the use of Factor Xa to expose NCys on phage. (b) Factor Xa cleavage efficiency over time monitored using ELISA with anti-HA tag antibodies. 6 h incubation gave the lowest reading and hence was chosen for all library construction work in this study.

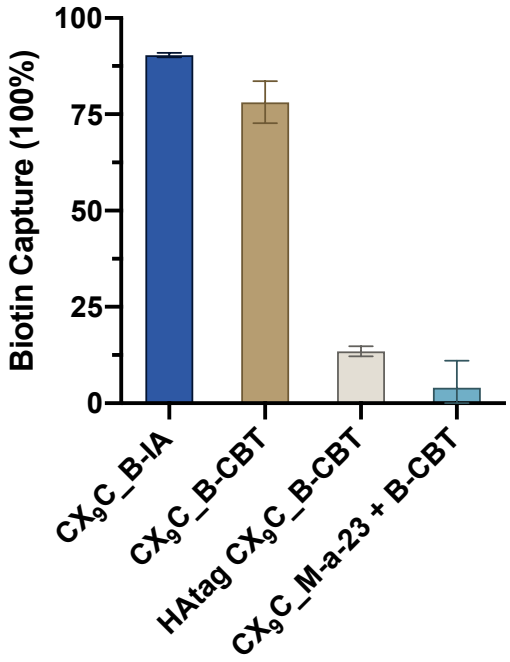


Figure S7. Phage pulldown assay to quantify the extent of chemical modifications. The results show that B-IA modification of the reduced phage was quite efficient, yielding 90% phage pulldown. This near quantitative phage modification justifies our use of **B-IA** as a positive control in the ELISA assay (Figure 2C of the main text). Importantly, **B-CBT** modification of the Factor Xa-cleaved and reduced phage gave 80% pulldown. In contrast, **B-CBT** treatment of phage without Factor Xa cleavage gave only ~13% phage pulldown. These data demonstrate the selectivity of **B-CBT** for NCys and confirm the efficient phage cleavage by Factor Xa. Also contrasting the efficient pulldown elicited by **B-CBT**, little pulldown was observed for the cleaved and reduced phage treated with **M-a-23** followed by **B-CBT**, indicating efficient phage modification by **M-a-23** as well.

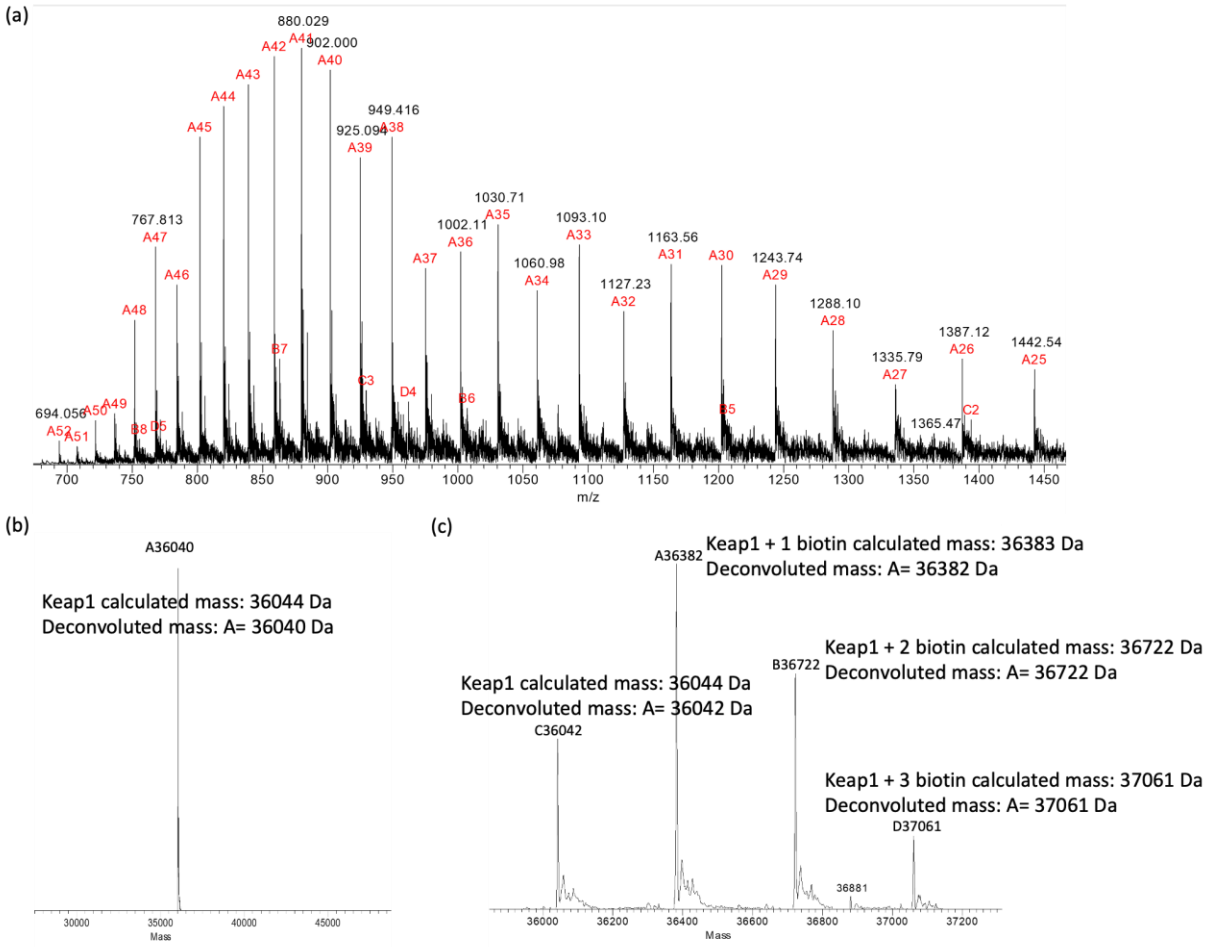


Figure S8. (a) Raw and (b) deconvoluted mass data of recombinant Keap1. (c) Deconvoluted mass data of biotinylated Keap1, which show a biotinylation efficiency around 80%.

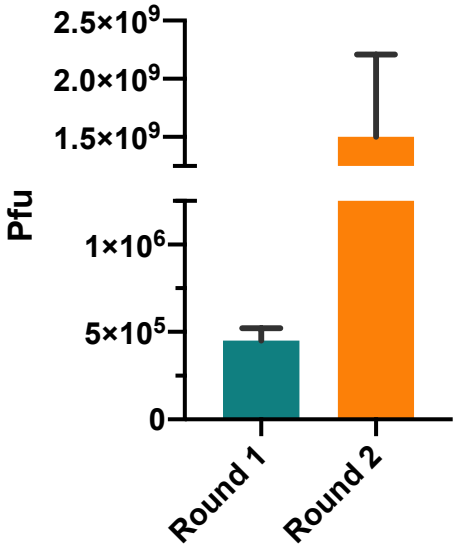
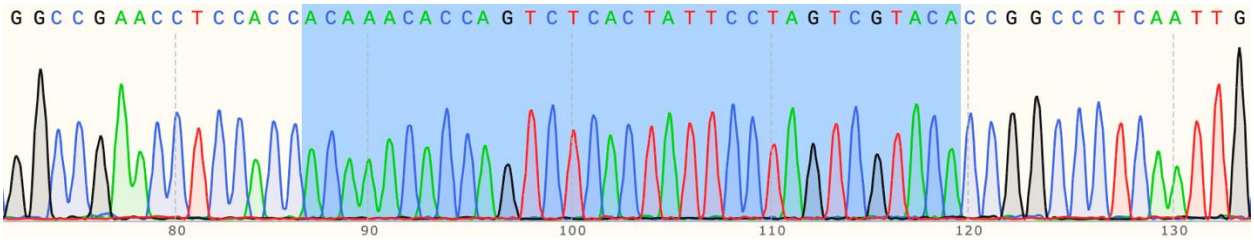
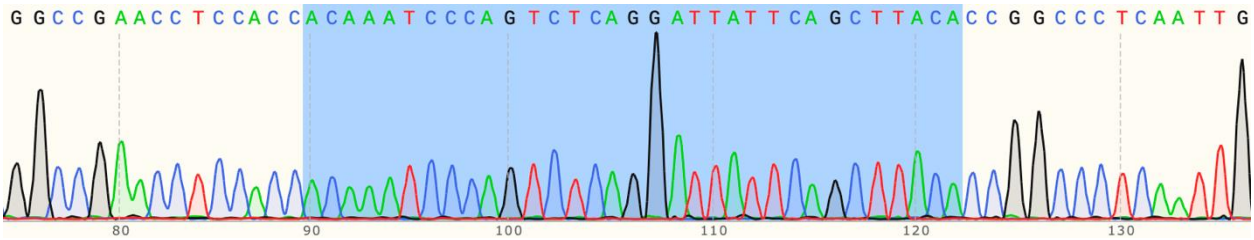


Figure S9. Phage count after Round 1 and Round 2 of selection against Keap1. The dramatic increase in output population after Round 2 indicates successful enrichment of Keap1 binding peptides.

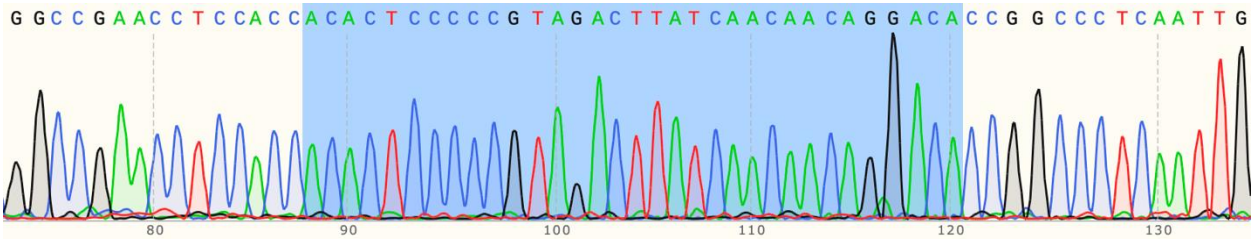
K1: CTTRNSETGVC



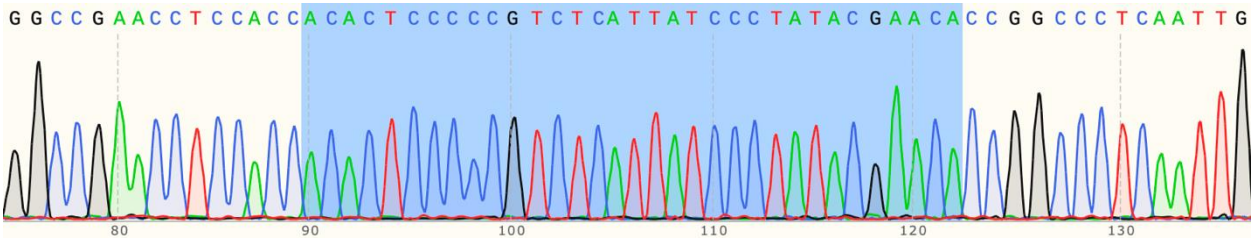
K2: CKLNNPETGIC



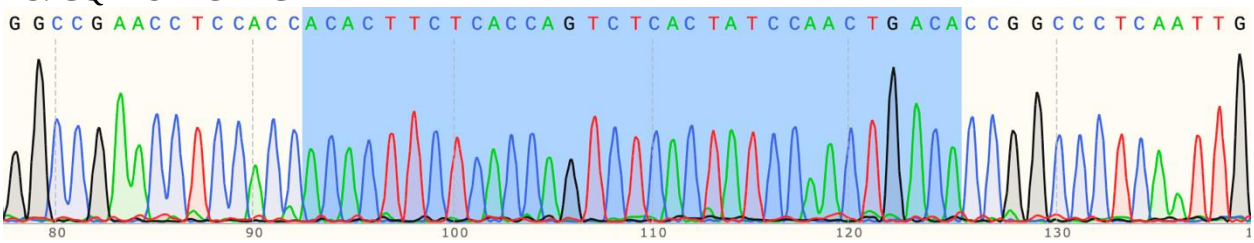
K3: CPVVDKSTGEC



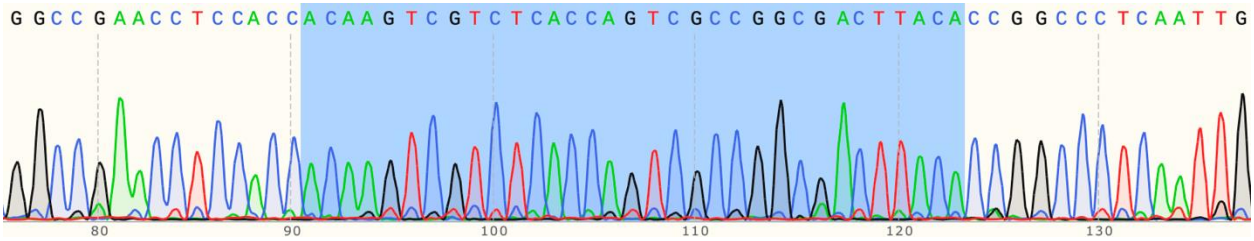
K4: CSYRDNETGEC



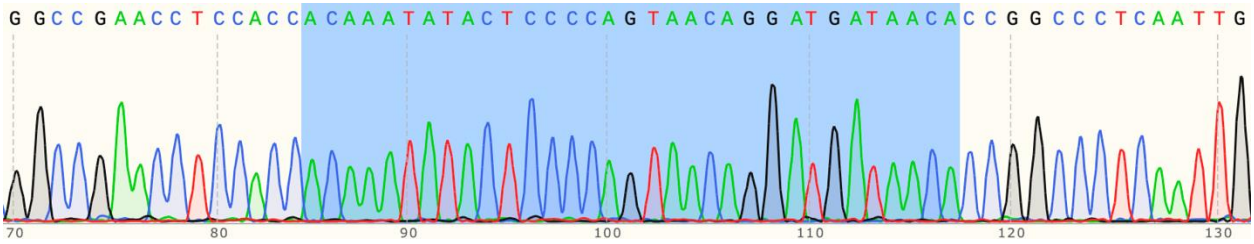
K5: CQLDSETGEKC



K6: CKSPATGETTC



K7: CYHPVTGEYIC



K8: CVESATGEHLC

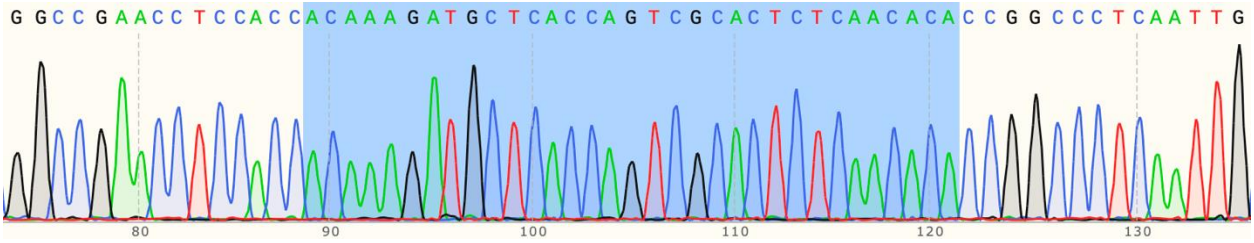


Figure S10. DNA sequencing result of the isolated phage from Keap1 screening.

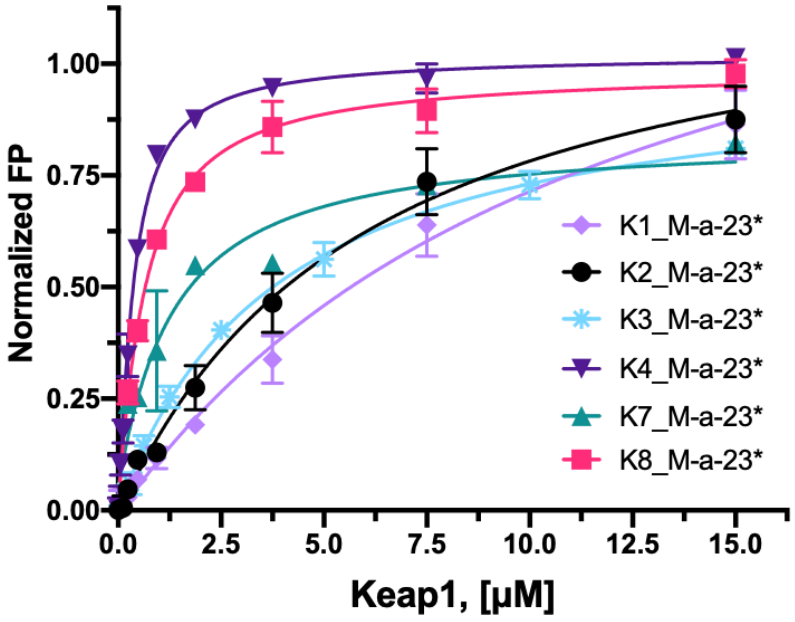


Figure S11. Fluorescence polarization-based titration to measure peptides' binding to the target protein Keap1. The binding curve of **K5_M-a-23*** is shown in Figure 3D of the main text and the binding curve of **K6_M-a-23*** is shown in Figure **S12**. Each data point presents the mean value of three intendent measurements. * indicates FAM labeled peptide.

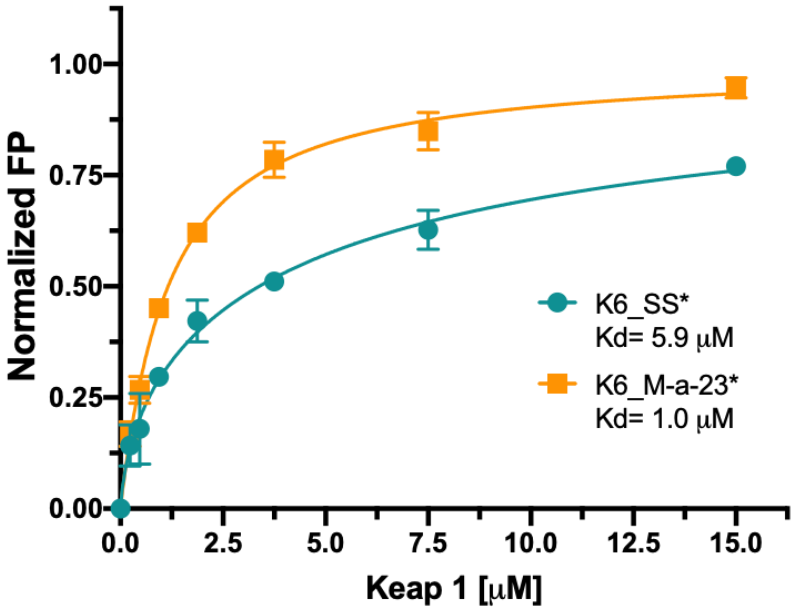


Figure S12. Comparison of the Keap1 binding curves of **K6_SS*** and **K6_M-a-23*** showing the superior potency of the **M-a-23** cyclized peptide. Each data point presents the mean value of three independent measurements. * indicates FAM labeled peptide.

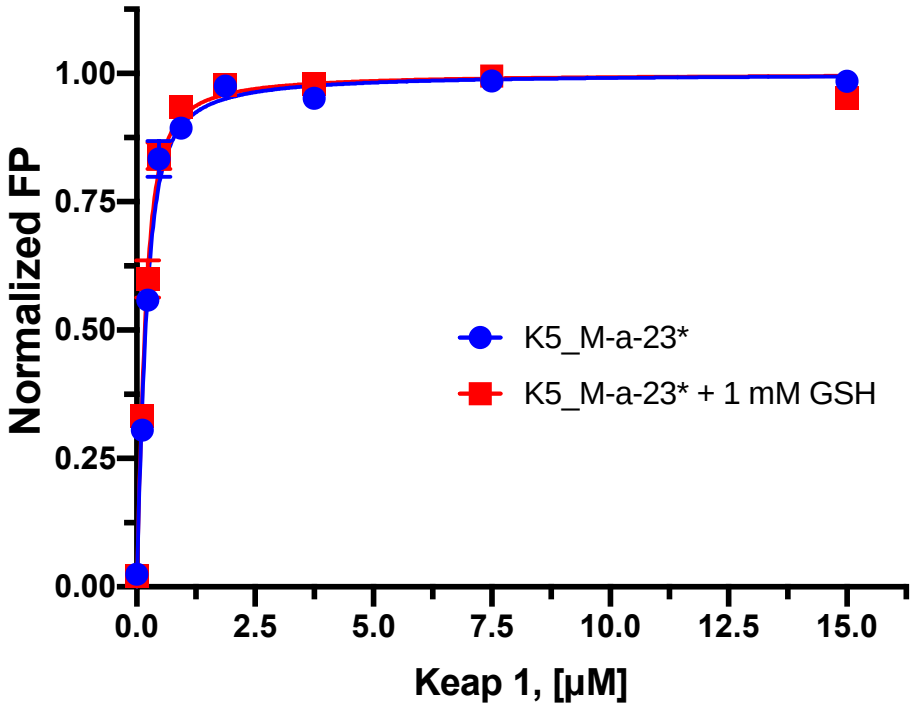


Figure S13. Keap1 binding curves of **K5_M-a-23*** in the presence and absence of a 1 mM glutathione redox buffer (GSH: GSSG, 9:1). The overlapping curves showcase the robustness of the M-a-23 cyclized peptide under redox conditions.

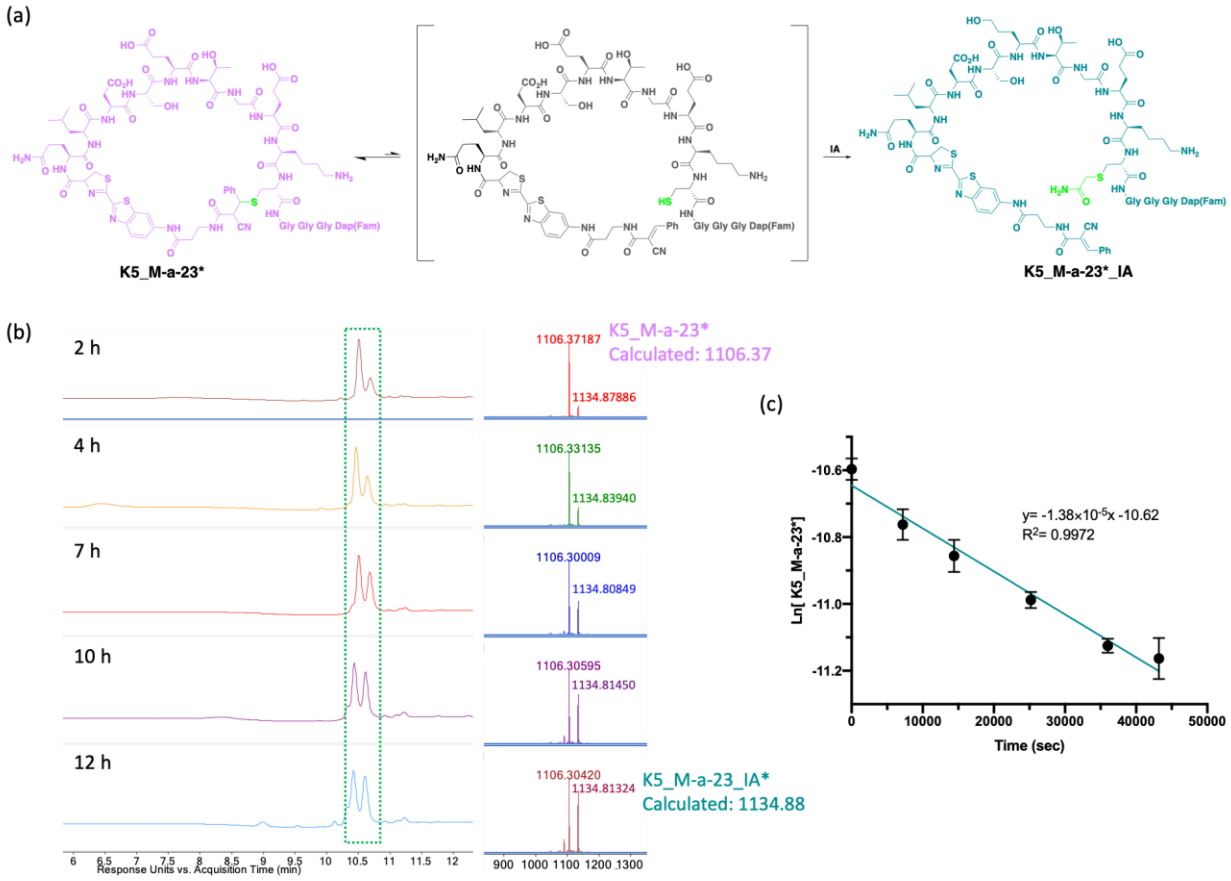


Figure S14. Determining the rate of ring opening of **K5_M-a-23*** using IA alkylation. (a) Illustration of IA alkylation of **K5_M-a-23*** upon ring opening. (b) LC-MS analysis of the peptide alkylation over time. Chromatograms show absorbance at 254 nm and the corresponding mass data are shown on the right. **K5_M-a-23*** (*m*/*z* for $[M+2]^{2+}$ expected: 1106.3710, found: 1106.3719), **K5_M-a-23_IA*** (*m*/*z* for $[M+2]^{2+}$ expected: 1134.8817, found: 1134.8789) (c) Curve fitting according to a pseudo first-order reaction mechanism yields a rate of opening to be: 1.38×10^{-5} s^{-1} (half-life of ~20 hours). The concentration of **K5_M-a-23*** over time was determined by the height of mass peaks.

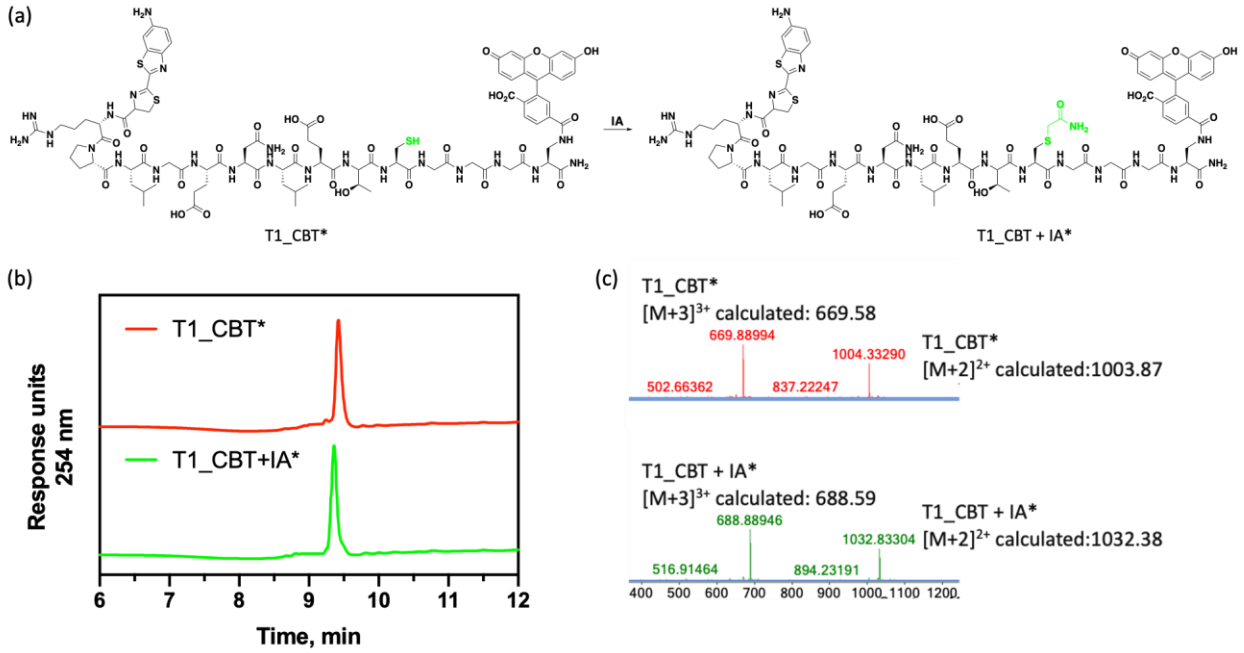


Figure S15. Cysteine alkylation of a CBT modified CX_9C peptide. (a) Illustration of **IA** alkylation of **T1_CBT***. (b)(c) LC-MS analysis of alkylation. Peptide **T1_CBT*** (40 μM) incubated with **IA** (500 μM) at room temperature for 2 h. **T1_CBT*** *m/z* for $[M+2]^{2+}$ expected: 1003.87, found: 1004.32; *m/z* for $[M+3]^{3+}$ expected: 669.58, found: 669.89; **T1_CBT + IA*** m/z for $[M+2]^{2+}$ expected: 1032.38, found: 1032.83; *m/z* for $[M+3]^{3+}$ expected: 688.59, found: 688.89.

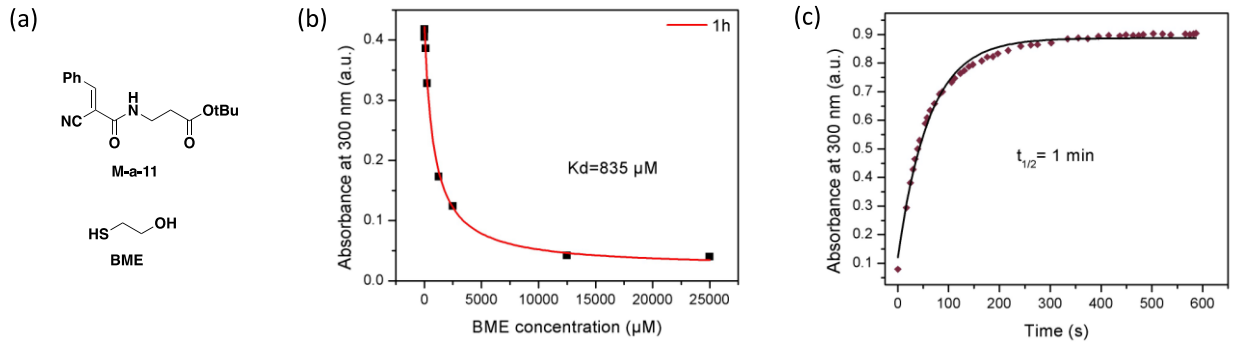


Figure S16. Kinetic characterizations of α-cyanoacrylamide-thiol reaction. (a) Chemical structure of **M-a-11** and BME. (b) Titration (binding) curve of **M-a-11** with BME. 25 μM **M-A-11** (25 μM) was incubated with BME (0, 25, 125, 250, 1250, 2500, 12500, 25000 μM) in PBS buffer (pH=7.4) at room temperature for 1 h, then monitored by UV spectrum. Curving fitting gave the apparent k_d value of 835 μM. (c) Dissociation kinetics of the conjugate of **M-a-11** and BME. **M-A-11** (100 mM) incubated with BME (100 mM) in DMSO:PBS buffer (pH=7.4, 3:1) at room temperature for 2 h, and then diluted to 50 μM. The dissociation reaction was monitored by recording the UV absorption at 300 nm. Fitting the curve gives a half-life of ~1 min and the dissociation rate constant (k_{-1}) was determined to be1.5×10^{-2} s^{-1}.[1]

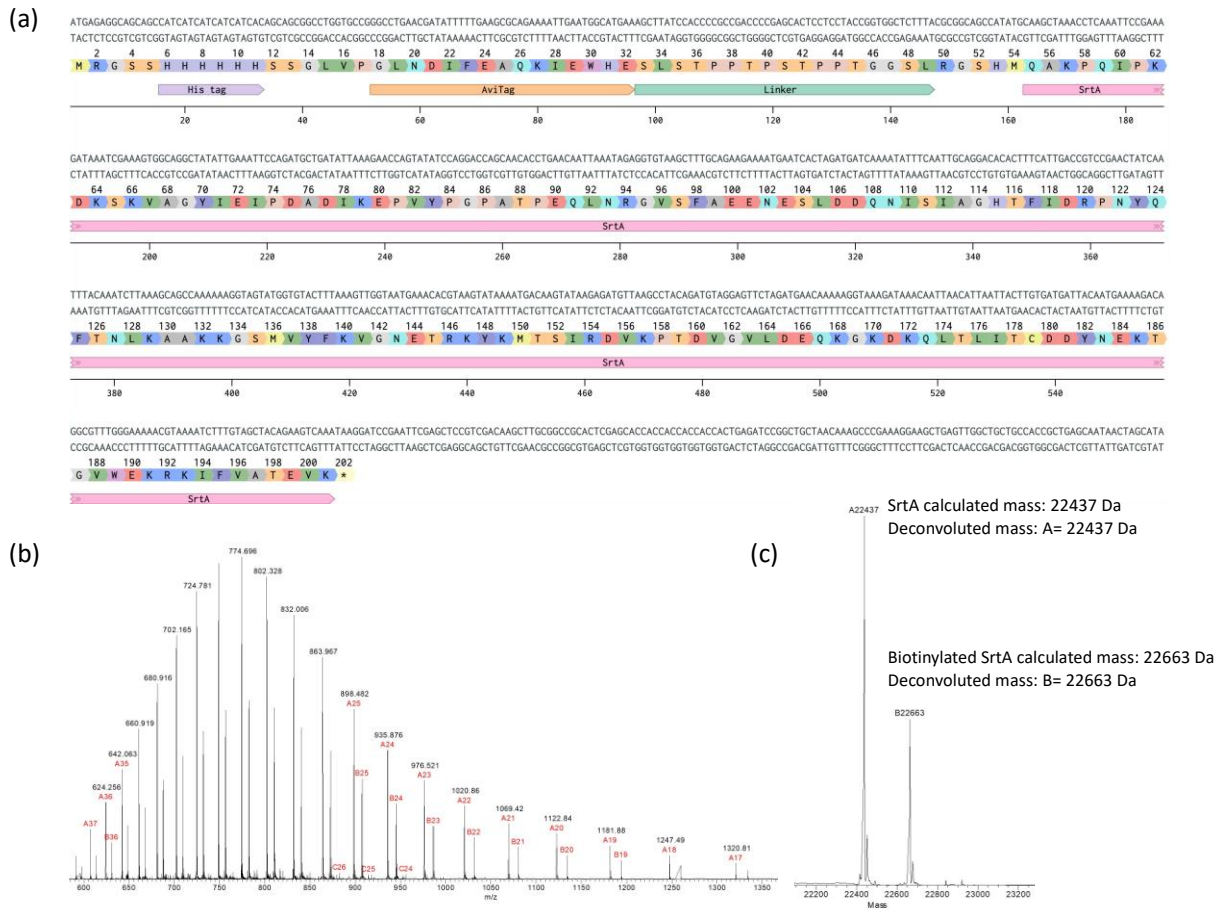
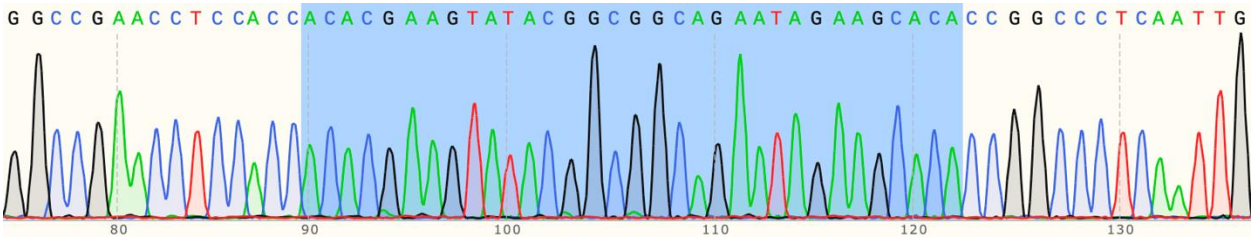
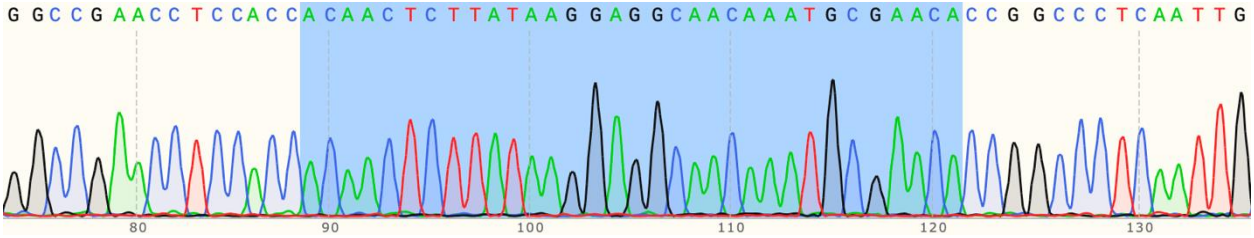


Figure S17. Recombinant expression of biotinylated SrtA. (a) Expression construct for producing biotinylated SrtA using AviTag. A HisTag is installed for protein purification and an AviTag is installed to enable protein biotinylation in situ. A rigid proline linker is inserted in between the AviTag and SrtA to improve the biotinylation efficiency. (b) Raw MS data of expressed biotinylated SrtA after purification. (c) Deconvolution mass data of biotinylated SrtA showing a biotinylation efficiency around 40%.

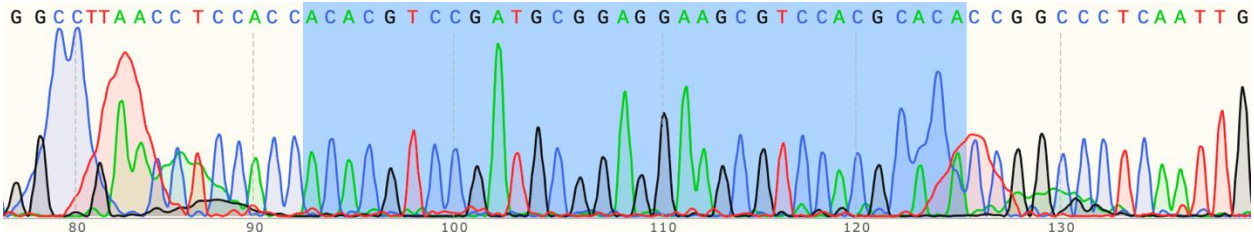
S1: CASILPPYTSC



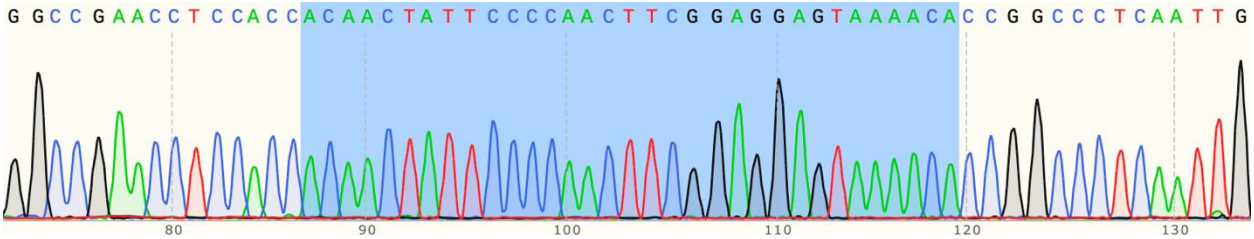
S2: CSHLLPPYKSC



S3: CAWTLPPHRTC



S4: CFTPPKLGNSC



S5: CDKIHSRLGNC

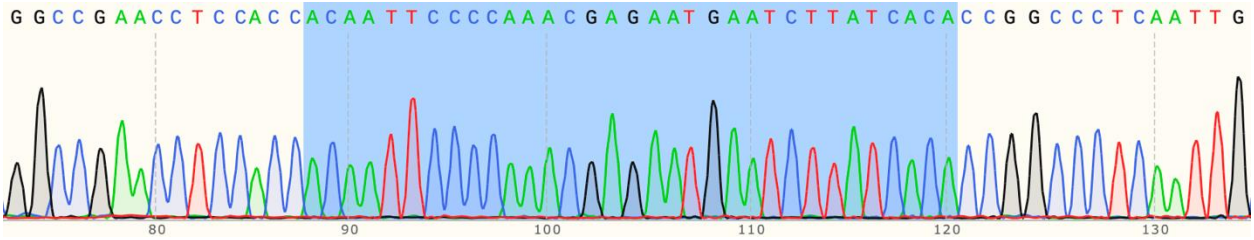


Figure S18. DNA sequencing result of the isolated phage from Sortase A screening.

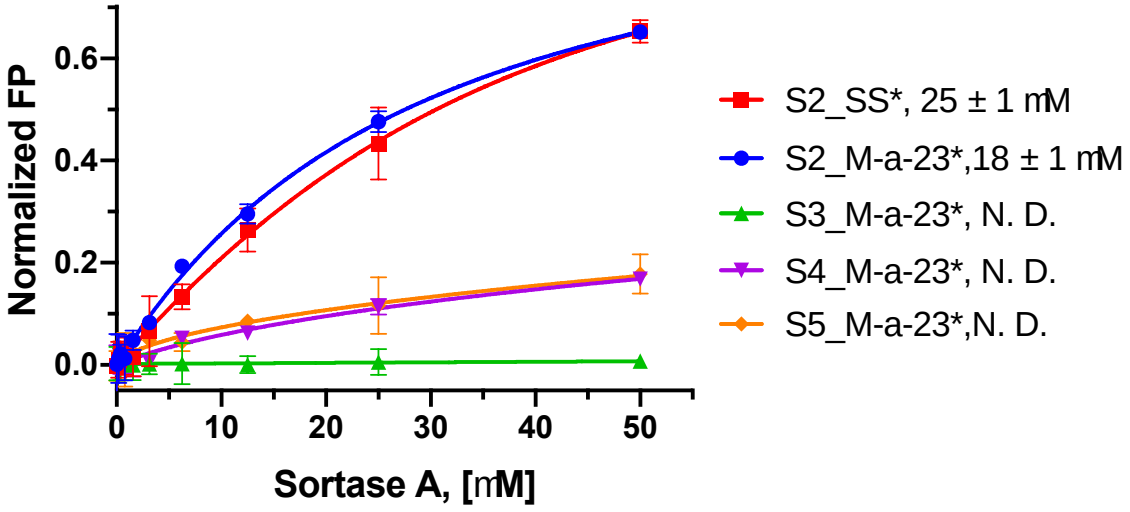


Figure S19. SrtA binding curves of the peptide hits identified from phage display. The data were generated through SrtA titration monitored by fluorescence polarization of FAM labeled peptides. Each data point presents the mean value of three intendent measurements. *indicates FAM labeled peptide.

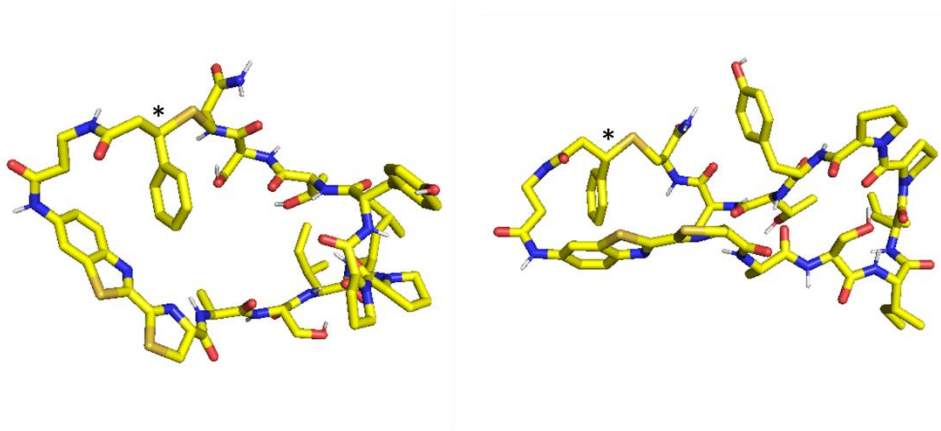


Figure S20. Computationally generated models of **S1_M-a-23-2** showing the R (left) and S (right) diastereomers that resulted from Michael addition-based peptide cyclization. *denotes the newly generated chiral center. Computational evaluation of the isomers revealed a lower Gibbs free energy for the R-isomer by -4.1 kcal/mol. Details of the computational studies can be found in Section IX of this document.

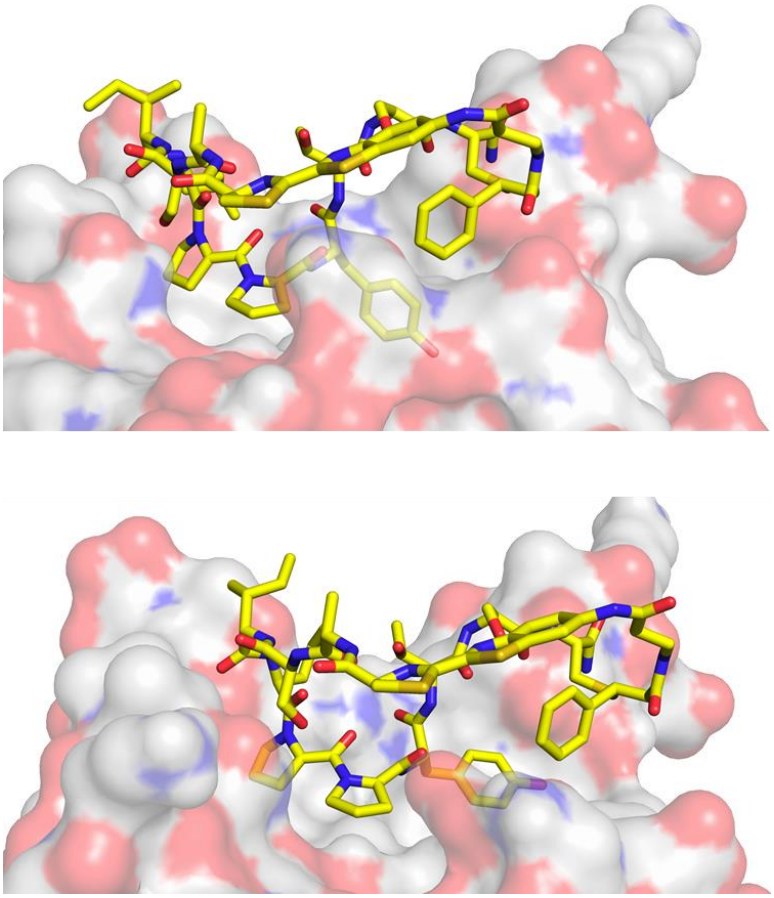


Figure 21. Docked poses of **S1_M-a-23-2** showing that the peptide's PPY motif binds into the active site of SrtA. The lower energy diastereomer (R-isomer, Figure S20) of this cyclic peptide was used for the docking studies. Detailed docking protocols can be found in Section IX of this Supporting Information.

II. General Information

Unless noted otherwise, all chemicals were obtained from commercial sources and used as received without further purification. All Fmoc-protected amino acids and HBTU were purchased from Chem-Impex International (Wood Dale, IL) or Advanced Chemtech (Louisville, KY). 5(6)-Carboxyfluorescein was purchased from Acros Organics (Germany). Rink Amide MBHA resin was purchased from NovaBiochem (San Diego, CA). **B-IA** was purchased form Thermofisher (cat. no. 21334). **B-CBT** was synthesized as previously reported [2]. Dynabeads™ M-280 Streptavidin (cat. no. 11205-D) was purchased from Invitrogen (USA). Peptide synthesis was carried out on a Tribute peptide synthesizer (Protein Technologies, Tucson, AZ). Peptides were purified on a Waters PrepLC system using a Phenomenex Jupiter C18 column (Torrance, CA). ^{1}H NMR spectra were collected using a VNMRS 500 MHz or 600 MHz NMR spectrometer. NMR data were processed using MestReNova software. LC-MS data were collected using an Agilent 6230 LC TOF mass spectrometer.

III. Protein Expression

a. Expression of Keap1

The plasmid that contains the DNA region coding for Keap1 (residues 321-624) was built by deleting the DNA region coding for Keap1 (residues 1-320) of plasmid pET28a-His6-Keap1 (Addgene, #62454) through a Q5 mutagenesis kit with the following primer:

Deleted_F: 5'-CATCCCTGAGCCCTGGAAATACAAGTTTTCTCCG3'

Deleted_R: 5'-GCGCCCAAGGTGGGCCGC-3'

The sequence of the vector was verified by Sanger sequencing. The plasmid was transformed into BL21(DE3) competent cells for expression. For protein expression, a single colony from a plate was inoculated into a culture tube with 5 mL LB medium containing 50 μg/mL Kanamycin, which was then incubated overnight at 37°C with shaking at 250 rpm. Then this overnight culture was inoculated into 500 mL of the same medium in a 2.5 L flask. The cell culture was incubated at 37°C with shaking at 250 rpm until the O.D. reached 0.4-0.6. IPTG (1 mM) was then added to induce Keap1 expression. After shaking at room temperature for 16 h, the cells were harvested by centrifugation (5,000 g for 15 min) and then lysed in a lysis buffer (20 mM Tris, 300 mM NaCl, 10 mM imidazole) via sonication on ice. The insoluble cell debris was removed by centrifuging at

10,000 g for 15 min and the clear supernatant was purified with Ni-NTA agarose resin. Finally, the protein was desalted by running through a NAP-10 column (GE healthcare) and stored in PBS buffer at -80 °C until further use.

For the biotinylation of Keap1, 5 μM of Keap1 was incubated with 2-fold molar excess of Sulfo-NHS-Biotin (ThermoFisher 21217) in PBS buffer (pH= 6.5) at 4°C for overnight. The biotinylation of Keap1 was confirmed by LC-MS (Figure S8) and the unreacted free Sulfo-NHS-Biotin was removed by a NAP-5 column (GE healthcare).

b. Expression of biotinylated SrtA

The biotinylated SrtA (biotin-SrtA) expression vector pET28a-AviTag-Linker-SrtAdelta59 was built by inserting the AviTag and proline-rich linker sequence into the pET28a-SrtAdelta59 through a Q5 mutagenesis kit in two steps. First, the AviTag was inserted with primers

AviTag_F: 5'-GCAGAAAATTGAATGGCATGAACGCGGCAGCCATATGCAAGC-3'

AviTag_R: 5'-GCTTCAAAAATATCGTTCAGGCCCGGCACCAGGCCGCTGCT-3'

The sequence of the plasmid was verified by Sanger sequencing, and then the linker coding sequence was inserted in between the AviTag and SrtA with the following primers:

Linker_F: 5'-CACTCCTCCTACCGGTGGCTCTTTACGCGGCAGCCATATGCAA-3'

Linker_R: 5'CTCGGGGTCGGCGGGGTGGATAAGCTTTCATGCCATTCAATTTTCTGCGC-3'

The sequence of the pET28a-AviTag-Linker-SrtAdelta59 vector was verified by Sanger sequencing. The plasmid was transformed into CVB-T7 POL competent cells for expression. An overnight preculture in 5 mL LB medium containing 50 μg/mL Kanamycin and 10 μg/mL Chloramphenicol was used for inoculating 500 mL LB medium containing 50 μg/mL Kanamycin. The cells were cultured at 37°C with shaking at 250 r.p.m. until the O.D. reaches 0.4-0.6. IPTG (1 mM) and biotin (50 μM) were then added to induce the expression of BirA and SrtA. After shaking at room temperature for 16 h, the cells were harvested by spinning at 5,000 g for 15 min. The purification was done following the same protocol described above for other SrtA variants.

c. Expression of SrtA without AviTag

The SrtA binding studies of the peptide hits were carried using SrtA without the AviTag, which was expressed in *E. coli* strain BL21(DE3) using the cytoplasmic expression plasmid pET28a-SrtAdelta59 (Addgene, #51138). For protein expression, a single colony from a plate was inoculated into a culture tube with 5 mL LB medium containing 50 μg/mL Kanamycin, which was

then incubated overnight at 37°C with shaking at 250 rpm. Then this overnight culture was inoculated into 500 mL of the same medium in a 2.5 L flask. The cell culture was incubated at 37°C with shaking at 250 rpm until the O.D. reached 0.4-0.6. IPTG (1 mM) was then added to induce SrtA expression. After shaking at room temperature for 16 h, the cells were harvested by centrifugation (5,000 g for 15 min) and then lysed in a lysis buffer (20 mM Tris, 300 mM NaCl, 10 mM imidazole) via sonication on ice. The insoluble cell debris was removed by centrifuging at 10,000 g for 15 min and the clear supernatant was purified with Ni-NTA agarose resin. Finally, the protein was desalted by running through a NAP-10 column (GE healthcare) and stored in DPBS buffer at -80 °C until further use.

IV. Peptide Synthesis

All peptides were synthesized on a peptide synthesizer using a Rink Amide resin and the standard Fmoc/tBu protocol. Fmoc-Dap(Alloc)-OH was used to introduce an Alloc-protected residue on the C-terminus to facilitate on-resin coupling of fluorophore. The Dap residue was preceded by a triple glycine linker and then the peptide hit sequence at the N-terminus. 5(6)-FAM or biotin was conjugated to the peptide on resin by removing the Alloc protecting group with $Pd(PPh_3)_4$ (50 mg) and phenylsilane (0.3 mL) in DCM (2 mL) followed by subsequent HBTU-mediated coupling in 0.4 M NMM/DMF. The peptides were cleaved off resin with a cleavage cocktail containing 88% TFA, 5% H_2O, 2% triisopropylsilane, 5% phenol. The crude peptides obtained from ether precipitation were purified by RP-HPLC.

For peptide modification, the peptide was treated with **M-a-23** or **M-a-23-2** (1.2 equiv) in the presence of TCEP (2 eq) in an DMF/ PBS buffer (v/v=1/4, pH 7.4) for 2 hours at room temperature and purified via RP-HPLC.

For cyclization of **K5_M-a-23-2**, 1 mg of HPLC purified peptide was dissolved in PBS buffer, followed by incubating at room temperature under nitrogen protection for four days. The cyclization efficiency was determined by incubation with **IA**. **IA** (1 mM) was added to 10 μL of the reaction mixture and incubated at room temperature for 2 h, and then monitored by LC-MS.

All peptides were characterized with LC-MS to confirm their identities and excellent purities

(>95%) (Section XII of supporting information).

V. Construction of Phage Libraries

The Ph.D. Peptide Display Cloning System (NEB #E8101S) was used to construct the peptide libraries. For the library construction, the following library-coding sequence was used: CATGTTTCGGCCGAACCTCCACCACAMNNMNNMNNMNNMNNMNNMNNMNNMNNMNN ACACCGGCCCTCAATTGCAGCGTAGTCTGGAACGTCGTAGGGGTAAGAGTGAGAAT AGAAAGGTACCCGGG. The library-coding DNA was prepared by annealing the purchased oligonucleotide with an M13 extension primer. Then the annealed duplex was extended using Klenow fragment (NEB #M0210), digested with EagI-HF (NEB #R3505)/KpnI-HF (NEB #R3142) and then purified by gel. The purified DNA was ligated with gel-purified, EagI-HF/KpnI-HF digested M13KE_K10R vector. The ligation mixture was then electroporated into TOP10 competent cells. Titering of the electroporation sample revealed a library size of 4.5×10^8 pfu. Finally, phage amplification was accomplished by incubating the electroporated cells at 37 °C with shaking at 250 r.p.m for 4.5-5 h. Phage was recovered from the supernatant by adding 1/6 volume of 20% PEG/2.5 M NaCl. The precipitated phage was resuspended in TBS buffer pH 7.4 and stored at 4°C until further use.

VI. Chemical Modification of Phage Libraries

20 μL of the prepared phage library (10^{13} pfu/mL) was diluted with 80 μL Factor Xa cleavage buffer (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$), then 0.5 μL of Faxtor Xa (NEB#P8010) was added and incubated at room temperature for 6 h. The cleavage phage was precipitated with 1/6 volume of 20% PEG/2.5 M NaCl on ice for 1 h, then the precipitated phage was pelleted at 4°C by centrifugation at 10,000 g for 20 min. The supernatant was discarded, the phage pellet was re-suspended in 100 μL of buffer R (20 mM ammonium bicarbonate, pH= 8.0) and reduced with 1 mM TCEP. The reaction was carried out at room temperature for 1 h. The reduced phage particles were precipitated with 1/6 volume of 20% PEG/2.5 M NaCl on ice for 1 h, then the precipitated phage was pelleted at 4°C by centrifugation at 10,000 g for 20 min. The supernatant was discarded and the phage pellet was re-suspended in 90 μL of PBS buffer (pH= 7.4), then 10 μL of **M-a-23** (1.5 mM in DMF) was added. The reaction was mixed and incubated at room temperature for 2 h. Then 1 μL of free cysteine (250 mM in PBS) was added, the reaction was mixed and incubated at

room temperature for another 2 h. The phage was precipitated using 1/6 volume of 20% PEG/2.5 M NaCl solution and the phage pellet was re-suspended in 100 μL PBS for phage selection.

We used a phage ELISA assay to quantitatively assess the efficiency of **M-a-23** mediated peptide cyclization. Six different chemically modified libraries were prepared separately. (1) 10 μL of Factor Xa cleaved phage was diluted with 90 μL PBS buffer (pH= 8.5), then incubated with 1 mM **B-IA** for 2 hours followed by precipitation; (2) 10 μL of the Factor Xa cleaved phage library was reduced with 1 mM TCEP in 90 μL of buffer R (20 mM ammonium bicarbonate, pH= 8.0). The reaction was carried out at room temperature for 1 h. The reduced phage particles were precipitated and re-suspended in 100 μL PBS buffer (pH= 8.5), then incubated with 1 mM **B-IA** for 2 h, followed by precipitation; (3) 10 μL of the Factor Xa cleaved phage library was reduced with 1 mM TCEP as mentioned above. The reduced phage particles were precipitated and re-suspended in 90 μL of PBS buffer (pH= 7.4), then 10 μL of **B-CBT** (1.5 mM in DMF) was added. The reaction was mixed and incubated at room temperature for 2 h. Then 1 μL of free cysteine (250 mM in PBS) was added, the reaction was mixed and incubated at room temperature for 2 h, followed by precipitation; (4) 10 μL of the Factor Xa cleaved phage library was reduced with 1 mM TCEP as mentioned above. The reduced phage particles were precipitated and re-suspended in 90 μL of PBS buffer (pH= 7.4), then 10 μL of **M-a-23** (1.5 mM in DMF) was added. The reaction was mixed and incubated at room temperature for 2 h. Then 1 μL of free cysteine (250 mM in PBS) was added, the reaction was mixed and incubated at room temperature for 2 h, followed by precipitation. The precipitated phage was re-suspended in 90 μL of PBS buffer (pH= 7.4), then 10 μL of **B-CBT** (1.5 mM in DMF) was added. The reaction was mixed and incubated at room temperature for 2 h. Then 1 μL of free cysteine (250 mM in PBS) was added, the reaction was mixed and incubated at room temperature for 2 h, followed by precipitation; (5) 10 μL of the Factor Xa cleaved phage library was reduced with 1 mM TCEP as mentioned above. The reduced phage particles were precipitated and re-suspended in 90 μL of PBS buffer (pH= 7.4), then 10 μL of **M-a-23** (1.5 mM in DMF) was added. The reaction was mixed and incubated at room temperature for 2 h. Then 1 μL of free cysteine (250 mM in PBS) was added, the reaction was mixed and incubated at room temperature for 2 h, followed by precipitation. The precipitated phage was re-suspended in100 μL PBS buffer (pH= 8.5), then incubated with 1 mM **B-IA** for 2 h, followed by precipitation; (6) 10 μL of the Factor Xa cleaved phage library was reduced with 1

mM TCEP as mentioned above. The reduced phage particles were precipitated and re-suspended in 90 μL of PBS buffer (pH= 7.4), then 10 μL of **CBT** (1.5 mM in DMF) was added. The reaction was mixed and incubated at room temperature for 2 h. Then 1 μL of free cysteine (250 mM in PBS) was added, the reaction was mixed and incubated at room temperature for 2 h, followed by precipitation. The precipitated phage was re-suspended in100 μL PBS buffer (pH= 8.5), then incubated with 1 mM **B-IA** for 2 h, followed by precipitation. All the chemically modified libraries were re-suspended in 100 μL of PBS buffer (pH= 7.4) and the subjected to the ELISA assay.

For the ELISA assay, streptavidin (0.1 mg/mL in 0.1 M $NaHCO_3$ pH 8.6, 100 μL/well) was added into 96-well plate (Corning^{TM}, 3361) and incubated overnight at 4°C. Wells incubated with buffer only were used as a negative control. After incubation, the solution in wells was removed and the wells were blocked with 5mg/mL BSA in PBS (room temperature, 1h) and then washed six times with PBST. Chemically modified phage samples (100 μL, 10^9 pfu/mL) were added to the wells and incubated for 1 h at room temperature. After 6 times of washing with PBST, an anti-M13 antibody-HRP fusion (1:10,000 dilution) was added and incubated at room temperature for 1 h. The wells were washed with PBST for 6 times and then 100 μL TMB (Thermo scientific, 34028) was added into the plate. After incubation at room temperature for 15 min, 100 μL 2M sulfuric acid was added to stop the reaction. The absorbance at 450 nm was read for all treated wells. All experiments were repeated at least three times and consistent results were obtained.

For the phage pull-down assay, chemically modified phage was quantified through titering and then diluted to 10^7 pfu/mL. Then 100 μL of the diluted phage solution was mixed with 100 μL blocking buffer (2 mg/mL BSA, 0.2% Tween-20 in PBS) and incubated for 30 min at room temperature. In parallel, pre-washed streptavidin beads (20 μL for each phage sample) were incubated with the same buffer at room temperature for 30 min before mixing with the blocked phage samples. After incubation at room temperature for 30 min, the beads together with the biotinylated phage were precipitated using a magnetic stand and the phage count in the supernatant was determined via titering. The percentage of biotin capture was calculated as:

Biotin capture (%) = [(input-output)/input]×100%

VII. Phage Panning Against Target Proteins

20 μL of streptavidin-coated magnetic beads (Dynabeads M-280 streptavidin, Invitrogen) were washed three times with 1 mL of DPBS (for SrtA) or PBS (for Keap1). The beads were re-suspended completely with 100 μL DPBS (for SrtA) or PBS (for Keap1), to which a biotinylated target protein (0.4 μg of biotinylated SrtA or 0.8 μg of biotinylated Keap1) was added. The mixture was incubated on a slowly rotating wheel for 30 min at room temperature. The beads were washed three times with 1 mL of DPBS (for SrtA) or PBS (for Keap1), re-suspended in 450 μL of a blocking buffer (see below for detailed composition), and then incubated on a slowly rotating wheel for 30 min at room temperature. In parallel, a phage library was also blocked for 30 min at room temperature. Then the library was mixed with the magnetic beads and incubated at room temperature for 30 min. After that, the unbounded phage was removed. The beads were washed eight times with a washing buffer (see below for detailed composition) and twice with 1 mL DPBS (for SrtA) or PBS (for Keap1). After washing, the beads were re-suspended in 100 μL of elution buffer (0.2 M glycine-HCl, pH= 2.2). After 5-10 min incubation, the supernatant was separated from the beads, neutralized with 400 μL of neutralization buffer (1 M Tris-HCl, pH=9.0). A small aliquot of the supernatant was subjected to titering to quantify the output phage population and the remaining was amplified to prepare for next rounds of selection.

For SrtA:
Blocking buffer: 1 mg/mL BSA, 0.1% Tween-20 in DPBS, pH=7.4
Washing buffer: 0.1% Tween-20 in DPBS, pH=7.4

For Keap1:
Blocking buffer: 1 mg/mL BSA, 0.5% Tween-20 in PBS, pH=7.4
Washing buffer: 0.5% Tween-20 in PBS, pH=7.4

VIII. Fluorescence Polarization Assay

Both fluorescence polarization binding assay and competition assay were performed in 0.1% DPBST buffer (for SrtA) or PBST (for Keap1), and the fluorescence polarization values were measured in a black 96-well plate (Corning™ 3915) using a SpectraMax M5 Microplate Reader (Molecular Devices). The experiments were conducted in triplicates, and the results from the

triplicates were averaged and analyzed. Each experiment was repeated at least three times, which gave consistent results. Representative results are presented in the paper with the FP value normalized against a fitted maximum.

For the fluorescence polarization binding assay, a FAM labeled peptide was mixed with varying concentration of target protein (0 to 50 μM). The final peptide concentration was 200 nM in all samples. After incubated at room temperature for 30 min, the fluorescence polarization values were measured. Dissociation constants (K_d) were determined by plotting the fluorescence polarization value as a function of protein concentration, and the plots were fitted to the following equation.

y=FPmin+(FPmax-FPmin)*(K_d+Lst+x-sqrt((K_d+Lst+x)^2-4*Lst*x))/(2*Lst).

The Lst is the concentration of the FAM-labeled peptide and the x stands for the concentration of the protein.

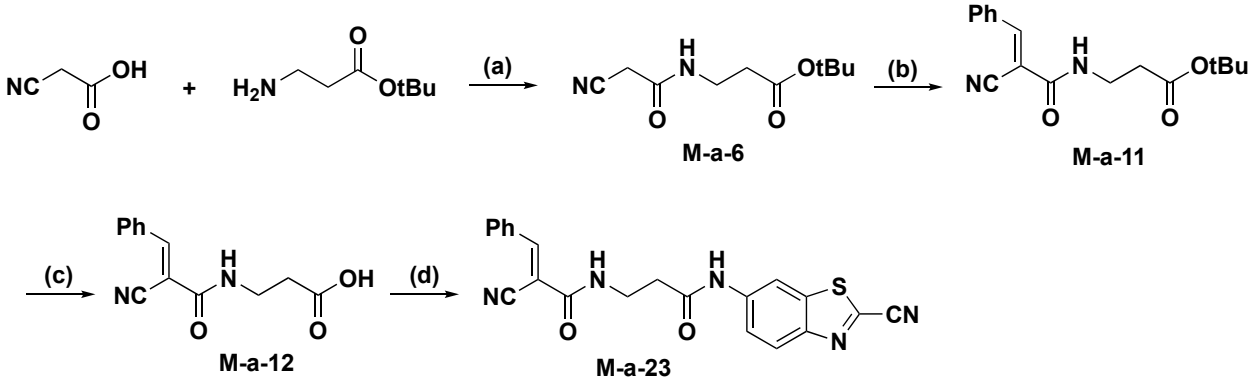
For the competition assay, the concentration of the Keap1 and a FAM-labeled peptide was kept at 300 nM and 200 nM, respectively. This mixture was incubated (30 min) with a peptide competitor at varied concentrations and then the fluorescence polarization value was recorded. The IC_{50} was determined by fitting the dose-response curves to absolute IC_{50} via nonlinear regression using Prism. We note that under our experimental conditions, the IC_{50} values can be approximated as K_d as the reporter peptide was used as much lower concentrations.

IX. Protocols for Computational Studies

Peptide models were prepared using the Avogadro program and saved in pdb-file format. The diastereomeric cyclic peptides **K5_M-a-23-2** and **S1_M-a-23-2** were geometry optimized using the B3LYP/6-31G* method. The optimizations were carried out in water solution simulated by the PCM solvation method. Frequency calculations were carried out to make sure the optimized structures are minima on the potential energy surface and the generated normal mode frequencies were used to estimate the Gibbs free energies at 298 K and 1 atm. The calculations were carried out with the Gaussian 16 program.

The docking calculations were carried out using these optimized geometries and the AutoDock Vina program. The Auto Dock Tool utility program prepare_ligand4.py (v 1.10) was used to create the ligand input file for the docking calculations in pdb qt file format. The target protein structures were prepared from the Brookhaven Protein Databank file with code 1T2W for SrtA and 5WFV for Keap1. The molecular docking program Vina (AutoDockVina 1.1.2 (May 11, 2011) was used for the molecular docking calculations. The molecular docking space was chosen to be 40 by 40 by 40 Å, encompassing the entire target protein. All molecular docking parameters were set to their default values. Superposition of the docked pose of K5 ligand stereoisomer 1 and Nrf2 using the common sequence ETGE and their backbone atoms resulted in a computed RMSD-value of 0.74 Angstrom. The unit-weighted coordinates were used in the superposition.

X. Chemical synthesis of M-a-23 and M-a-23-2



Scheme S1. Synthesis of **M-a-23**. Reaction conditions: (a) EDC, HOBt, DIPEA, DCM, r.t. 2 h; (b) PhCHO, NEt_3, IPA, 60℃ , 1 h, 58% over two steps; (c) DCM/TFA(50/50), 1 h; (d) CBT-NH_2, ClCOO-iBu, NMP, THF, 75% over two steps.

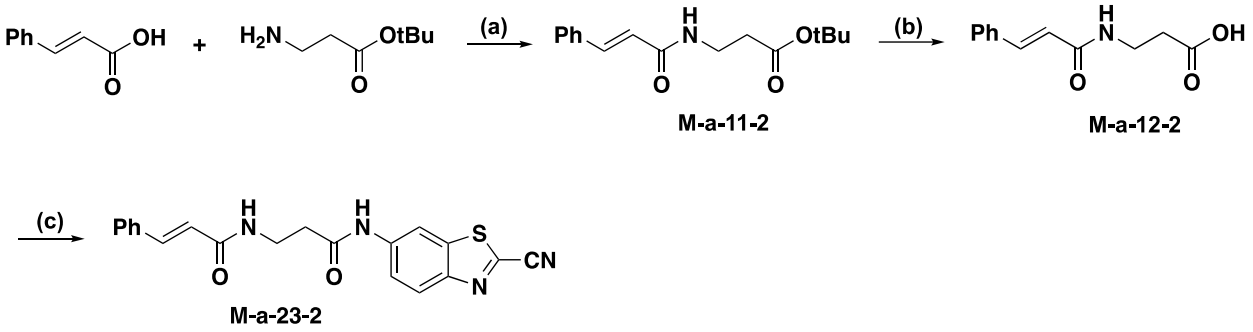
Synthesis of **M-a-11**:

EDC (1.45 g, 7.5 mmol) and HOBt (1.15 g, 7.5 mmol) was added to a mixture of cyanoacetic acid (0.425 g, 5 mmol) and DIPEA (1.75 mL, 10 mmol) in DCM at 0 ℃. 15 min later, beta-alanine tert-butyl ester hydrochloride (1 g, 5.5 mmol) was added to the mixture. After stirring at room temperature for 2 h, The mixture was washed with 1N HCl, sat. $NaHCO_3$, brine and dried over anhydride sodium sulfate. The solvent was removed under reduced pressure to give the desired

product **M-a-6**, which was directly used for next step without further purification. To a solution of **M-a-6** (106 mg, 0.5 mmol) in 2-propanol (1.5 mL) was added benzaldehyde (80 mg, 0.75 mmol) and triethylamine (76 mg, 0.75mmol). The reaction was stirred for 1 hour at 60℃. The solvent was removed and purified by chromatography on silica gel to give the desired product as a white solid. Yield: 110 mg (58% over steps). ^{1}H NMR (600 MHz, DMSO-*d6*) δ 8.45 (t, J= 6 Hz, 1 H), 8.14 (s, 1 H), 7.92 (m, 2 H), 7.56 (m, 3 H), 3.40 (m, 2 H), 2.46 (m, 2 H), 1.39 (s, 9 H);.1317. ^{13}CNMR (150 MHz, DMSO-*d6*) δ 170.91, 161.28, 151.14, 132.84, 132.31, 130.45, 129.72, 116.72, 106.60, 80.48, 36.47, 35.09, 28.18. MS: calcd. For $C_{17}H_{20}N_2NaO_3^+$[(M+Na)$^+$]: 323.2366; LC-MS found: m/z 323.1304.

Synthesis of **M-a-23**:

The protection group tBu in compound **M-a-11**(90 mg, 0.3 mmol) was removed with a mixture of DCM/TFA (1:1, 4 mL). After 1 h at room temperature, the solvent was then removed under vacuum to give **M-a-12**, which was directly used for next step without further purification. The isobutyl chlorformate (20 mg, 0.15 mmol) was added to the mixture of **M-a-12** (48.8 mg, 0.2mmol) and N-methyl morpholine (30 mg, 0.3 mmol) in THF (5 mL) at 0℃ and the reaction mixture was stirred at 0℃ for 30 min. Then 2-cyano-6-aminobenzothiazole (17.5 mg, 0.1 mmol) was added to the reaction mixture and stirred for another 2 hours at 0℃ then at room temperature for overnight. Saturated $NaHCO_3$ was added and the reaction mixture was extracted with ethyl acetate. The combined organic phase was dried by Na_2SO_4. The solvent was removed and purified by chromatography on silica gel to give the desired product as a white solid. Yield: 31 mg (75% over two steps). ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.50 (s, 1 H), 8.74 (s, 1 H), 8.59 (t, J= 5 Hz, 1 H), 8.17 (m, 2 H), 7.92 (m, 2 H), 7.75 (m, 1 H), 7.56 (m, 3 H), 3.55 (m, 2 H), 2.69 (t, J= 5 Hz, 2 H); ^{13}CNMR (125 MHz, DMSO-*d6*) δ 170.54, 161.52, 151.02, 148.01, 140.11, 137.17, 135.34, 132.82, 132.34, 130.44, 129.71, 125.23, 121.19, 116.78, 114.05, 111.64, 106.78, 36.70, 36.52. MS: calcd. For $C_{21}H_{16}N_5O_2S^+$[(M+H)$^+$]: 402.1019; LC-MS found: m/z 402.0991.



Scheme S2. Synthesis of **M-a-23-2**. Reaction conditions: (a) EDC, HOBt, DIPEA, DCM, r.t. 2 h, 82%; (b) DCM/TFA (50/50), 1 h, 98%; (c) CBT-NH_2, ClCOO-iBu, NMP, THF, 75%;

Synthesis of **M-a-12-2**:

EDC (1.45 g, 7.5 mmol) and HOBt (1.15 g, 7.5 mmol) was added to a mixture of *trans*-cinnamic acid (0.79 g, 5 mmol) and DIPEA (1.75 mL, 10 mmol) in DCM at 0 ℃. 15 min later, beta-alanine tert-butyl ester hydrochloride (1 g, 5.5 mmol) was added to the mixture. After stirring at room temperature for 2 h, The mixture was washed with 1N HCl, sat. $NaHCO_3$, brine and dried over anhydride sodium sulfate. The solvent was removed under reduced pressure to give the desired product **M-a-11-2**, which was directly used for next step without further purification. The protection group tBu in compound **M-a-11-2** (165 mg, 0.6 mmol) was removed with a mixture of DCM/TFA (1:1, 5mL). After 1 h at room temperature, the solvent was then removed under vacuum to give **M-a-12-2**, which was directly used for next step without further purification. Yield: 128 mg (80% over two steps). ^{1}H NMR (600 MHz, DMSO-*d6*) δ 12.22 (s, 1H), 8.16 (t, J= 6 Hz, 1 H), 7.53 (m, 2 H), 7.37 (m, 4 H), 6.62 (d, J= 18 Hz, 1H), 3.34 (m, 2 H), 2.43 (t, J= 6 Hz, 2 H). ^{13}CNMR (150 MHz, DMSO-*d6*) δ 173.37, 165.44, 139.07, 135.37, 129.86, 129.37, 127.94, 122.59, 35.44, 34.34. MS: calcd. for $C_{12}H_{14}NO_3^+$[$(M+H)^+$]: 220.0968; LC-MS found: m/z 220.0925.

Synthesis of **M-a-23-2**:

The isobutyl chlorformate (20 mg, 0.15 mmol) was added to the mixture of **M-a-12-2** (43.8 mg, 0.2 mmol) and N-methyl morpholine (30 mg, 0.3 mmol) in THF (5 mL) at 0℃ and the reaction mixture was stirred at 0℃ for 30 min. Then 2-cyano-6-aminobenzothiazole (17.5 mg, 0.1 mmol) was added to the reaction mixture and stirred for another 2 hours at 0℃ then at room temperature for overnight. Saturated $NaHCO_3$ was added and the reaction mixture was extracted with ethyl

acetate. The combined organic phase was dried by Na_2SO_4. The solvent was removed and purified by chromatography on silica gel to give the desired product as a white solid. Yield: 28 mg (75%). ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.47 (s, 1H), 8.73 (s, 1H), 8.24 (t, J= 6 Hz, 1 H), 8.16 (d, J= 6 Hz, 1 H), 7.74 (m, 1 H), 7.53 (m, 2 H), 7.37 (m, 4 H), 6.63 (d, J= 18Hz, 1 H), 3.48 (m, 2 H), 2.63 (t, J= 6 Hz, 2 H); ^{13}CNMR (125 MHz, DMSO-*d6*) δ 170.66, 165.54, 147.98, 140.17, 139.08, 137.17, 135.35, 135.29, 129.88, 129.38, 127.95, 125.23, 122.61, 121.15, 114.05, 111.56, 36.98, 25.61. MS: calcd. for $C_{20}H_{17}N_4O_2S^+$[$(M+H)^+$]: 377.1067; LC-MS found: m/z 377.0976.

XI. Reference

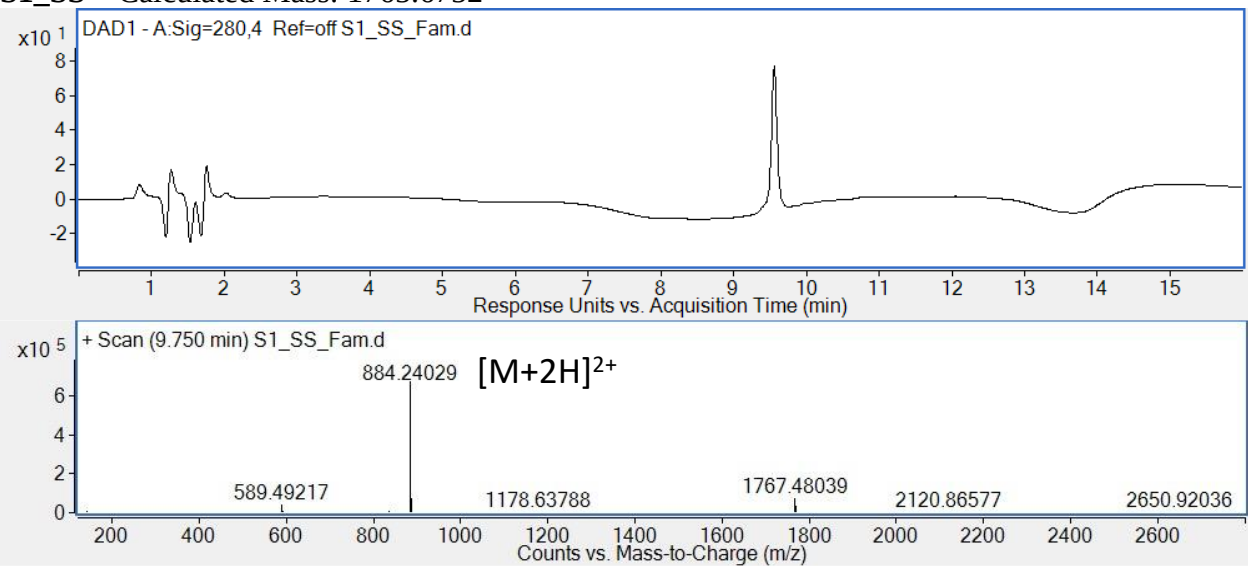
[1]. Li, K.; Weidman, C. ; Gao, J. Dynamic Formation of Imidazolidino Boronate Enables Design of Cysteine-Responsive Peptides. *Org. Lett.* **2018**, 20, 20-23.

[2]. Wang, W.; Gao, J. N, S-Double Labeling of N-Terminal Cysteines via an Alternative Conjugation Pathway with 2-Cyanobenzothiazole. *J. Org. Chem.* **2020**, 85, 1756−1763.

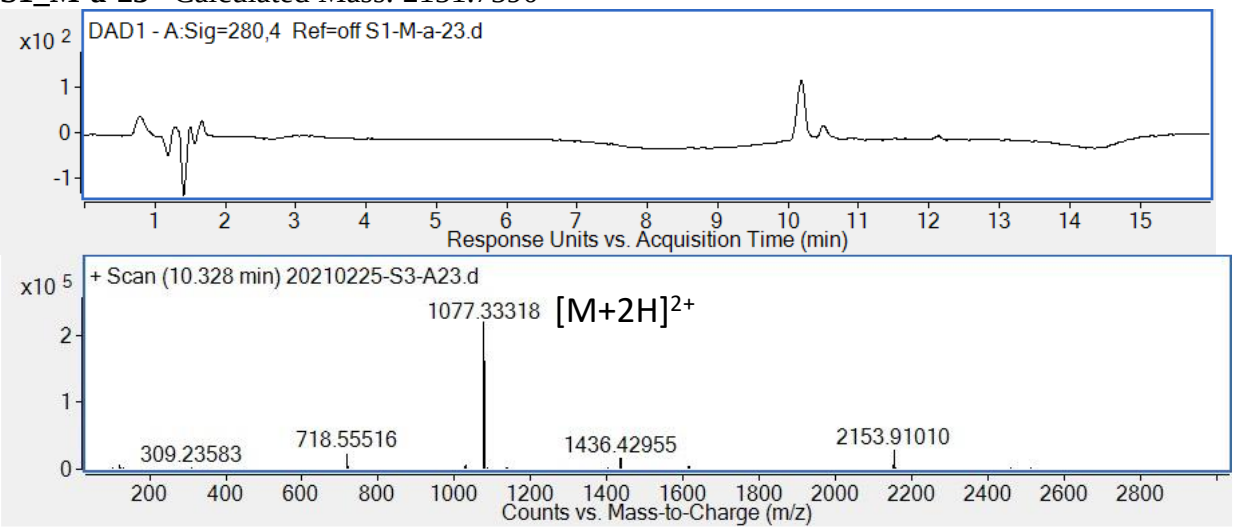
XII. LC-MS trace and ESI-MS$^+$ of Purified Peptides

Note: **M-a-23** cyclized peptides gave two peaks on the LC-MS trace corresponding to the diastereomers that resulted from the Michael addition reaction. * indicates FAM labeled peptide.

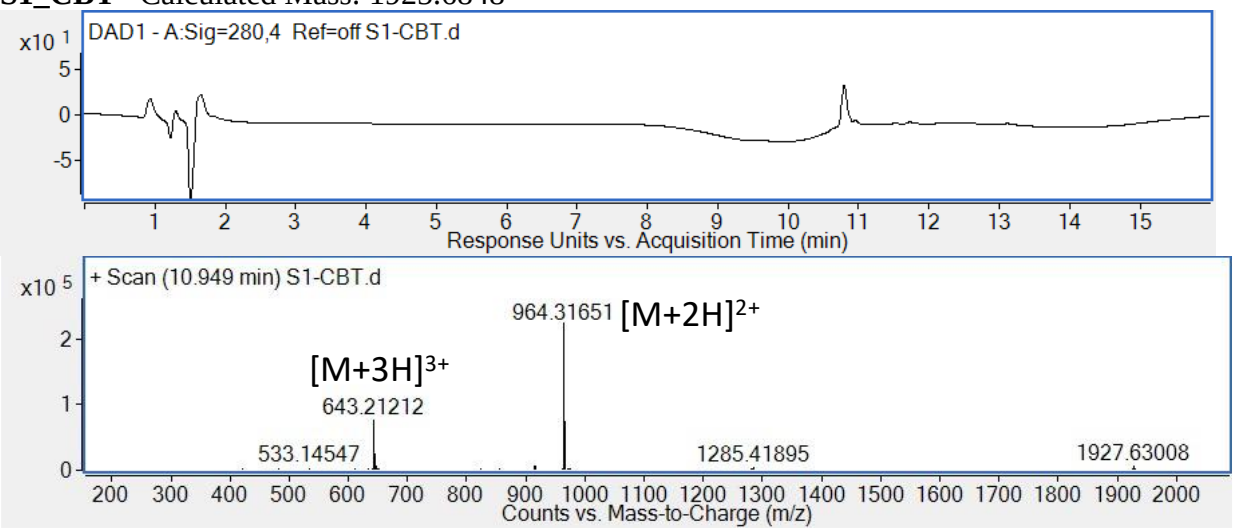
S1_SS* Calculated Mass: 1765.6752



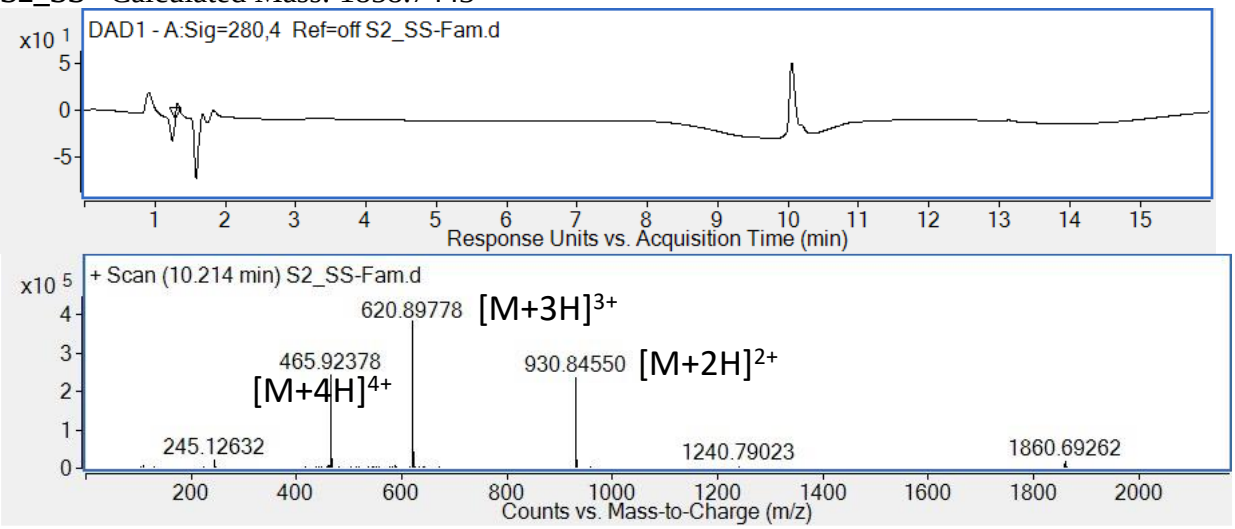
S1_M-a-23* Calculated Mass: 2151.7590



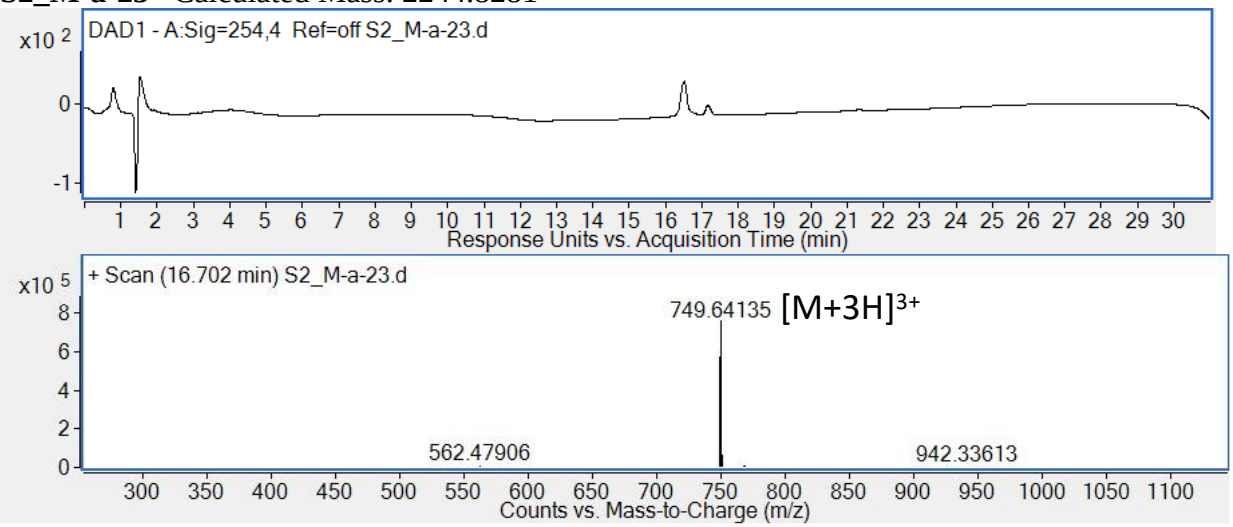
S1_CBT* Calculated Mass: 1925.6848



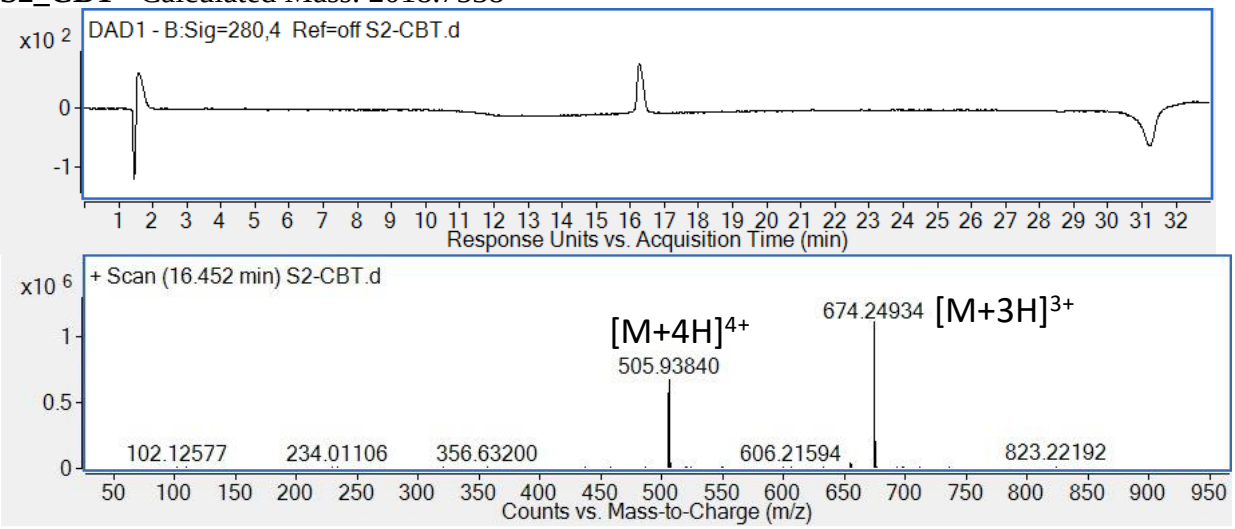
S2_SS* Calculated Mass: 1858.7443



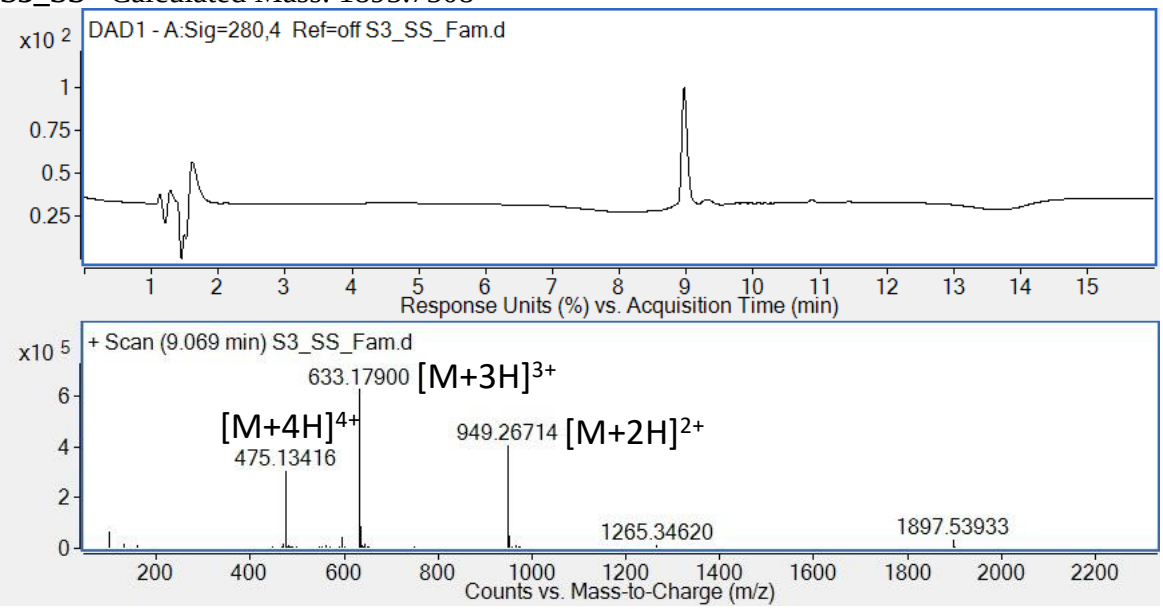
S2_M-a-23* Calculated Mass: 2244.8281



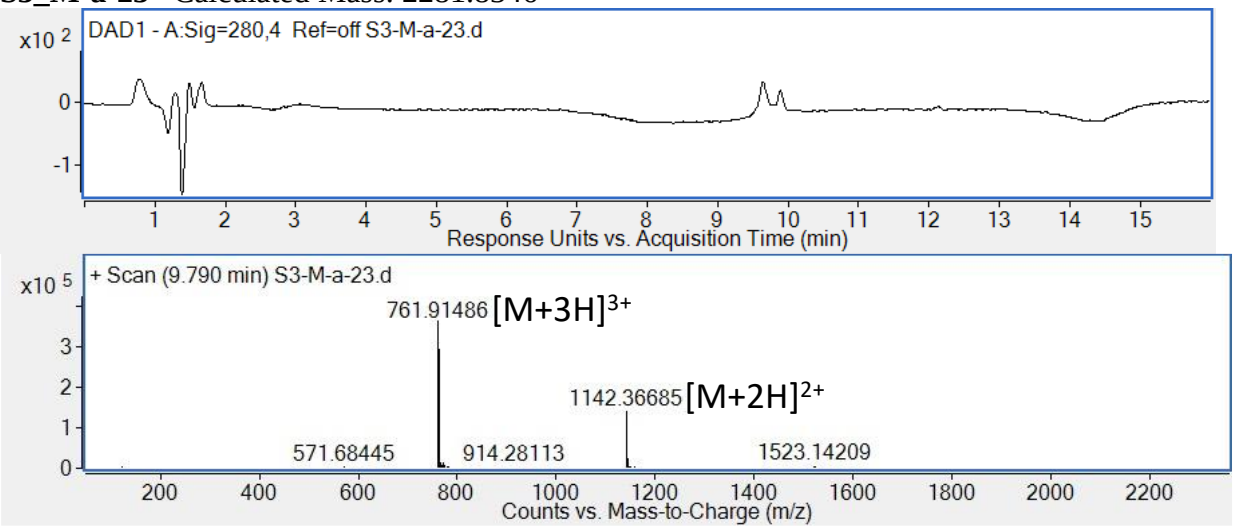
S2_CBT* Calculated Mass: 2018.7538



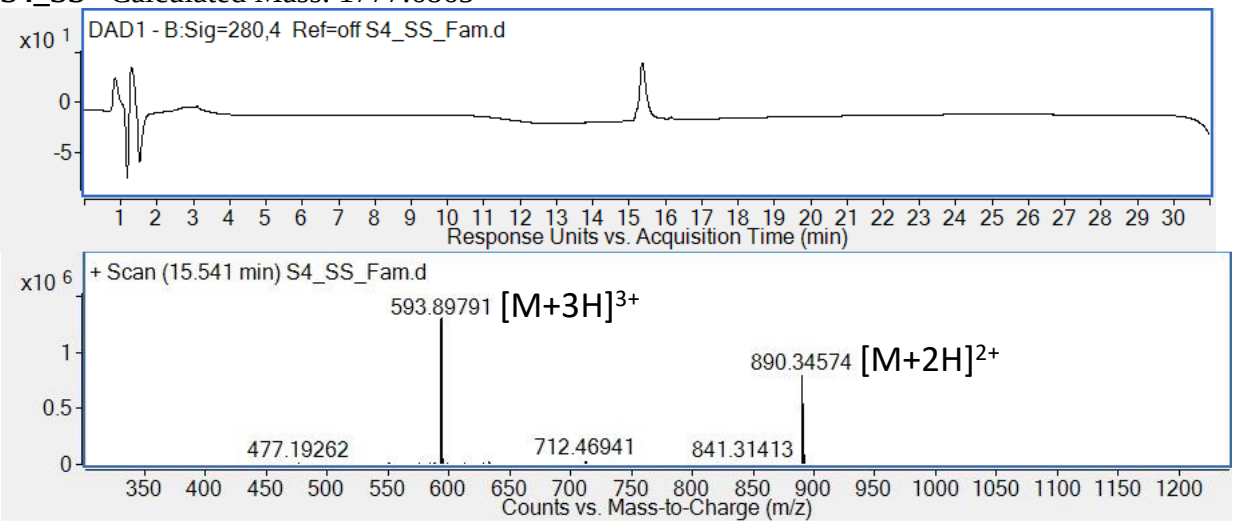
S3_SS* Calculated Mass: 1895.7508



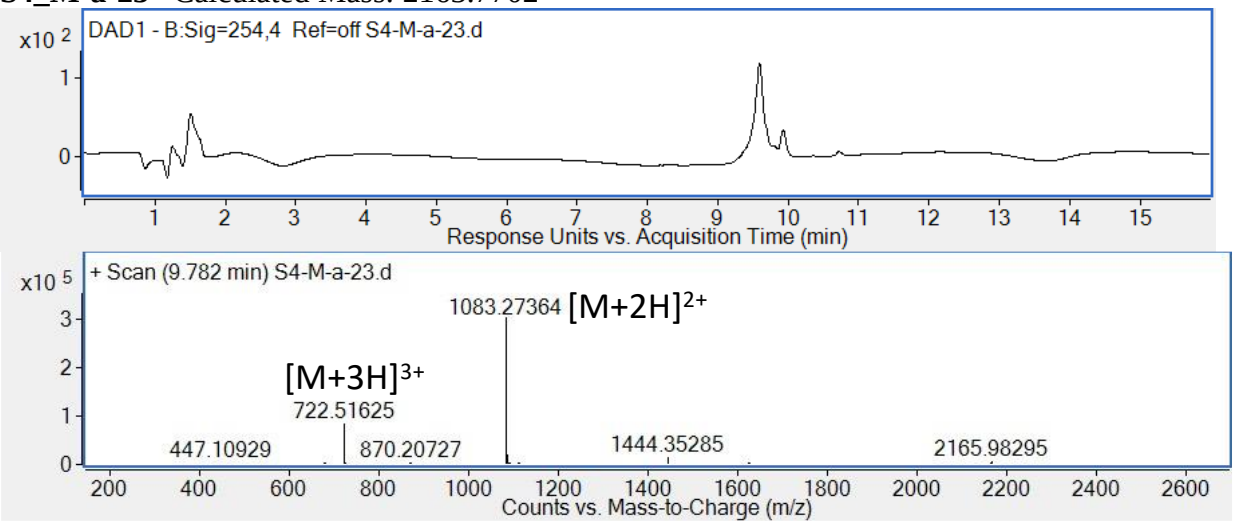
S3_M-a-23* Calculated Mass: 2281.8346



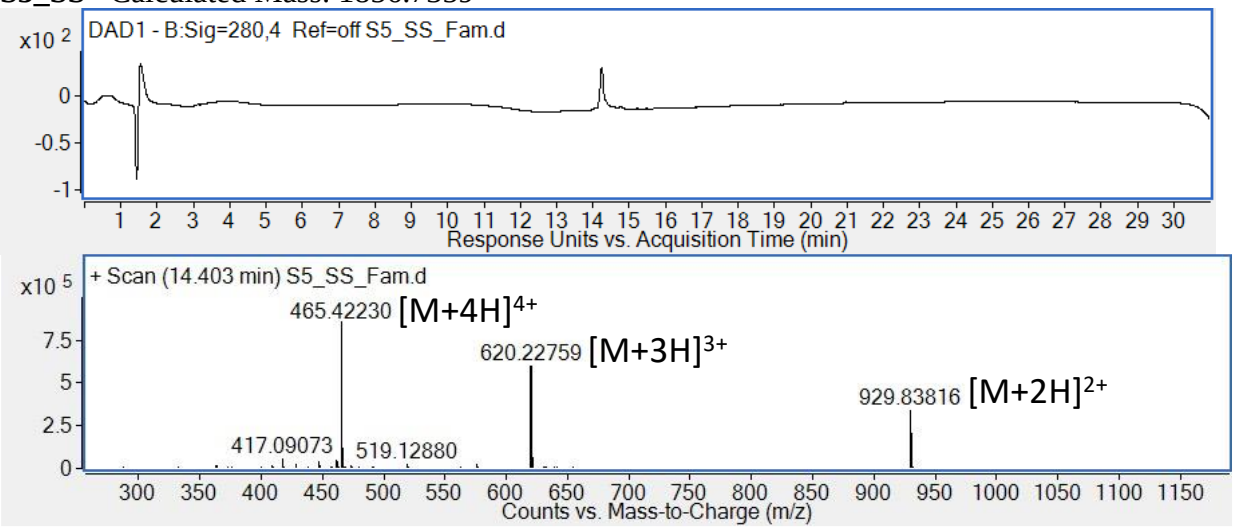
S4_SS* Calculated Mass: 1777.6865



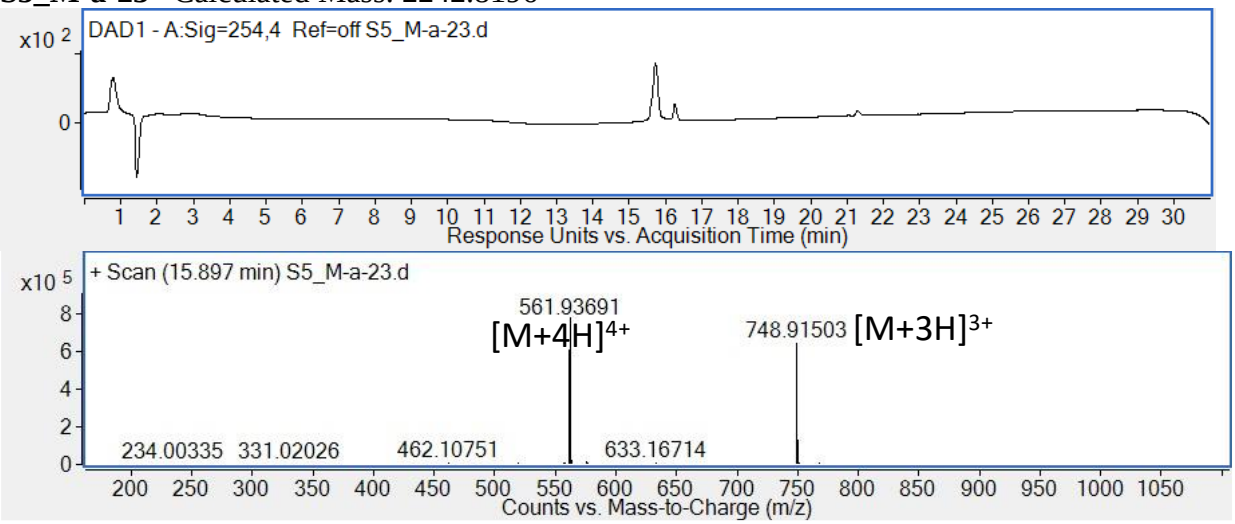
S4_M-a-23* Calculated Mass: 2163.7702



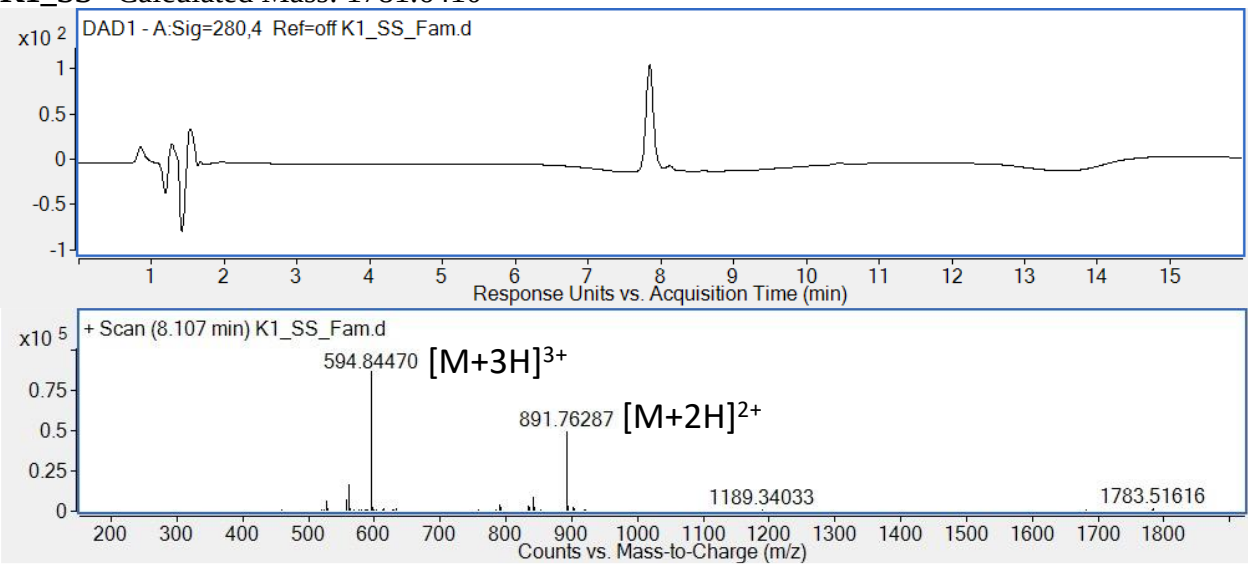
S5_SS* Calculated Mass: 1856.7359



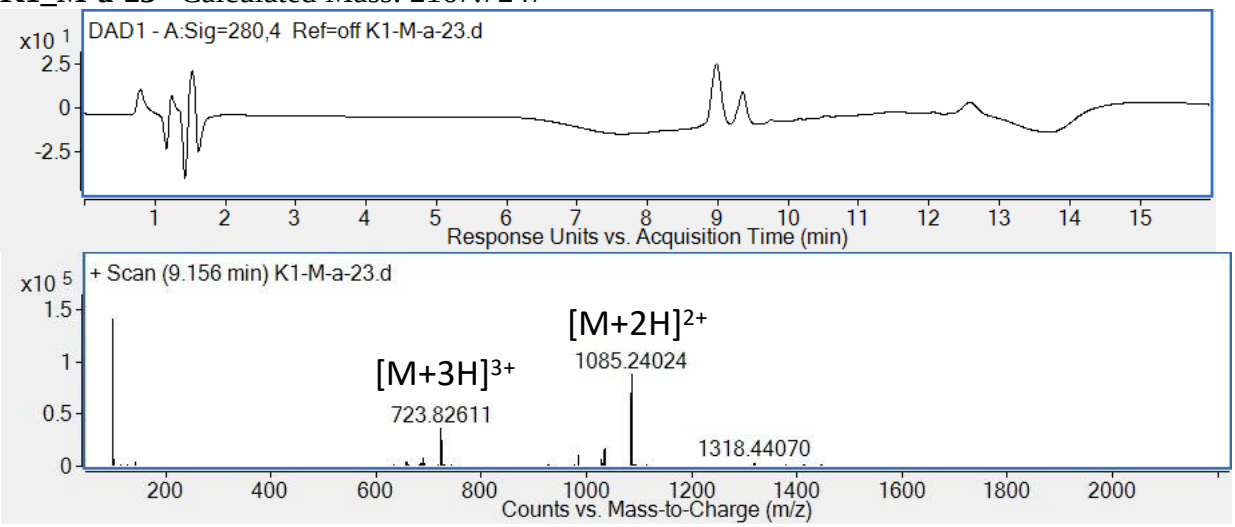
S5_M-a-23* Calculated Mass: 2242.8196



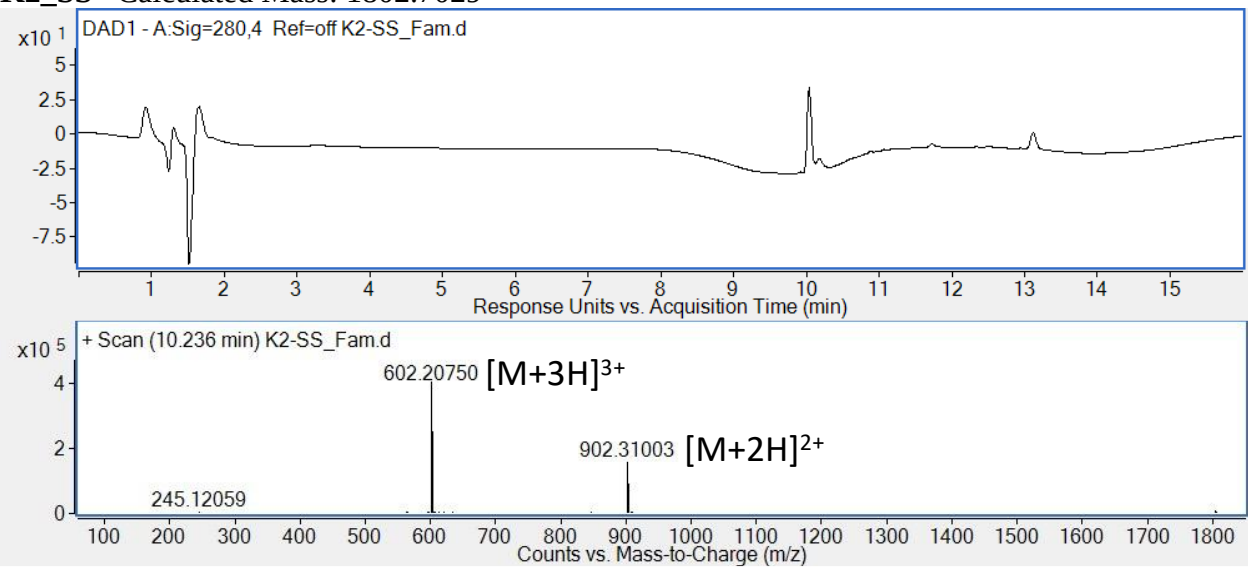
K1_SS* Calculated Mass: 1781.6410



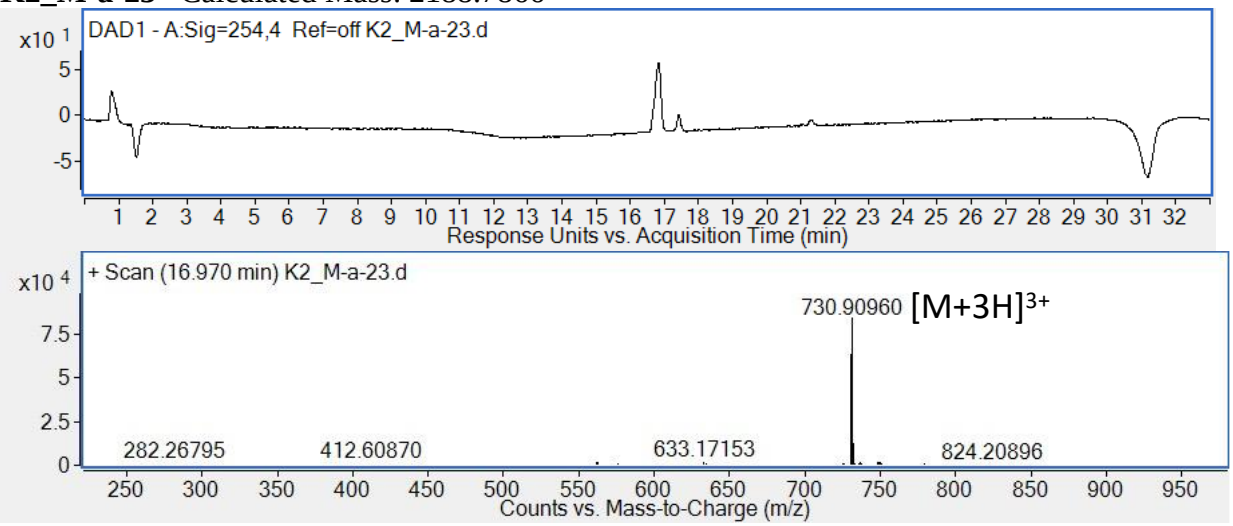
K1_M-a-23* Calculated Mass: 2167.7247



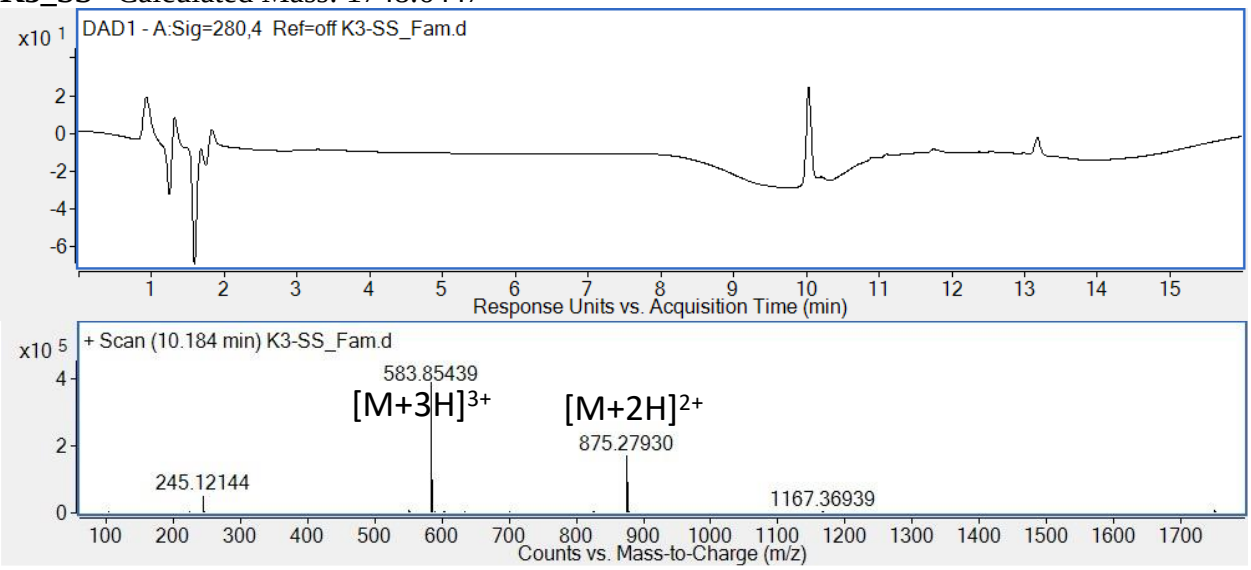
K2_SS* Calculated Mass: 1802.7029



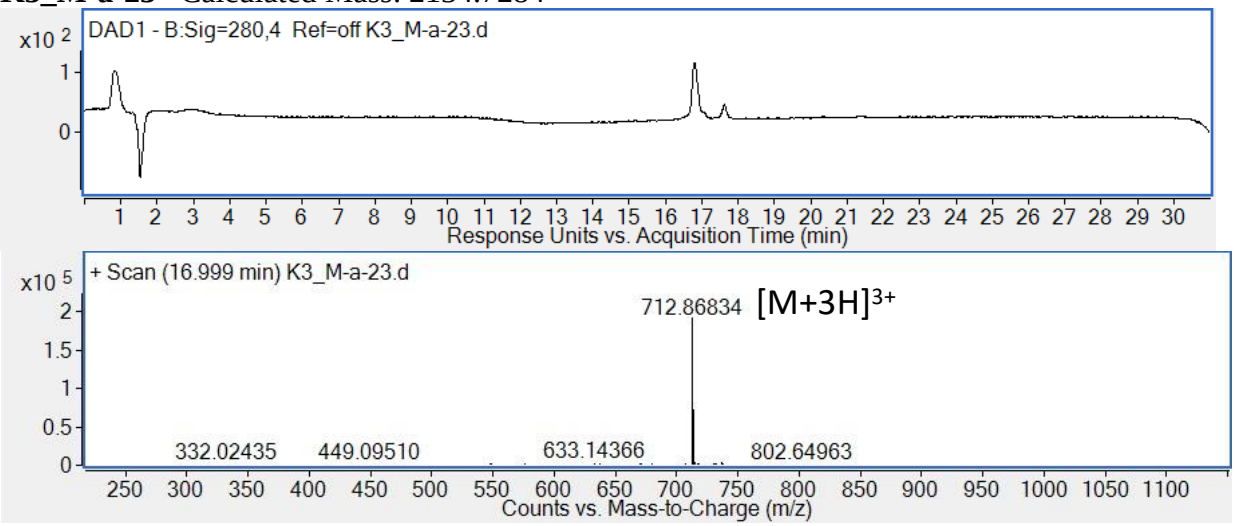
K2_M-a-23* Calculated Mass: 2188.7866



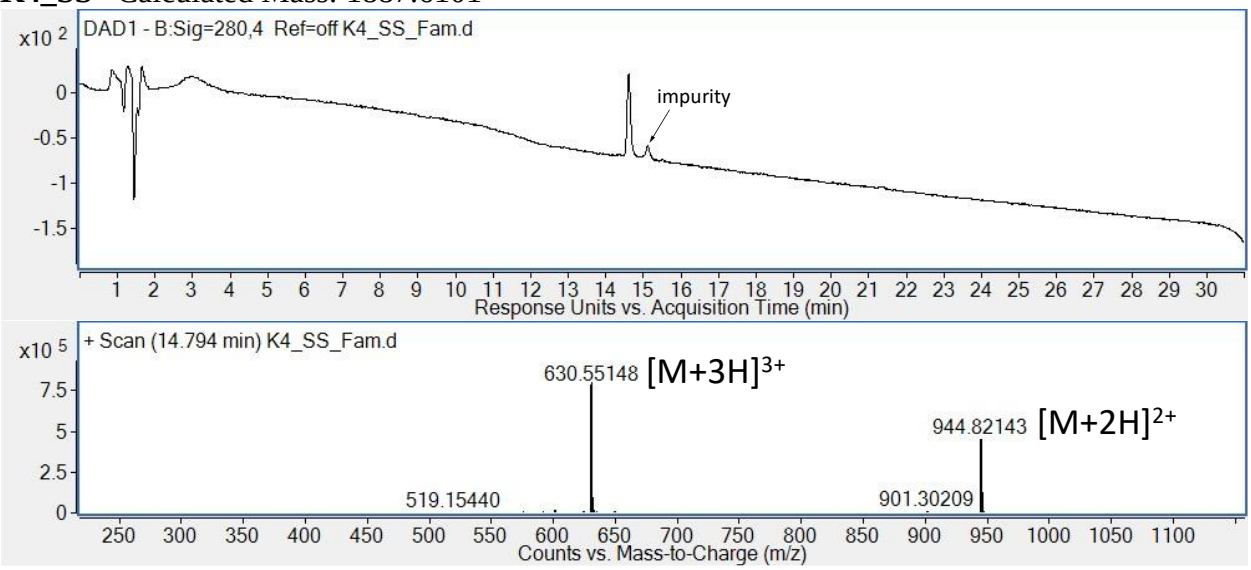
K3_SS* Calculated Mass: 1748.6447



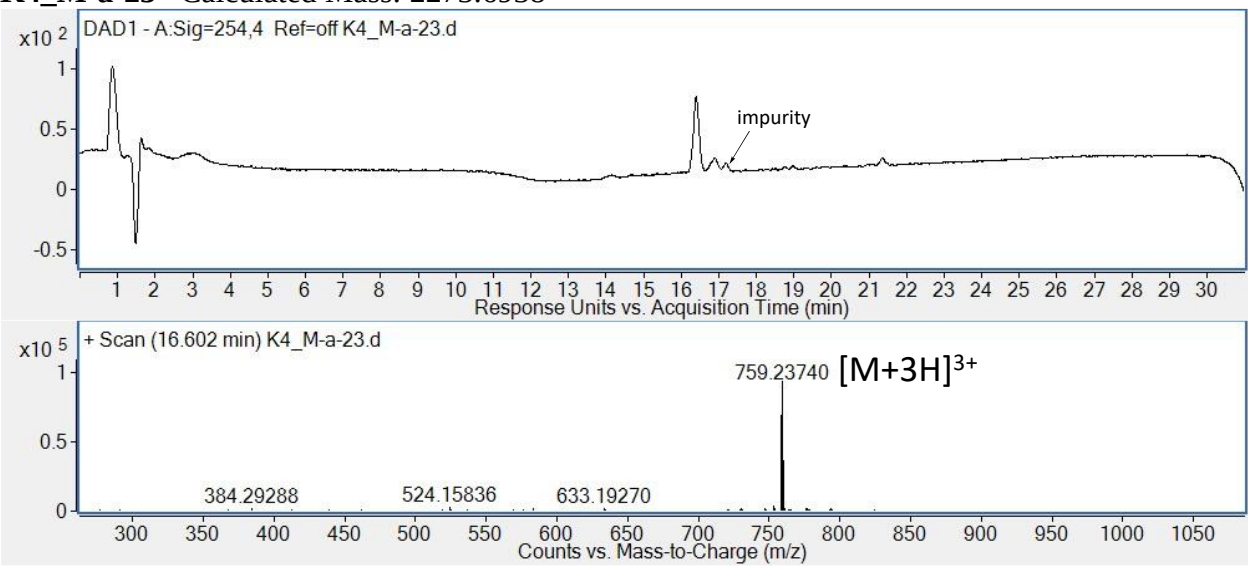
K3_M-a-23* Calculated Mass: 2134.7284



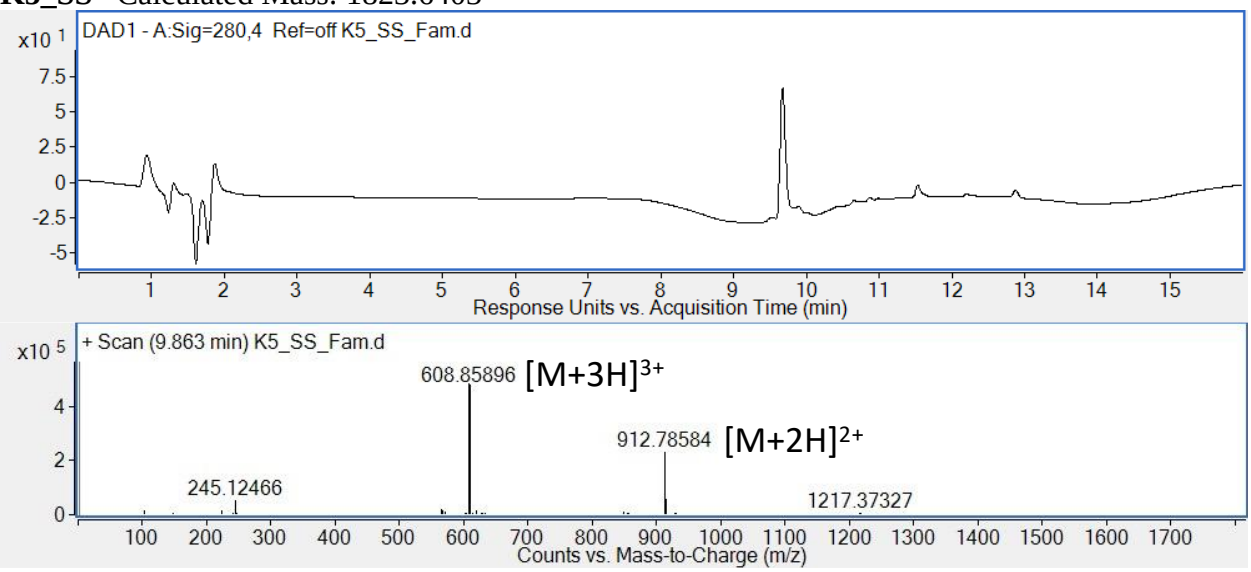
K4_SS* Calculated Mass: 1887.6101



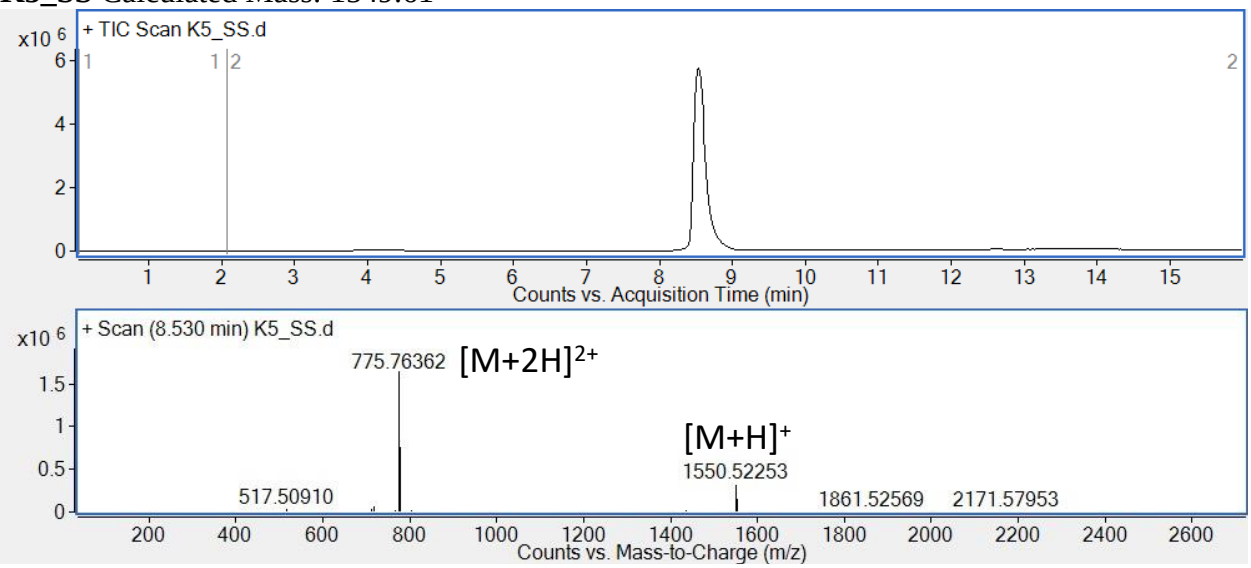
K4_M-a-23* Calculated Mass: 2273.6938



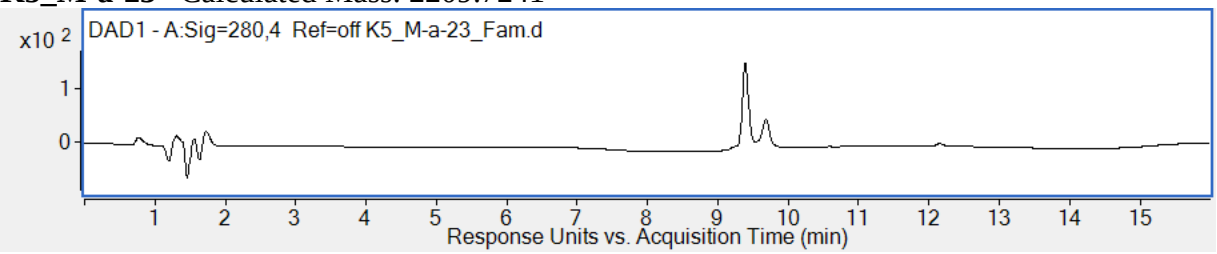
K5_SS* Calculated Mass: 1823.6403

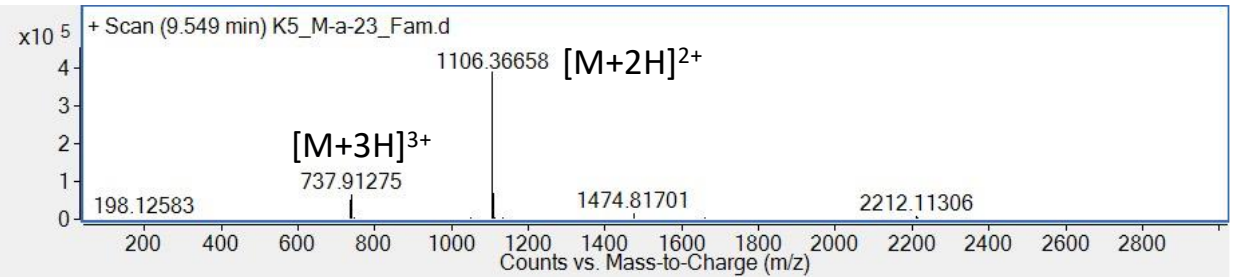


K5_SS Calculated Mass: 1549.61

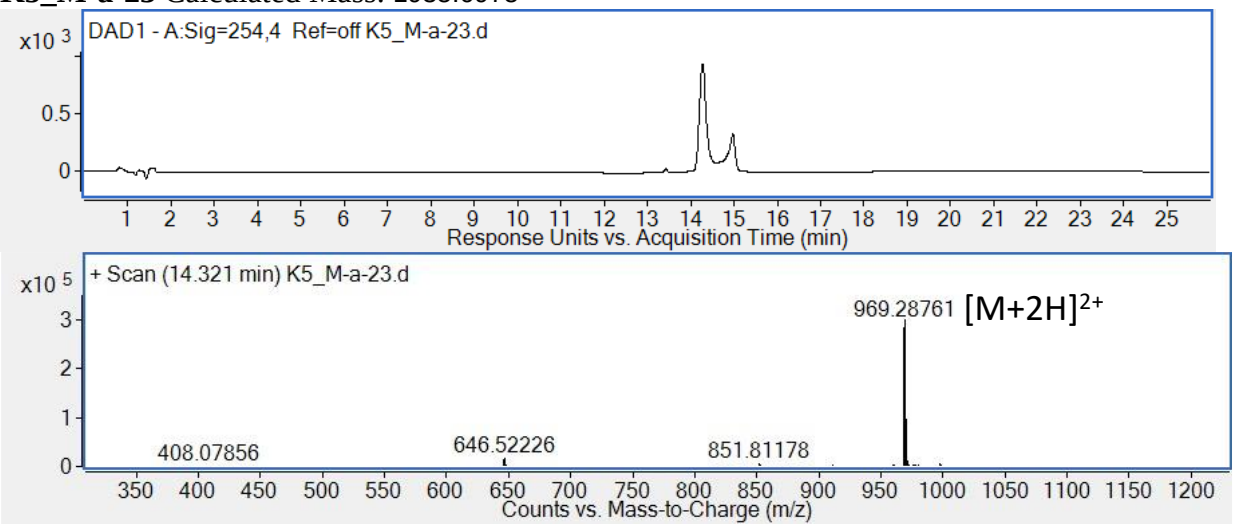


K5_M-a-23* Calculated Mass: 2209.7241

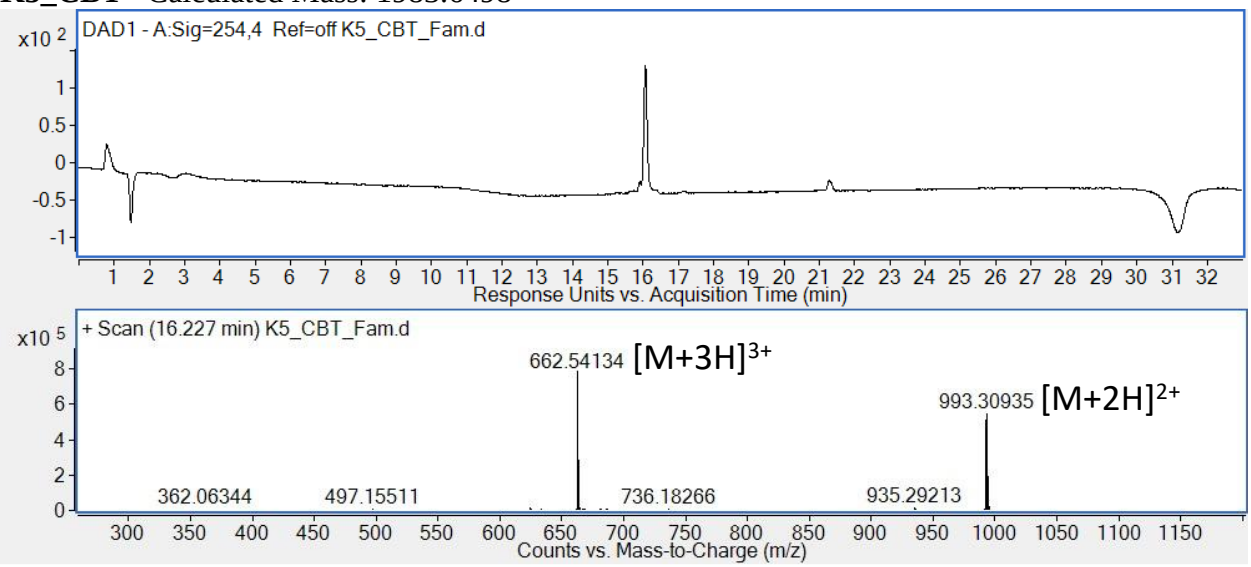




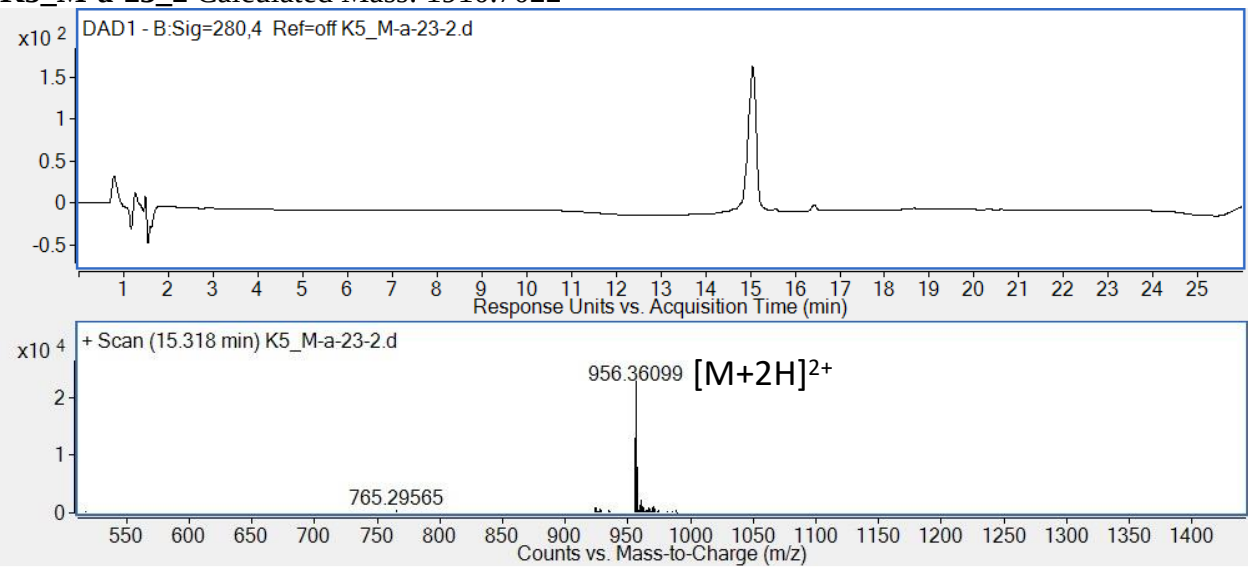
K5_M-a-23 Calculated Mass: 1935.6975



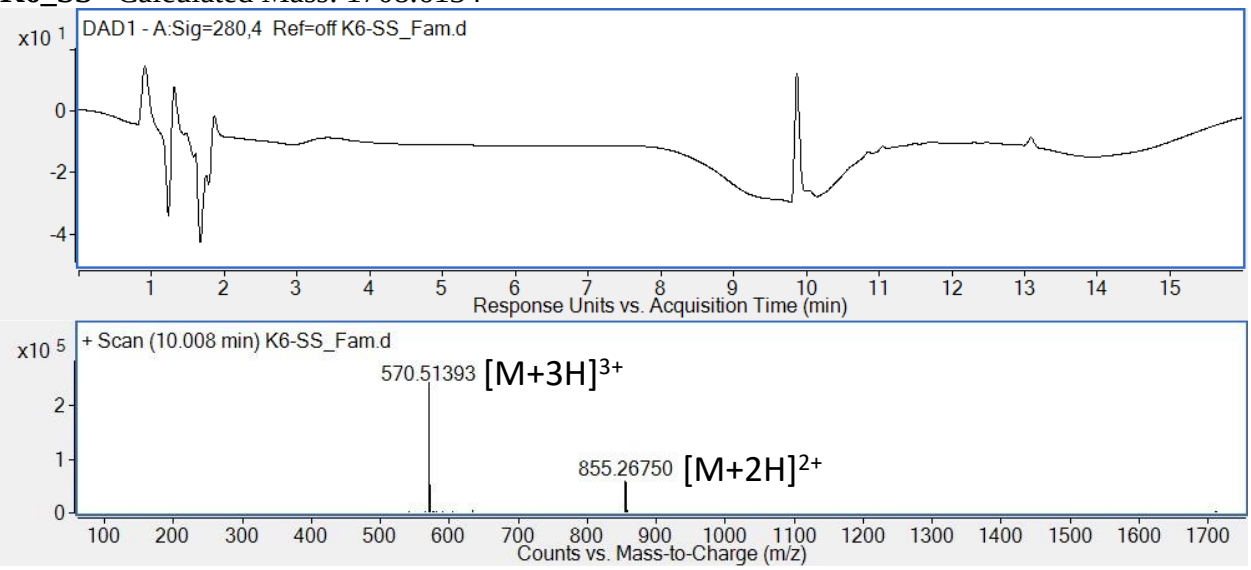
K5_CBT* Calculated Mass: 1983.6498



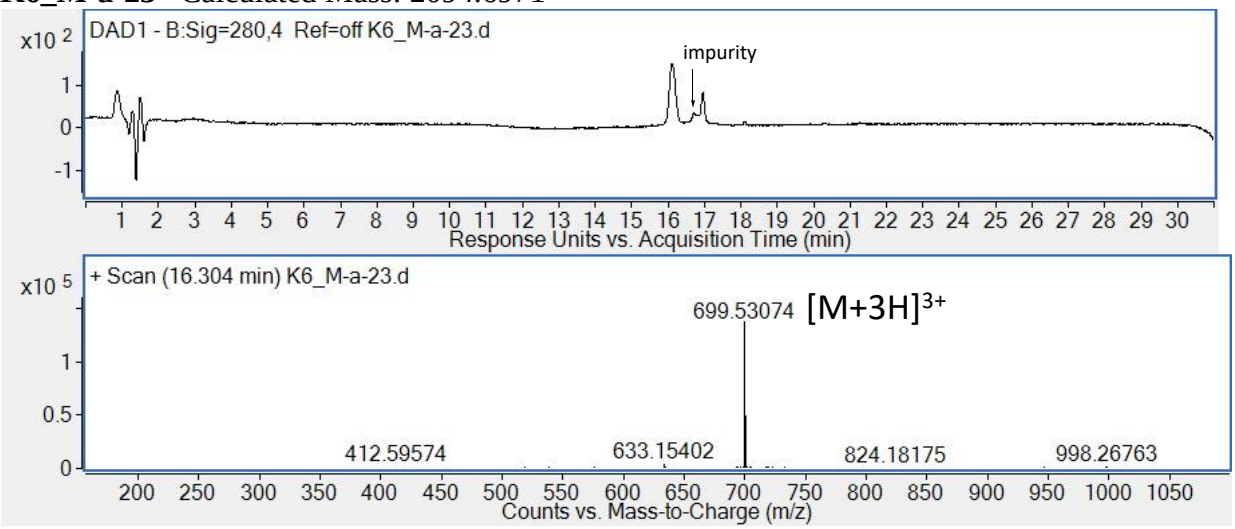
K5_M-a-23_2 Calculated Mass: 1910.7022



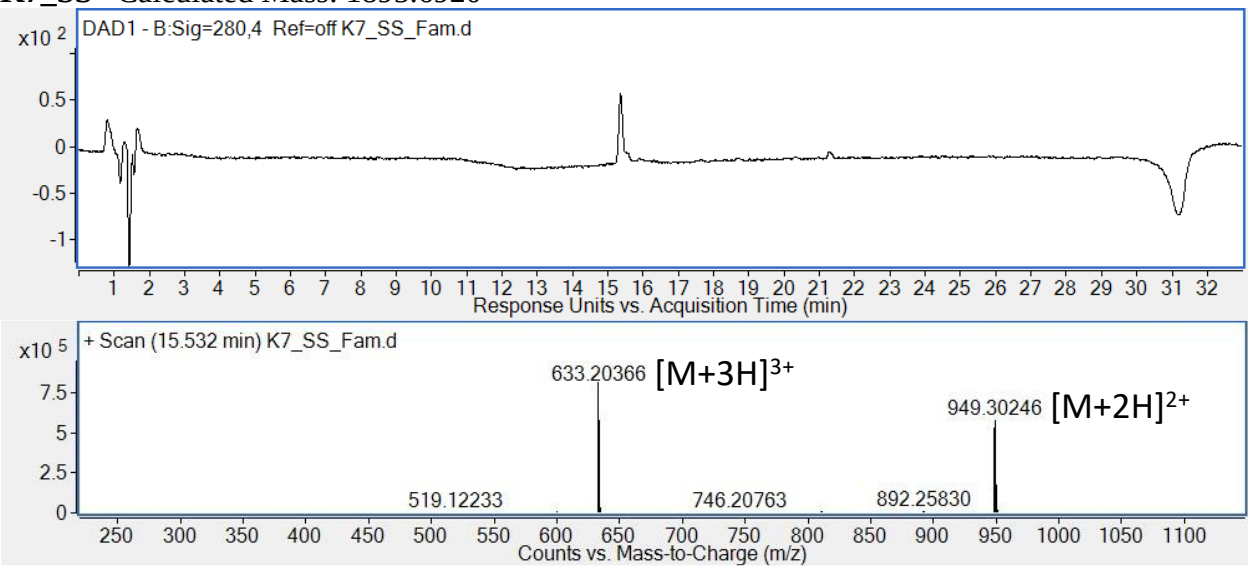
K6_SS* Calculated Mass: 1708.6134



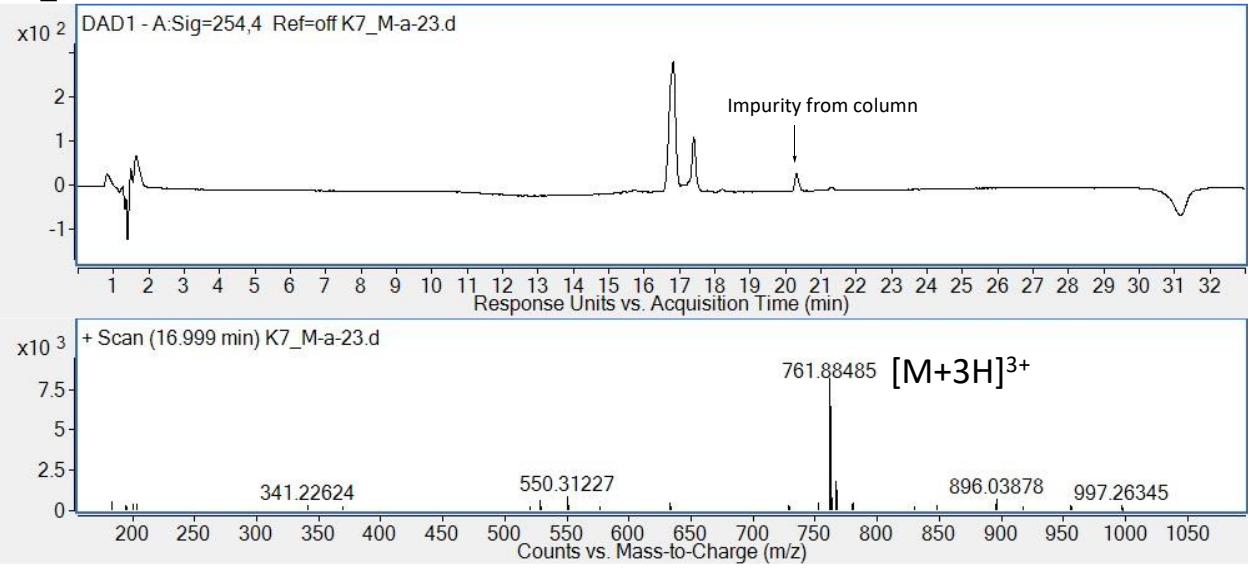
K6_M-a-23* Calculated Mass: 2094.6971



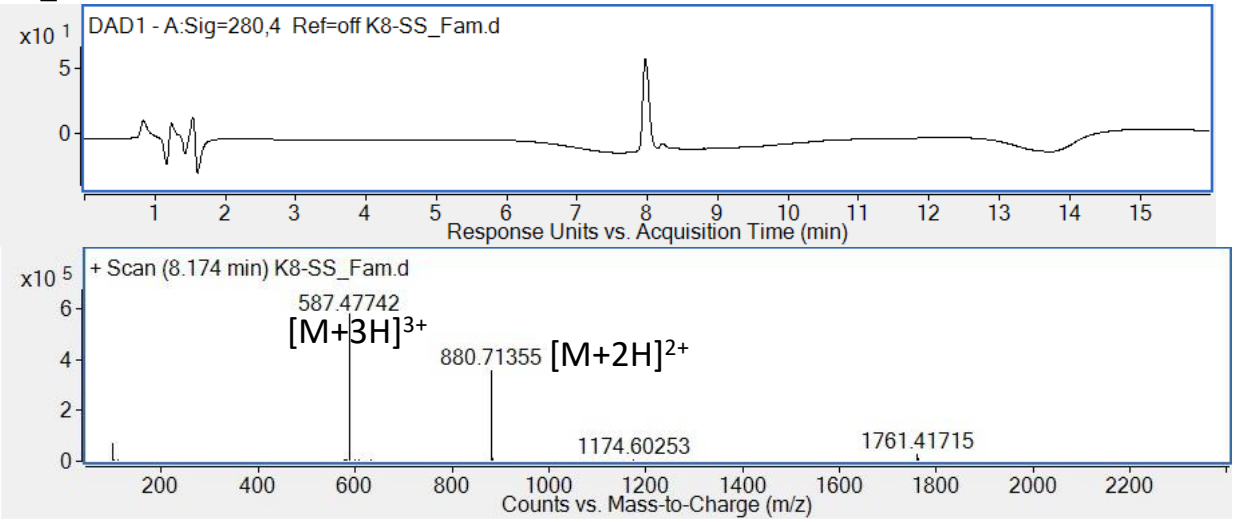
K7_SS* Calculated Mass: 1895.6920



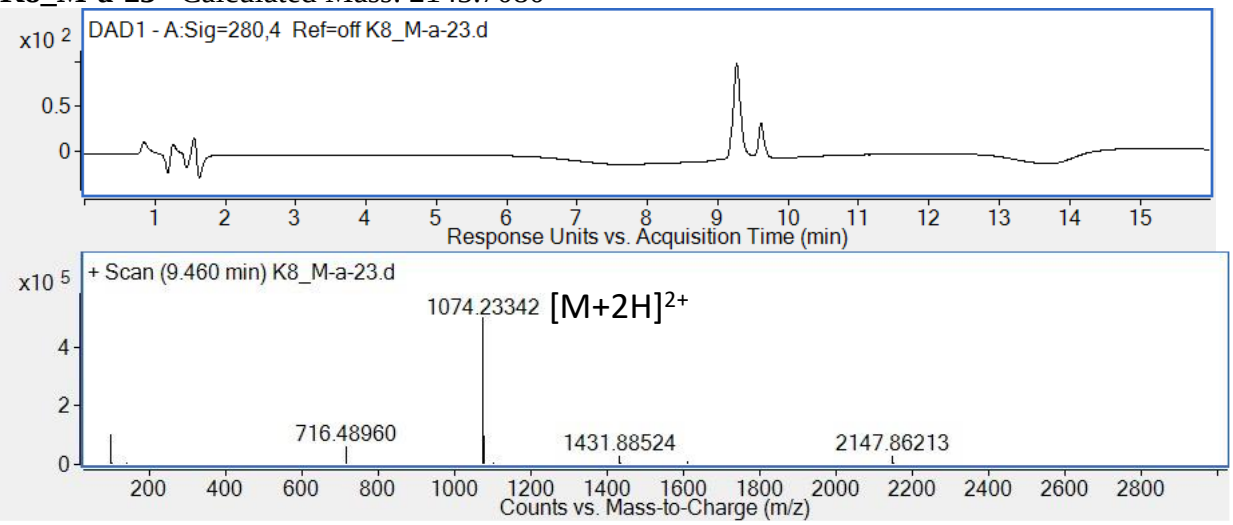
K7_M-a-23* Calculated Mass: 2281.7757



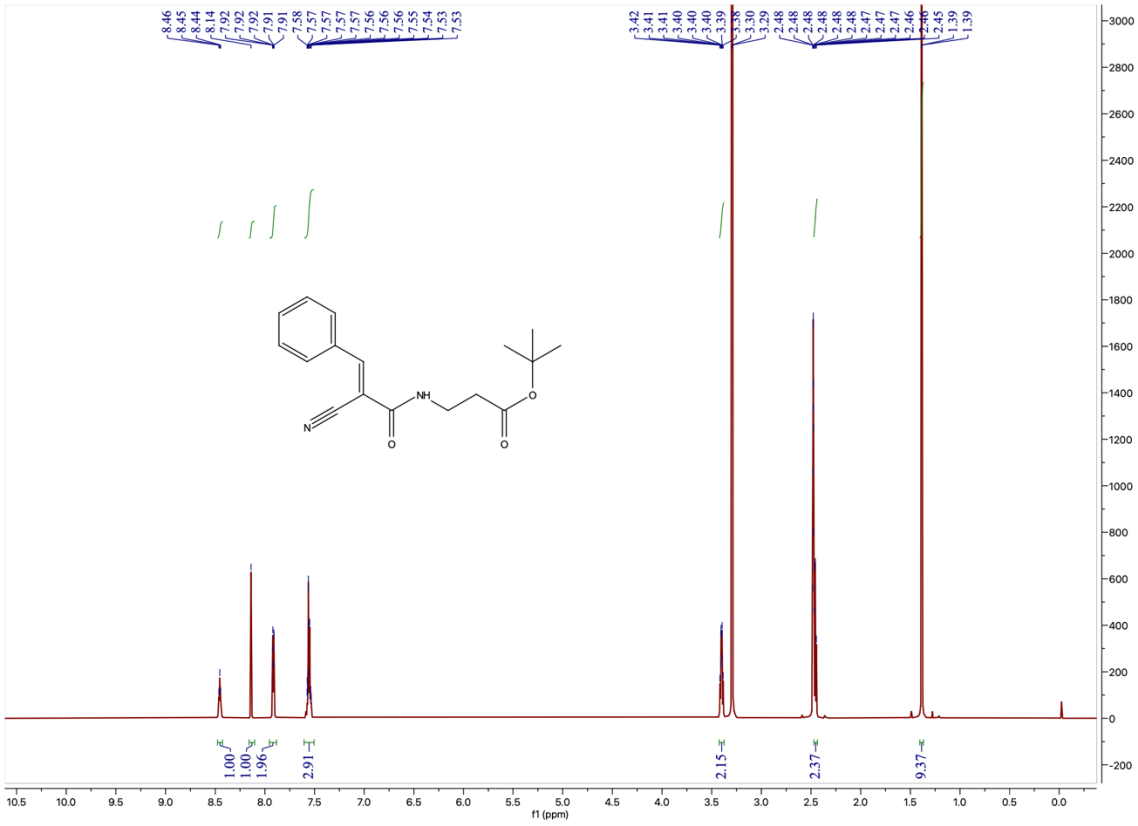
K8_SS* Calculated Mass: 1759.6243

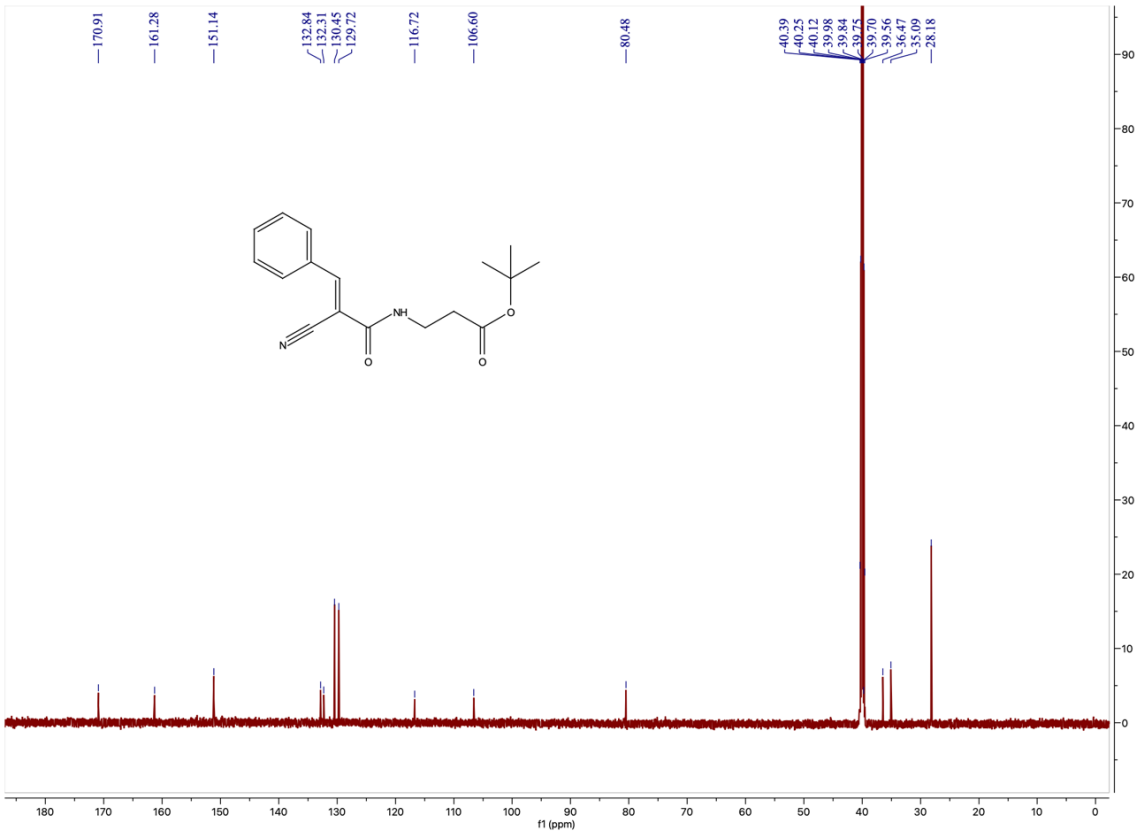


K8_M-a-23* Calculated Mass: 2145.7080



XIII. ^{1}H NMR and ^{13}C NMR Spectra

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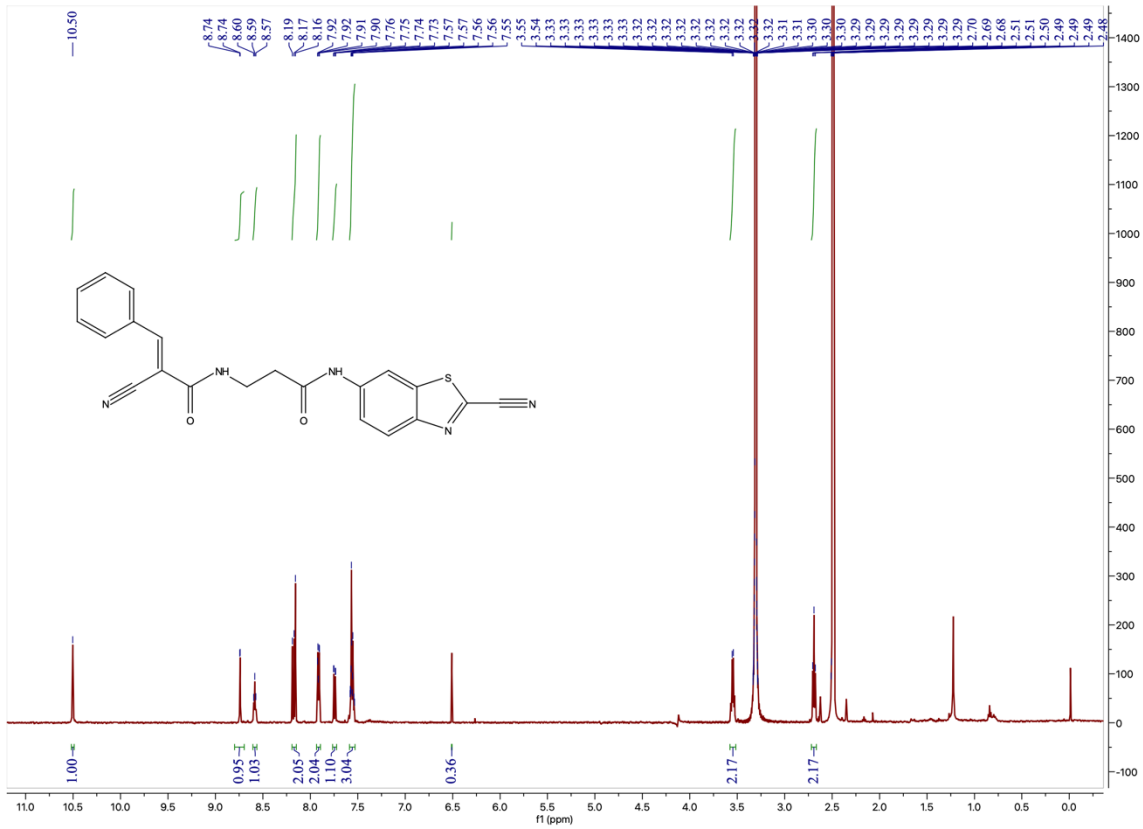
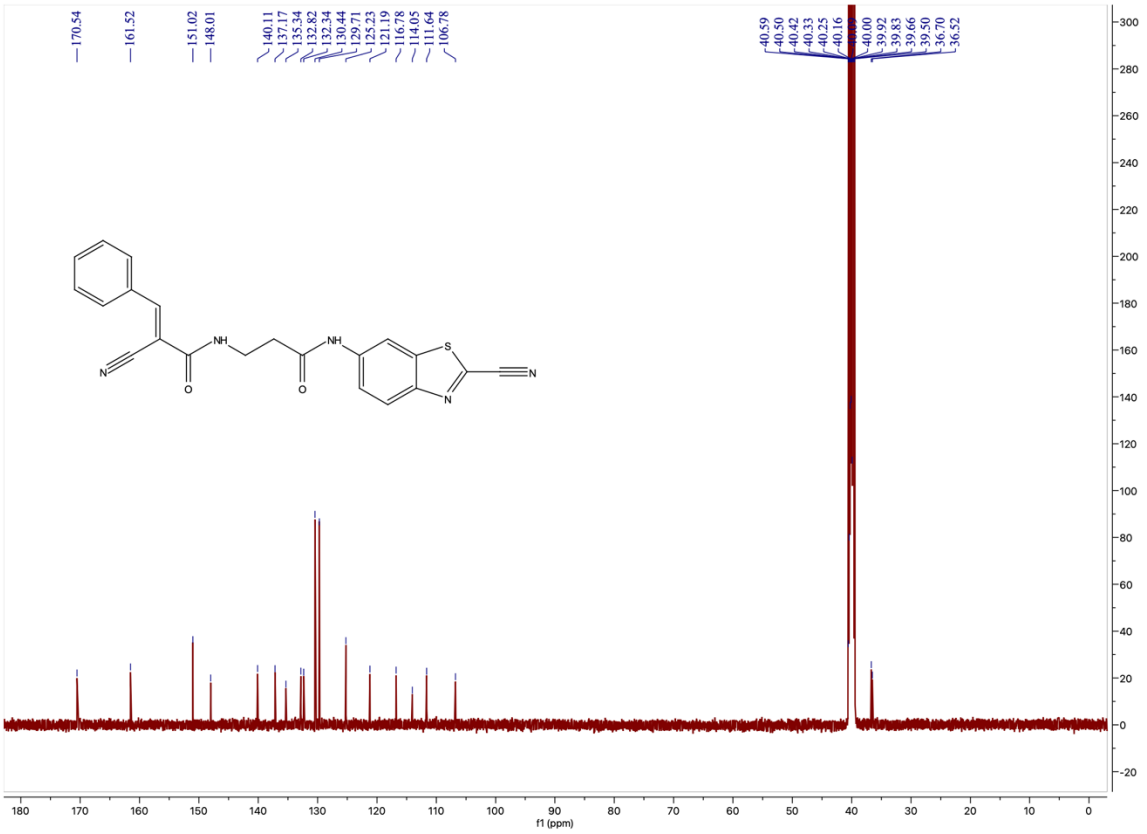
161.28
151.14
132.84
132.31
130.45
129.72
116.72
106.60
80.48
40.39
40.25
40.12
39.98
39.84
39.75
39.70
39.56
36.47
35.09
28.18
NH
N
O
O
O
f1 (ppm)


10.50
8.74
8.74
8.60
8.59
8.57
8.19
8.17
8.16
7.92
7.92
7.91
7.90
7.76
7.75
7.74
7.73
7.57
7.57
7.56
7.56
7.55
3.55
3.54
3.33
3.33
3.33
3.33
3.32
3.32
3.32
3.32
3.32
3.32
3.32
3.32
3.32
3.31
3.31
3.30
3.30
3.30
3.29
3.29
3.29
3.29
3.29
3.29
3.29
3.29
2.70
2.69
2.68
2.51
2.51
2.50
2.49
2.49
2.49
2.48
NH
NH
S
N
N
N
O
O
1.00
0.95
1.03
2.05
2.04
1.10
3.04
0.36
2.17
2.17
f1 (ppm)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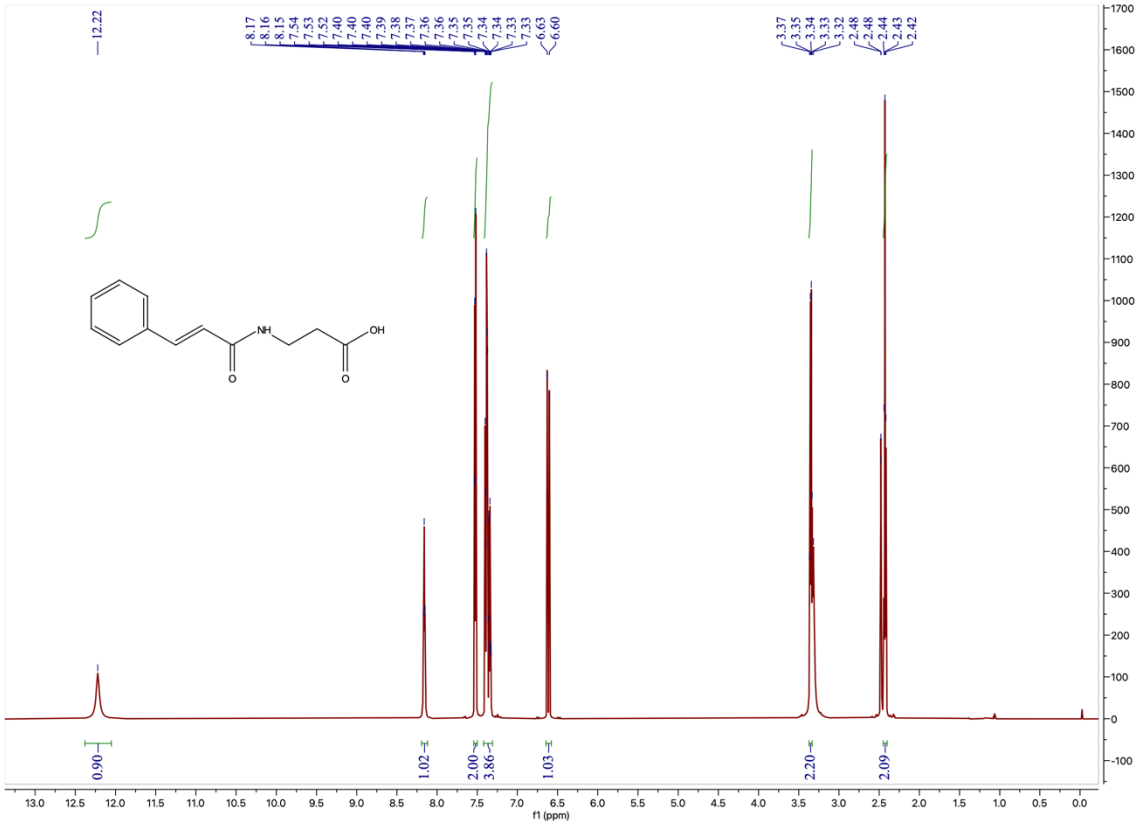
161.52
151.02
148.01
140.11
137.17
135.34
132.82
132.34
130.44
129.71
125.23
121.19
116.78
114.05
111.64
106.78
40.59
40.50
40.42
40.33
40.25
40.16
40.00
39.92
39.83
39.66
39.50
36.70
36.52
NH
NH
O
O
S
N
N
f1 (ppm)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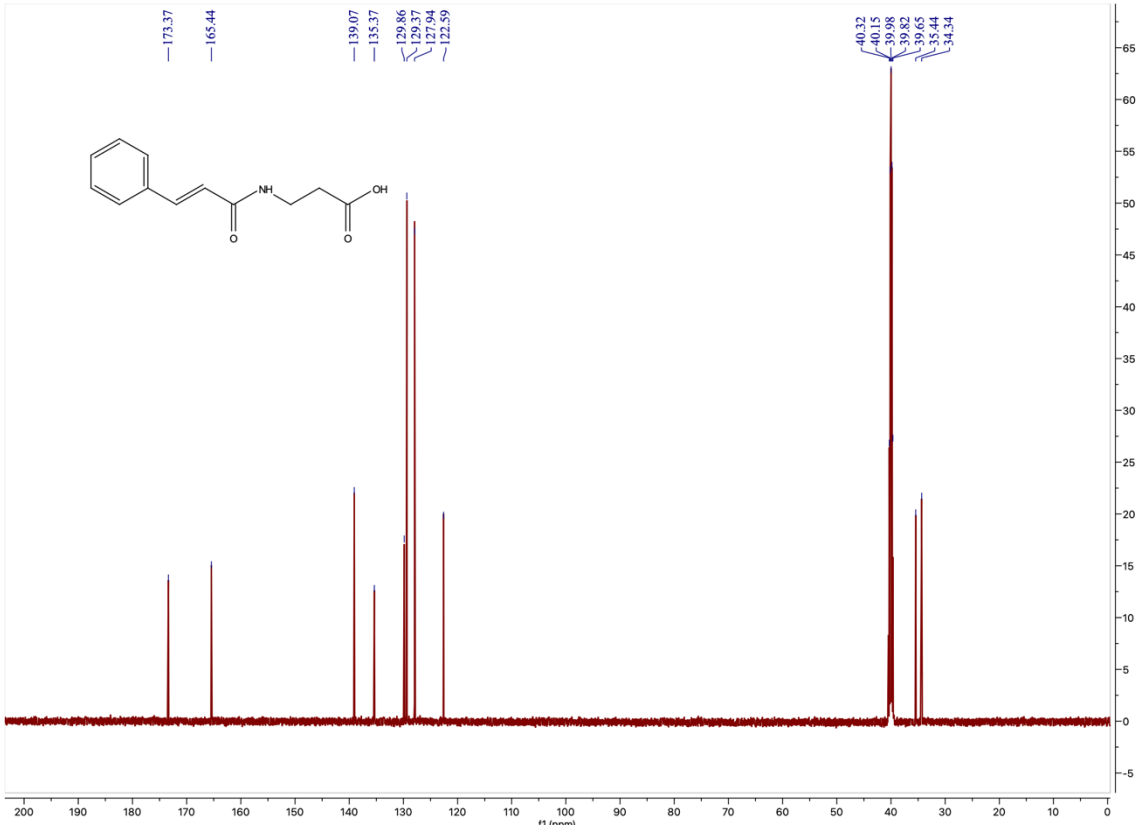
8.16
8.15
7.54
7.53
7.52
7.40
7.40
7.40
7.39
7.38
7.37
7.36
7.36
7.35
7.35
7.34
7.34
7.33
7.33
6.63
6.60
3.37
3.35
3.34
3.33
3.32
2.48
2.48
2.44
2.43
2.42
NH
OH
O
O
0.90
1.02
2.00
3.86
1.03
2.20
2.09
f1 (ppm)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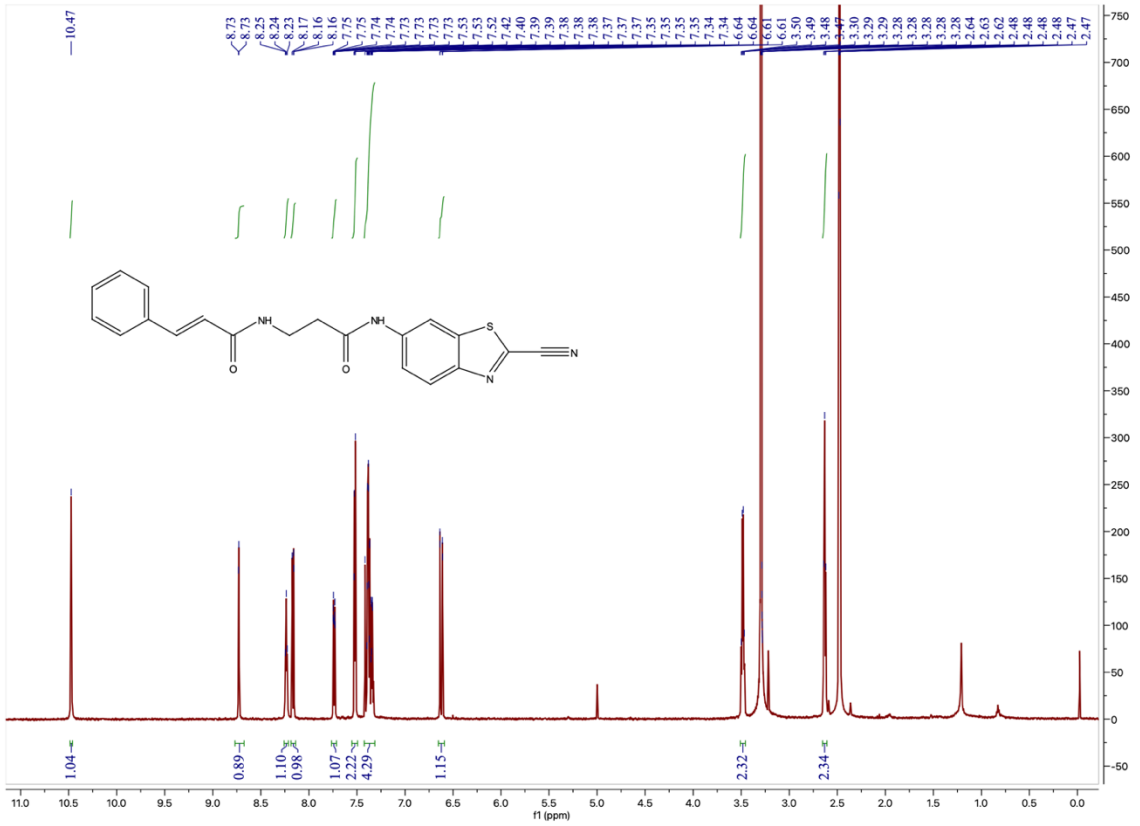
139.07
135.37
129.86
129.37
127.94
122.59
40.32
40.15
39.98
39.82
39.65
35.44
34.34
NH
OH
O
O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8.73
8.25
8.24
8.23
8.17
8.16
7.75
7.74
7.73
7.53
7.52
7.42
7.40
7.39
7.38
7.37
7.35
6.64
6.61
3.50
3.49
3.48
3.30
3.29
3.28
2.64
2.63
2.62
2.48
2.47
NH
NH
S
N
N
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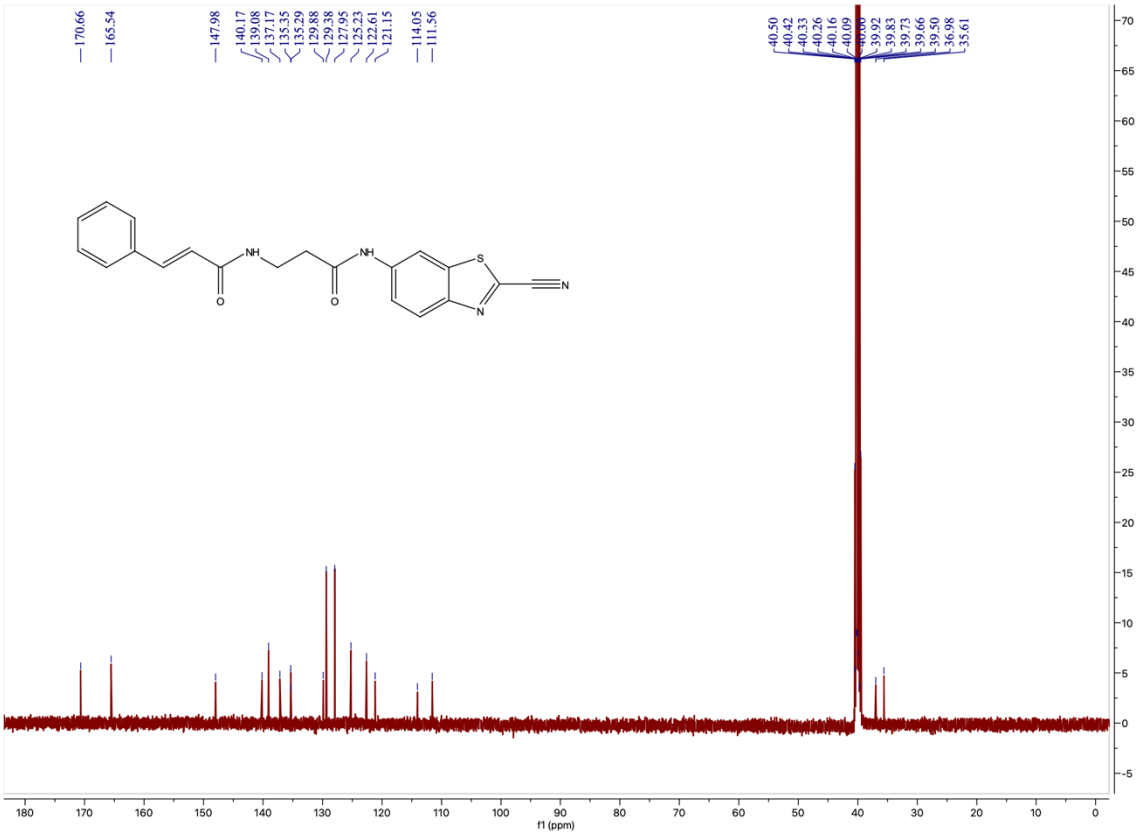
0.89
1.10
0.98
1.07
2.22
4.29
1.15
2.32
2.34
f1 (ppm)


170.66
165.54
147.98
140.17
139.08
137.17
135.35
135.29
129.88
129.38
127.95
125.23
122.61
121.15
114.05
111.56
40.50
40.42
40.33
40.26
40.16
40.09
40.00
39.92
39.83
39.73
39.66
39.50
36.98
35.61
NH
NH
O
O
S
N
N
f1 (ppm)