Supporting information

I. General

All reagents were purchased from commercial suppliers: Sigma Aldrich, TCI, Alfa Aesar and used without further purification. All solvents were reagent or HPLC (Fisher) grade. All reactions were carried under air, unless indicated otherwise. Small molecule reaction progress was monitored by TLC on pre-coated silica plates (Merck, TLC Silica gel 60 F254) and spots were visualized by UV, iodine or other suitable stains. The column chromatography was carried out on gravity columns using 230- 400 or 100-200 mesh silica gel from Merck.

II. Materials

Fmoc-amino acids, Rink amide resin, N,N′-diisopropylcarbodiimide (DIC), 3-[bis(dimethylamino)methyliumyl]-3H-benzotriazol-1-oxide hexafluorophosphate (HBTU), 1-hydroxy-7-azabenzotriazole (HOAt) and N,N-diisopropylethylamine (DIEA) were obtained from CreoSalus (Louisville, Kentucky). Piperidine, trifluoroacetic acid (TFA), and di-tert-butyl dicarbonate (BOC_2O) were obtained from Alfa Aesar (Ward Hill, Massachusetts). N,N-dimethylformamide (DMF), dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), tetrahydrofuran (THF) were obtained from VWR (100 Matsonford Road Radnor, Pennsylvania).

III. Purification. HPLC

Purification was performed using high performance liquid chromatography (HPLC) on an Agilent 1100/1200 series HPLC equipped with a 5 μm particle size, C-18 reversed-phase column. All separations involved a mobile phase of 0.1% formic acid (v/v) in water (solvent A) and 0.1% formic acid (v/v) in acetonitrile (solvent B). The HPLC method employed a linear gradient of 0–80% solvent B over 30 minutes at ambient temperature with a flow rate of 1.0 mL min^{-1}. The separation was monitored by UV absorbance at both 220 nm unless otherwise noted.

IV. Instrumentation and sample analysis.

NMR. ^{1}H and ^{13}C spectra were acquired at 25 °C in DMSO-d_6, $CDCl_3$ using an Agilent DD2 (600 MHz) spectrometer with a 3-mm He triple resonance (HCN) cryoprobe. All ^{1}H NMR chemical shifts (δ) were referenced relative to the residual DMSO-d_6 peak at 2.50 ppm, $CDCl_3$ peak at 7.26 ppm or internal tetramethylsilane (TMS) at 0.00 ppm. ^{13}C NMR chemical shifts were referenced to DMSO-d_6 at 39.52 ppm and $CDCl_3$ at 77.2 ppm. ^{13}C NMR spectra were proton decoupled. NMR spectral data are reported as chemical shift (multiplicity, coupling constants (J), integration). Multiplicity is reported as follows: singlet (s), broad singlet (br s), doublet (d), doublet of doublets (dd), doublet of triplets (td), triplet (t) and multiplet (m). Coupling constant (J) in hertz (Hz).

V. LC/MS.

Mass spectrometry was performed using an Agilent 1100 high performance liquid chromatograph coupled to an Agilent MSD VL mass spectrometer.

HRMS and MS/MS. High resolution MS data were acquired on a Q-ToF mass spectrometer using positive polarity electrospray ionization (+ESI). Tandem MS experiments were performed using collision-induced dissociation (CID) with N_2 as the collision gas.

Analytical HPLC. Peptide purification and compositions were evaluated by analytical HPLC 100 series equipped with a 4.6 mm C-18 reversed-phase column. All separations involved mobile phase of 0.1% formic acid (FA) (v/v) in water (solvent A) and 0.1% FA (v/v) in acetonitrile (solvent B). Analytical HPLC method use a linear gradient of 0-80% 0.1% FA (v/v) acetonitrile in 0.1% aqueous FA over 30 min at room temperature with a flow rate of 1.0 mL min-1. The eluent was monitored by absorbance at 220 nm unless otherwise noted.

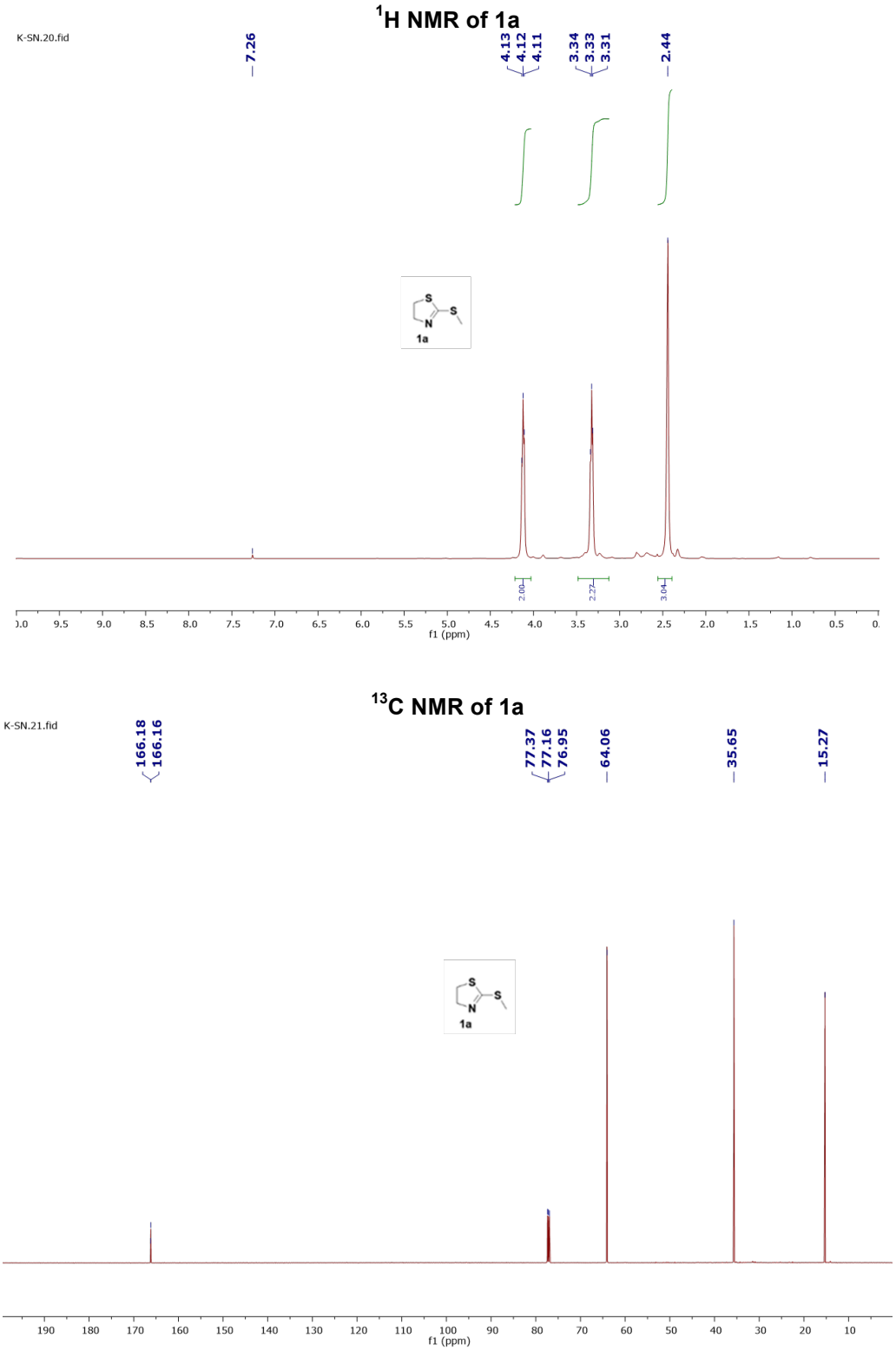
VI. Analytical Methods.

High resolution LC-MS conditions for all purified peptides: Analyses were performed on an ultra-performance LC system (ACQUITY, Waters Corp., USA) coupled with a quadrupole time-of-flight mass spectrometer (Q-ToF Premier, Waters) with electrospray ionization (ESI) in positive mode using Mass lynx software (V4.1). Unless otherwise mentioned a sample was injected either onto a C4 column (Phenomenex Aeris™ 3.6 μm WIDEPORE C4 200 Å, LC Column 50 x 2.1 mm) with beginning gradient- Time- 0 min 10% B; 5 min 28% B; 20 min 38% B; 22 min 90% B; C18 column (ACQUITY UPLC BEH 1.7 μm 1x 50 mm) with a 200 μL/min flow rate of mobile phase of solution A (95% H_2O, 5% acetonitrile and 0.1% formic acid) and solution B (95% acetonitrile, 5% H_2O, and 0.1% formic acid) in ESI positive mode.

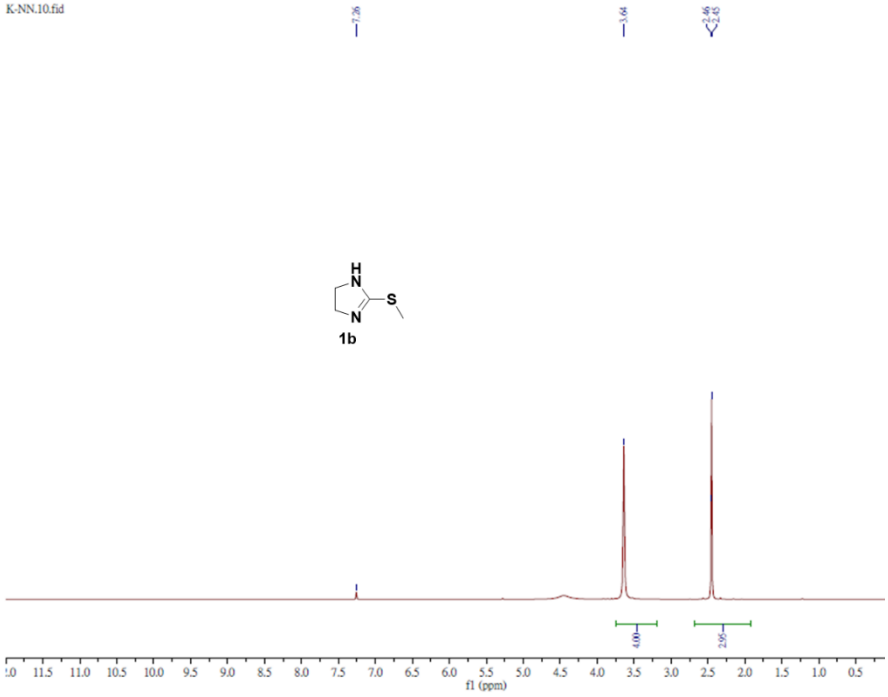
VII. Fmoc Solid-Phase Peptide Synthesis (Fmoc-SPPS).[1]

Peptides were synthesized manually on a 0.25 mm scale using Rink amide resin. Resin was swollen with DCM for 1 h at room temperature. Fmoc group was deprotected using 20% piperidine–DMF for 5 min to obtain a deprotected peptide-resin. First Fmoc-protected amino acid (1.25 mm/5 equiv.) was coupled using HOAt (1.25 mm/5 equiv.) and DIC (1.25 mm/5 equiv.) in DMF for 15 min at room temperature. Fmoc-protected amino acids (0.75 mm/3 equiv.) were sequentially coupled on the resin using HBTU (0.75 mm/3 equiv.) and DIEA (1.5 mm/6 equiv.) in DMF for 5 min at room temperature. Peptides were synthesized using standard protocols. Any Fmoc-protected amino acid added after Fmoc-proline was subjected to the conditions of the first amino acid coupling. Peptides were cleaved from the resin using a cocktail of 95:2.5:2.5, trifluoroacetic acid : triethylsilane : water for 2 h. The resin was removed by filtration and the resulting solution was concentrated. The residue was diluted with ACN/water mixture. The resulting solution was purified by HPLC.

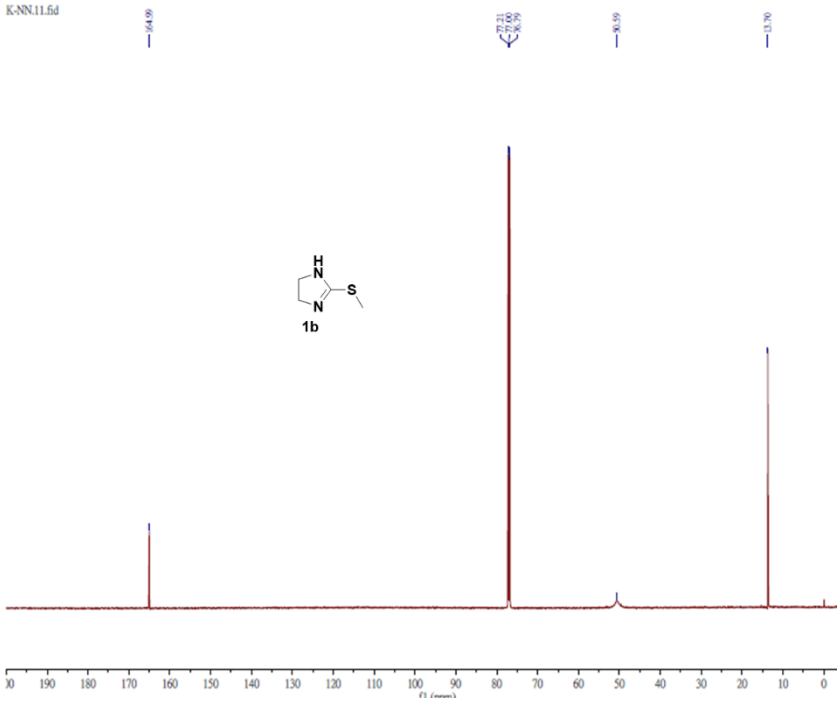
VIII. Supplementary Figure 1: Synthesis of heteroaromatic probes (1a-1o, N_3-1o). Compounds 1a, 1b, 1c and 1i were synthesized by the known methods[2,3]. 1e, 1h, and 1d were purchased form TCI.



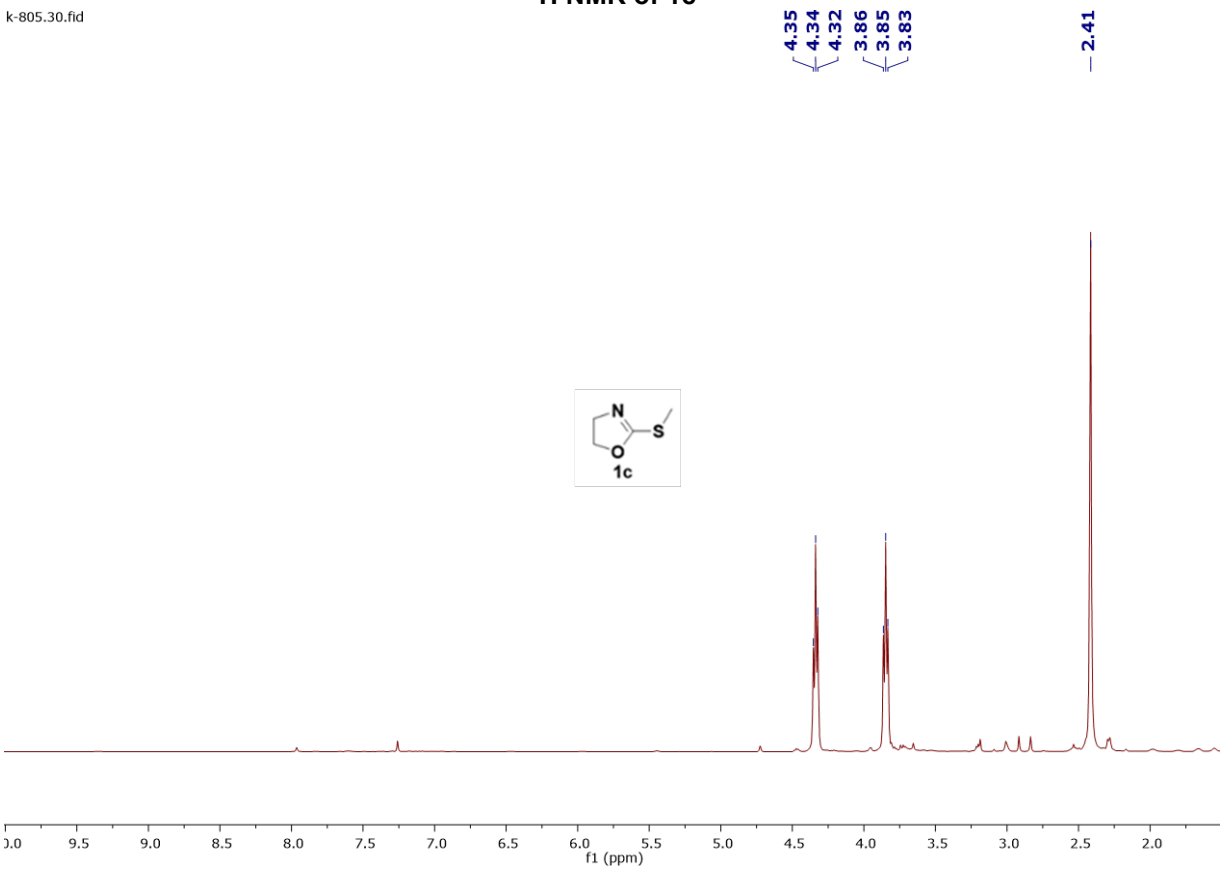
^{1}H NMR of 1b



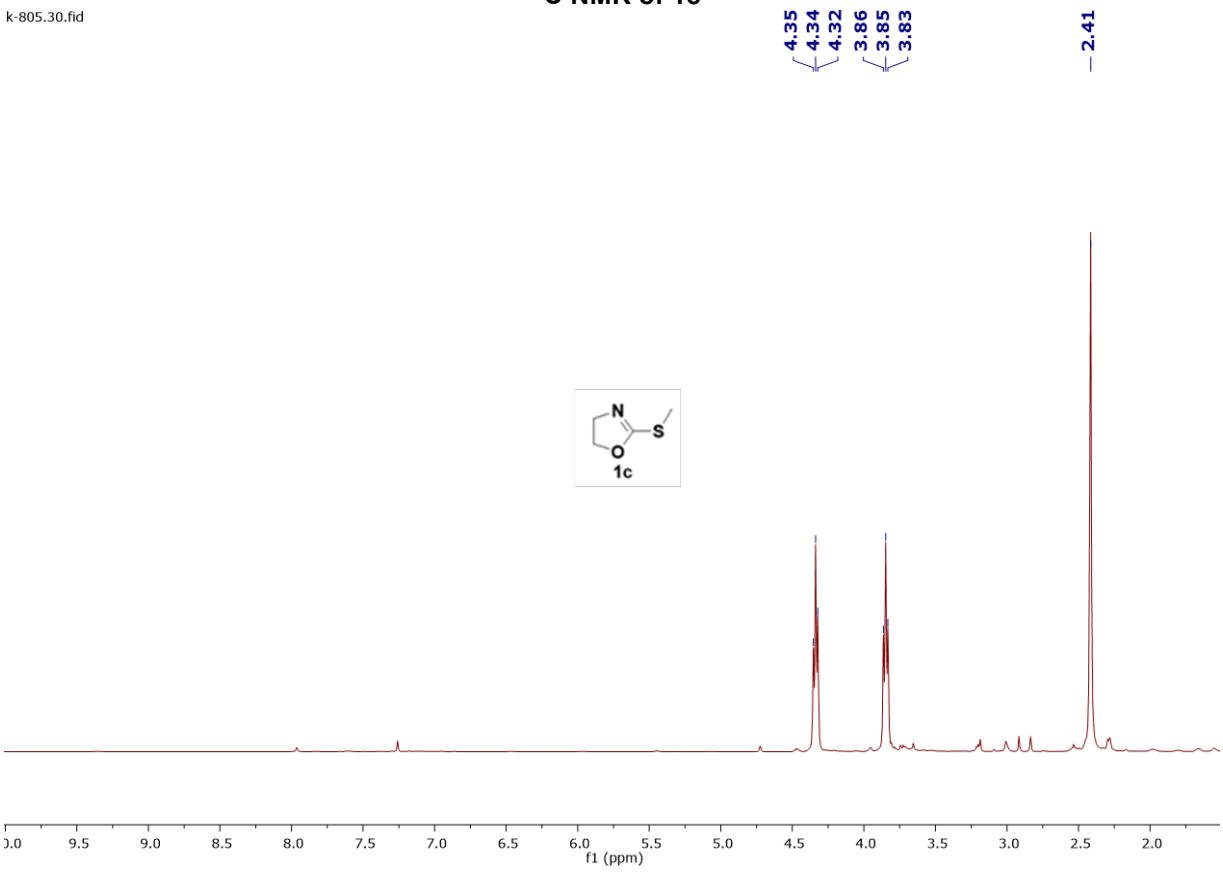
^{13}C NMR of 1b



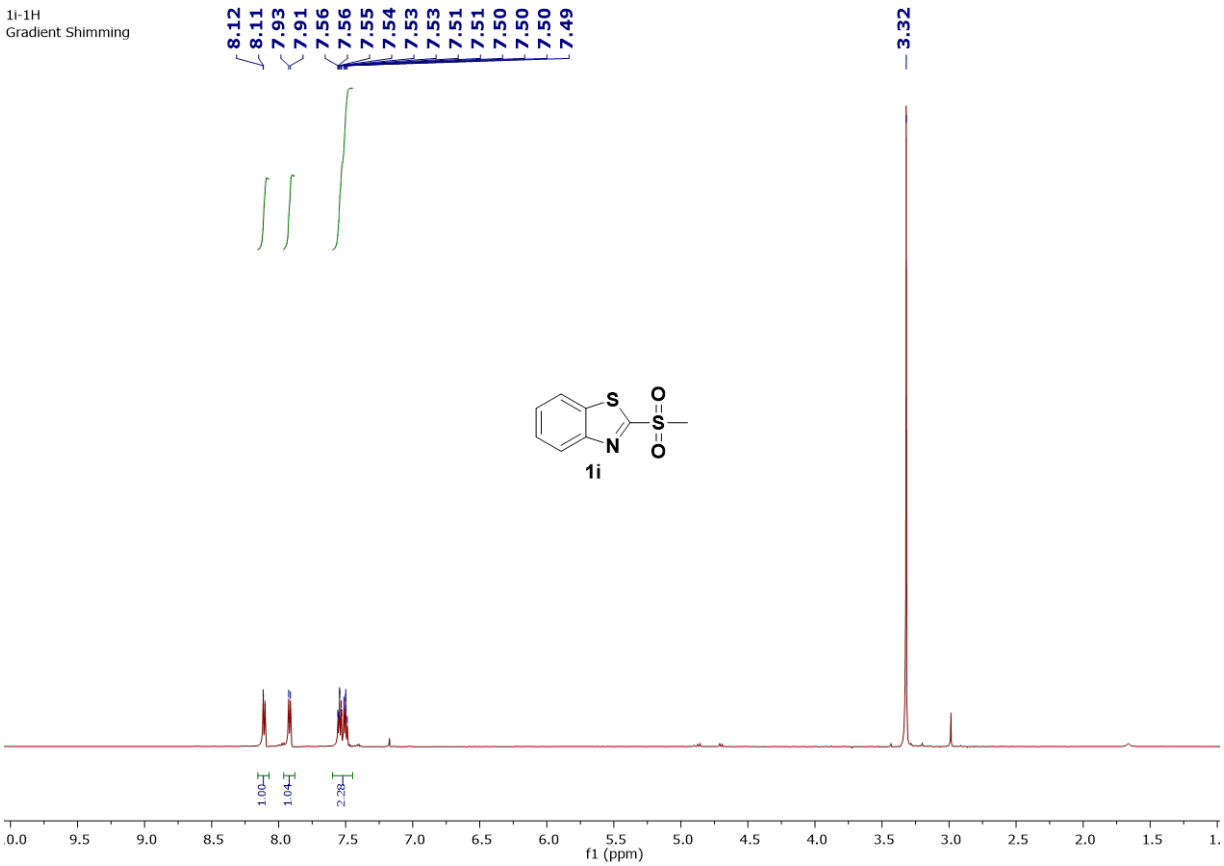
^{1}H NMR of 1c



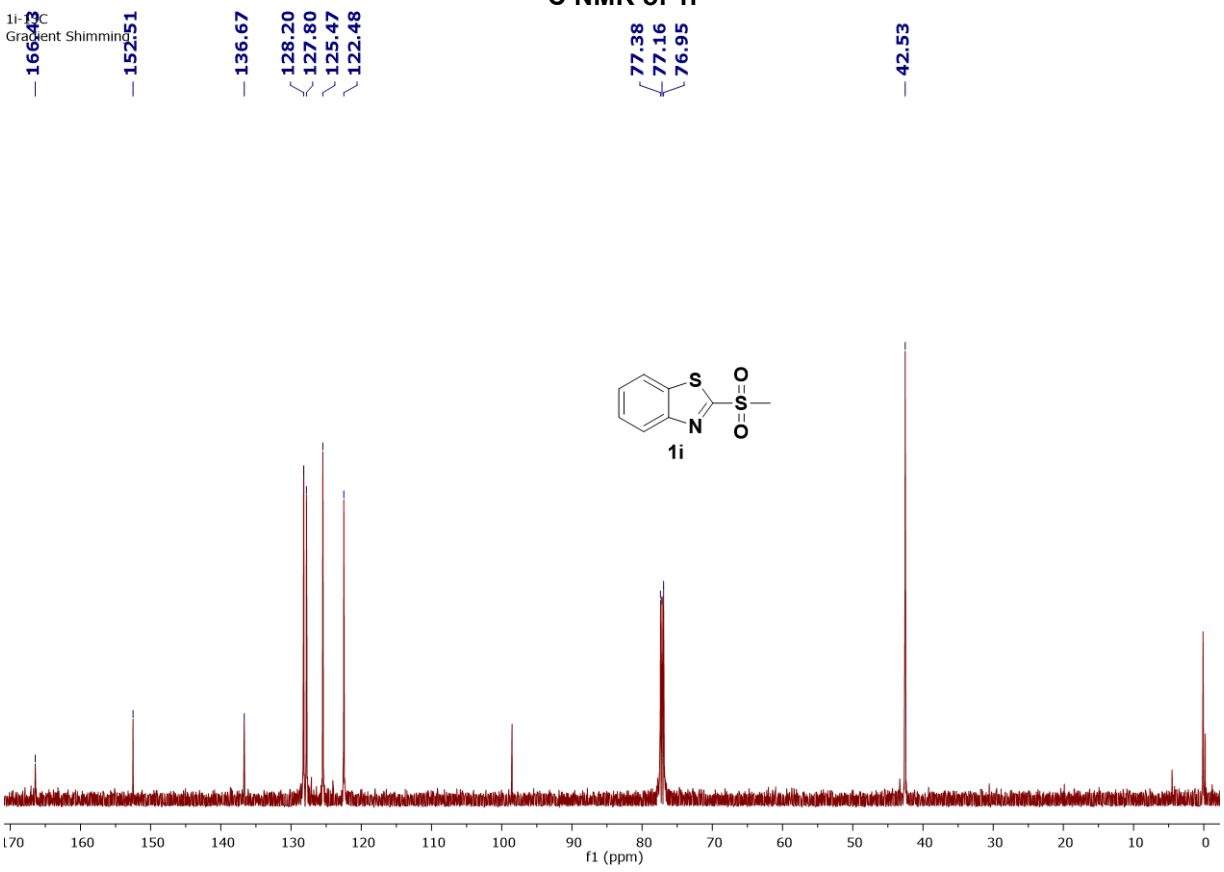
^{13}C NMR of 1c



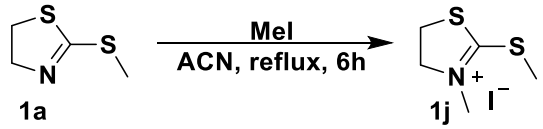
^{1}H NMR of 1i



^{13}C NMR of 1i

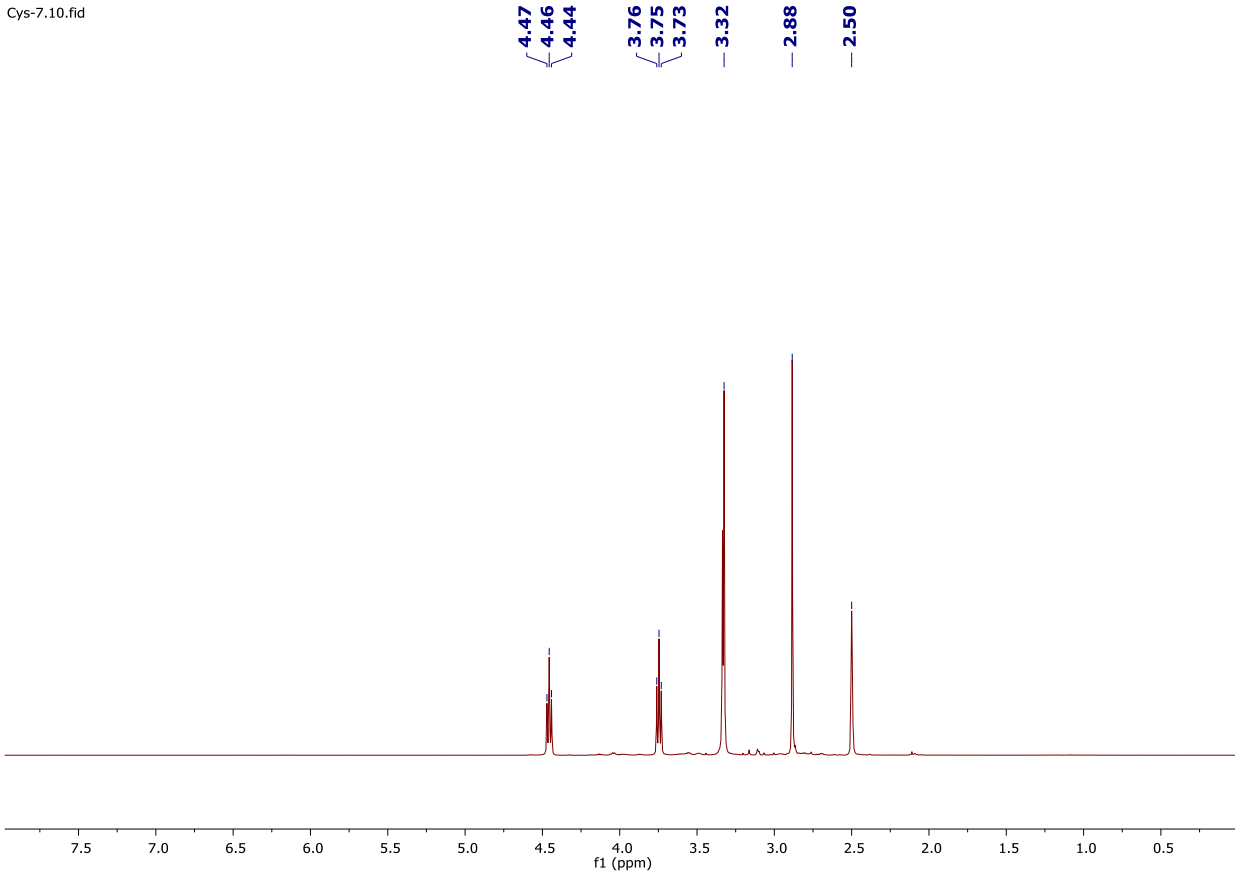


Synthesis of compound 1j.

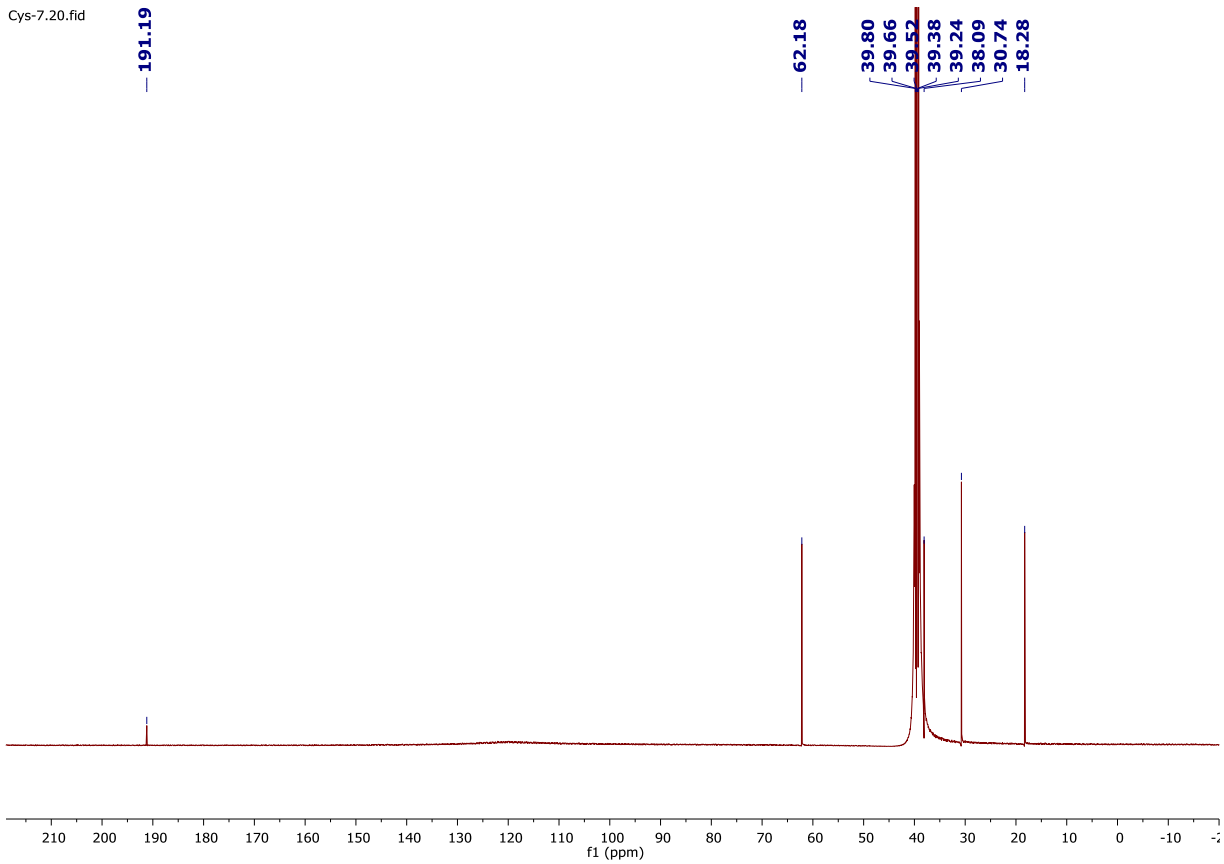


To a solution of 2-(methylthio)-4,5-dihydrothiazole **1a** (300 mg, 2.20 mmol) in ACN (5 mL), methyl iodide (137 µL, 2.20 mmol) was added. The reaction mixture was refluxed for 6 hours. The reaction solution was cooled down to room temperature and concentrated by rotary evaporation to obtain the yellow powder. The crude powder was purified by recrystallization with ethanol / hexane (1:10) to afford pure **1j** as yellow powder (91 %, 295 mg). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 4.47 (t, J = 8.8 Hz, 2H), 3.76 (t, J = 8.8 Hz, 2H), 3.34 (s, 3H), 2.90 (s, 3H). **^{13}C NMR** (150 MHz, DMSO-d_6): δ 191.72, 62.70, 38.62, 31.27, 18.81.

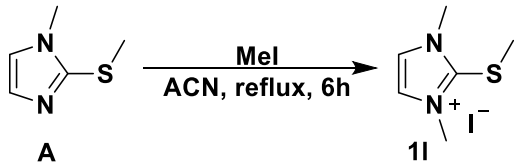
^{1}H NMR of 1j



^{13}C NMR of 1j

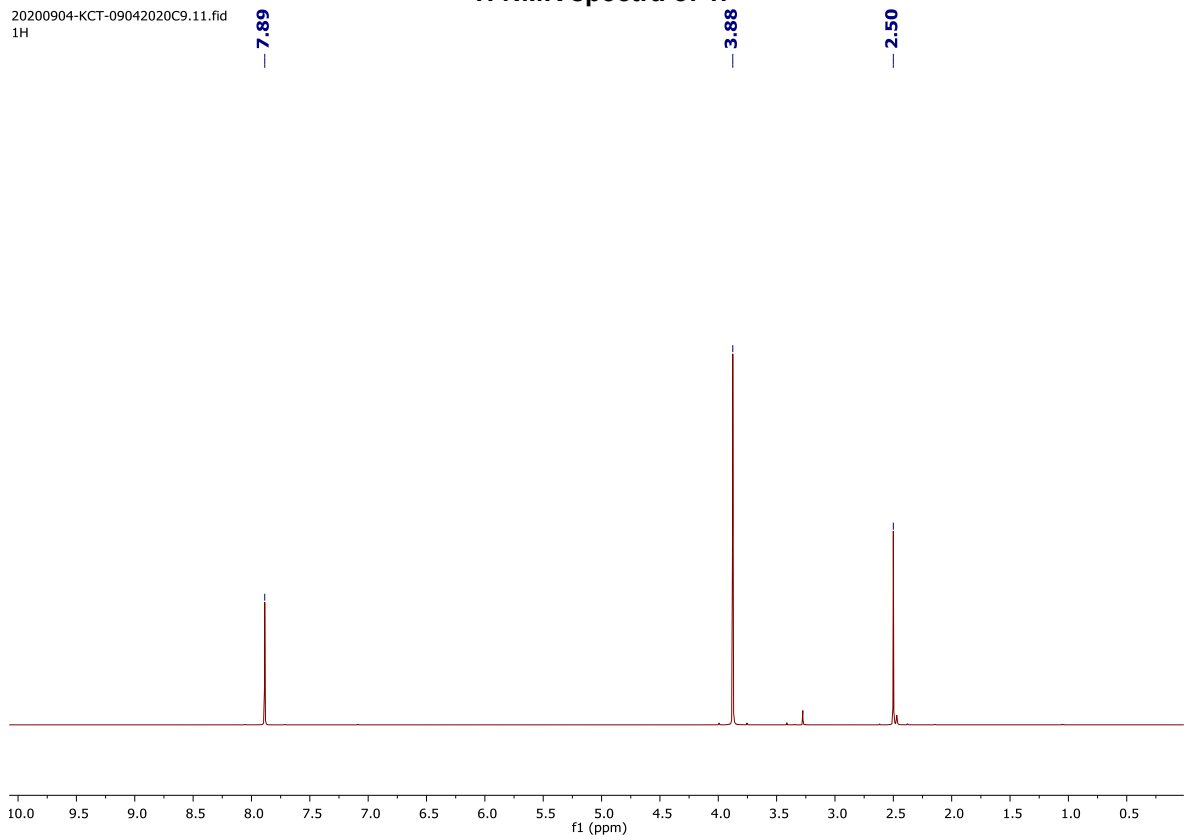


Synthesis of compound 1l

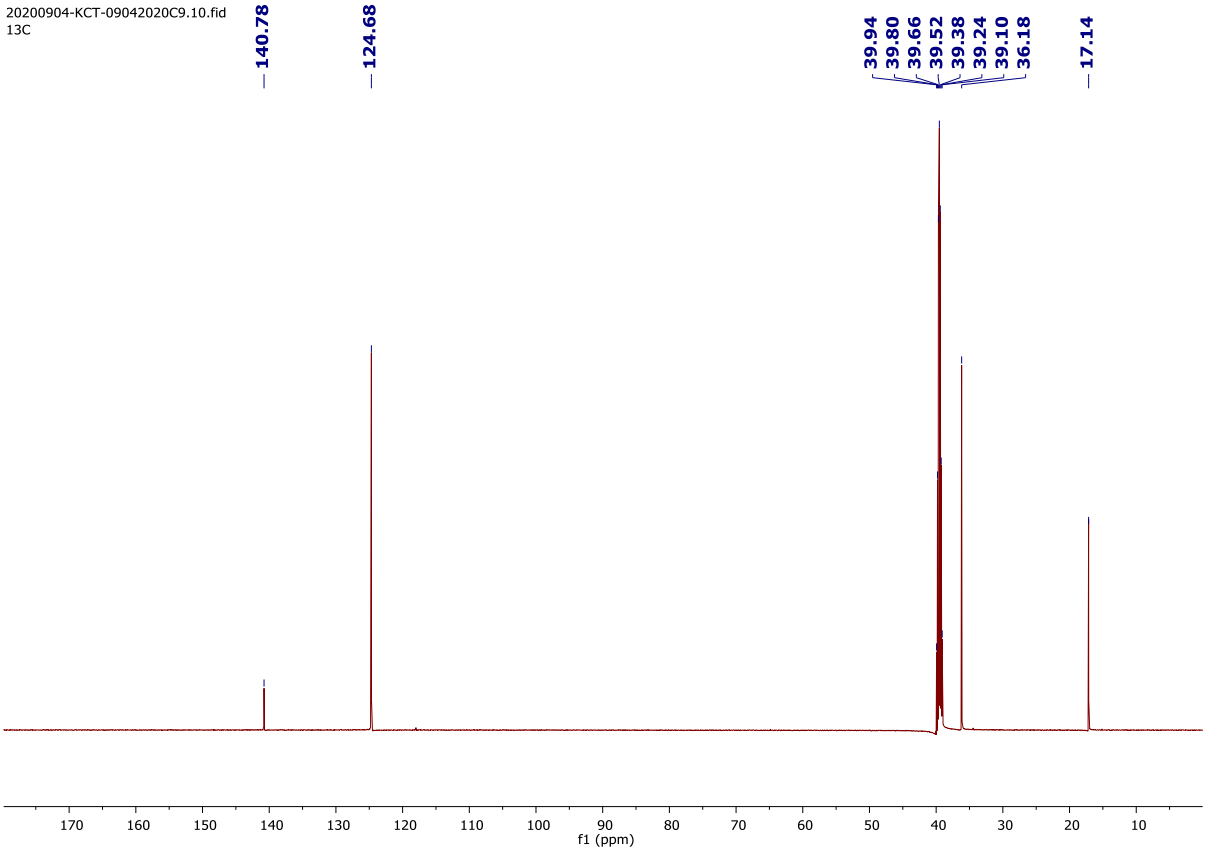


To a solution of 1-Methyl-2-(methylthio)imidazole **A** (200 mg, 1.56 mmol) in dry acetonitrile (5 mL), CH_3I (486 µL, 7.8 mmol) was added. The reaction mixture was refluxed for 6 hours. The reaction solution was cooled to room temperature and concentrated by rotary evaporation to afford the white solid. The crude was purified by recrystallization with ethanol / hexane (1:10) to afford pure **1l** as white powder (95 %, 211.9 mg). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 7.89 (s, 2H), 3.88 (s, 6H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 140.78, 124.68, 36.18, 17.14.

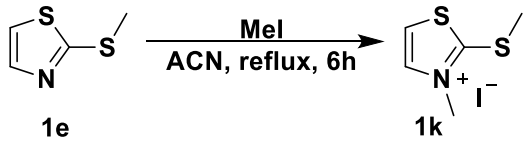
^{1}H NMR spectra of 1l



^{13}C NMR spectra of 1l

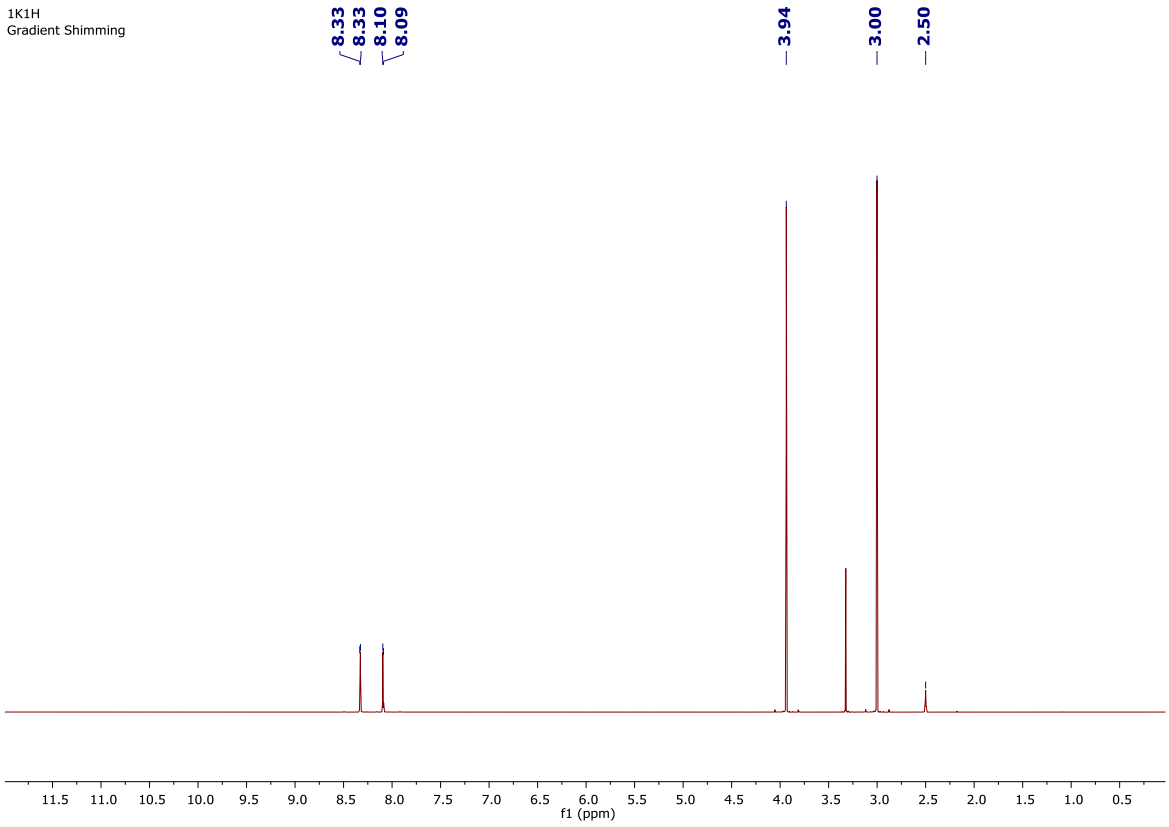


Synthesis of compound 1k

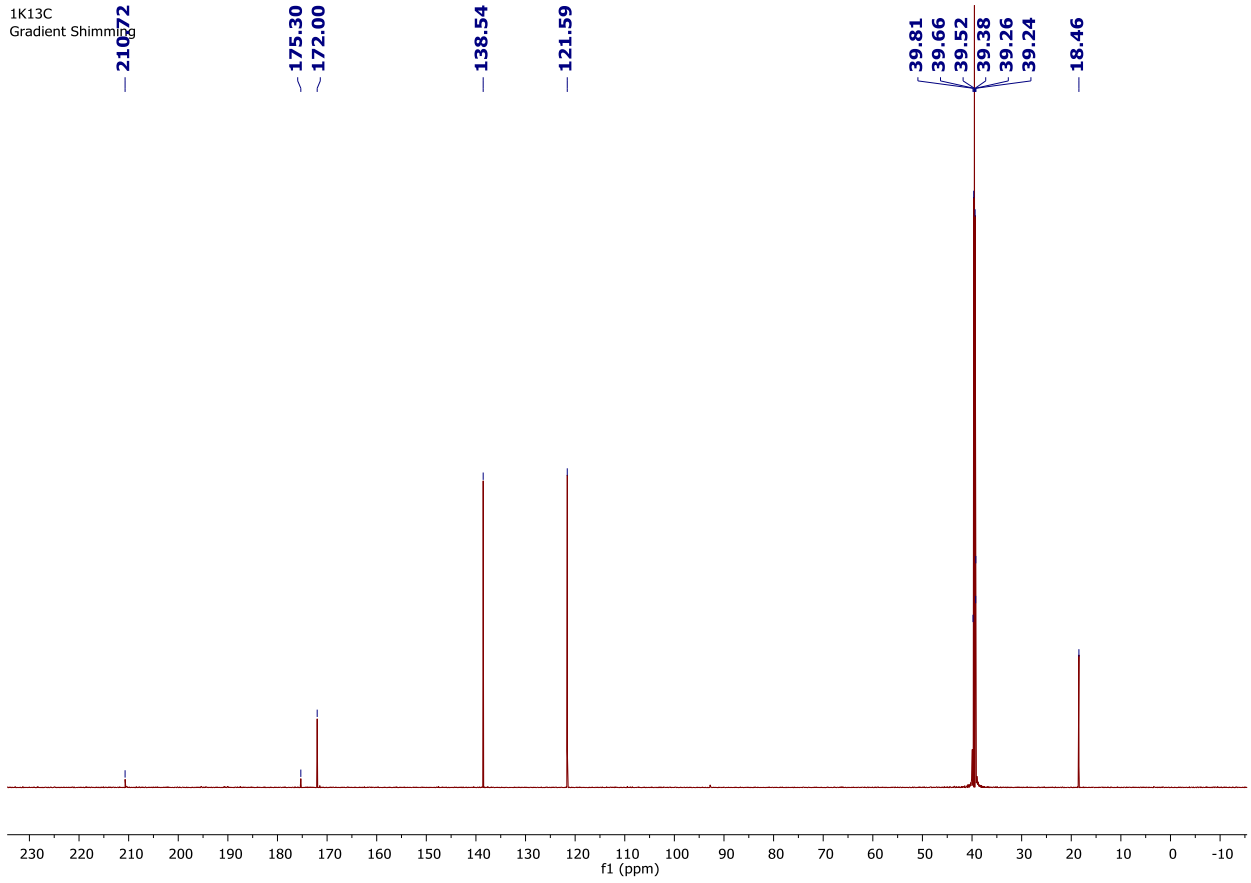


To a solution of 2-(methylthio)thiazole **1e** (203 mg, 1.56 mmol) in dry acetonitrile (5 mL), CH_3I (486 µL, 7.8 mmol) was added. The reaction mixture was refluxed for 6 hours. The reaction solution was cooled to room temperature and concentrated by rotary evaporation to afford the white solid. The crude was purified by recrystallization with ethanol / hexane (1:10) to afford pure **1l** as white powder (91 %, 386 mg). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.34 (d, J = 4.0 Hz, 1H), 8.10 (d, J = 4.0 Hz, 1H), 3.92 (s, 3H), 2.99 (s, 3H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 211.47, 175.09, 172.00, 138.36, 121.86, 18.85.

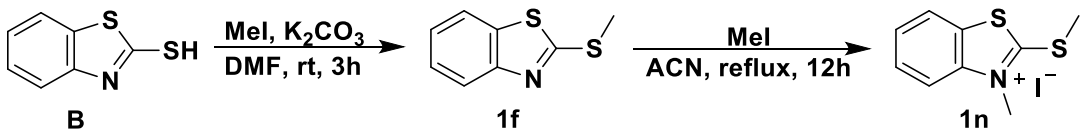
^{1}H NMR spectra of 1k



^{13}C NMR spectra of 1k



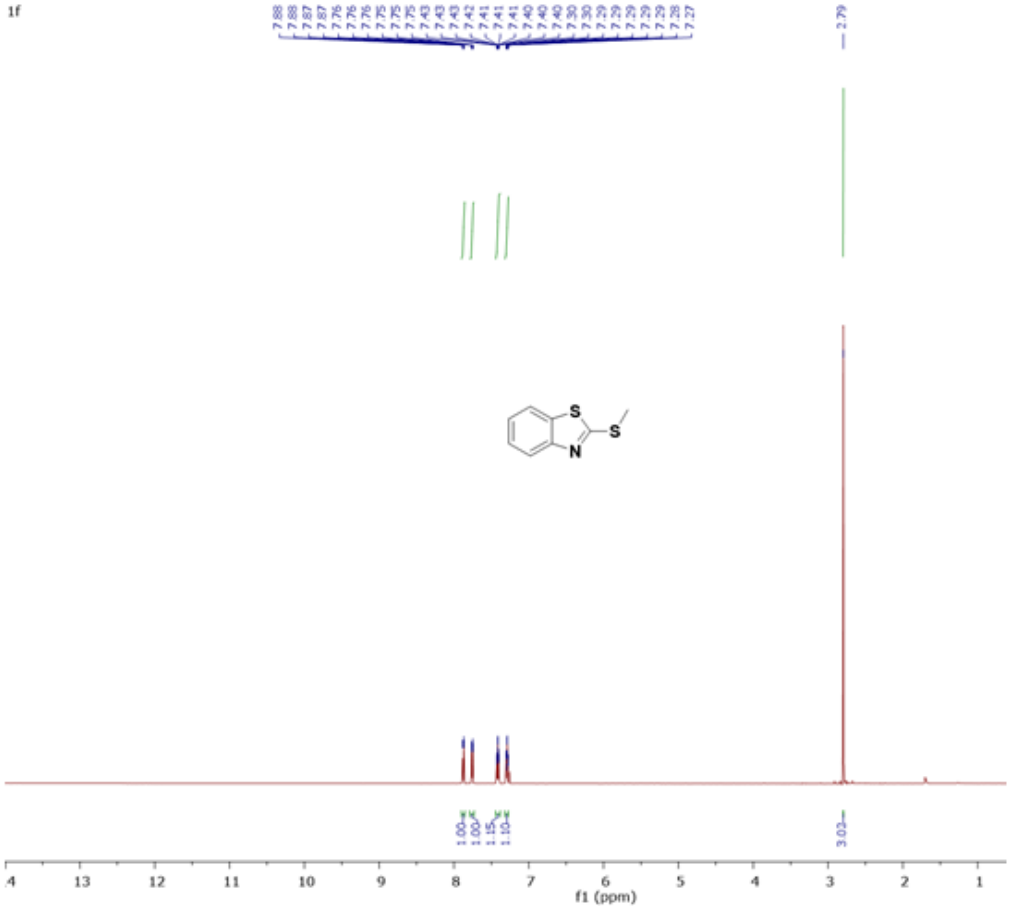
Synthesis of compound 1n



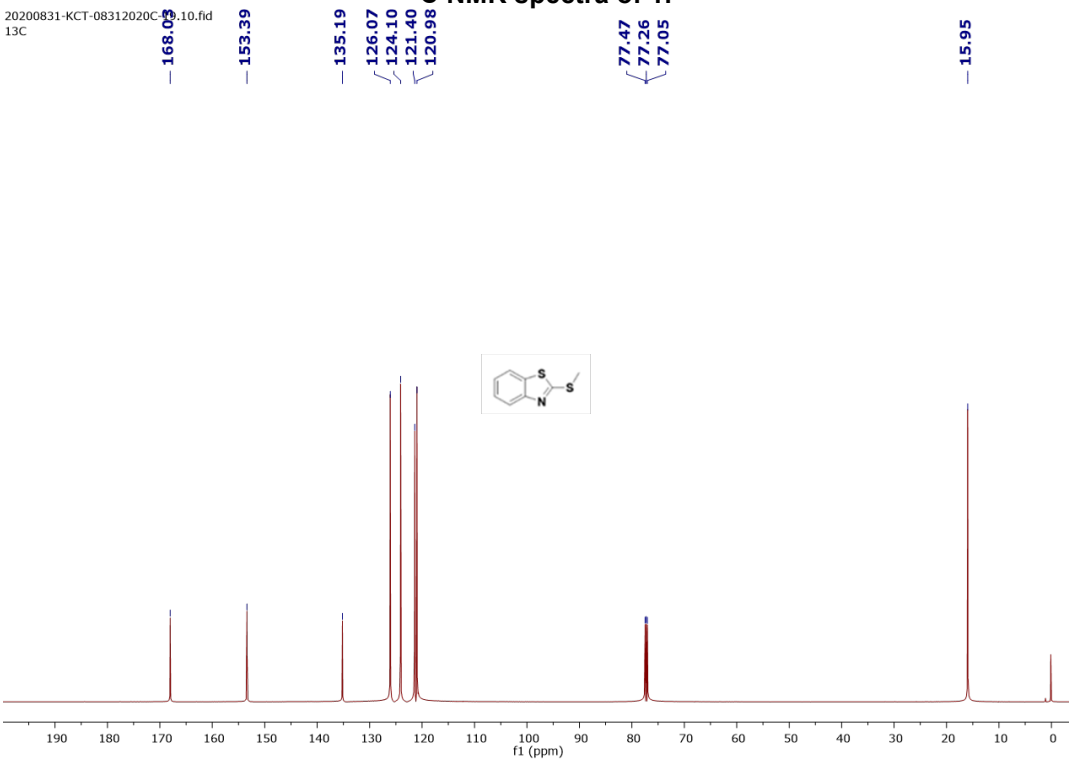
Synthesis of compound 1f

Mercaptobenzothiazole **B** (3 g, 18.0 mmol), potassium carbonate (2.48 g, 18.0 mmol), and methyl iodide (1.1 mL, 18.0 mmol) were added sequential into dry DMF at 0 ^{o}C. The mixture was warmed to room temperature and stirred for 3 hours. The reaction mixture was washed with ethyl acetate and brine for 3 times. The organic layer was collected, dried over anhydrous $MgSO_4$, filtered, and concentrated under the reduced pressure. The residue was purified by the column chromatography (hexane: ethyl acetate 3:1) to yield compound **1f** as colorless crystal (2.77 g, 85 %). **^{1}H NMR** (600 MHz, $CDCl_3$) δ 7.88 (d, *J* = 8 Hz, 1 H), 7.75 (d, *J* = 8 Hz, 1 H), 7.42 (t, *J* = 8 Hz, 1 H), 7.29 (d, *J* = 8 Hz, 1 H), 2.97 (s, 3 H)**. ^{13}C NMR** (151 MHz, $CDCl_3$) δ 167.94, 153.29, 135.09, 125.97, 124.00, 121.30, 120.88, 15.85.

^{1}H NMR spectra of 1f



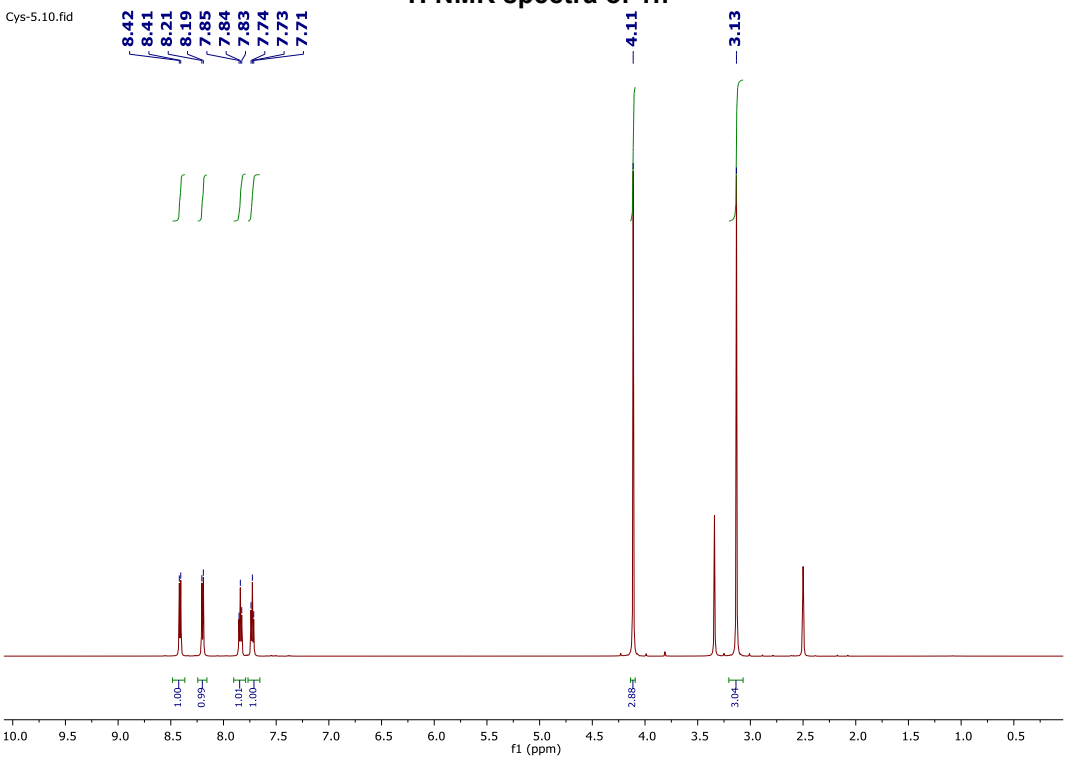
^{13}C NMR spectra of 1f



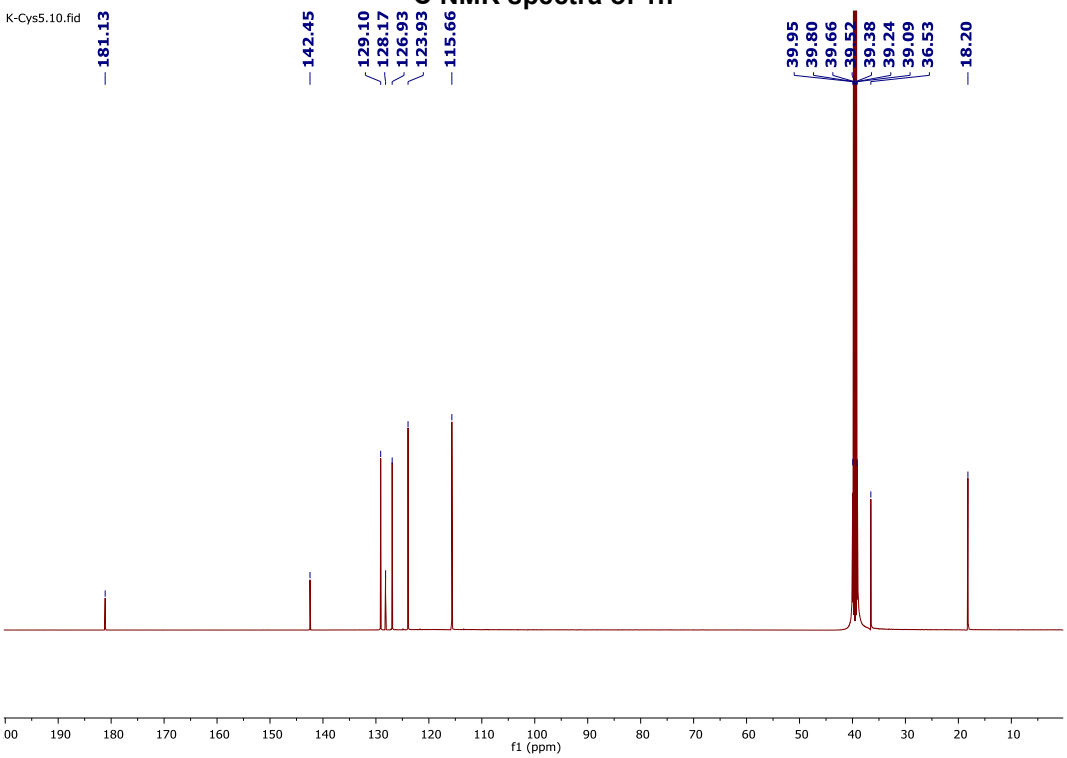
Synthesis of compound 1n

To a mixture of 2-(Methylthio)benzothiazole **1f** (1.5 g, 8.28 mmol) and MeI (2.6 mL, 41.43 mmol) in dry ACN was refluxed for 12 hours. The reaction mixture was cooled to room temperature and solvent was removed by rotary evaporation. The recrystallization was applied in EtOH : Hexane (1:20) to obtained pure compound **1n** as yellow powder (92 %, 1.49 g). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.42 (d, J = 8.1 Hz, 1H), 8.21 (d, J = 8.5 Hz, 1H), 7.88 – 7.82 (m, 1H), 7.74 (t, J = 7.7 Hz, 1H), 4.11 (s, 3H), 3.13 (s, 3H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 181.13, 142.45, 129.10, 128.18, 126.93, 123.94,115.66, 36.53, 18.20.

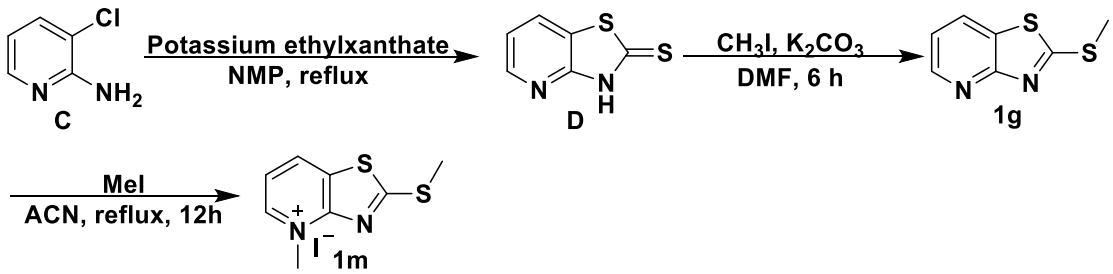
^{1}H NMR spectra of 1n



^{13}C NMR spectra of 1n



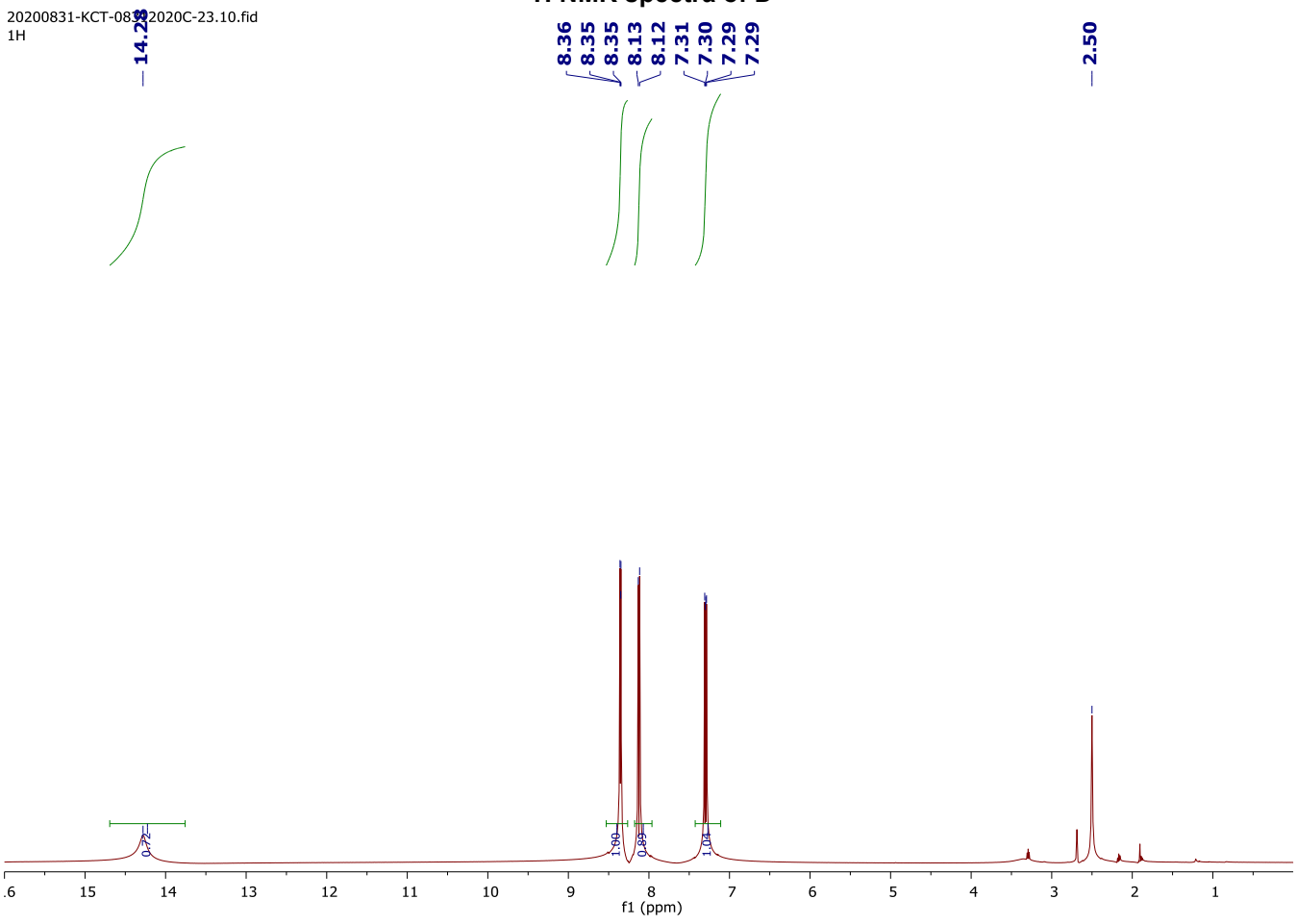
Synthesis of compound 1m



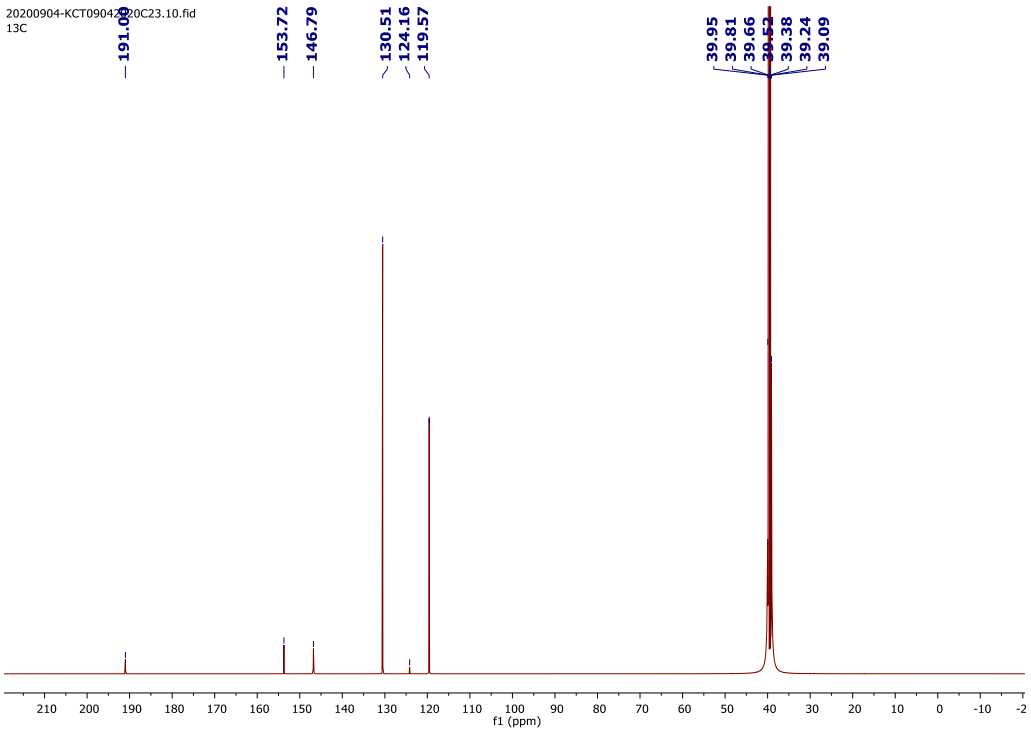
Synthesis of compound D

2-Amino-3-chloro-pyridine **C** (1 g, 7.78 mmol) was dissolved in NMP (15 mL) and potassium ethyl xanthate (1.87 g, 11.6 mmol) was added. The solution was heated to 160 °C for 12 h. The solution was then cooled to RT and treated with glacial acetic acid (5 mL) and diluted with water (100 mL). The resulting precipitate was filtered off and washed with diethyl ether for three times. The off-white precipitate was dried under high vacuum to obtain compound **D** as off-white powder (65 %, 850 mg). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 14.28 (s, 1H), 8.35 (dd, J = 4.9, 1.5 Hz, 1H), 8.12 (dd, J = 7.9, 1.5 Hz, 1H), 7.30 (dd, J = 7.9, 4.9 Hz, 1H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 191.01, 153.73, 146.80, 130.52, 124.17, 119.58, 39.94.

^{1}H NMR spectra of D



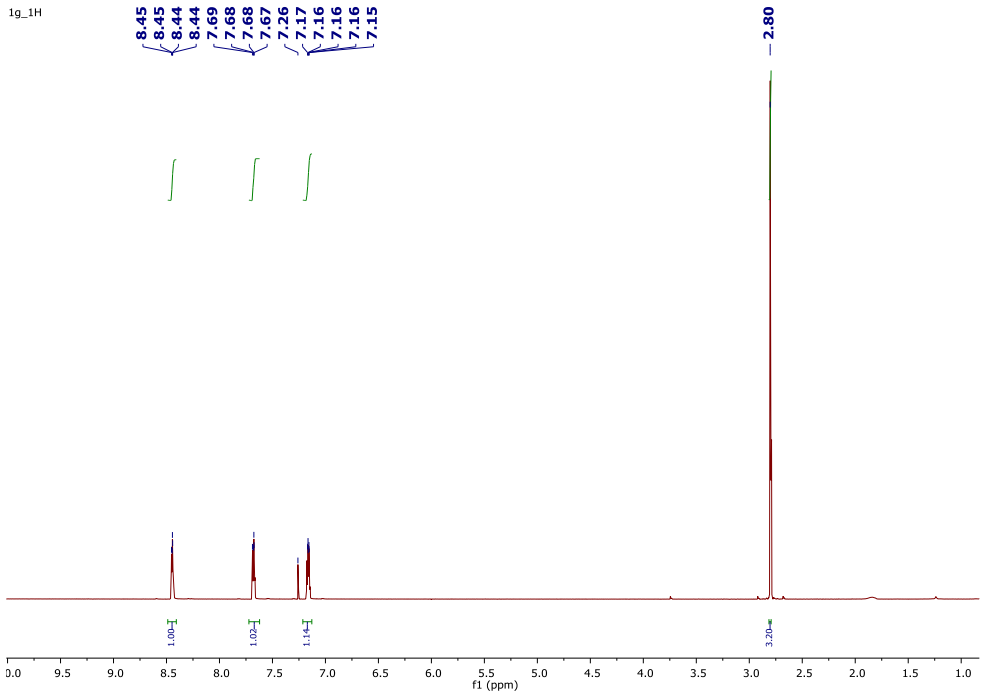
^{13}C NMR spectra of D



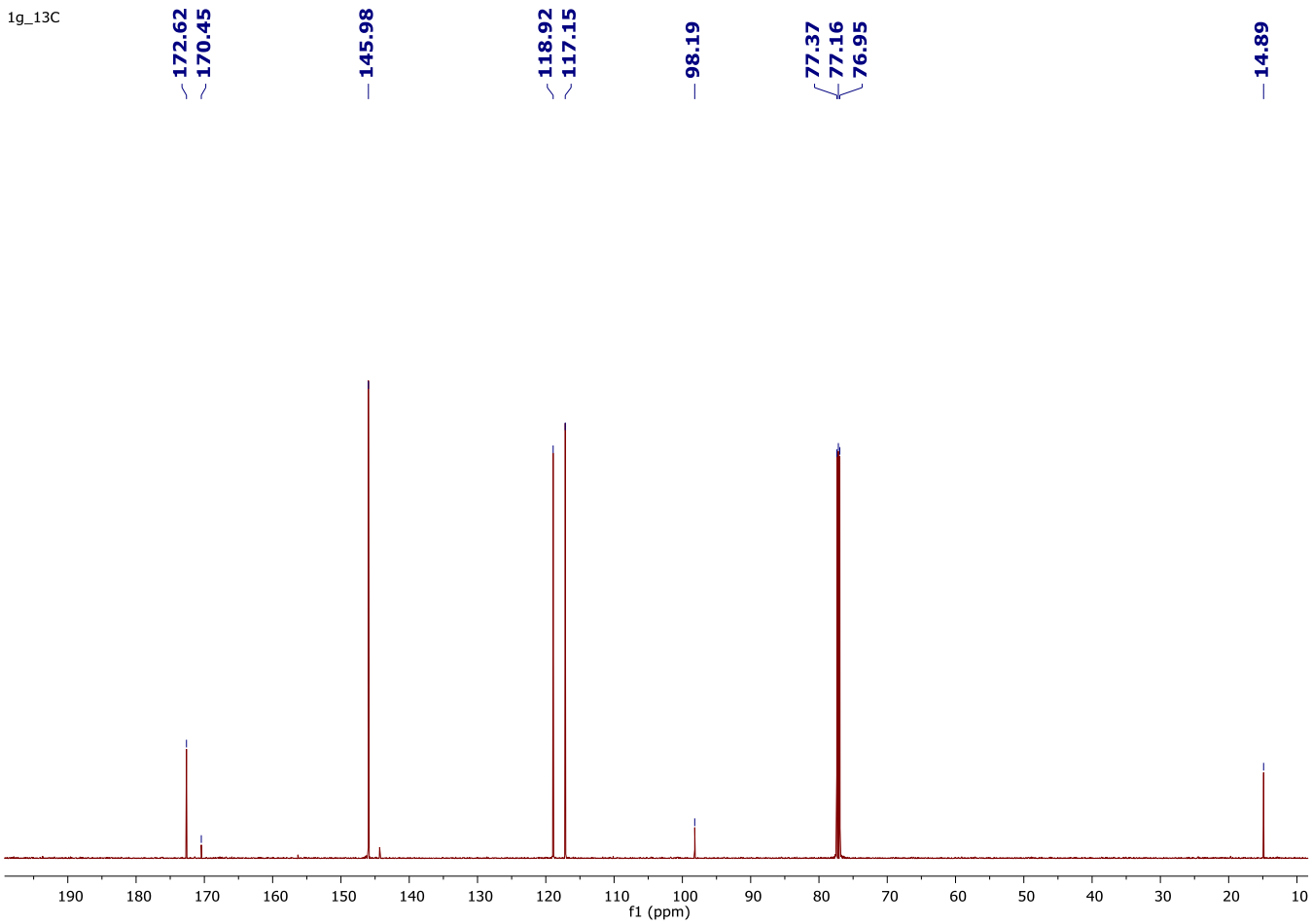
Synthesis of compound 1g

To solution of thiazolo[4,5-b]pyridine-2(3H)-thione **D** (400 mg, 2.38 mmol) in dry DMF (10 mL) was added K_2CO_3 (328 mg, 2.38 mmol), MeI (149 µL, 2.38 mmol) sequentially at 0 ^{o}C, then the reaction was warmed to room temperature and stirred for 6 h. The reaction mixture was washed with ethyl acetate and brine for three times. The organic layer was collected and dried over anhydrous $MgSO_4$, filtered, and concentrated. The crude was purified by flash chromatography (hexane: ethyl acetate 2:1) to afford compound **1g** as colorless crystal (73 %, 312 mg). **^{1}H NMR** (600 MHz, $CDCl_3$) δ 8.58 (dd, J = 4.7, 1.7 Hz, 1H), 8.07 (dd, J = 7.9, 1.7 Hz, 1H), 7.19 (dd, J = 7.9, 4.7 Hz, 1H), 2.84 (s, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 172.62, 170.45, 145.98, 118.92, 117.15, 98.19, 77.37, 77.16, 76.95, 14.89.

^{1}H NMR spectra of 1 g



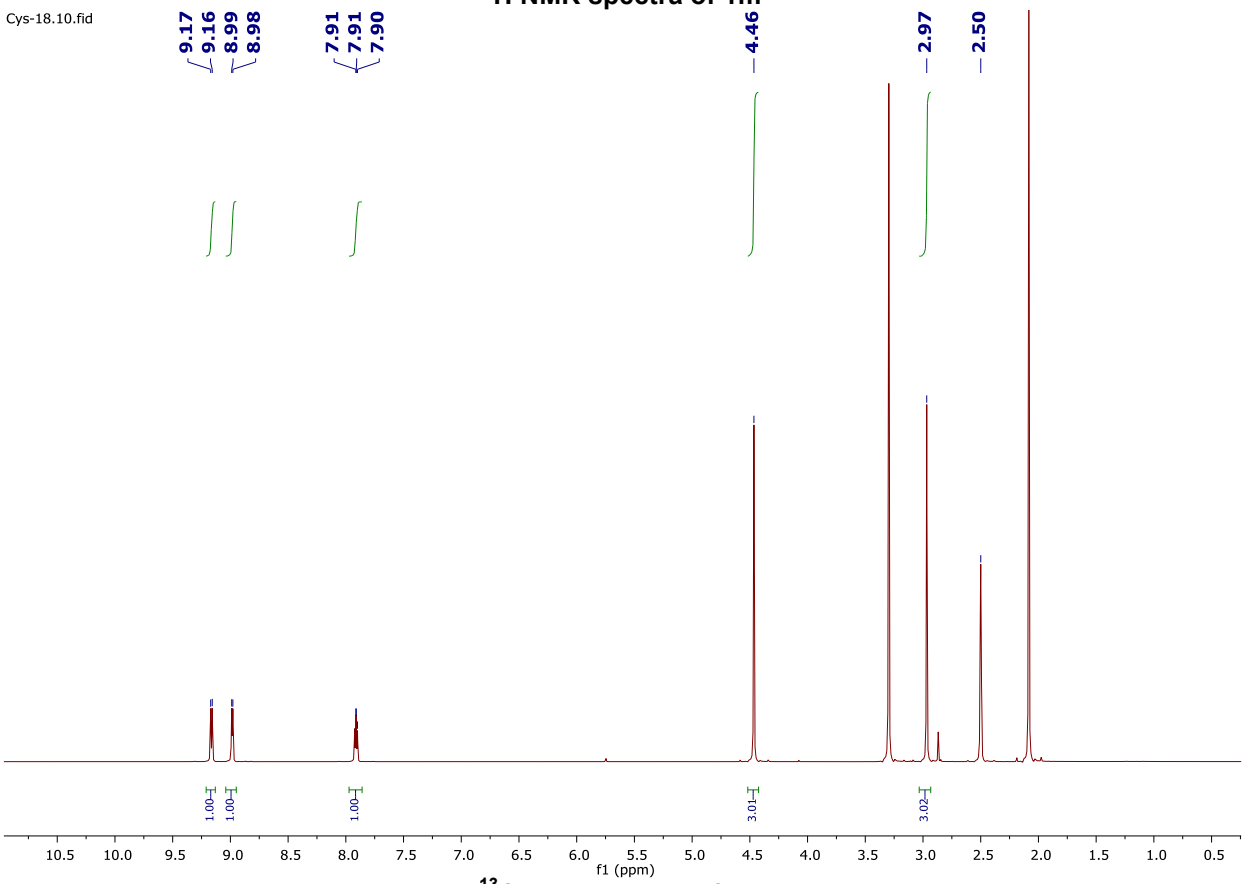
^{13}C NMR spectra of 1 g



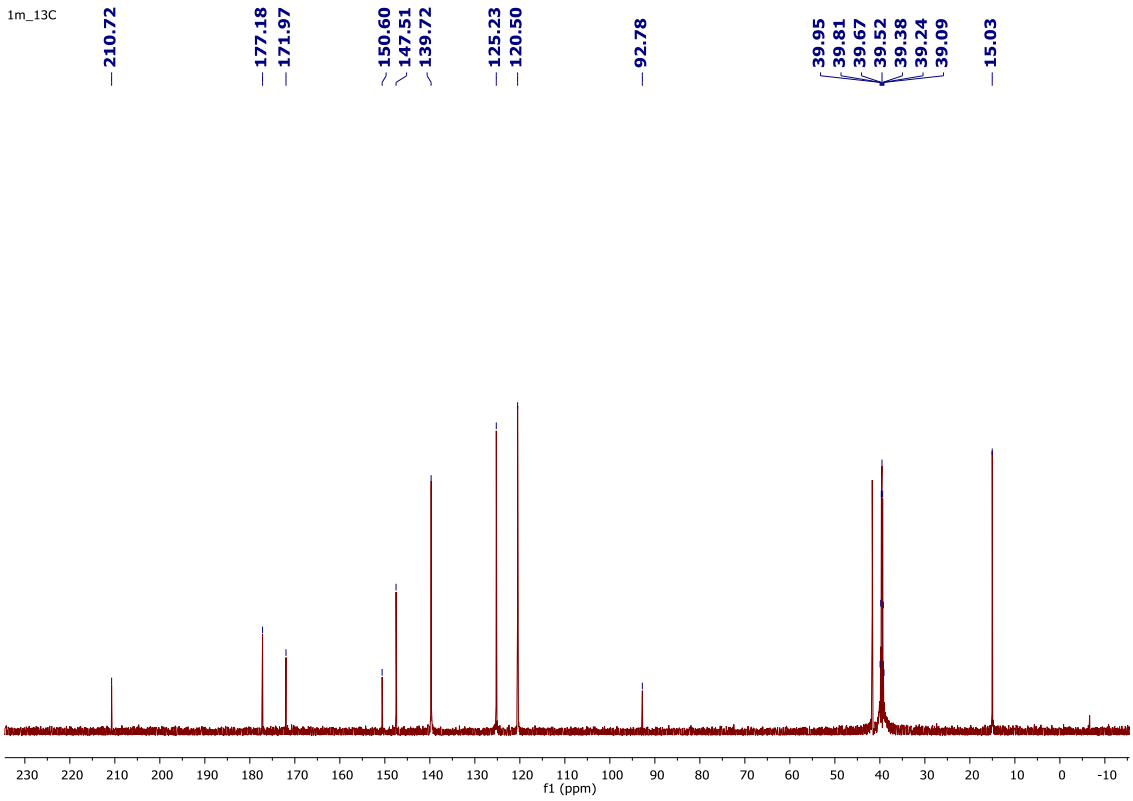
Synthesis of compound 1m

2-(Methylthio)thiazolo[4,5-b]pyridine **1g** (150 mg, 0.82 mmol) was dissolved in dry ACN (10 mL), and treated with methyl iodide (258 µL, 4.12 mmol). The reaction mixture was refluxed for 12 h. The mixture was cooled to room temperature and ACN was removed by rotary evaporation. The crude was purified by recrystallization with methanol / hexane (1:20) to obtain compound **1m** as bright yellow powder (91 %, 242 mg). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 9.18 (d, J = 8.1 Hz, 1H), 9.00 (d, J = 6.1 Hz, 1H), 7.92 (d, J = 1.9 Hz, 1H), 4.48 (s, 3H), 2.98 (s, 3H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 210.72, 177.17, 171.96, 150.60, 147.50, 139.71, 125.23, 120.49, 92.78, 41.69, 15.02.

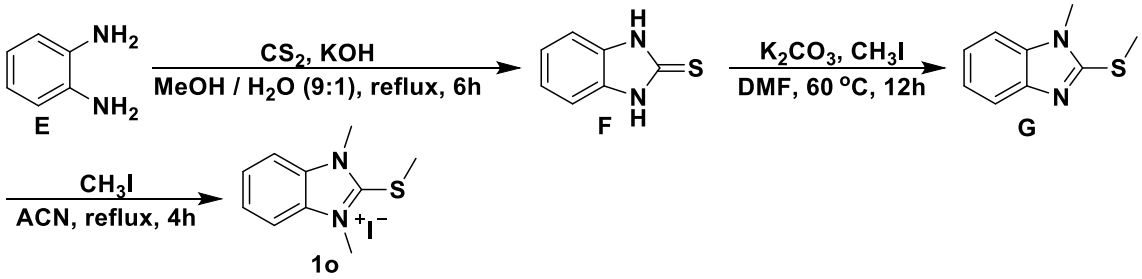
^{1}H NMR spectra of 1m



^{13}C NMR spectra of 1m



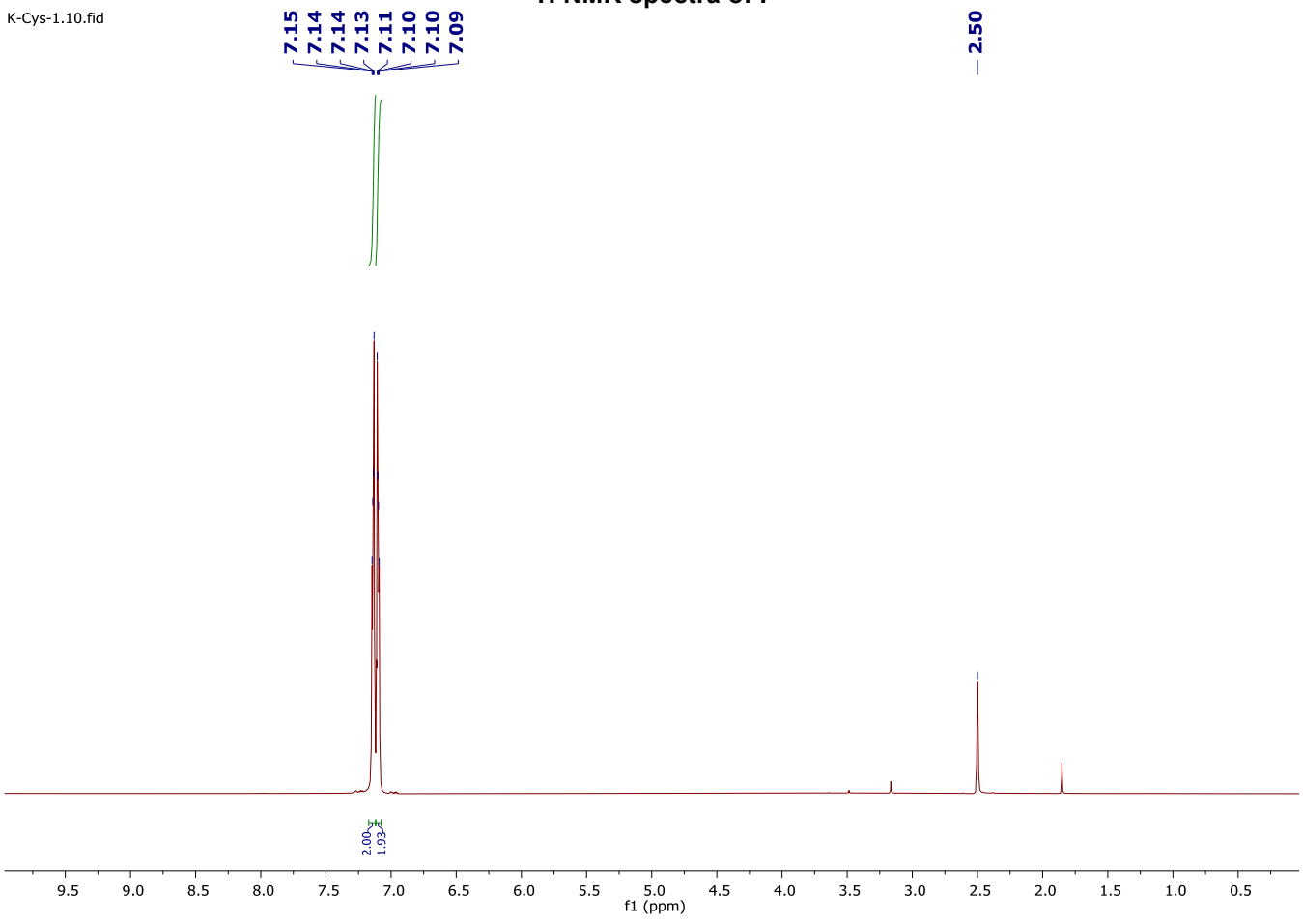
Synthesis of compound 1o

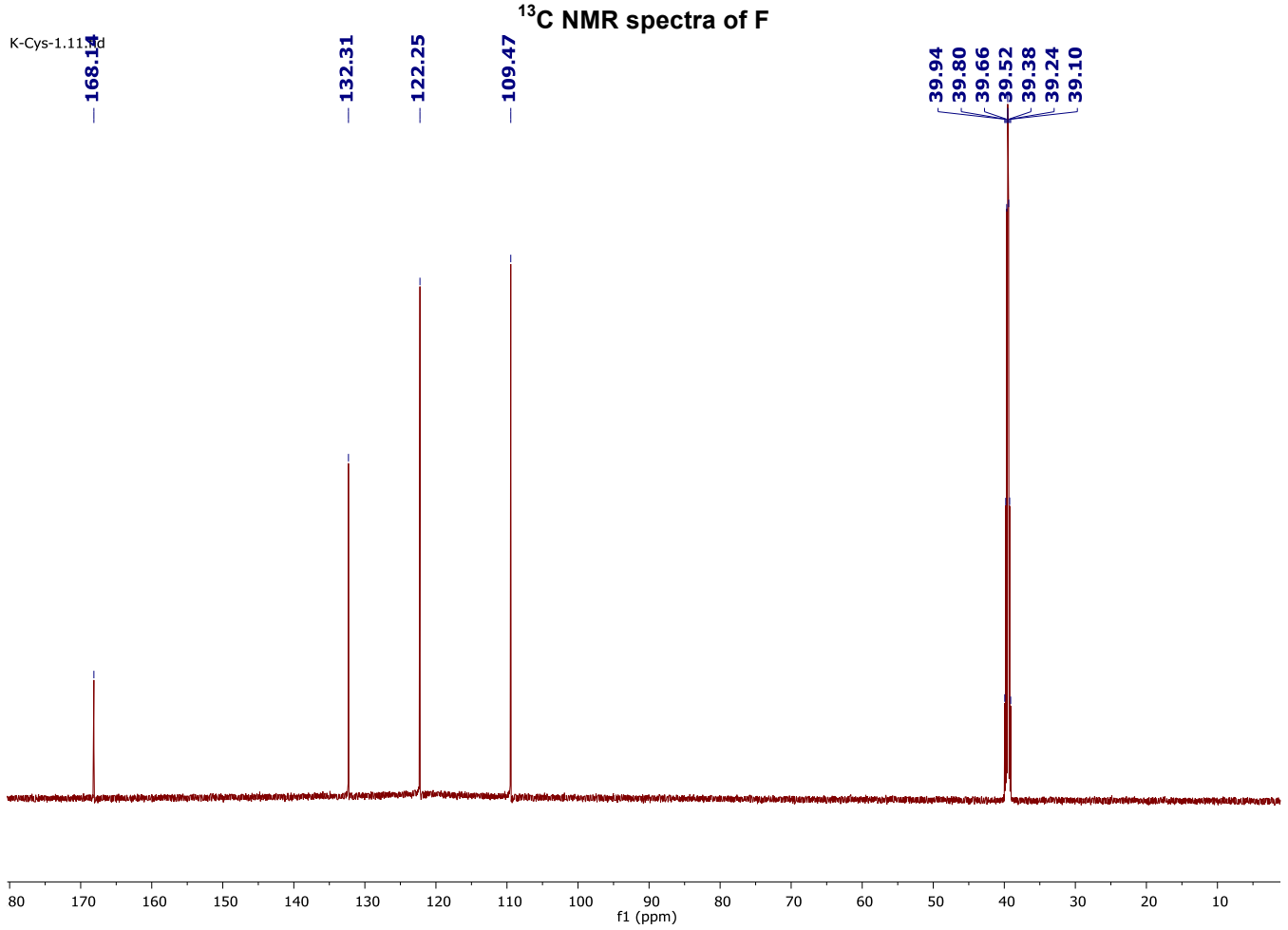


Synthesis of compound F

o-Phenylenediamine **E** (2 g, 18.5 mmol) was dissolved in methanol / water (9:1) and KOH (1.01 g, 18.5 mmol) and CS_2 (3.3 ml, 55.5 mmol) was added. The reaction mixture was refluxed for 6 h. The solution was then cooled to rt and treated with glacial acetic acid (8 mL) and diluted with water. The resulting precipitate was filtered off and washed with methanol for three times. The off-white powder was dried under high vacuum to obtain compound **F** as off-white powder (73 %, 2.02 g). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 7.13 – 7.11 (m,4 H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 168.14, 132.31, 122.25, 109.47.

^{1}H NMR spectra of F

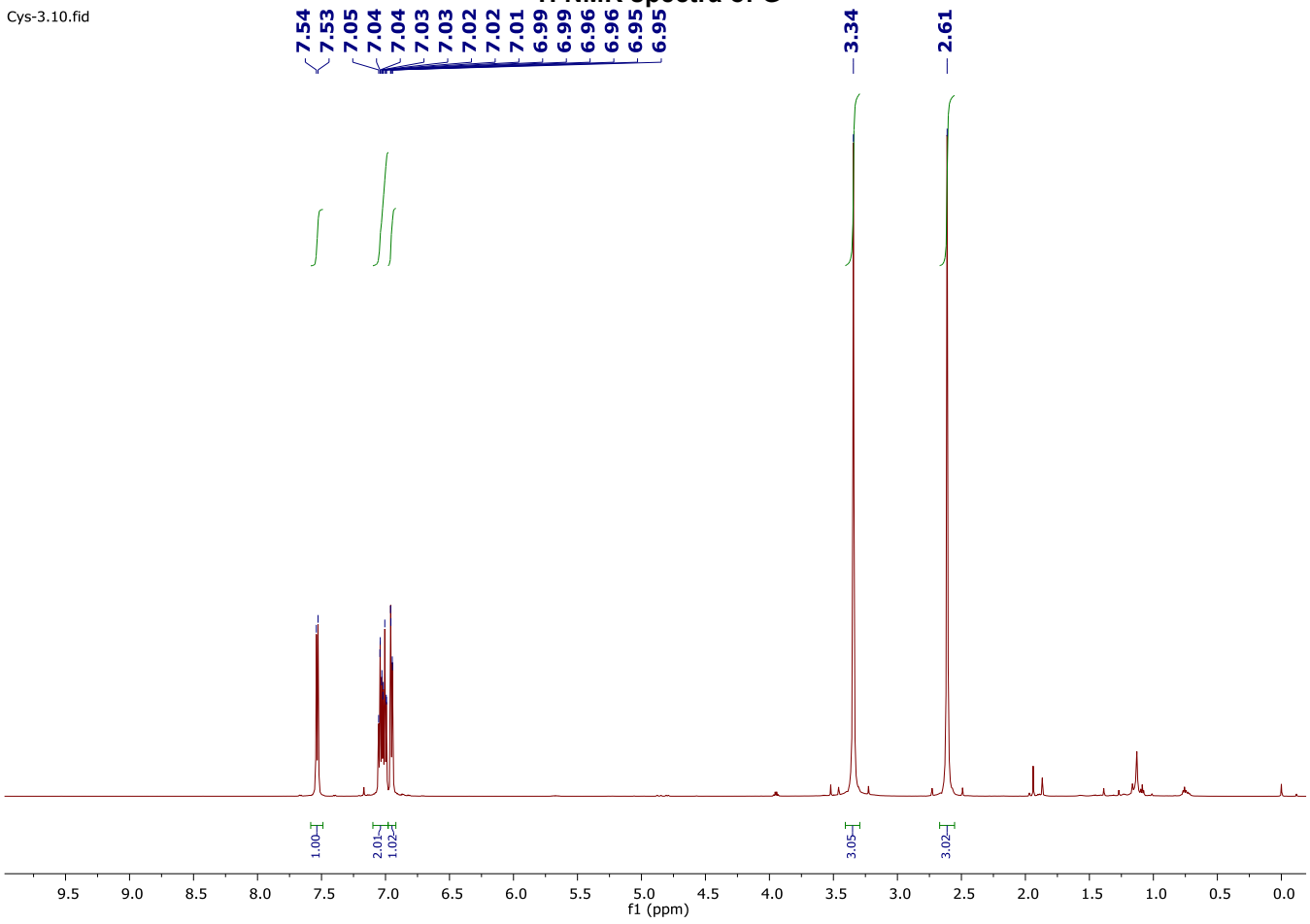



^{13}C NMR spectra of F
K-Cys-1.11.1d
168.14
132.31
122.25
109.47
39.94
39.80
39.66
39.52
39.38
39.24
39.10
80
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

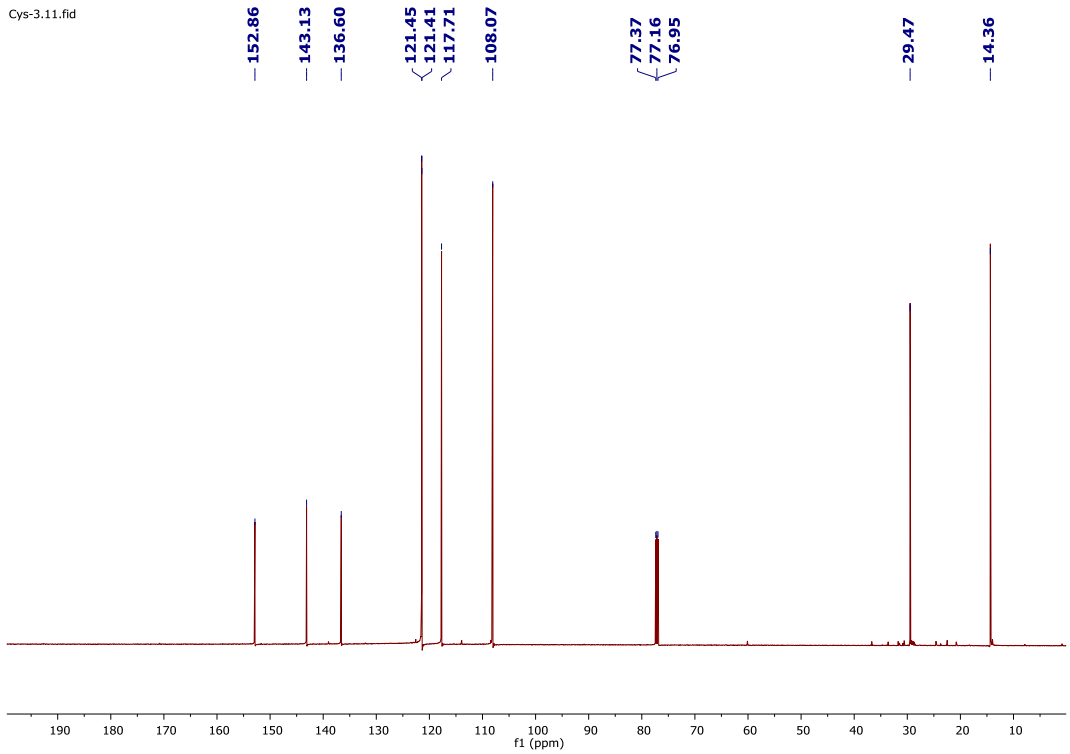
Synthesis of compound G

To a solution of 2-Benzimidazolethiol **F** (1.2 g, 8 mmol) in dry DMF (20 mL), K_2CO_3 (1.1 g, 8 mmol) and methyl iodide (2 mL, 32 mmol) were added sequentially. The reaction mixture was stirred at 60 ^{o}C for 12 hours. The reaction solution was washed with ethyl acetate and brine for 3 times. The residue was concentrated under reduced pressure and purified by the column chromatography (hexane: ethyl acetate 3:1) to obtain compound **G** as white solid (1.28 g, 90 %). **^{1}H NMR** (600 MHz, Chloroform-*d*) δ 7.53 (d, *J* = 8.0 Hz, 1H), 7.07 – 6.98 (m, 2H), 6.98 – 6.92 (m, 1H), 3.34 (s, 3H), 2.61 (s, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 152.86, 143.13, 136.60, 121.45, 121.41, 117.71, 108.07, 29.47, 14.36.

^{1}H NMR spectra of G



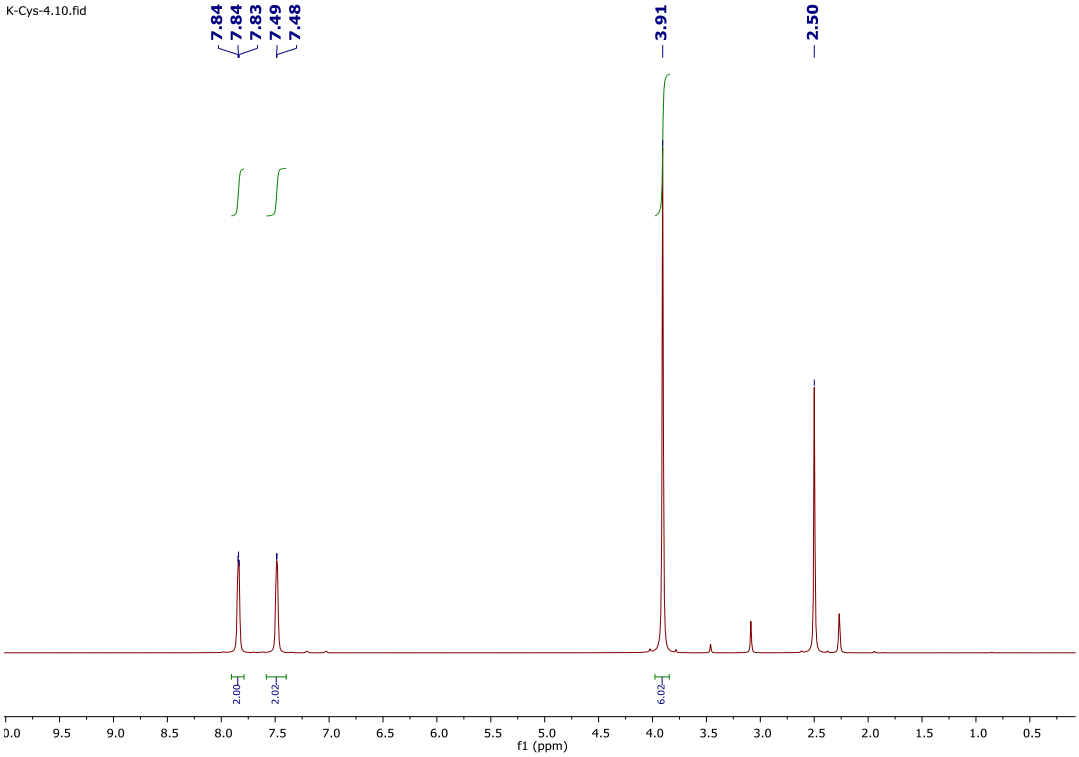
^{13}C NMR spectra of G



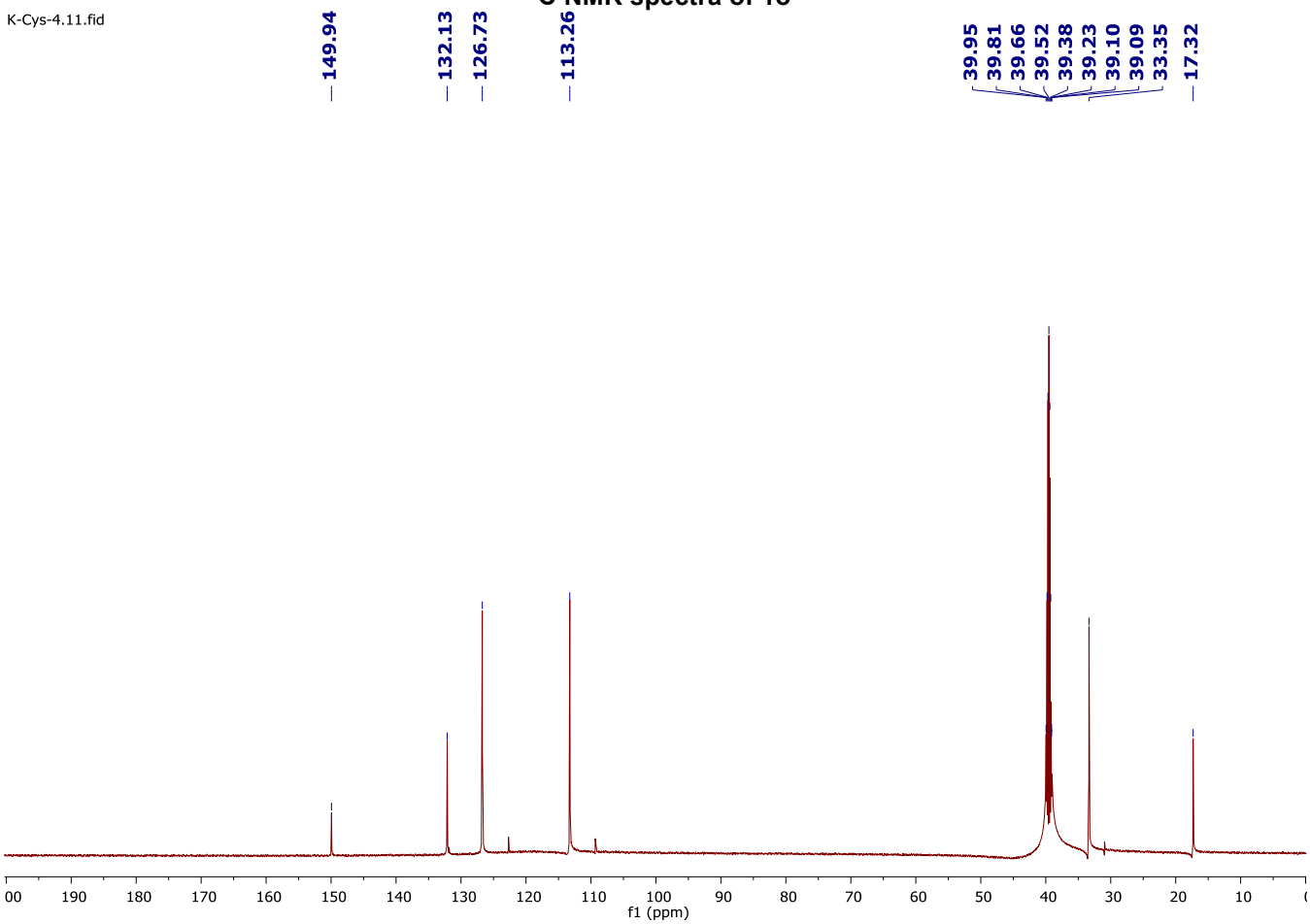
Synthesis of compound 1o

To a mixture of compound **G** (1g, 5.61 mmol) in the dry acetonitrile (20 mL), MeI (1.05 mL, 16.83 mmol) was added at room temperature under nitrogen. After 4 h the solvent was removed by rotary evaporation to afford crude powder. The crude was washed with diethyl ether three times, dried under vacuum to obtain pure compound **1o** as white powder (93 %, 1.67 g). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.07 (dd, J = 6.5, 3.3 Hz, 2H), 7.72 (dd, J = 6.5, 2H), 4.14 (s, 6H), 2.73 (s, 3H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 149.95, 132.13, 126.73, 113.27, 33.35, 17.32.

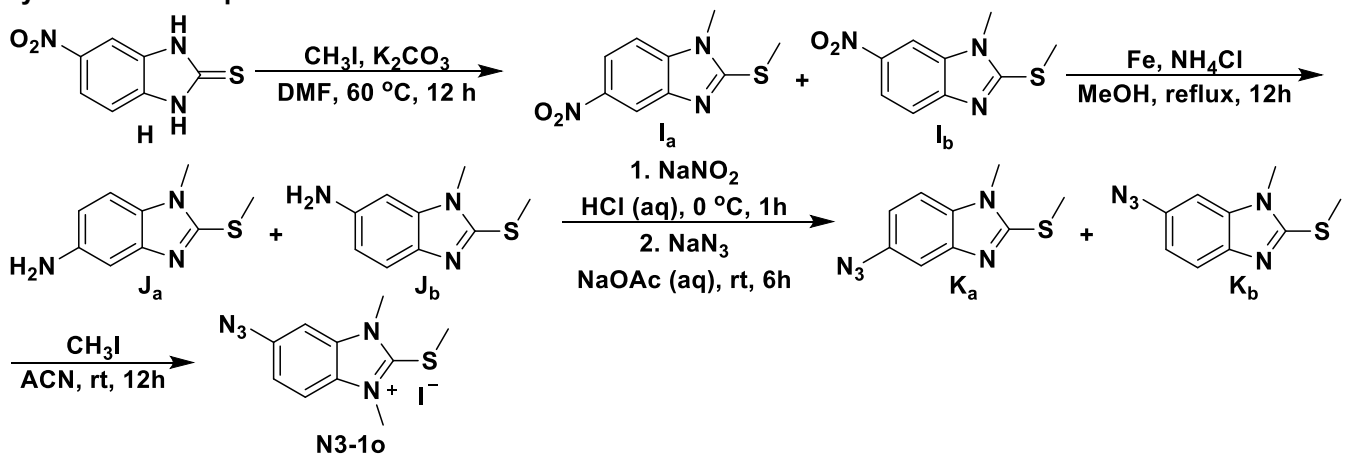
^{1}H NMR spectra of 1o



^{13}C NMR spectra of 1o

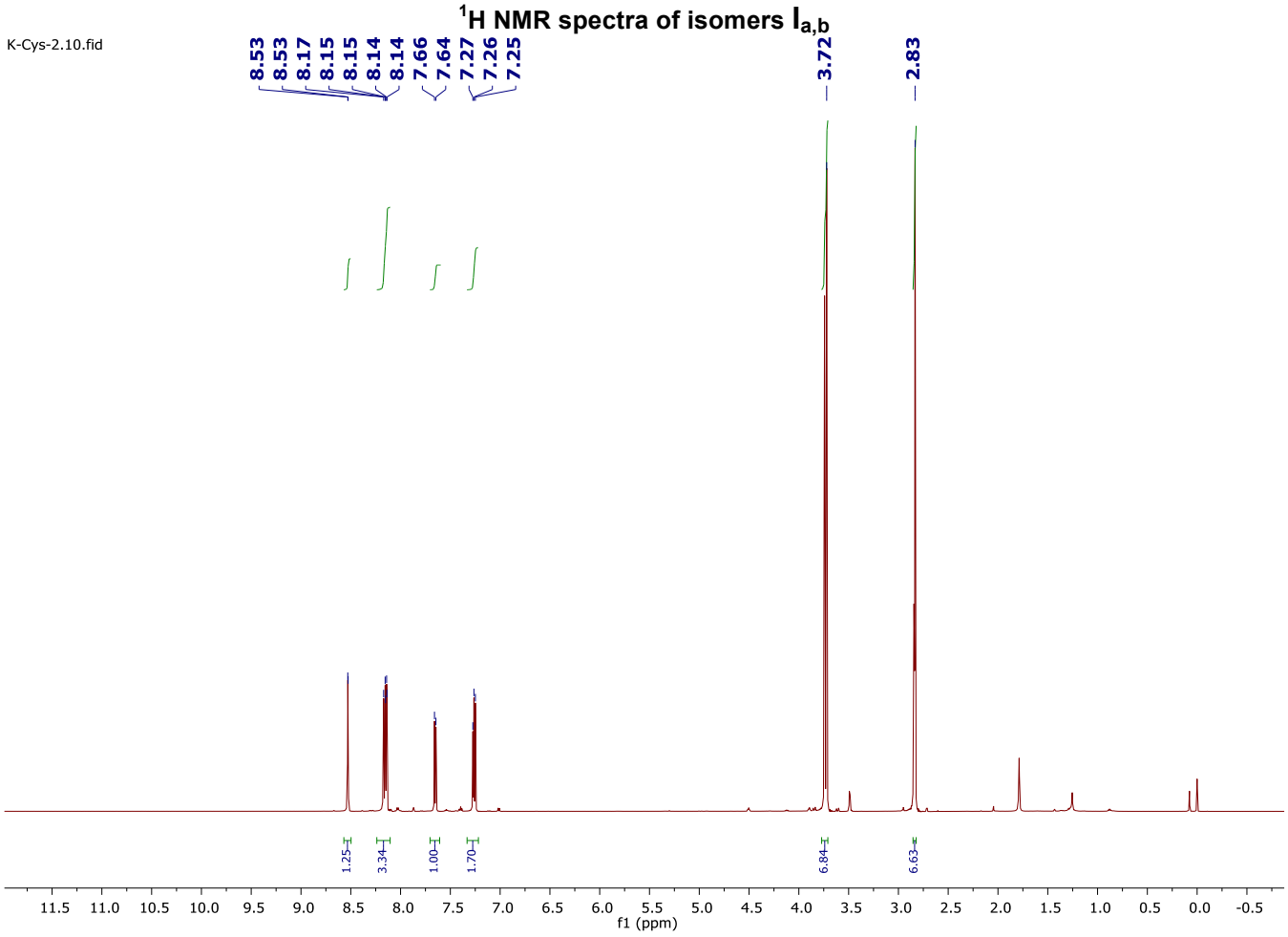


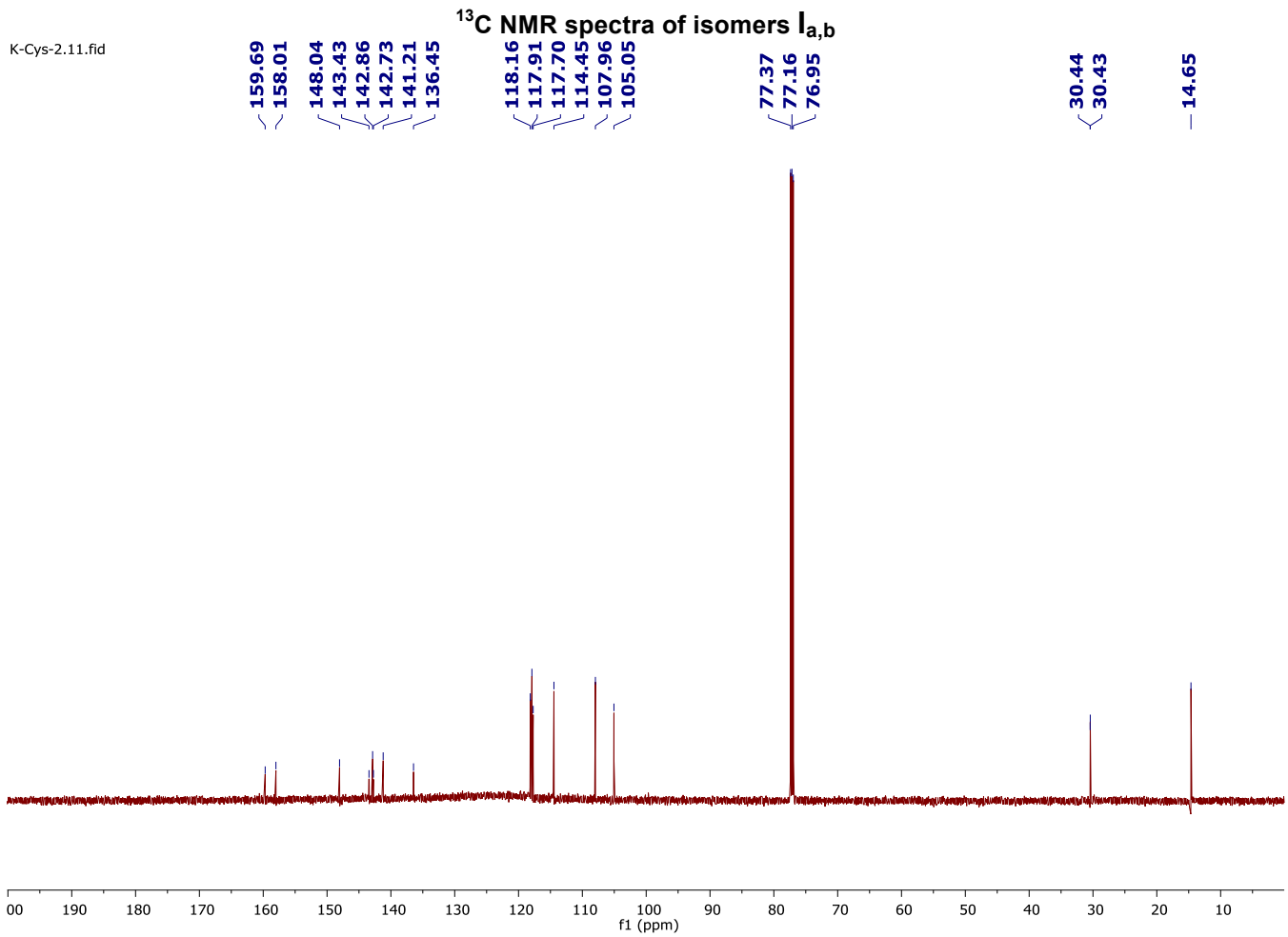
Synthesis of compound N3-1o



Synthesis of isomers $I_{a,b}$

To a stirred solution of 2-Mercapto-5-nitrobenzimidazole **H** (1 g, 5.12 mmol) in dry DMF (15 mL), K_2CO_3 (707.7 mg, 5.12 mmol) and MeI (320 µL, 5.12 mmol) were added consecutively at room temperature, then the reaction was heated to 60 °C for 12 h. The mixture was cooled to room temperature and extracted with ethyl acetate and brine for three times. The organic portion was collected and dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude was purified by flash chromatography (hexane: ethyl acetate 1:1) to afford isomers I_a and I_b as yellow powder (62 %, 707.9 mg). **^{1}H NMR** (600 MHz, Chloroform-*d*) δ 8.53 (d, *J* = 2.1 Hz, 1H), 8.19 – 8.12 (m, 3H), 7.65 (d, *J* = 8.7 Hz, 1H), 7.26 (d, *J* = 8.8 Hz, 1H), 3.73 (d, *J* = 12.7 Hz, 6H), 2.84 (d, *J* = 5.1 Hz, 6H). **^{13}C NMR** (151 MHz, Chloroform-*d*) δ 159.58, 157.90, 147.92, 143.32, 142.75, 142.62, 141.09, 136.34, 118.05, 117.79, 117.59, 114.34, 107.84, 104.94, 30.33, 30.31, 14.53.


^{1}H NMR spectra of isomers $I_{a,b}$
K-Cys-2.10.fid
8.53
8.53
8.17
8.15
8.15
8.14
8.14
7.66
7.64
7.27
7.26
7.25
3.72
2.83
1.25
3.34
1.00
1.70
6.84
6.63
11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

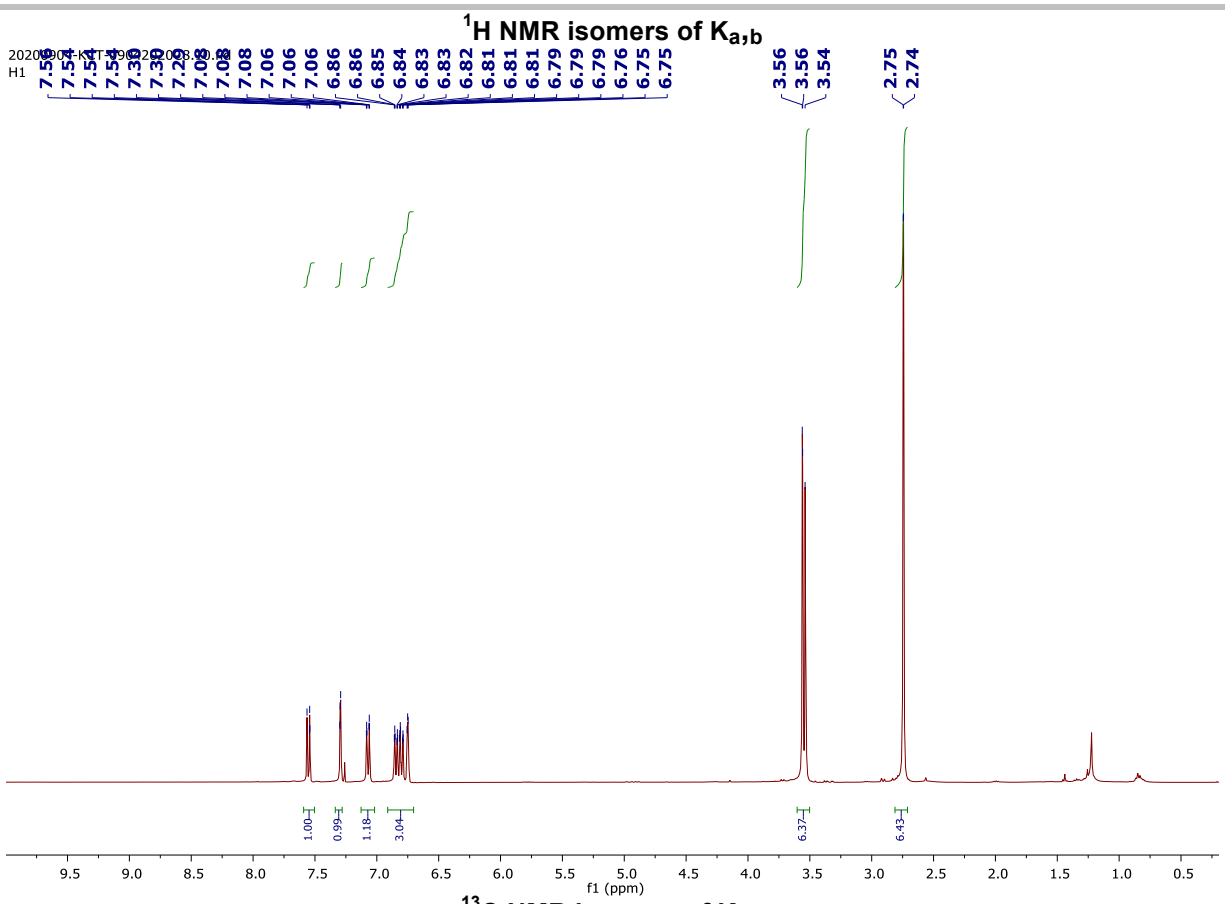


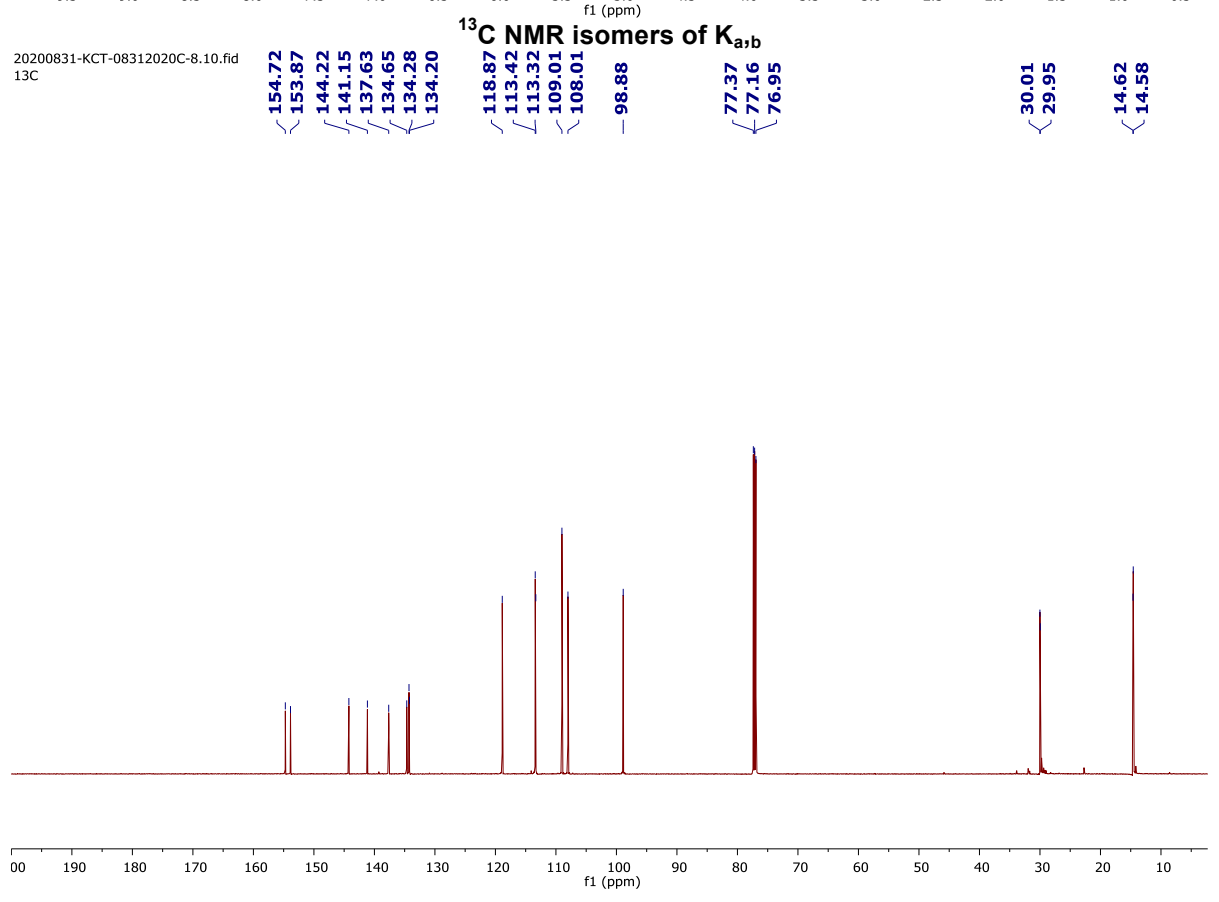
Synthesis of isomers $J_{a,b}$

A mixture of the isomers $\mathbf{I_a}$ and $\mathbf{I_b}$ (500 mg, 2.24 mmol), iron powder (125 mg, 2.24 mmol) and ammonia chloride (600 mg, 11.2 mmol) in methanol (50 mL) was vigorously stirred and refluxed for 12 h. The suspension was cooled and filtered through a pad of Celite. The filtrate was diluted with water and extracted with dichloromethane for 3 times. The combined organic layers were washed with saturated aqueous $NaHCO_3$ solution for 3 times, dried over sodium sulfate, and concentrated in vacuo to afford isomers $\mathbf{J_a}$ and $\mathbf{J_b}$ as purple oil (54 %, 233 mg). The isomers $\mathbf{J_a}$ and $\mathbf{J_b}$ were used directly without further purification.

Synthesis of isomers $K_{a,b}$

To a solution of isomers $\mathbf{J_a}$ and $\mathbf{J_b}$ (200 mg, 1.04 mmol) in 3M HCl (2.5 mL) was added a solution of $NaNO_2$ (71.7 mg, 1.04 mmol) in H_2O (2.5 mL) at 0°C. After 60 min at 0°C a solution of NaN_3 (202.7 mg,10.5 mmol) in saturated NaOAc solution (2.5 mL) was added dropwise and the mixture was stirred for 60 min at 0°C. The reaction was warmed to room temperature and allowed to stir for 6 h. The mixture was extracted with ethyl acetate and brine for three times, dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude mixture was purified by flash chromatography (hexane : ethyl acetate 5:1) to get isomers $\mathbf{K_a}$ and $\mathbf{K_b}$ as bright yellow oil (45 %, 102.5 mg). **^{1}H NMR** (400 MHz, Chloroform-*d*) δ 7.55 (d, *J* = 8.5 Hz, 1H), 7.29 (d, *J* = 1.9 Hz, 1H), 7.07 (ddd, *J* = 8.5, 2.1, 1.3 Hz, 1H), 6.82 (dddd, *J* = 17.9, 8.5, 2.1, 0.8 Hz, 2H), 6.75 (t, *J* = 1.7 Hz, 1H), 3.57 – 3.55 (m, 3H), 3.55 – 3.52 (m, 3H), 2.74 (d, *J* = 1.0 Hz, 6H). **^{13}C NMR** (151 MHz, Chloroform-*d*) δ 154.18, 153.33, 143.68, 140.61, 137.10, 134.11, 133.74, 133.66, 118.33, 112.88, 112.78, 108.47, 107.47, 98.34, 76.83, 76.62, 76.41, 29.47, 29.41, 14.08, 14.04.

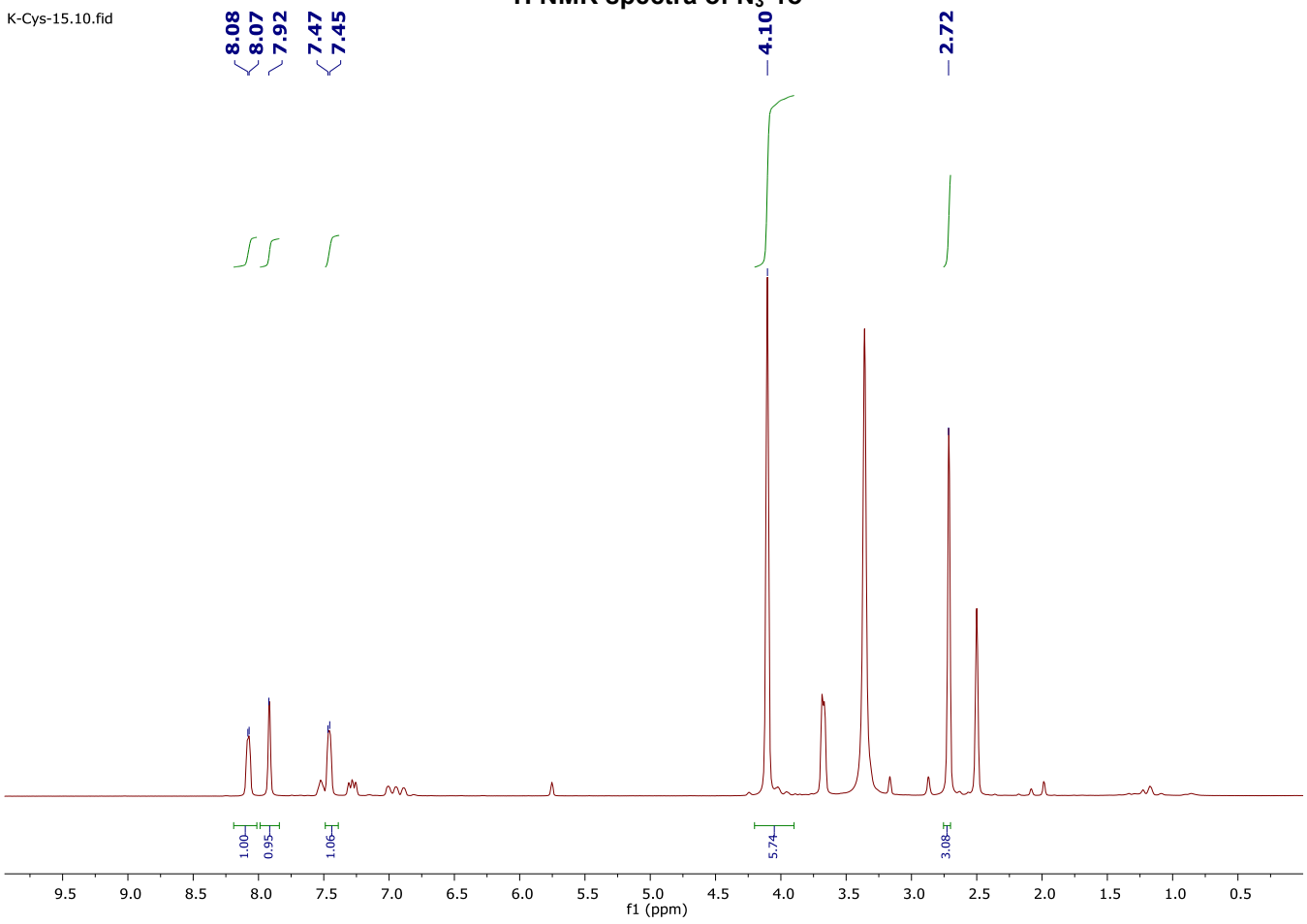

^{1}H NMR isomers of $K_{a,b}$
H1
7.56 7.54 7.54 7.54 7.31 7.31 7.22 7.08 7.08 7.08 7.06 7.06 7.06 6.86 6.86 6.85 6.84 6.83 6.83 6.82 6.81 6.81 6.81 6.79 6.79 6.79 6.76 6.75 6.75
3.56 3.56 3.54
2.75 2.74
1.00 0.99 1.18 3.04 6.37 6.43
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)

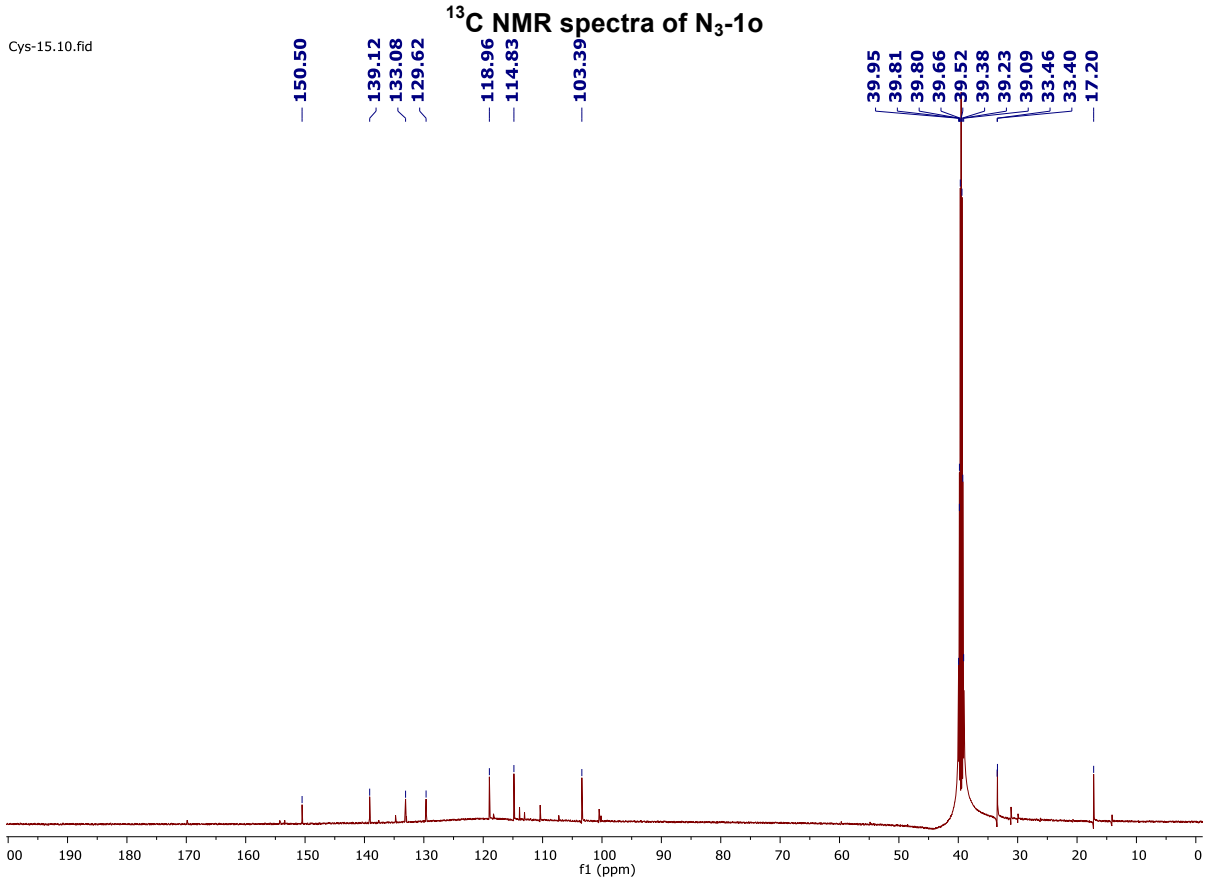

^{13}C NMR isomers of $K_{a,b}$
20200831-KCT-08312020C-8.10.fid
13C
154.72 153.87 144.22 141.15 137.63 134.65 134.28 134.20 118.87 113.42 113.32 109.01 108.01 98.88 77.37 77.16 76.95 30.01 29.95 14.62 14.58
00 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)

Synthesis of compound N_3-1o

To the isomers of $\mathbf{K_a}$ and $\mathbf{K_b}$ (50 mg, 0.22 mmol) in the dry acetonitrile (7 mL), was added MeI (71.3 µL, 1.14 mmol) under nitrogen at room temperature and reaction was stirred for 12 h. The solvent was removed by rotary evaporation to afford crude powder. The crude was washed with diethyl ether three times, dried under vacuum to obtain pure compound **N_3-1o** as bright yellow powder (85 %, 257.7 mg). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.12 – 8.03 (m, 1H), 7.92 (d, J = 4.5 Hz, 1H), 7.49 – 7.40 (m, 1H), 4.10 (s, 6H), 2.72 (s, 3H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 150.51, 139.12, 133.08, 129.63, 118.97, 114.84, 103.40, 33.46, 33.40, 17.20.

^{1}H NMR spectra of N_3-1o

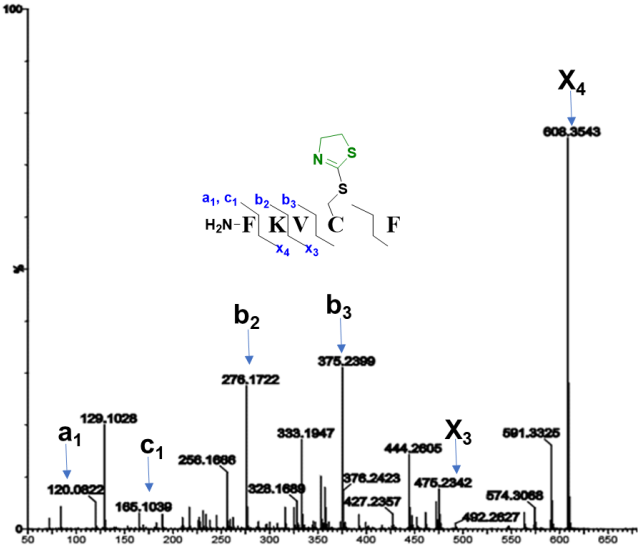




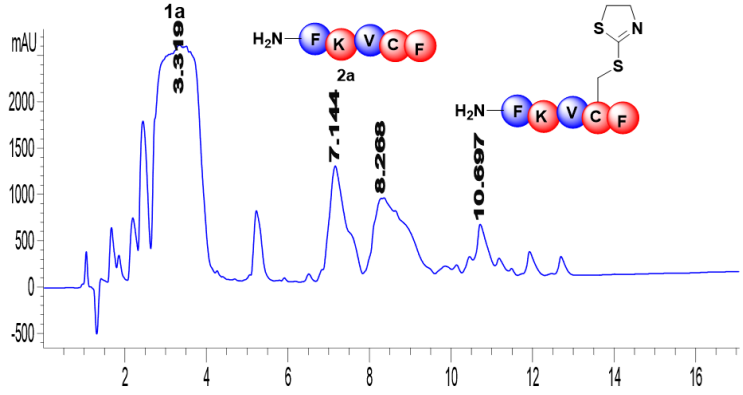
IX. Supplementary Figure 2: General procedure for the modification of FKVCF with probes 1a,1b, 1c, and 1i. To a 1 mg of FKVCF **2a** (4 mM) in 0.4 mL of 10 mM phosphate buffer (Nap, pH 7.5) was added **1a** or **1b** or **1c** or **1i** (10 equiv., 40 mM). The reaction was stirred at room temperature for 3h. The reaction was analyzed by HPLC and MS/MS. HPLC was carried out with 1% formic acid: water (solvent A): acetonitrile (solvent B); 0-80 % in 30 min, flow rate = 1.0 mL/min, detection wavelength 220 nm.

ESI MS/MS spectrum of 1a-FKVCF

Compound	Mass (m/z)
Unmodified peptide	641.34
Modified peptide	726.33

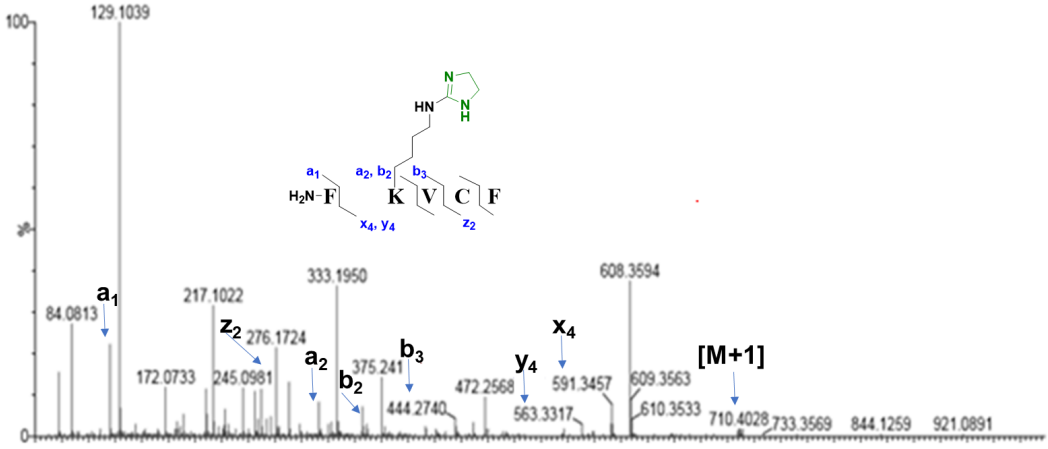


HPLC trace of 1a-FKVCF

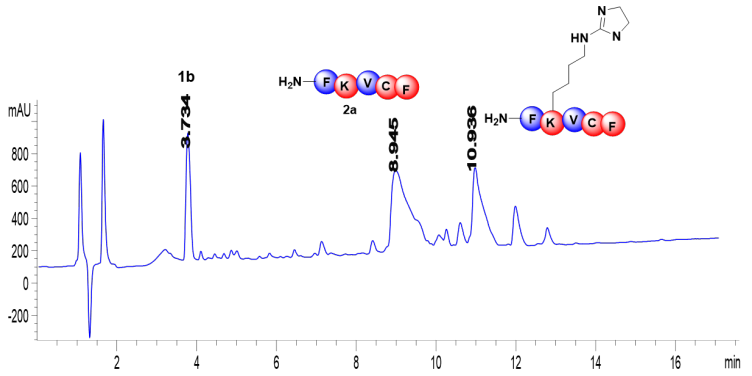


ESI MS/MS spectrum of 1b-FKVCF

Compound	Mass (m/z)
Unmodified peptide	641.34
Expected modified peptide	709.33
Observed modified peptide	710.4028

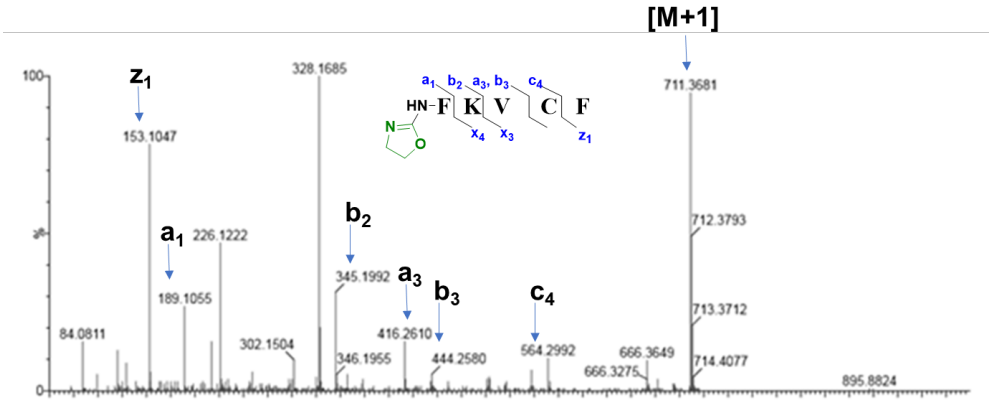


HPLC trace of 1b-FKVCF

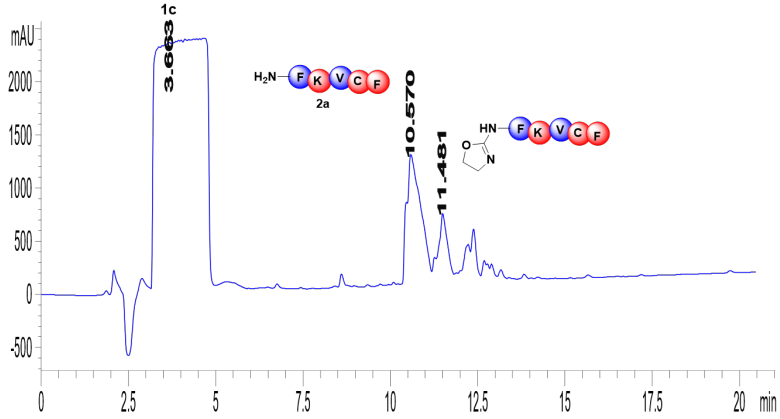


ESI MS/MS spectrum of 1c-FKVCF

Compound	Mass (m/z)
Unmodified peptide	641.34
Expected modified peptide	710.33
Observed modified peptide	711.3681

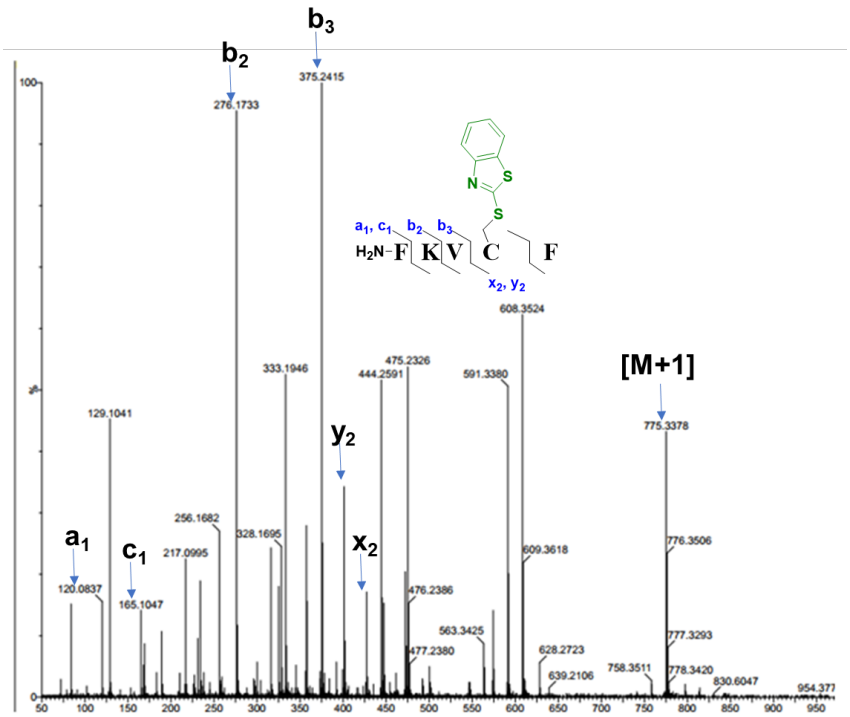


HPLC trace of 1c-FKVCF

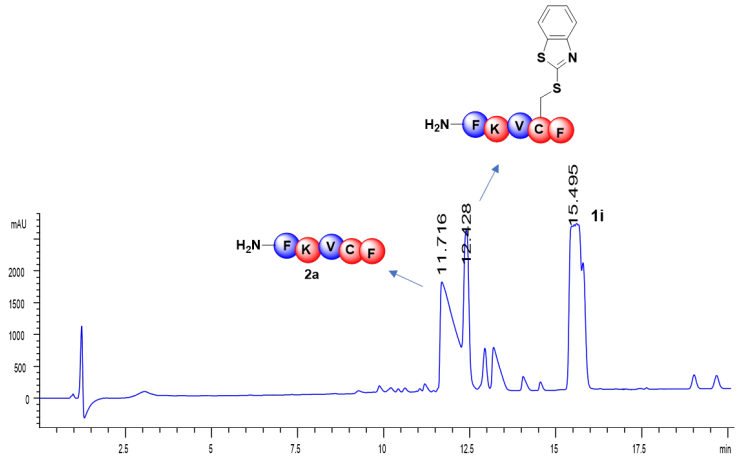


ESI MS/MS spectrum of 1i-FKVCF

Compound	Mass (m/z)
Unmodified peptide	641.34
Expected modified peptide	774.33
Observed modified peptide	775.3378



HPLC trace of 1i-FKVCF



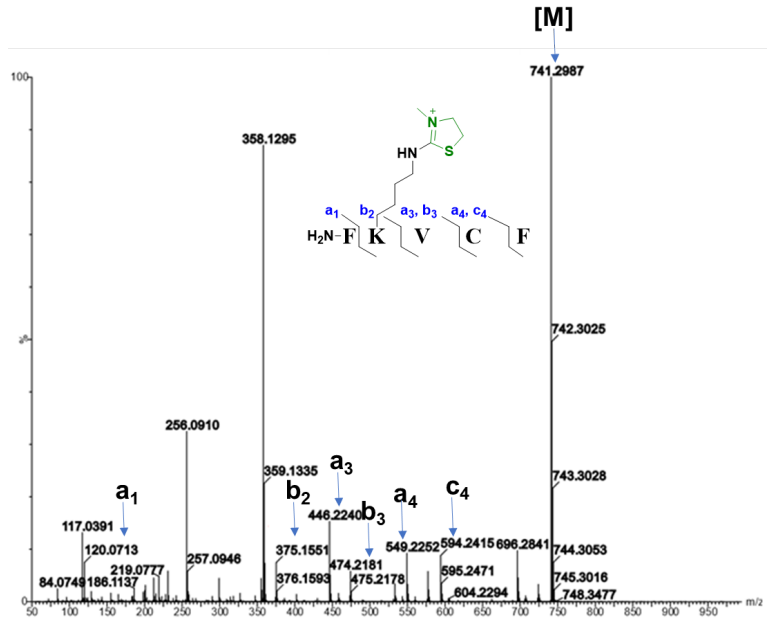
X. Supplementary Figure 3: Modification of Peptides FKVCF 2a with probes 1j-1n

To a 1 mg of FKVCF **2a** (4 mM) in 0.4 mL of 10 mM phosphate buffer (Nap, pH 7.5) was added probe (10 equiv., 40 mM). The reaction was stirred at room temperature for 3h. The reaction was analyzed by HPLC and MS/MS. HPLC was carried out with 1 % formic acid: water (solvent A): acetonitrile (solvent B); 0-80 % in 30 min, flow rate = 1.0 mL/min, detection wavelength 220 nm.

ESI MS/MS spectrum of FK(1j)VCF bioconjugated product

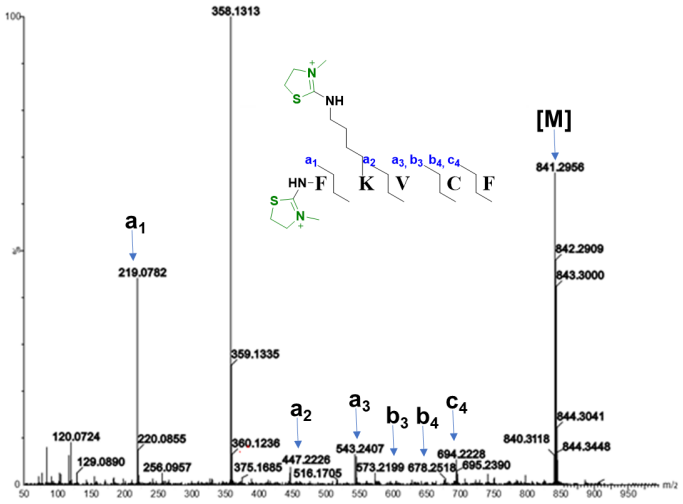
Compound	Mass (m/z)

Unmodified peptide	641.34
Expected modified peptide	741.36
Observed modified peptide	741.2987

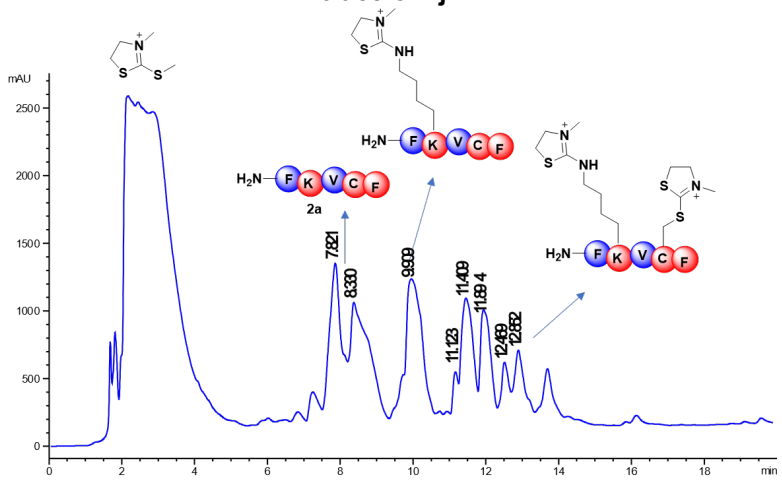


ESI MS/MS spectrum of FK(1j)VC(1j)F bioconjugated product

Compound	Mass (m/z)
Unmodified peptide	641.34
Expected modified peptide	841.38
Observed modified peptide	841.2956

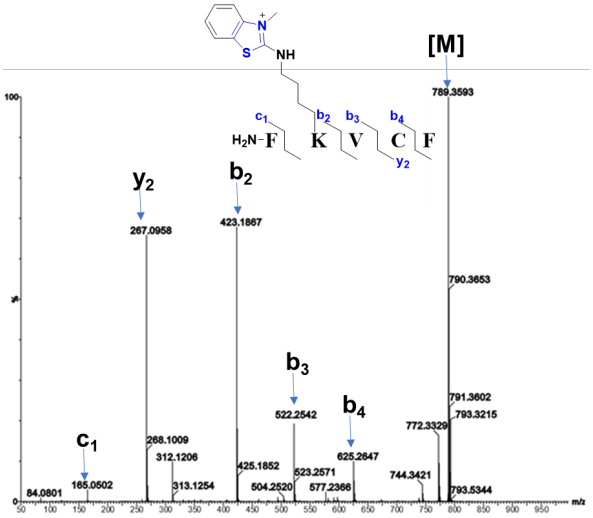


HPLC trace of 1j-FKVCF

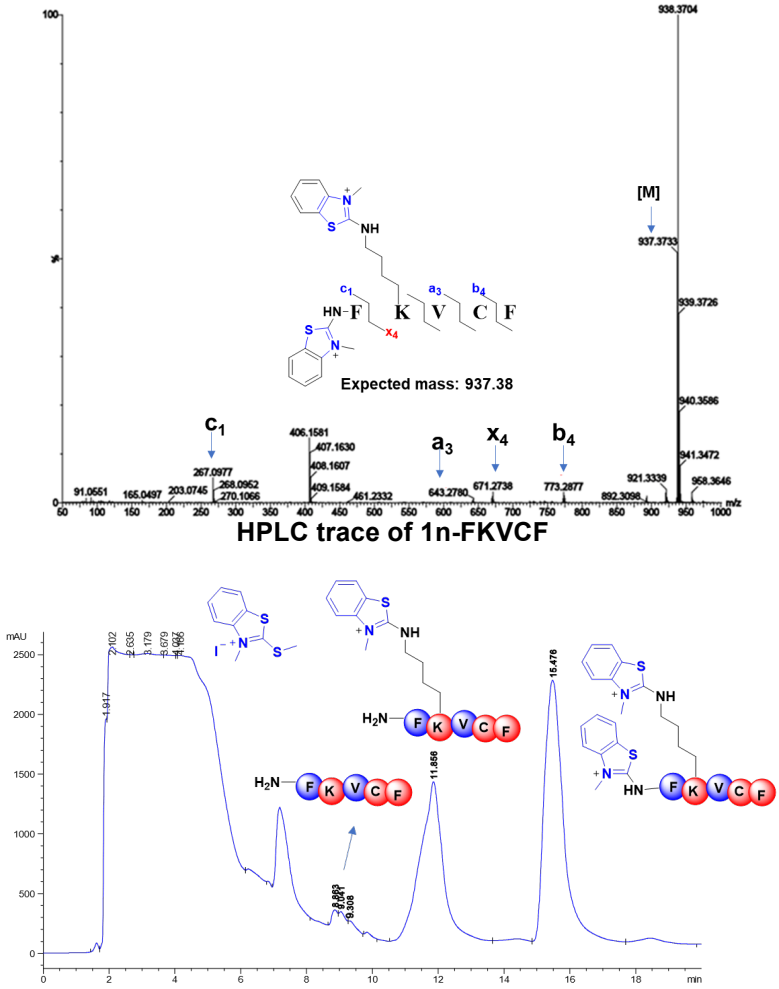


ESI MS/MS spectrum of FK(1n)VCF bioconjugated product

Compound	Mass (m/z)
Unmodified peptide	641.34
Mono-modified peptide	789.36
Double-modified peptide	937.38



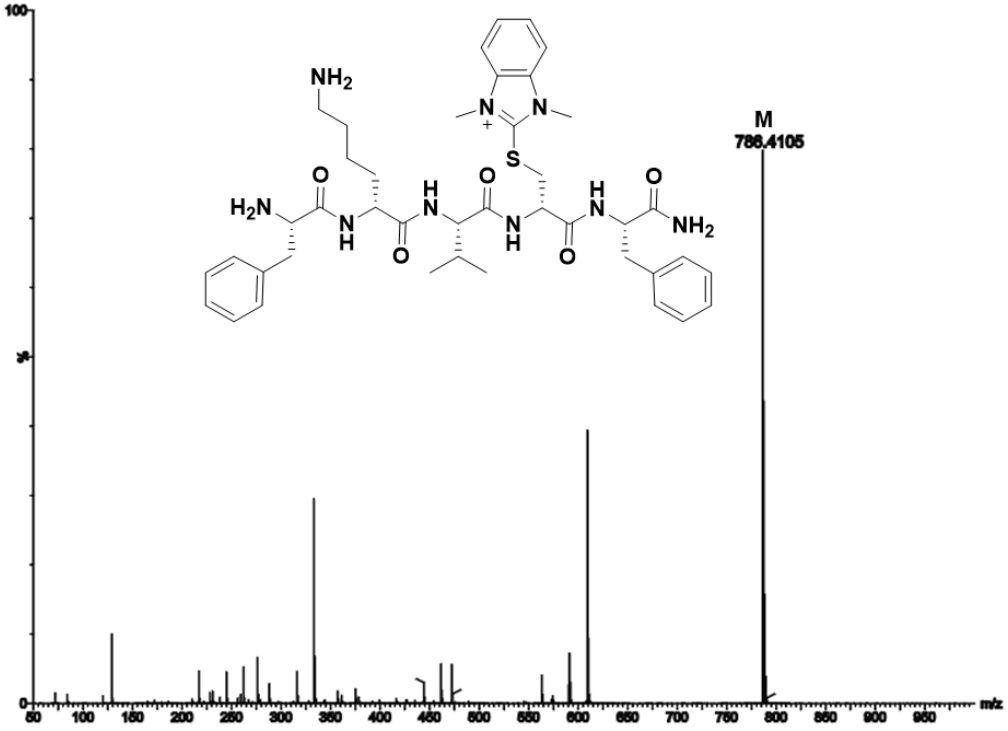
ESI MS/MS spectrum of (1n)FK(1n)VCF bioconjugated product



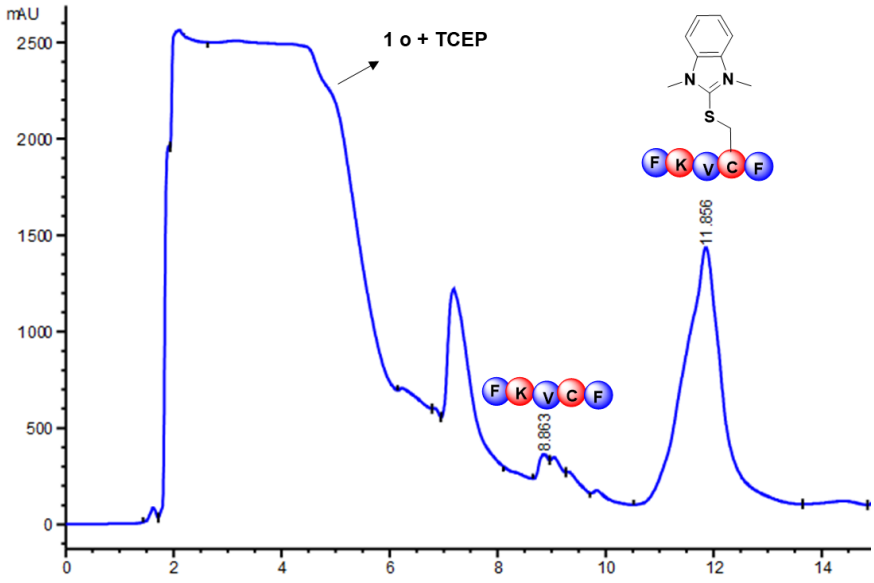
XI. Supplementary Figure 4 : General procedure for the modification of peptide with probe 1o.
To a 1 mg of FKVCF **2a** (4 mM) in 0.4 mL of 10 mM phosphate buffer (Nap, pH 7.5) was added probe **1o or 1m** (10 equiv., 40 mM). The reaction was stirred at room temperature for 3h. The reaction was analyzed by HPLC and MS/MS. HPLC was carried out with 1 % formic acid: water (solvent A): acetonitrile (solvent B); 0-80 % in 30 min, flow rate = 1.0 mL/min, detection wavelength 220 nm**.**

HRMS of FKVC(1o)F bioconjugated product

Compound	Mass (m/z)
Unmodified peptide	641.34
Expected modified peptide	786.41
Observed modified peptide	786.4105

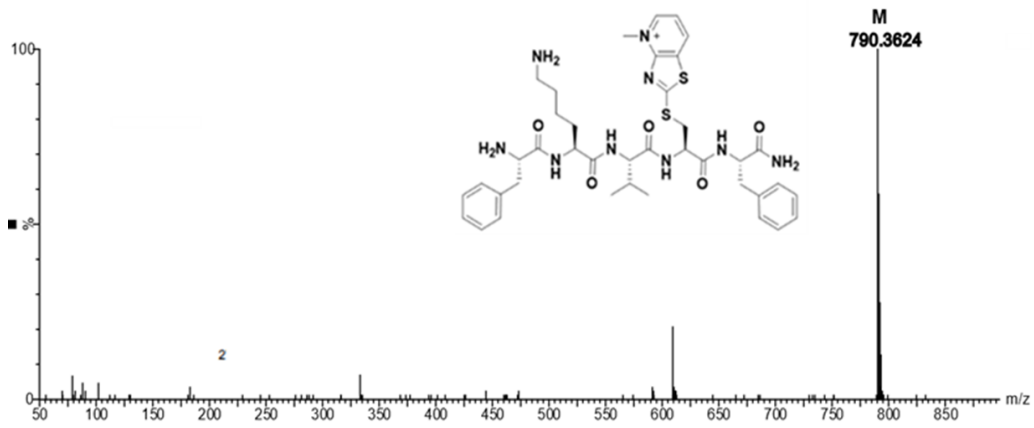


HPLC trace of FKVC(1o)F bioconjugated reaction

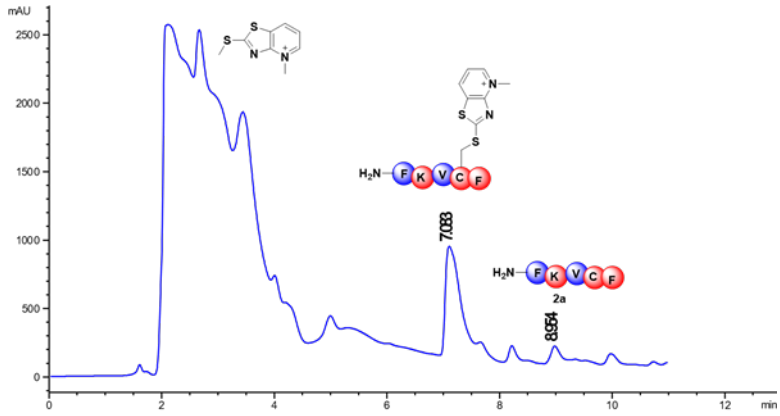


HRMS of FKVC(1m)F bioconjugated product

Compound	Mass (m/z)
Unmodified peptide	641.34
Expected modified peptide	790.35
Observed modified peptide	790.3624



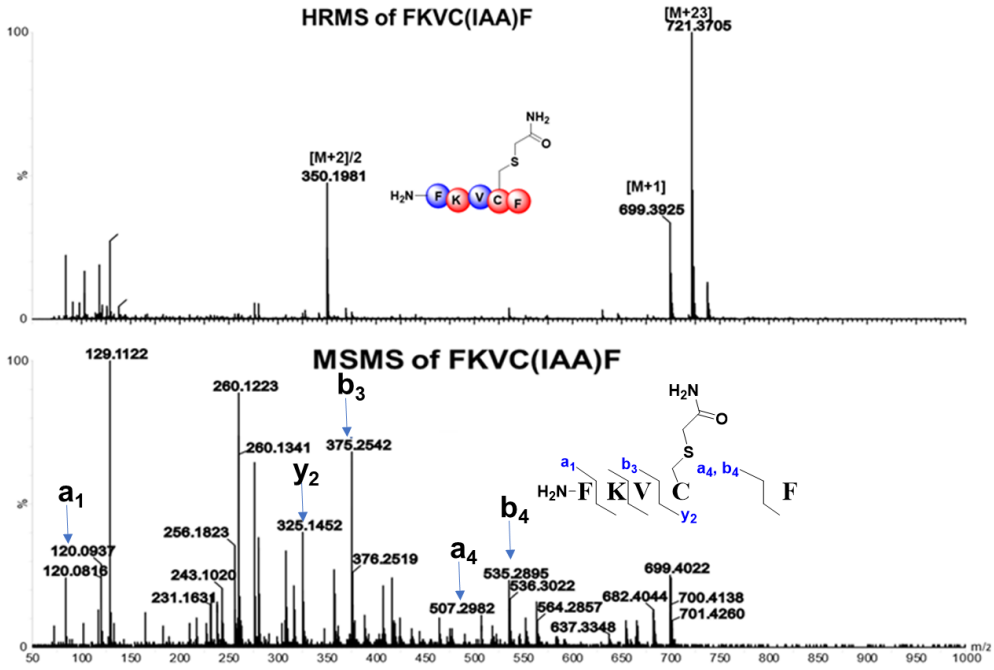
HPLC trace of FKVC(1m)F bioconjugated reaction

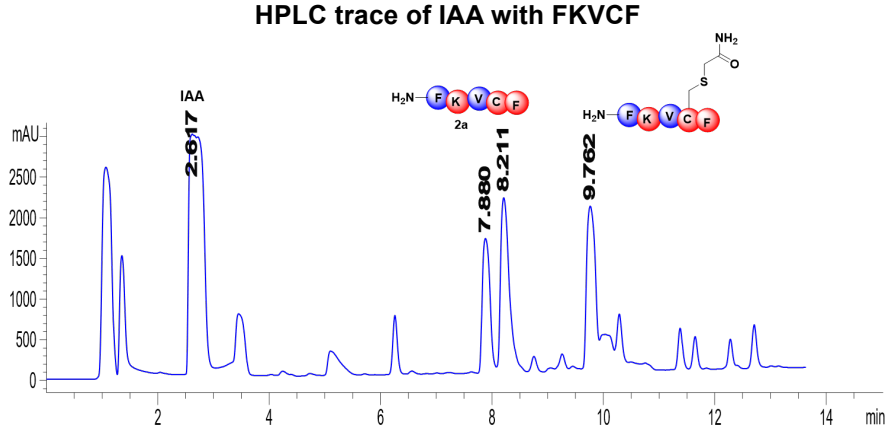


XII. Supplementary Figure 5: General procedure for the modification of peptides with IAA
FKVCF peptide **2a** (4 mM) was incubated with 10 eq of IAA (40 mM) in 10 mM phosphate buffer (Nap, pH 7.5) for 3h at room temperature. The reaction was analyzed by HPLC and MS/MS. HPLC was carried out with 1 % formic acid: water (solvent A): acetonitrile (solvent B); 0-80 % in 30 min, flow rate = 1.0 mL/min, detection wavelength 220 nm.

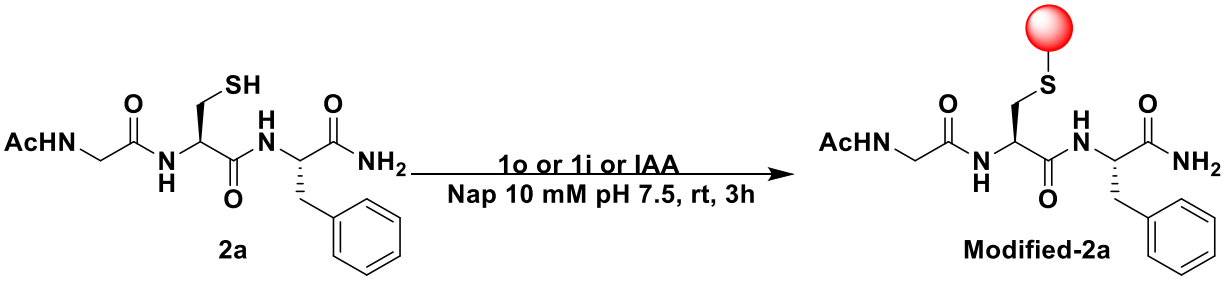
HRMS of FKVC(IAA)F bioconjugated product

Compound	Mass (m/z)
Unmodified peptide	641.34
Modified peptide	698.36
Expected modified peptide	698.36
Observed modified peptide	350.1981 [M+2]/2, 699.3925 [M+1], 721.3705 [M+Na]





XIII. Supplementary Figure 6: Optimization of bioconjugation reaction between peptide 2b and HAT probe 1o.

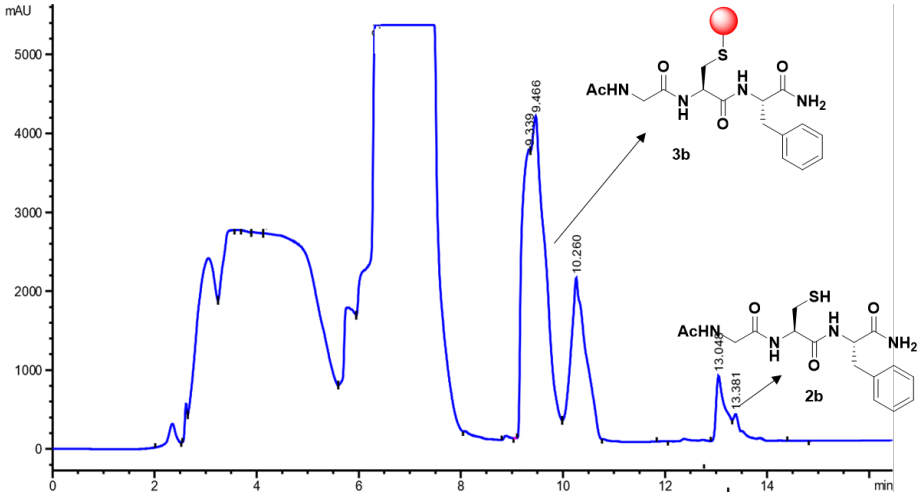


[a]Entry	Eq. of probe 1o	[b]Conversion %
1	1	61
	5	73

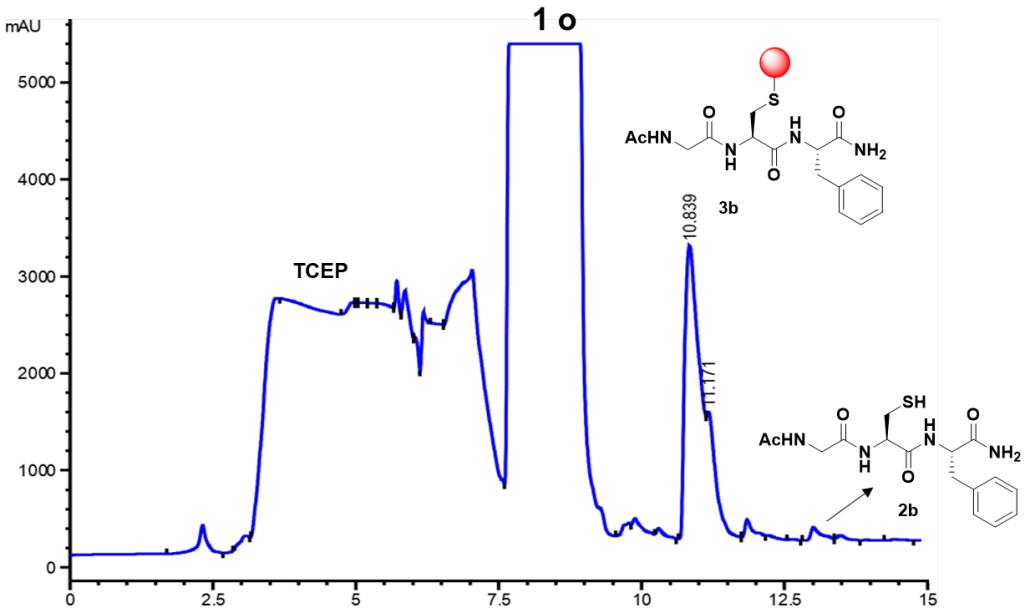
3	10	90
4	**25**	**>99**
5	10 (pH 6.5)	72
6	10 (pH 8.5)	36[c]
7	10 (pH 10.5)	30[d]
8	IAA, 25 eq (pH 7.5)	70
9	1i, 25 eq	53

[a]Reaction conditions: Peptide **2a** (0.0015 mmol, 3.75 mM) in 10 mM phosphate buffer of pH 7.5 (400 µL) and TCEP (0.05 M, 20 µL) and **1o** (1-25 equiv.) or **IAA** (25 equiv.), or **1i** (25 equiv.) was incubated for 3h at room temperature. [b]The conversion was determined by the HPLC analysis. [c]64 % of dehydroalanine. [d] 70 % of dehydroalanine.

HPLC trace of 1o (10 eq) with Ac-GCF 2b

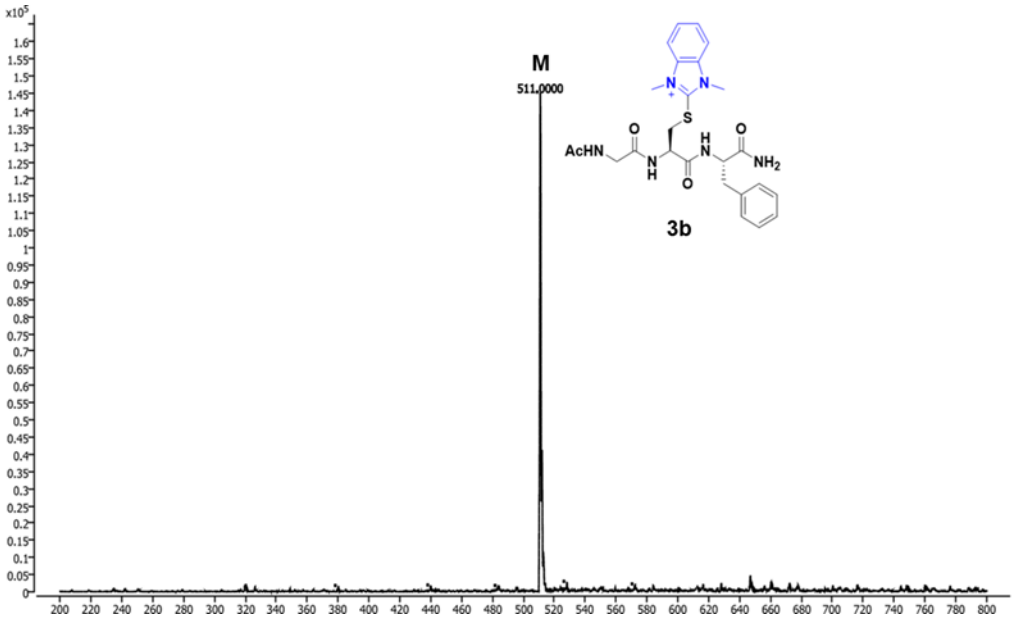


HPLC trace of 1o (25 eq) with Ac-GCF 2b

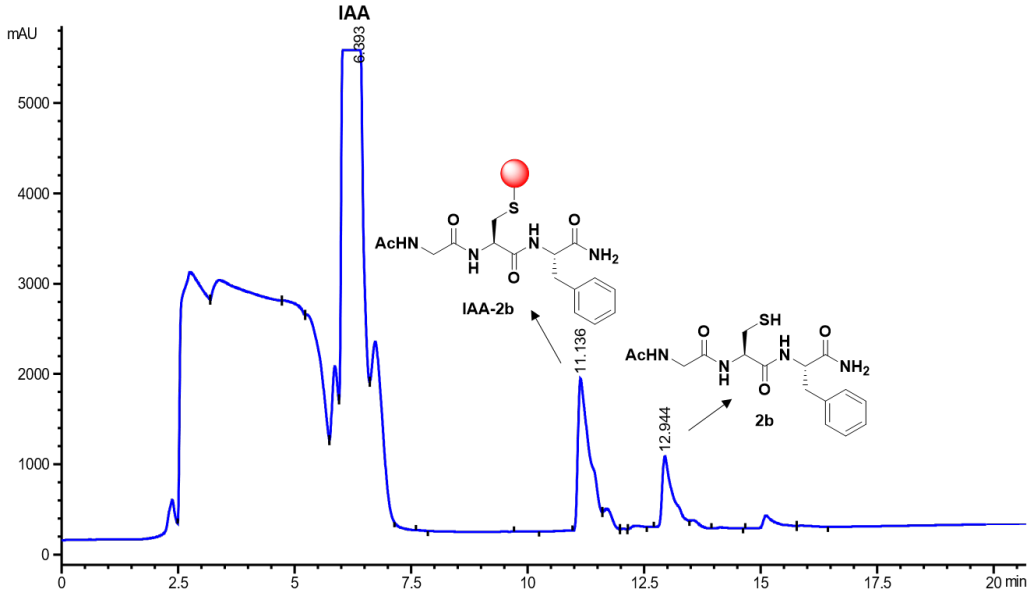


LC-MS spectrum of 3b

Compound	Mass (m/z)
Expected 3b	511.21
Observed 3b	511.00

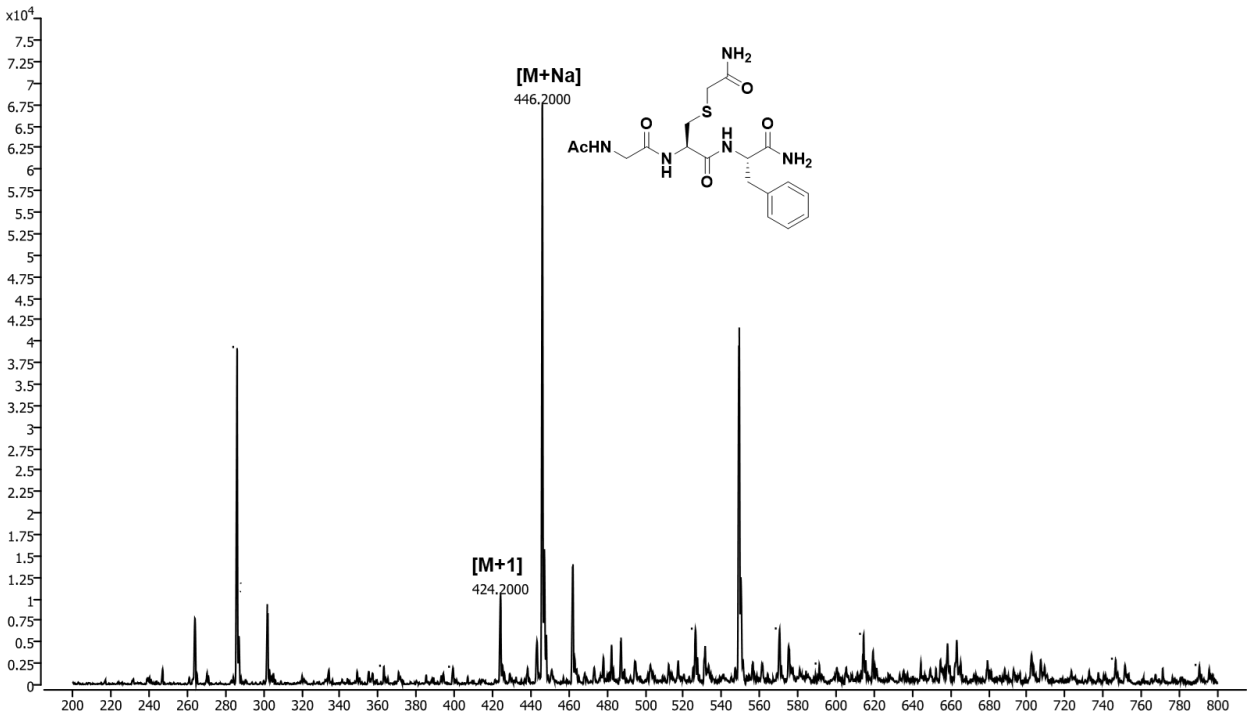


HPLC trace of IAA (25 eq) with Ac-GCF 2b

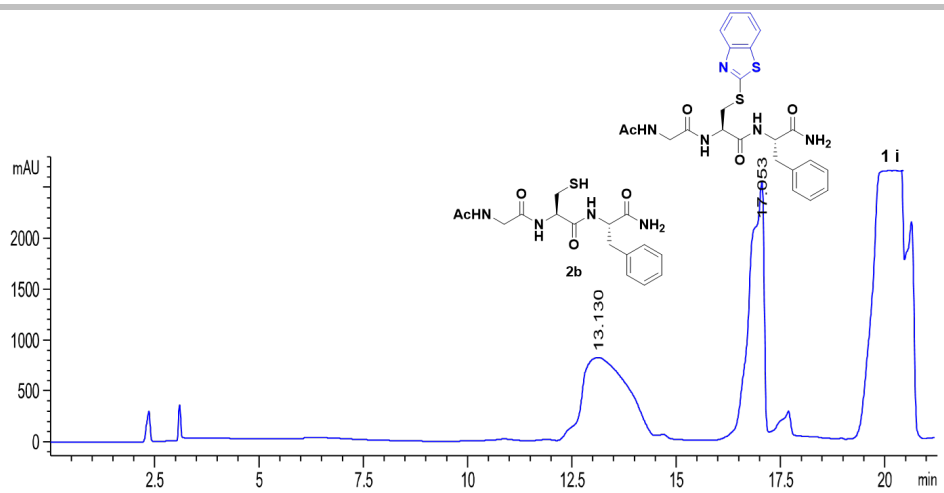


LC-MS spectrum of IAA-2a

Compound	Mass (m/z)
Expected IAA-2a	423.16
Observed IAA-2a	424.20 [M+1], 446.20 [M+Na]

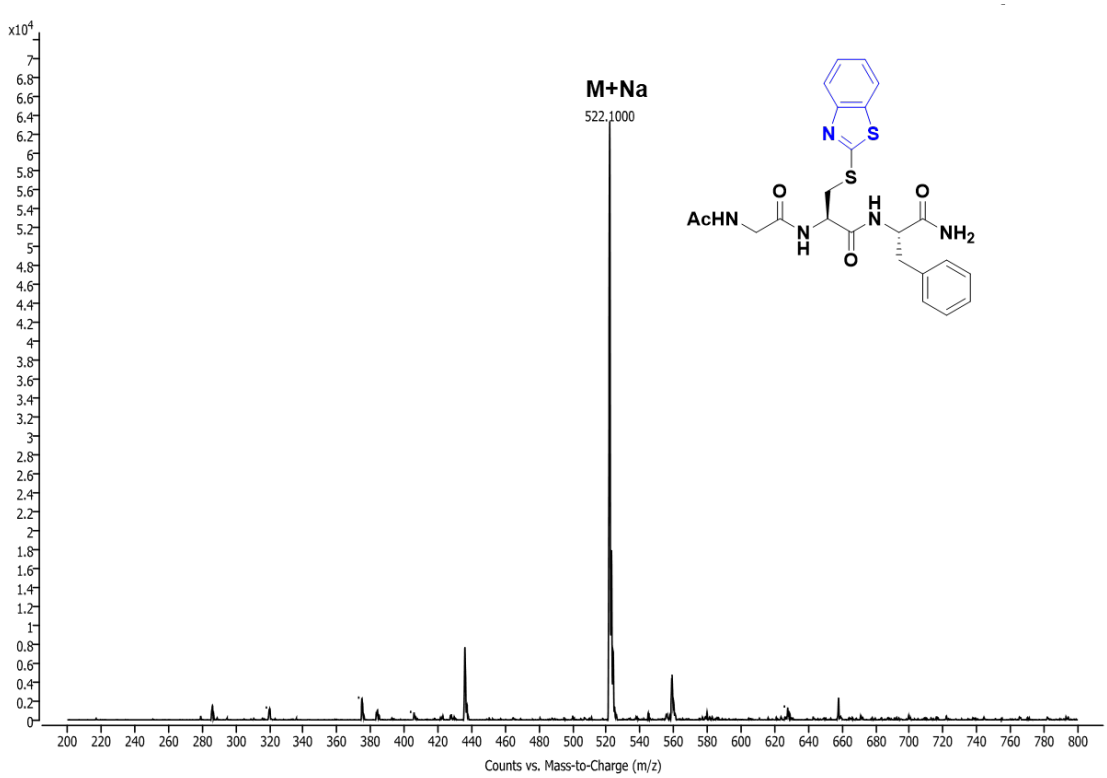


HPLC trace of 1i (25 eq) with Ac-GCF 2b

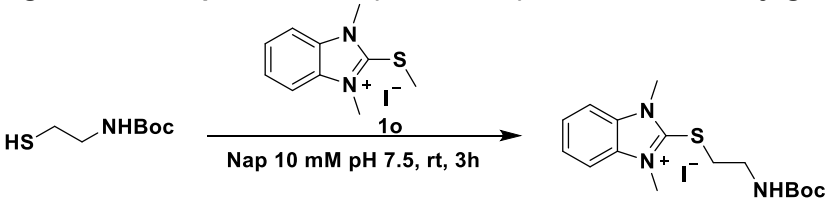


LC-MS spectrum of Ac-GCF with 1i

Compound	Mass (m/z)
Expected 1i-modified peptide	499.13
Observed 1i-modified peptide	522.10 [M+Na]



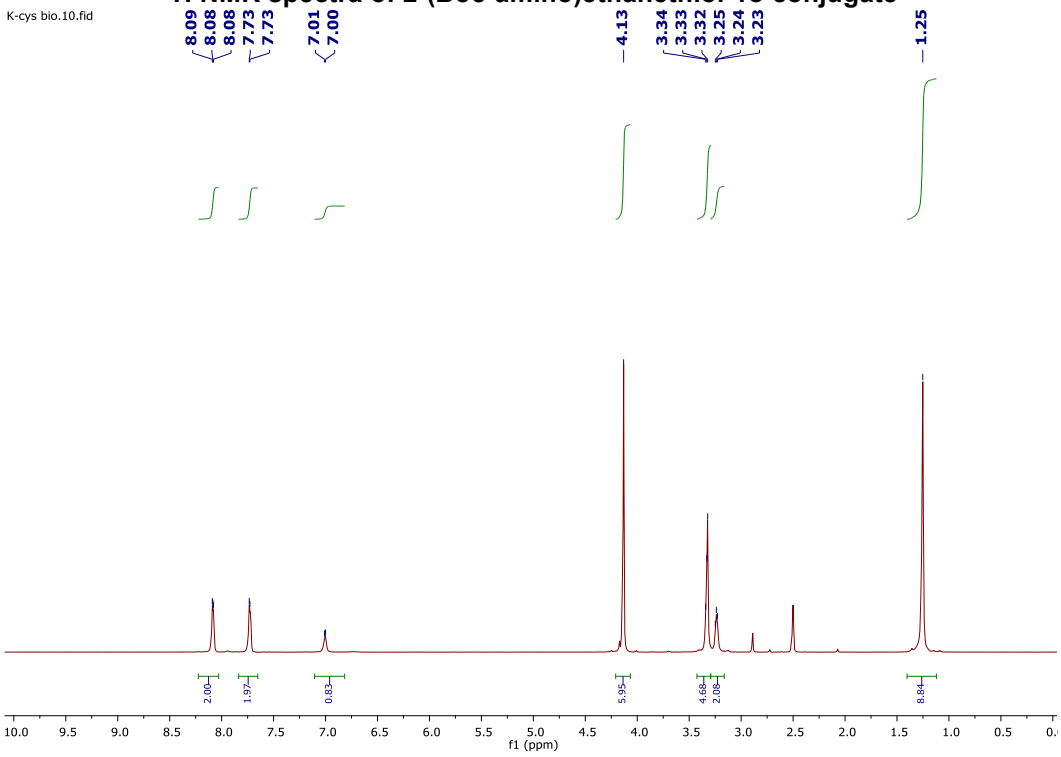
XIV. Supplementary Figure 7: NMR spectrum of 2-(Boc-amino)ethanethiol 1o conjugated compound



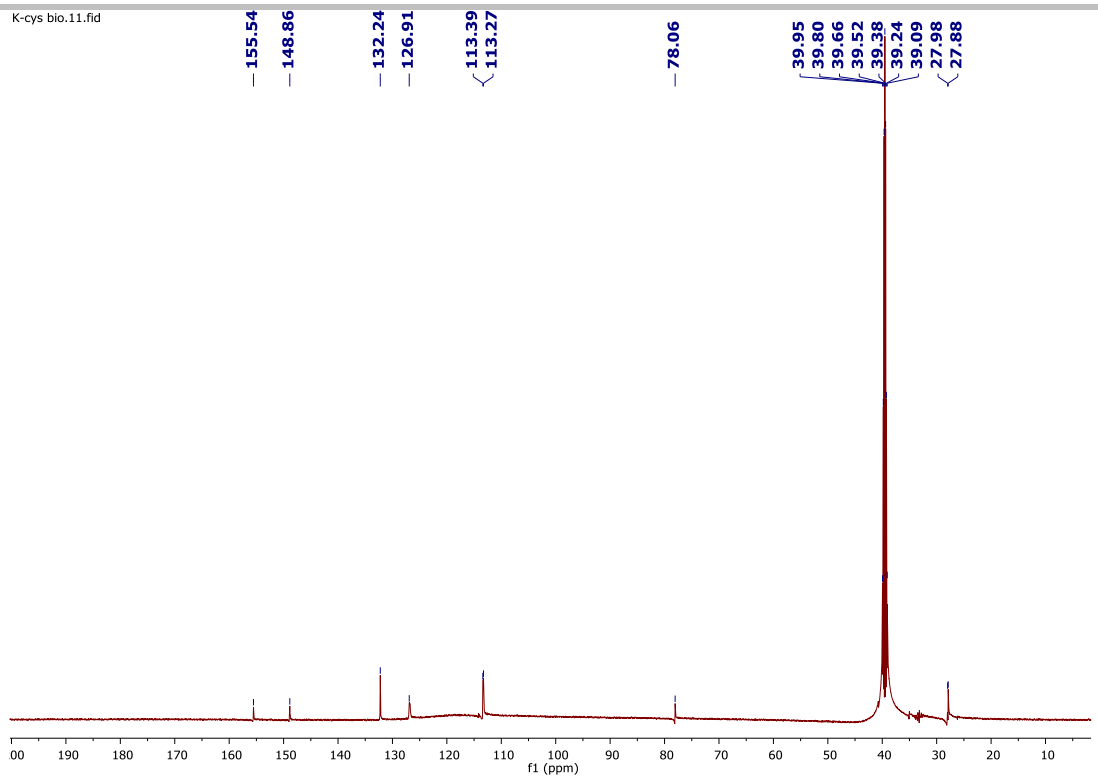
To a mixture of 2-(Boc-amino)ethanethiol (10 mg, 0.056 mmol) in Nap pH 7.5 (10 mM, 0.6 mL) **1o** (53.8 mg, 0.168 mmol) was added. The reaction mixture was incubated at room temperature for 3 h. The mixture was dried by lyophilization and purified by HPLC and characterized by NMR. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.09 – 8.08 (m, 2H), 7.73 – 7.72 (m, 2H), 7.01 – 7.00 (m, 1H), 4.13 (s, 6H), 3.32-3.24 (m, 4H), 1.27 (s, 9H). ^{13}C NMR (151

MHz, DMSO-*d6*) δ 155.55, 148.87, 132.24, 126.91, 126.77, 113.39, 113.27, 78.07, 35.05, 33.47, 33.19, 32.82, 32.68, 27.98, 27.89.

^{1}H NMR spectra of 2-(Boc-amino)ethanethiol-1o conjugate



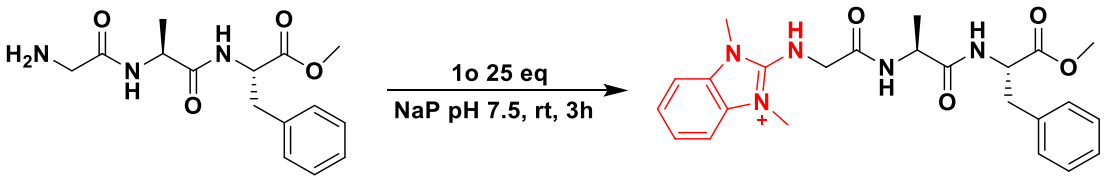
^{13}C NMR spectra of 2-(Boc-amino)ethanethiol-1o conjugate



XV. Supplementary Figure 8. General method for the verification of the chemo- and site-selective nature of probe 1o with GAFOMe.

Procedure for reactivity test of probe 1o with GAFOMe:

GAF-OMe (0.48 mg, 0.0015 mmol) was dissolved in 0.4 mL of 10 mM phosphate buffer (Nap, pH 7.5), then 25 equiv. of **1o** was added. The mixture was stirred at room temperature for 3 hours. The reaction was analyzed by HPLC and ESI-MS. HPLC was carried out with 1 % formic acid: water (solvent A): acetonitrile (solvent B); 0-80 % in 30 min, flow rate = 1.0 mL/min, detection wavelength 220 nm.



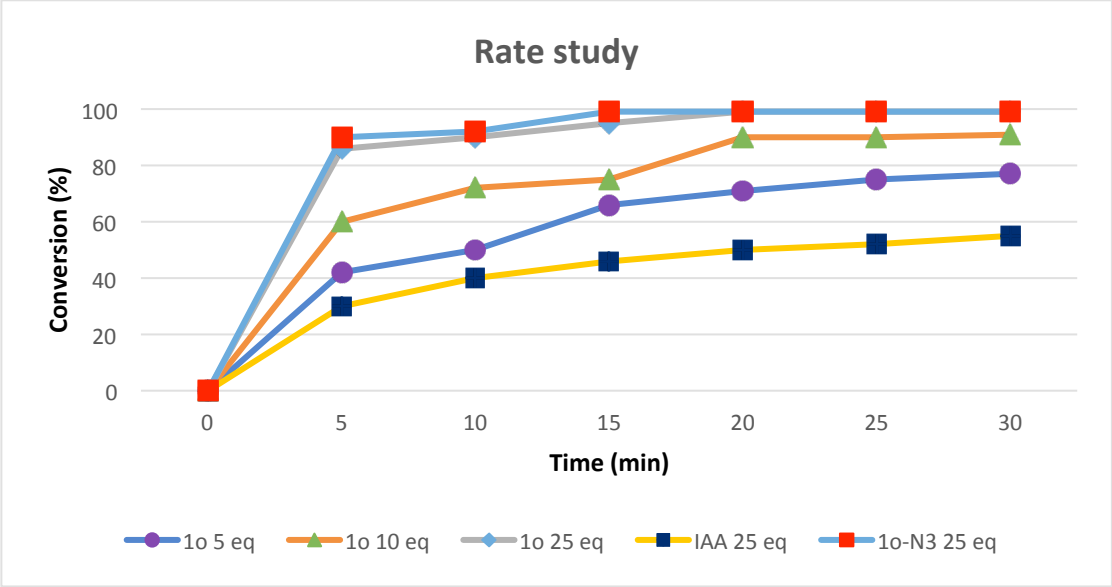
GAF-OMe **Modified GAF-OMe**

(The product was not observed)

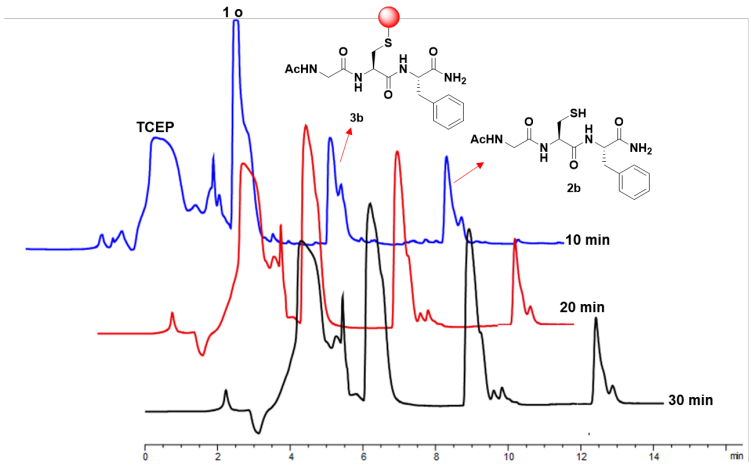
XVI. Supplementary Figure 9: Rate study of HAT bioconjugation.

Procedure for rate study of Ac-GCF with 1o, and N_3-1o. Comparison with IAA

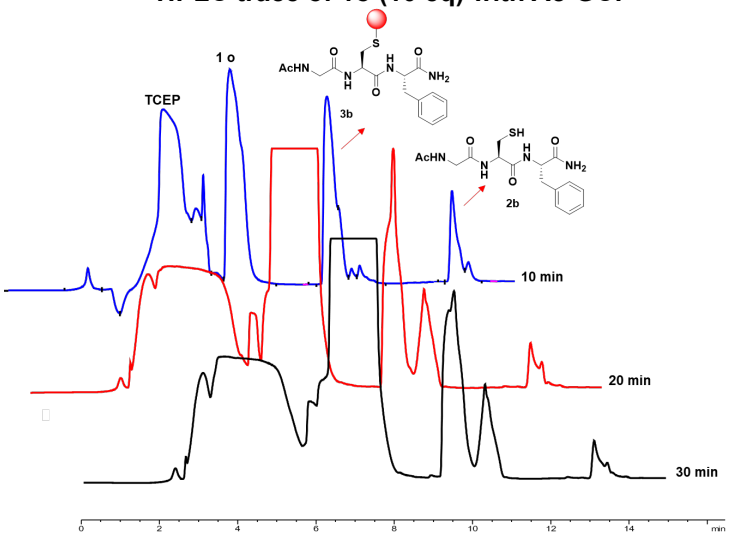
To a solution of Ac-GCF (1.14 mg, 3 mM) in 1 mL of 10 mM NaP (pH 7.5) was added probes (5-25 equiv., 15-75 mM). For time analysis, a sample (100 µL) was taken from the mixture after regular intervals of time and quenched by freezing the sample at -80 °C. The frozen samples were then lyophilized and dissolved in 100 µL of 1:1 H_2O/ACN and injected immediately into the HPLC for determining the % conversion to Ac-GCF-1o or Ac-GCF-N3-1o or Ac-GCF-IAA (X Terra C18 column {5 µm} with a gradient of 0 to 80% MeCN with 0.1% formic acid in 30 min). The rate study was done in triplet. We use average of three trials to plot the rate curve. 0 min sample is sample taken after addition of all the reagents of the bioconjugate reaction.



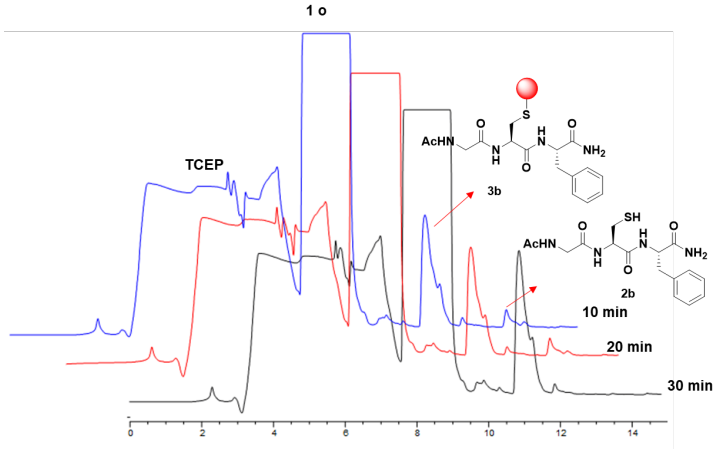
HPLC trace of 1o (5 eq) with Ac-GCF



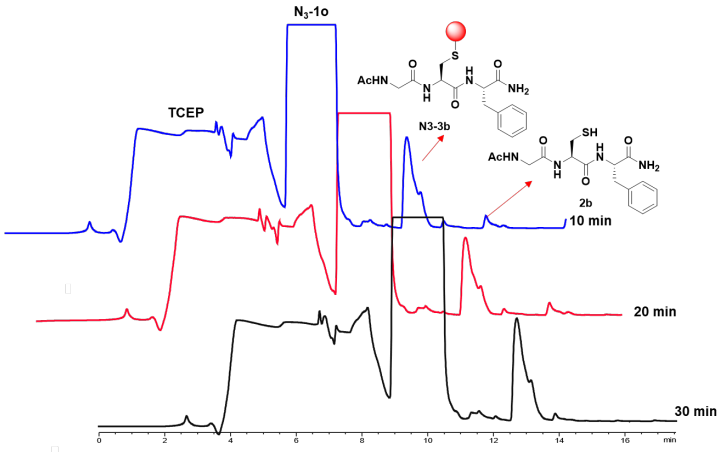
HPLC trace of 1o (10 eq) with Ac-GCF



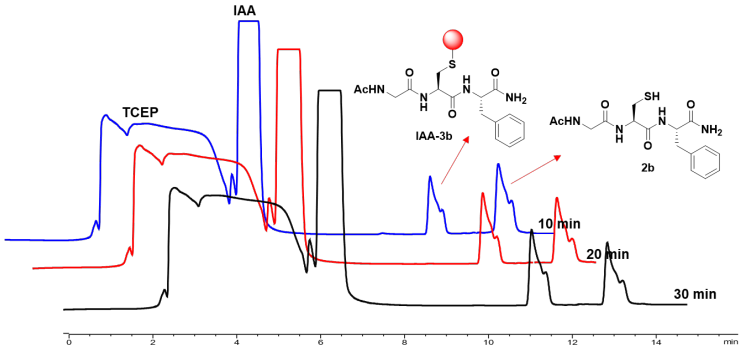
HPLC trace of 1o (25 eq) with Ac-GCF



HPLC trace of N_3-1o (25 eq) with Ac-GCF



HPLC trace of IAA (25 eq) with Ac-GCF

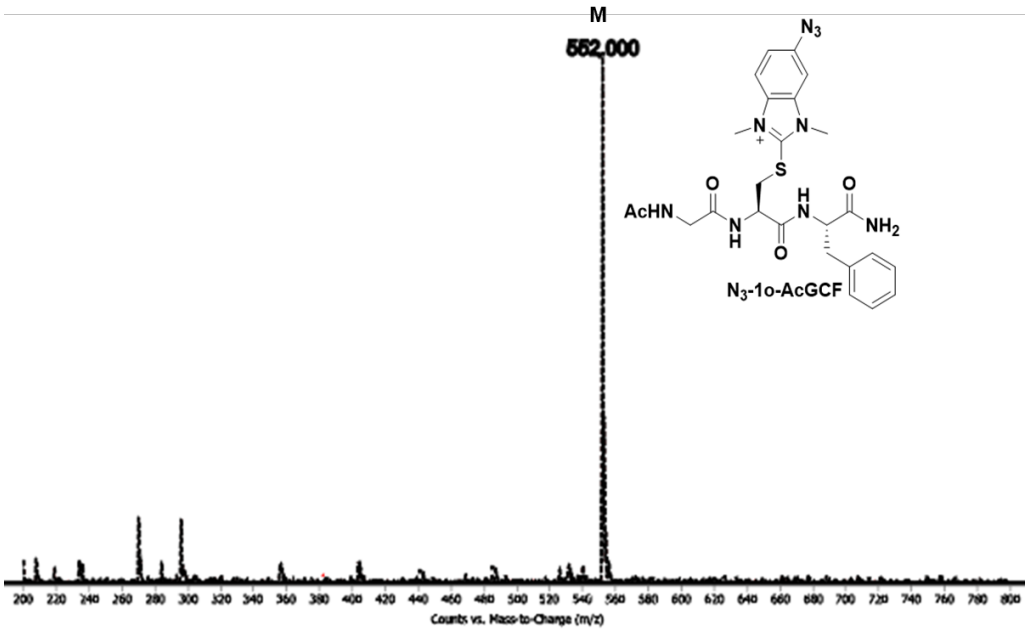


General procedure of Ac-GCF bioconjugation with probe N_3-1o

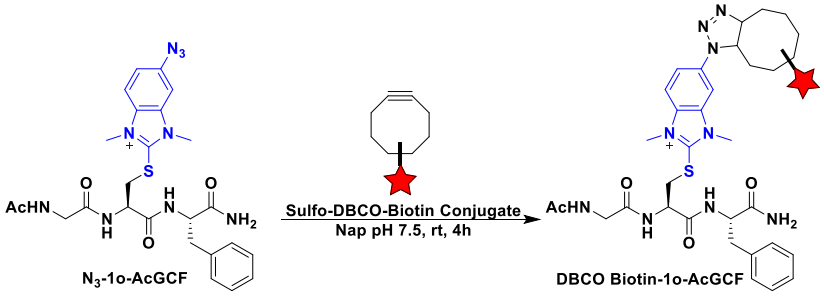
Ac-GCF **2a** (0.56 mg, 0.0015 mmol, mM) was dissolved in 0.4 mL of 10 mM phosphate buffer (Nap, pH 7.5), 20 μL of TCEP solution (pH 7.5, 50 mM) was added to prevent the disulfide bond formation, then 25 eq of **N_3-1o** (0.0375 mmol) was added. The mixture was stirred at room temperature for 3 hours. The reaction was analyzed by HPLC and ESI-MS. HPLC was carried out with 1 % formic acid: water (solvent A): acetonitrile (solvent B); 0-80 % in 30 min, flow rate = 1.0 mL/min, detection wavelength 220 nm.

ESI-MS of N_3-1o-AcGCF

Compound	Mass (m/z)
Expected N_3-1o-AcGCF	552.21
Observed N_3-1o-AcGCF	552.00

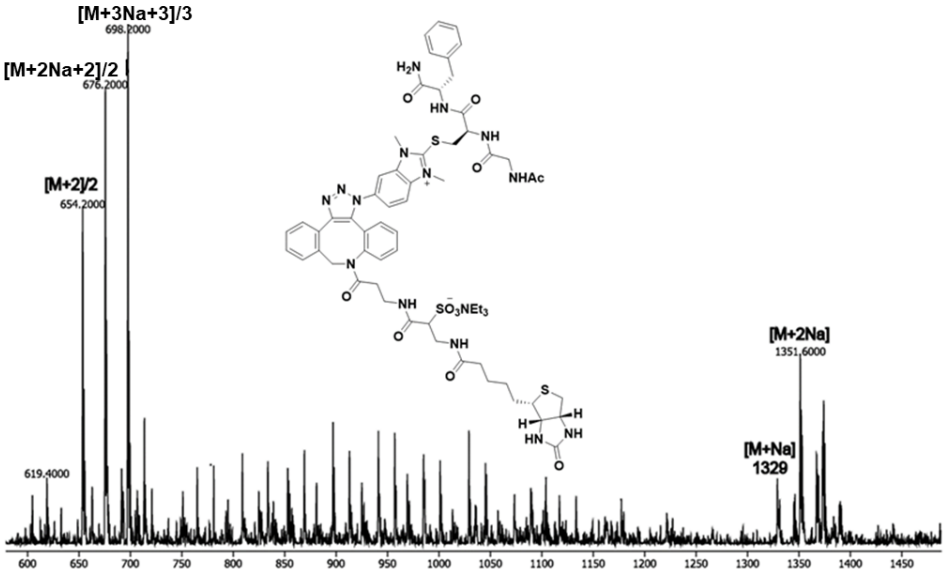


General procedure for the SPACC enrichment of modified peptides:
To a solution of pure N_3-1o-AcGCF conjugate compound (6.3 mM) in 10 mM Nap buffer pH 7.5 (400 µL), sulfo-DBCO-Biotin (1 eq., 6.3 mM) was added. The reaction was incubated at 25 ^{o}C for 4 h. The reaction mixture was analyzed by ESI-LCMS.

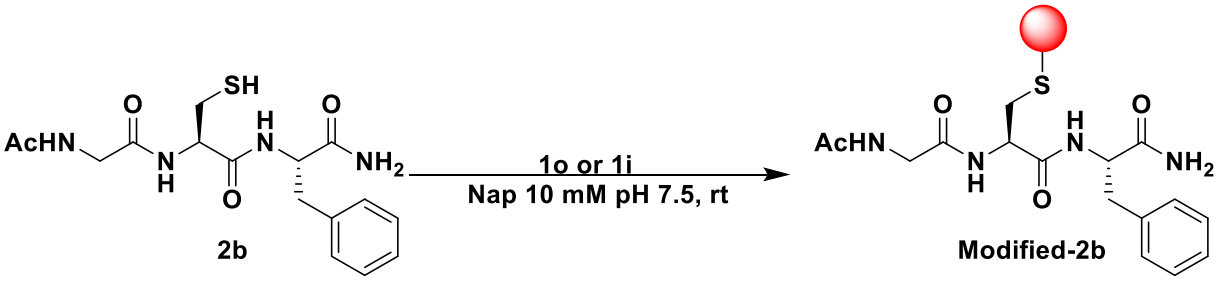


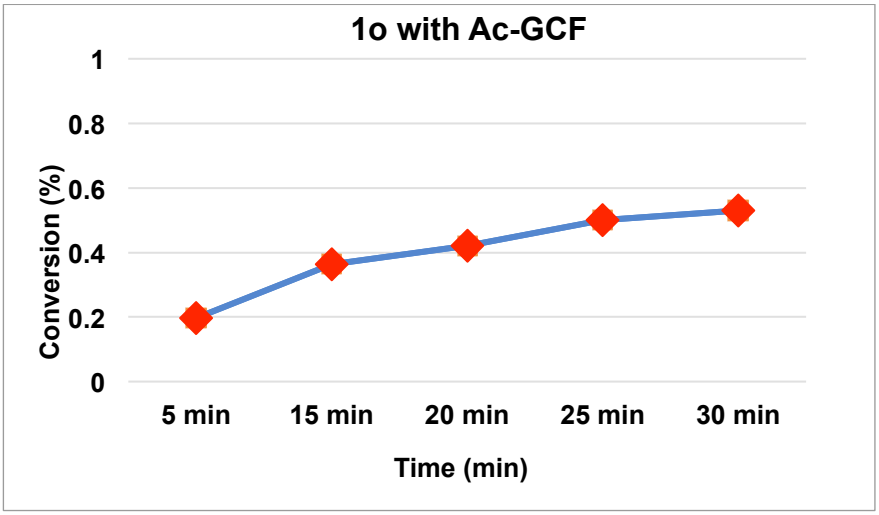
ESI-MS of DBCO-Biotin-1o-AcGCF

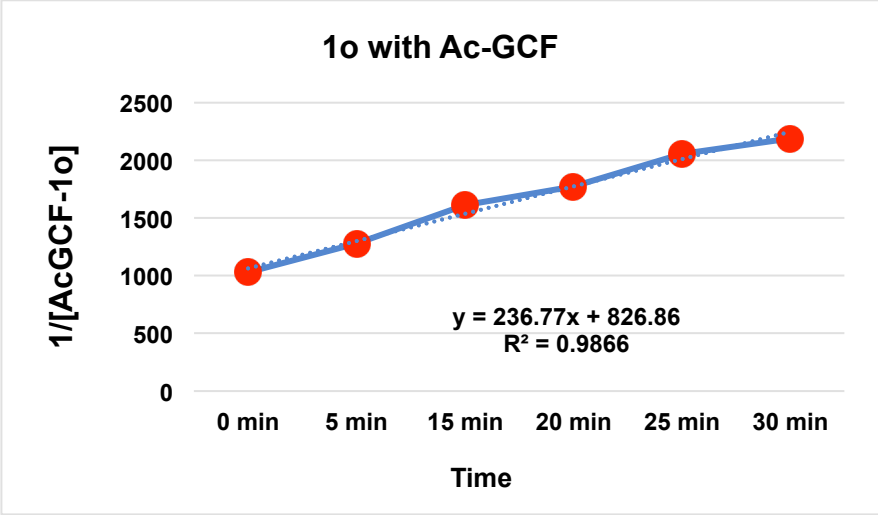
Compound	Mass (m/z)
Expected modified peptide	1306.22
Observed modified peptide	1329 [M+2Na], 1329 [M+Na], 698 [M+3Na+3]/3, 676 [M+2Na+2]/2, 654 [M+2]/2

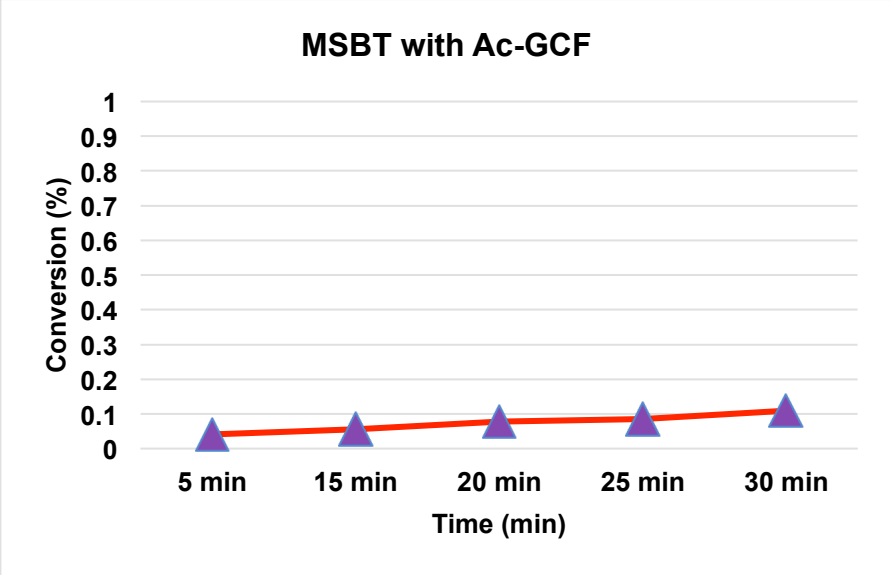


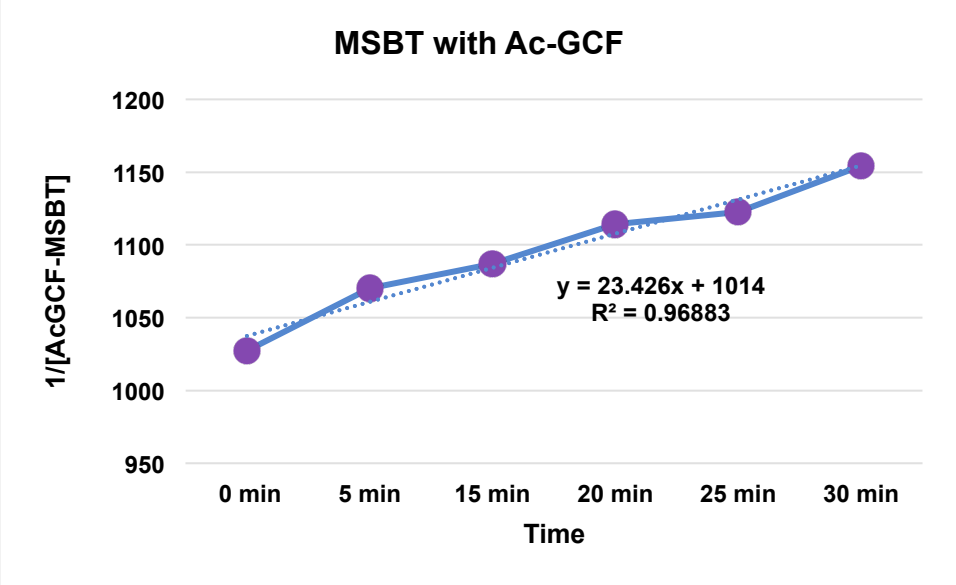
XVII. Supplementary Figure 10. Rate study of 1o and 1i with peptide Ac-GCF 2a.
To a solution of Ac-GCF **2c** (0.56 mg, 0.0015 mmol, 0.973 mM) in 1.6 mL of 10 mM phosphate buffer (Nap, pH 7.5) was added **1o** or **1i** (1 eq, 0.0015 mmol, 0.973 mM). For analysis, a sample (200 µL) was taken from the mixture after regular intervals of time and quenched by freezing the sample at -80 °C. The frozen samples were then lyophilized and dissolved in 100 µL of 1:1 H_2O/ACN and injected immediately into the HPLC for determining the % conversion to the modified product (X Terra C18 column {5 µm} with a gradient of 0 to 80 % MeCN with 0.1 % formic acid in 30 min). The rate study was done in triplicate. We use the average of three trials to plot the rate curve. 0 min sample was taken immediately after addition of all the reagents of the bioconjugation reaction. The result showed that 1o, 1i bioconjugate reactions are second order reaction with **k = 236.77 $M^{-1}S^{-1}$**, **23.43 $M^{-1}S^{-1}$** respectively.





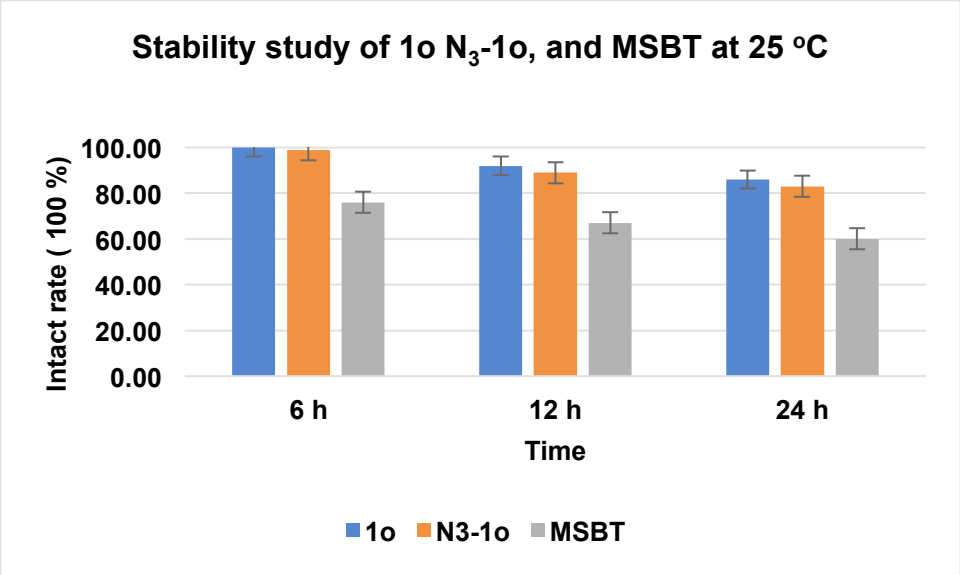




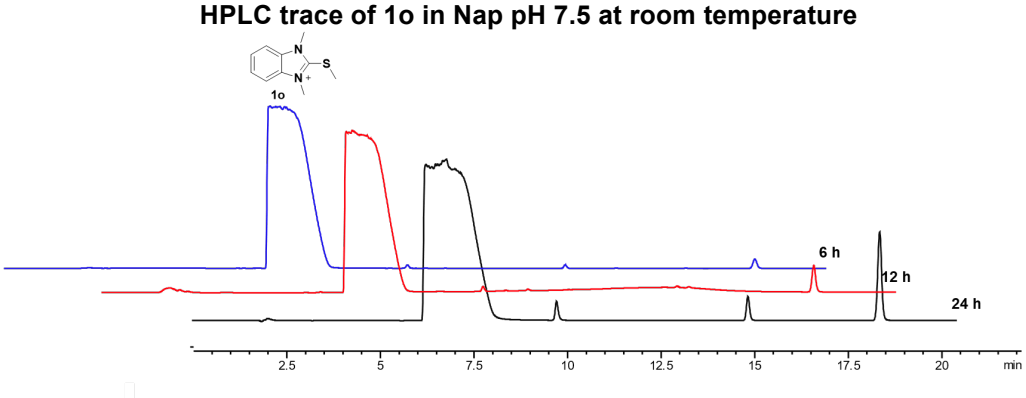


XVIII. Supplementary Figure 11. Stability study of 1o, N_3-1o and 1i
Procedure for stability study of probes. Probes **1o**, **N_3-1o** and **1i** (38.75 mM) was incubated in 400 µL of 10 mM Nap (pH 7.5) at room temperature. A sample (50 µL) was taken from the mixture and directly injected into

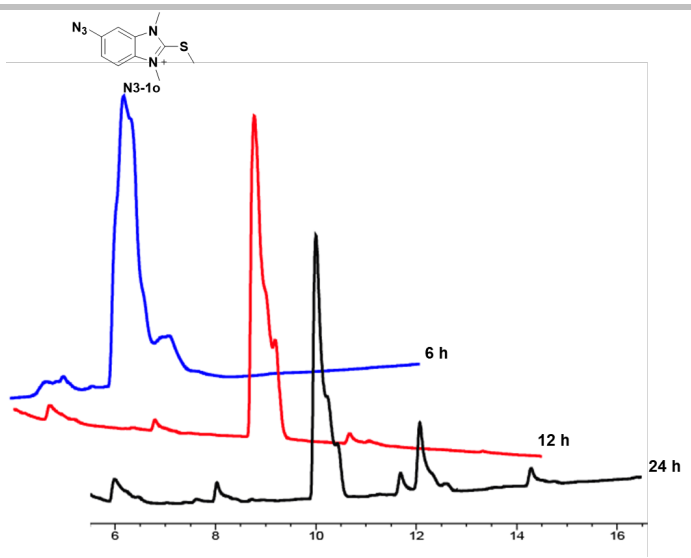
HPLC. The reaction was monitored by injecting samples in HPLC after regular intervals of time 6 h, 12 h and 24 h.



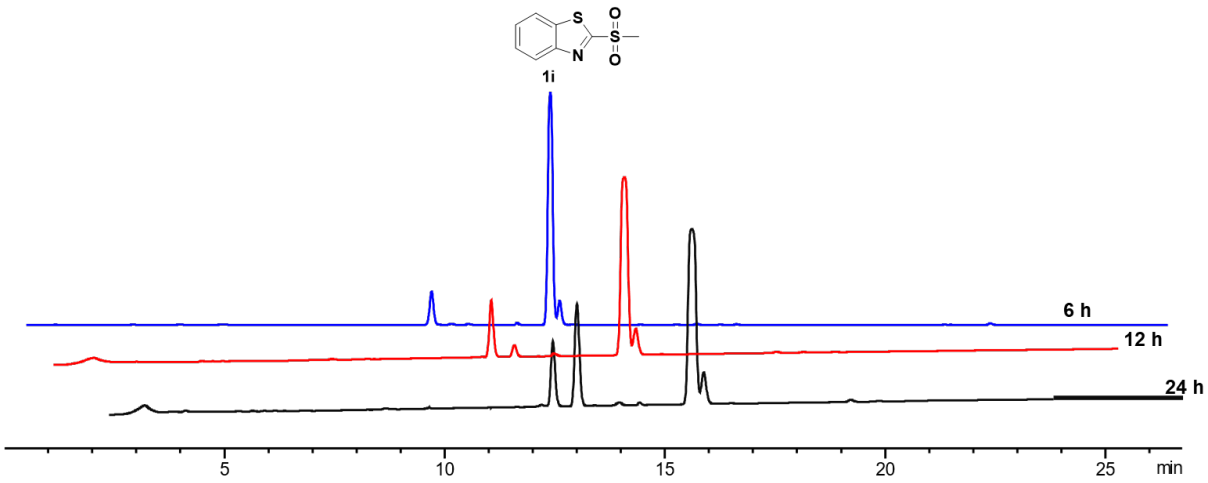
HPLC trace of 1o in Nap pH 7.5 at room temperature



HPLC trace of N3-1o in Nap pH 7.5 at room temperature



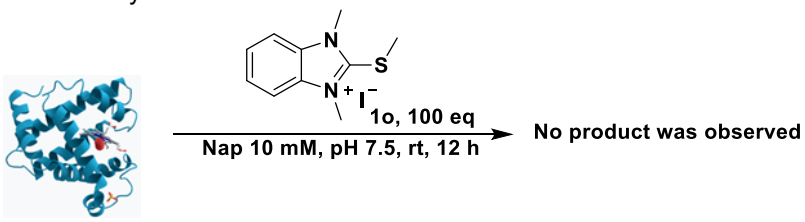
HPLC trace of 1i in Nap pH 7.5 at room temperature



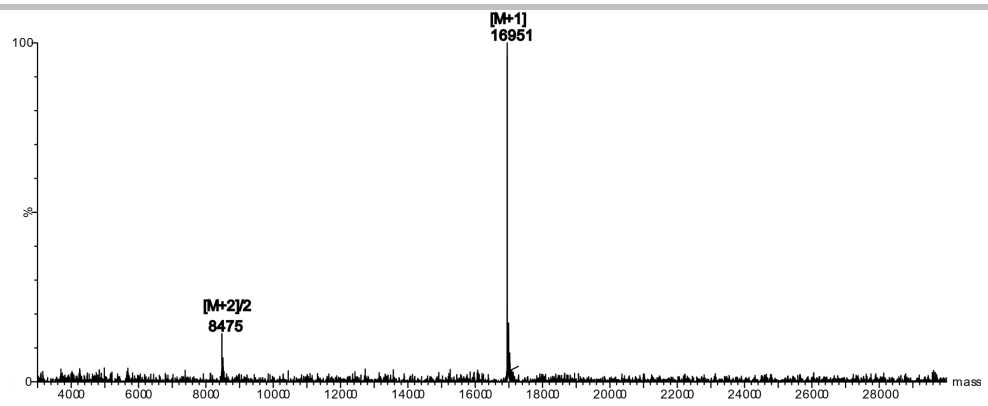
XIX. Supplementary Figure 12. Confirmation of the high chemo-selectivity of probe 1i, 1m, and 1o towards cysteine by reaction with proteins.

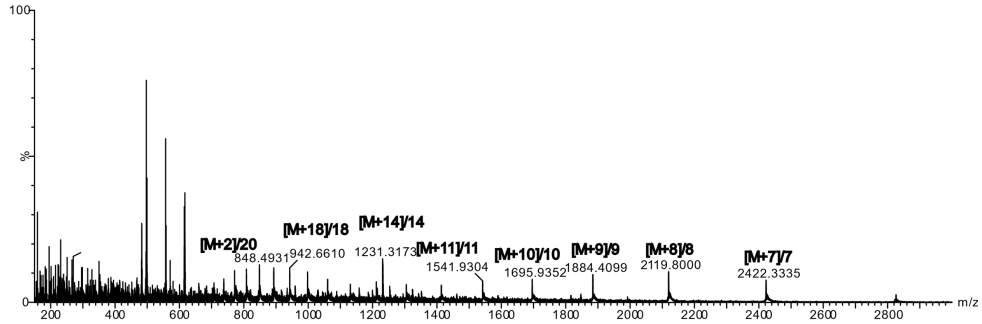
General procedure for bioconjugation of HAT probes with myoglobin

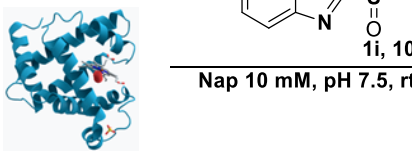
To a 1 mg of myoglobin (0.15 mM) in 400 µL 10 mM NaP (pH 7.5), probe **1i, or 1m or 1o** (100 equiv. 15 mM) was added. The reaction was incubated at room temperature for 12 h. The reaction mixture was purified by molecular weight cut off and characterized by LCMS.

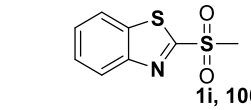


Compound	Mass (m/z)
Expected unmodified myoglobin	16950
Observed unmodified myoglobin	16951







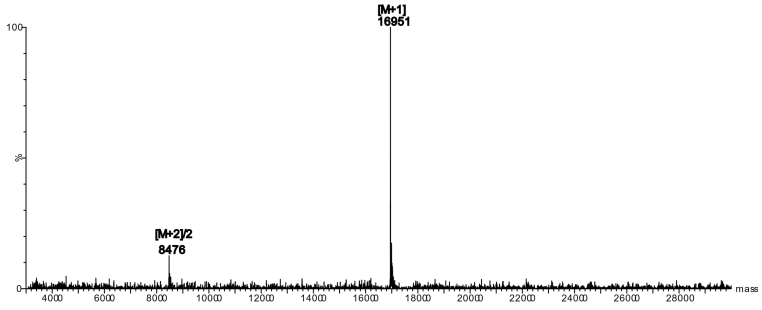


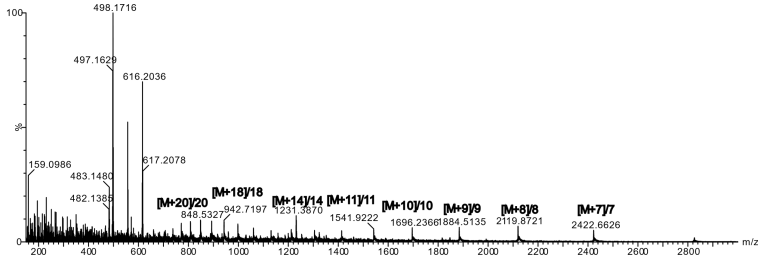
1i, 100 eq

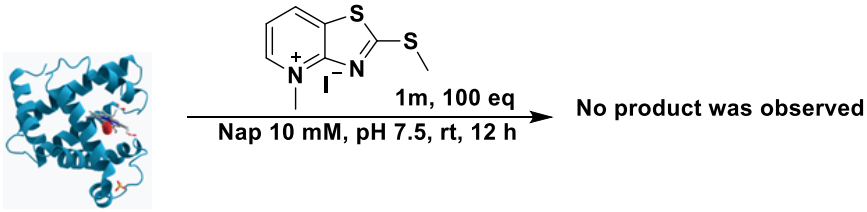
Nap 10 mM, pH 7.5, rt, 12 h

No product was observed

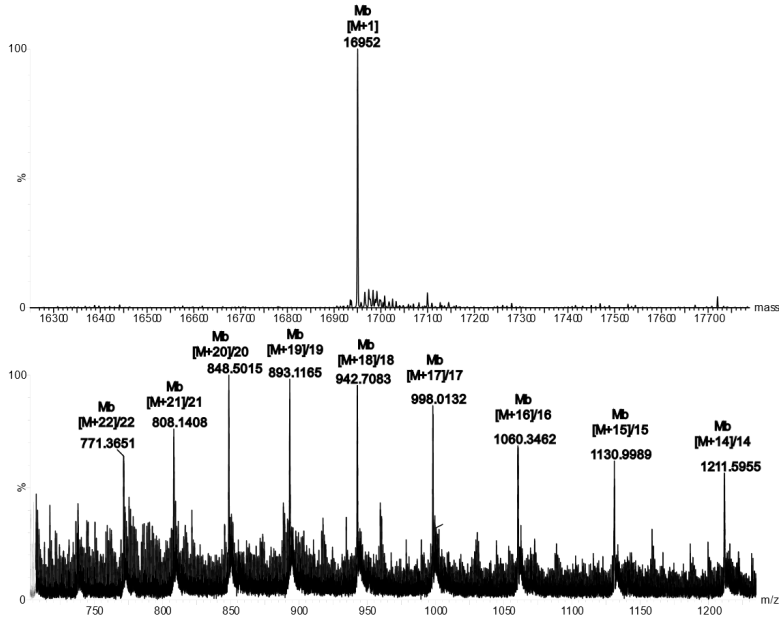
Compound	Mass (m/z)
Expected unmodified myoglobin	16950
Observed unmodified myoglobin	16951







Compound	Mass (m/z)
Expected unmodified myoglobin	16950
Observed unmodified myoglobin	16952

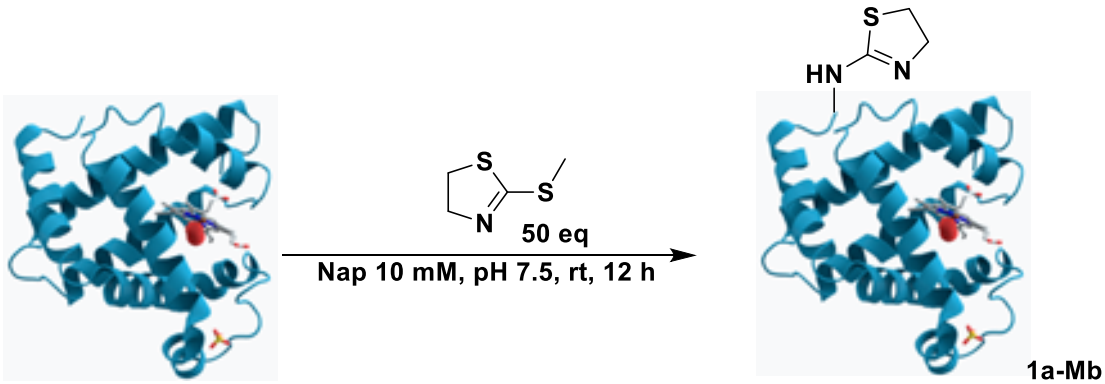


XX. Supplementary Figure 13. Modification of myoglobin Mb with different heteroaromatic azoline compounds 1a-1c, 1j, and 1n

Probe[a]	Eq	Time (h)	Site of modification	Conversion (%)[b]
1a	50	12	N-terminal	33
1b	50	12	Lysine	81
1c	50	12	N-terminal	70
1j	10	8	Lysine	56
1n	10	1	Lysine	99

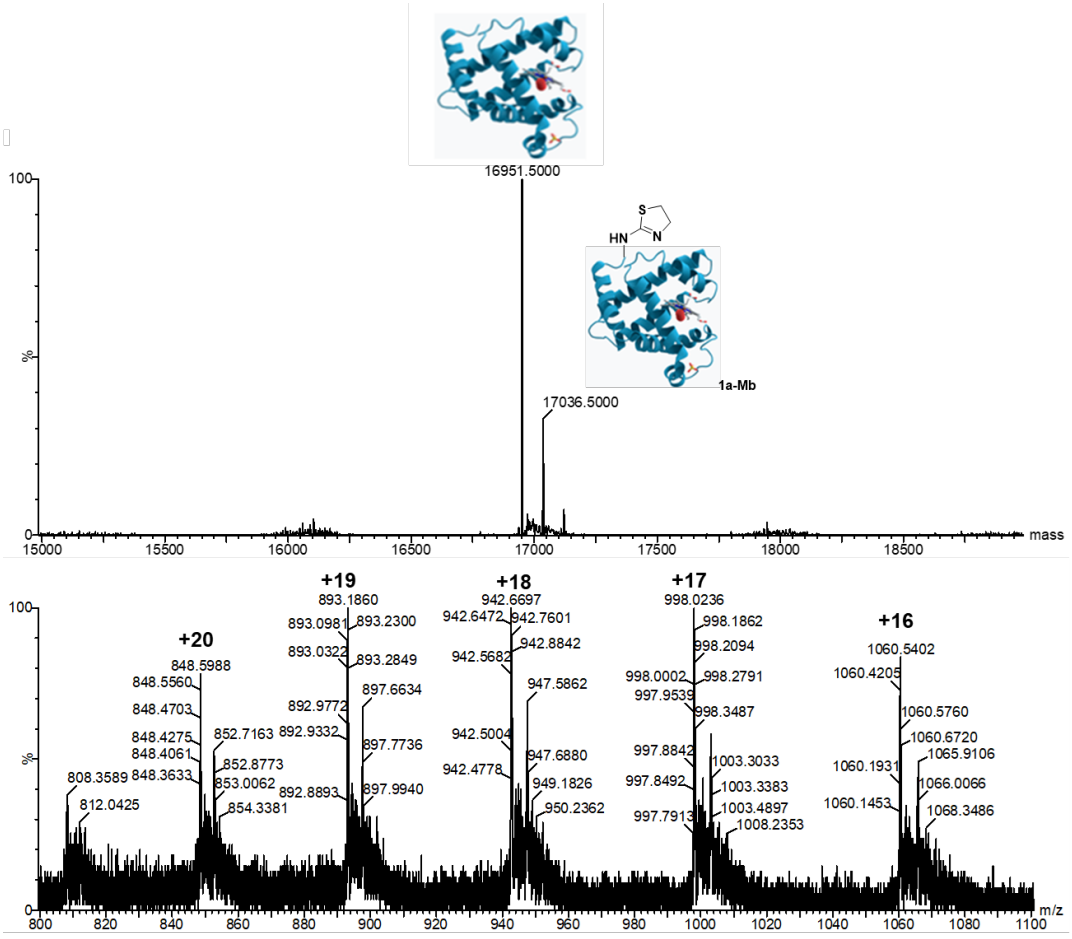
[a]Condition: Protein Mb (60 μmol, 0.15 mM) in 10 mM phosphate buffer of pH 7.5 (400 μL) and probe (10-50 equiv.) was incubated for 1-12 h at 25 °C. [b]The conversion was calculated based on the relative peak intensity of native protein and labeled protein in the deconvoluted mass spectrum.

Modification of Mb with 1a

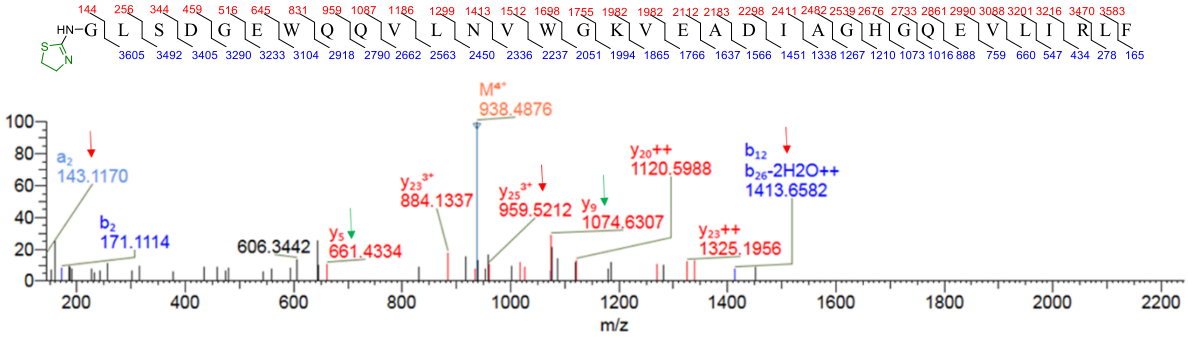


HRMS of 1a-Mb

Compound	Mass (m/z)
Expected unmodified myoglobin	16950
Observed unmodified myoglobin	16951.5000
Expected mono-modified myoglobin	17035
Observed mono-modified myoglobin	17036.5000



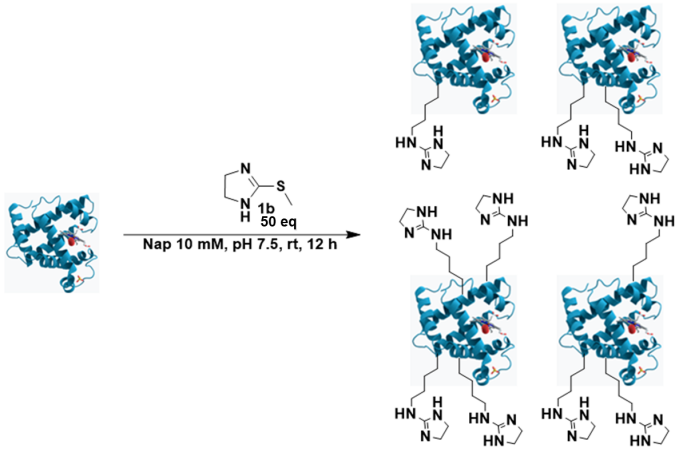
MS/MS spectrum 1a-Mb fragment



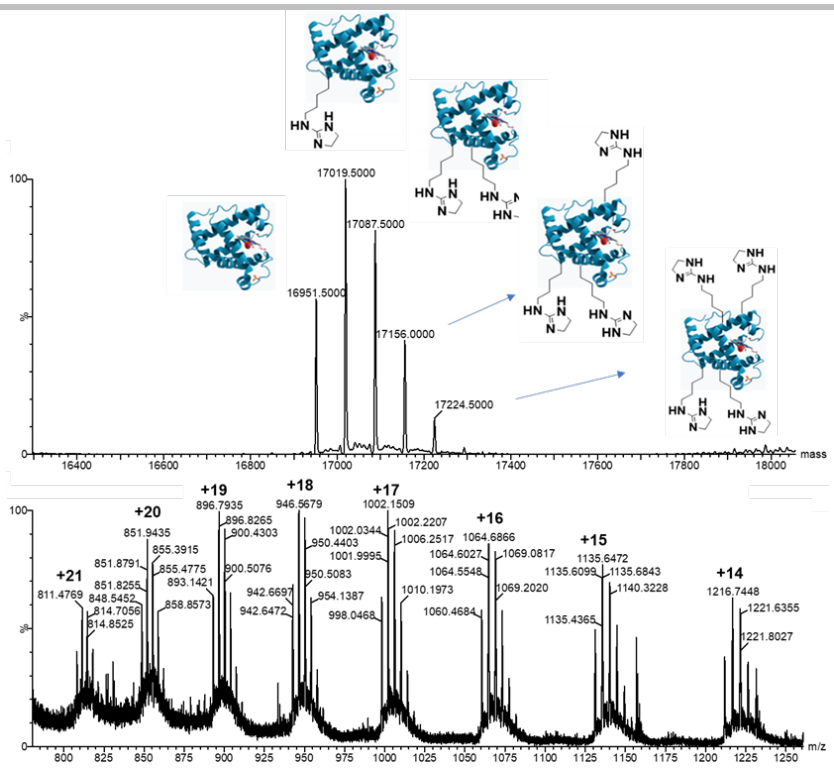
Modification of 1b-Mb

Number of modifications	Expected mass (m/z)	Observed mass (m/z)
1	17018	17019.5000
2	17086	17087.5000
3	17154	17156.0000

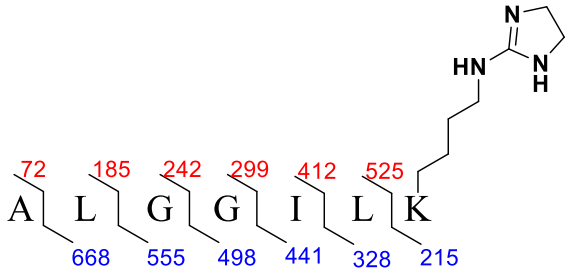
4	17222	17224.5000

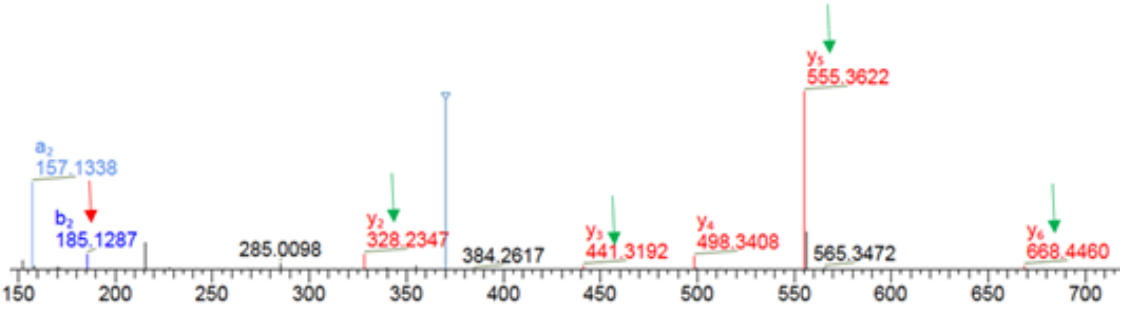


HRMS of 1b-Mb

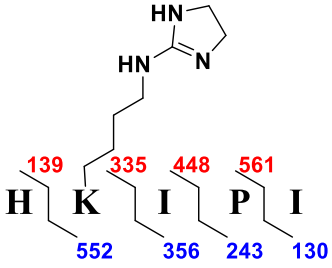


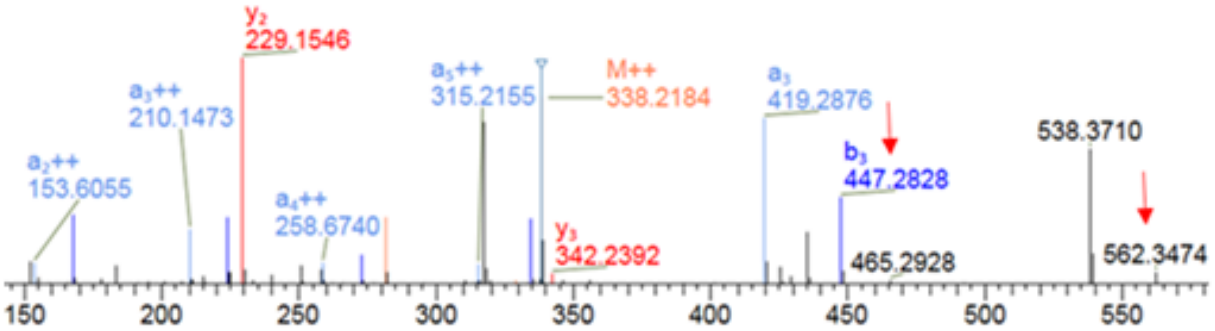
MS/MS spectrum 1b-Mb fragment I



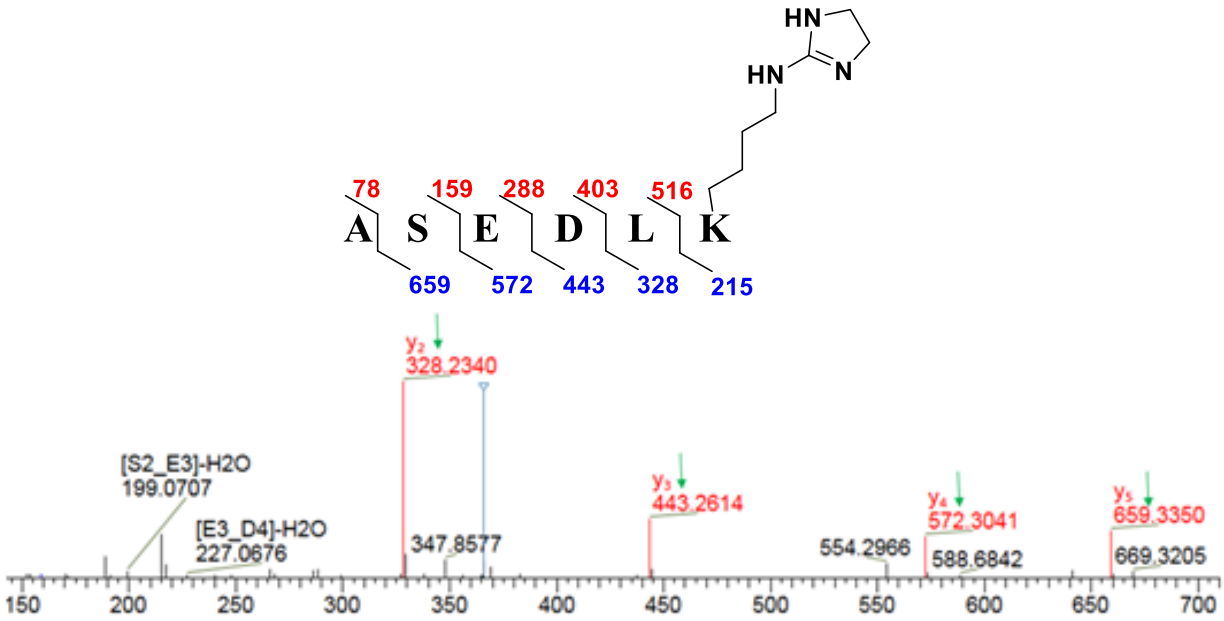


MS/MS spectrum 1b-Mb fragment II

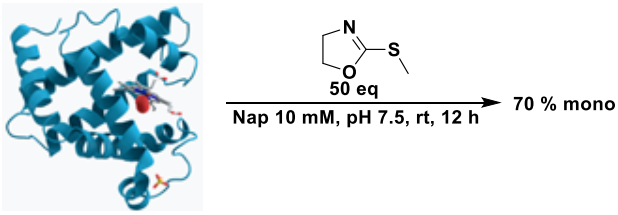




MS/MS spectrum 1b-Mb fragment III

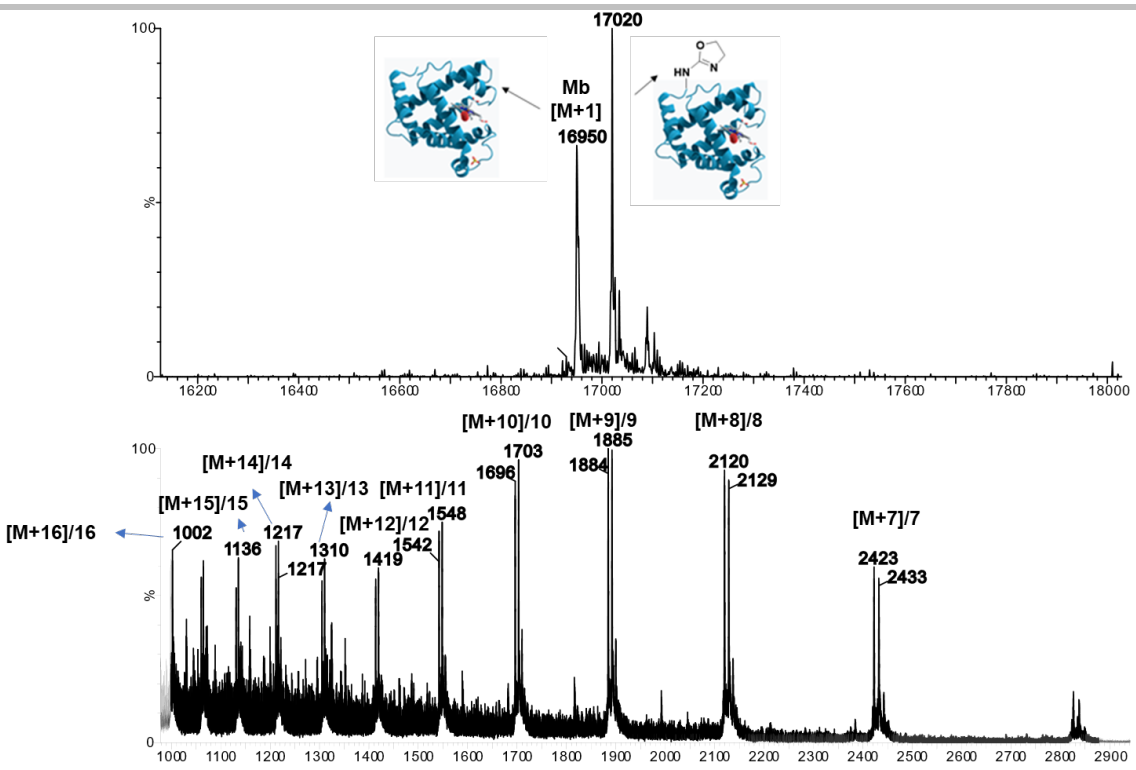


Modification of 1c with Mb

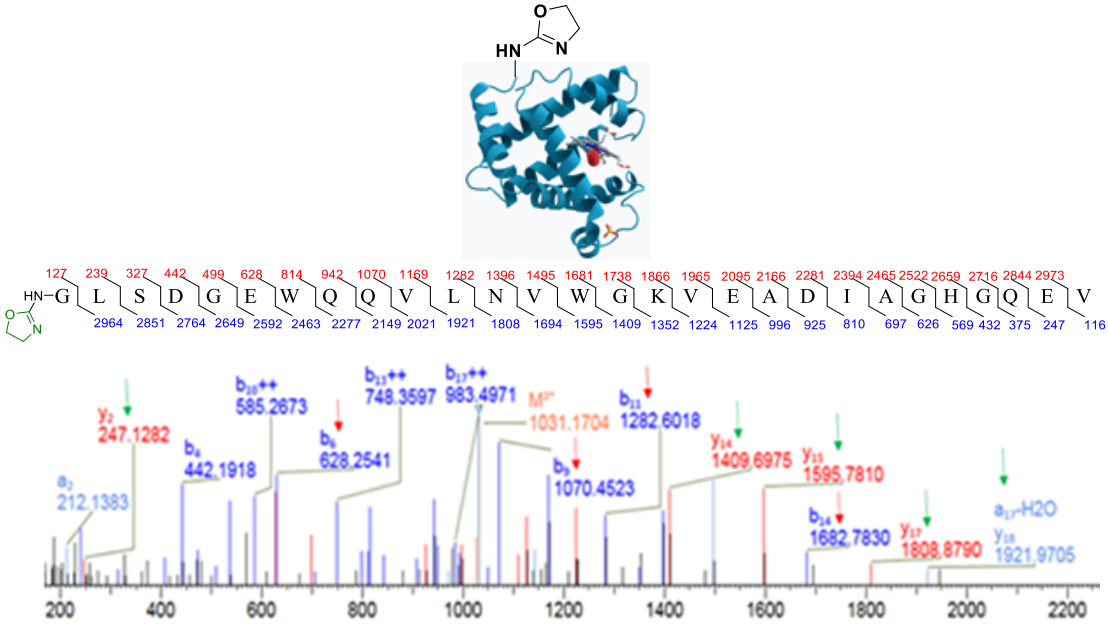


HRMS of 1c-Mb

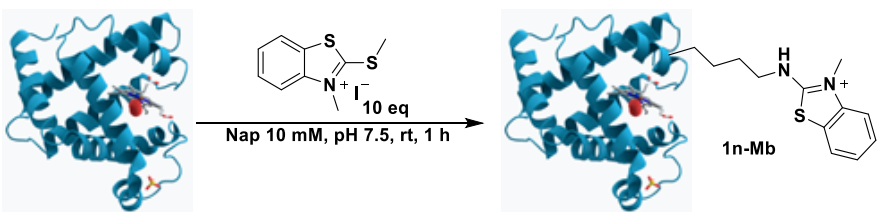
Compound	Mass (m/z)
Expected unmodified myoglobin	16950
Observed unmodified myoglobin	16950
Expected mono-modified myoglobin	17019
Observed mono-modified myoglobin	17020



ESI MS-MS spectrum of 1c-Mb

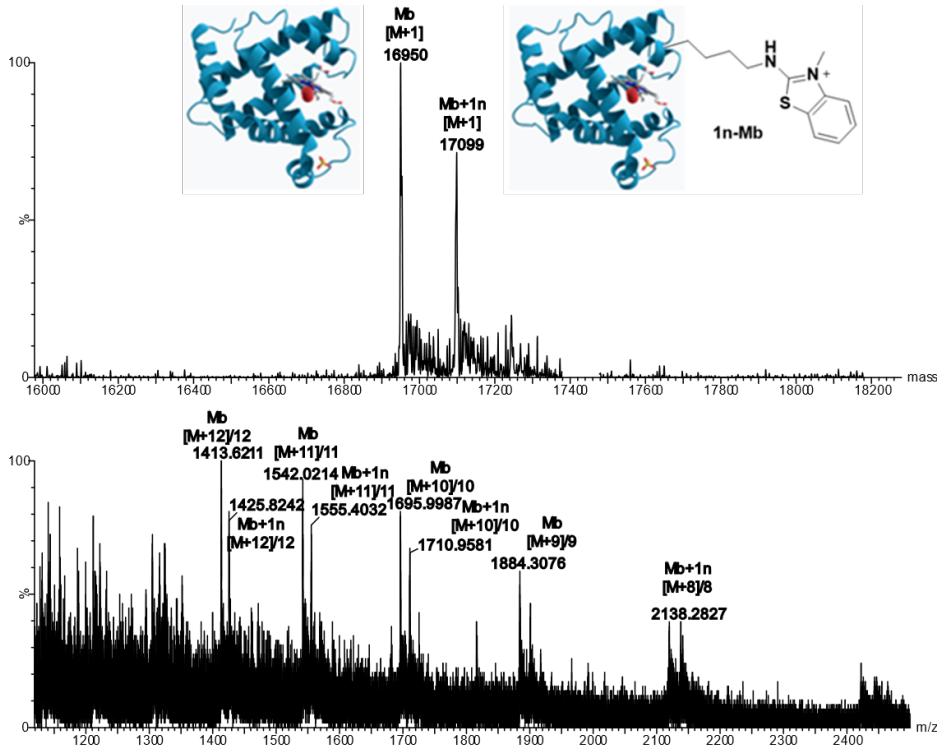


Modification of Mb with 1n

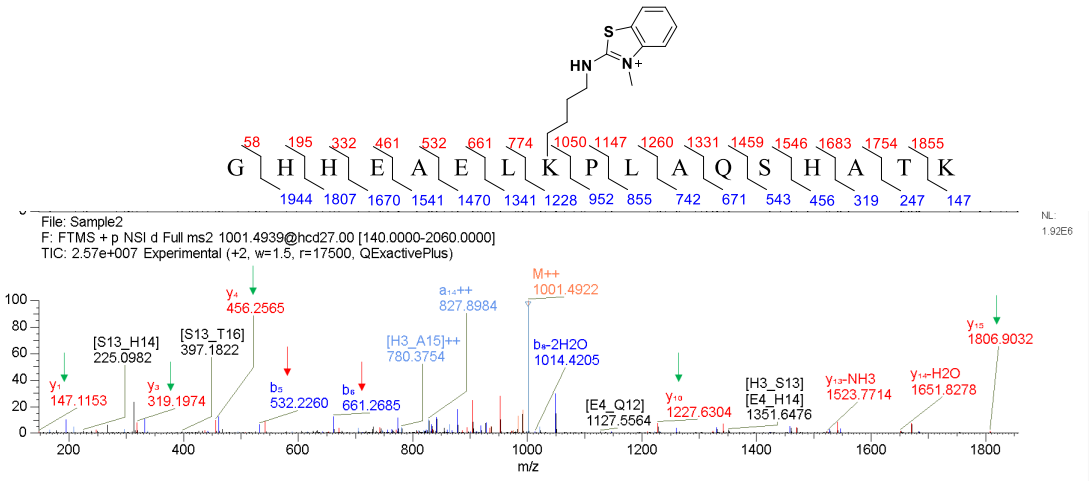


HRMS of 1n-Mb

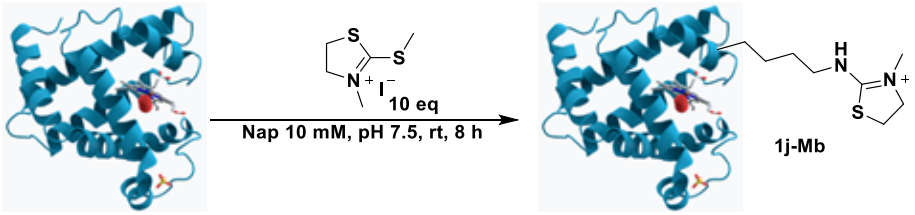
Compound	Mass (m/z)
Unmodified myoglobin	16950
Mono-modified myoglobin	17098
Observed mass	17099



MS/MS spectrum 1n-Mb fragment

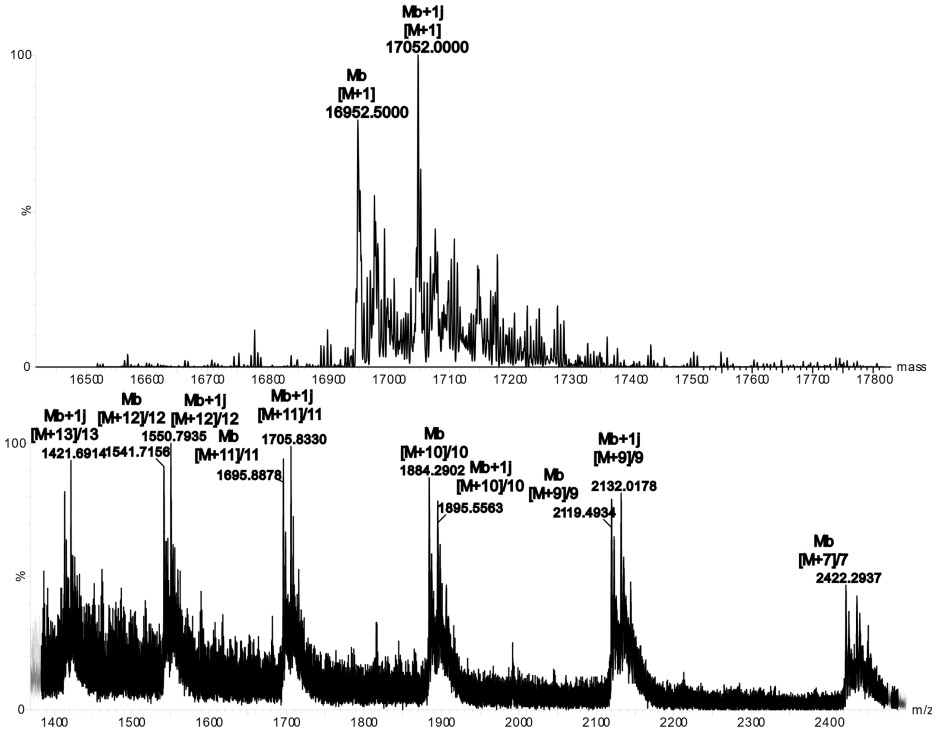


Modification of Mb with 1j

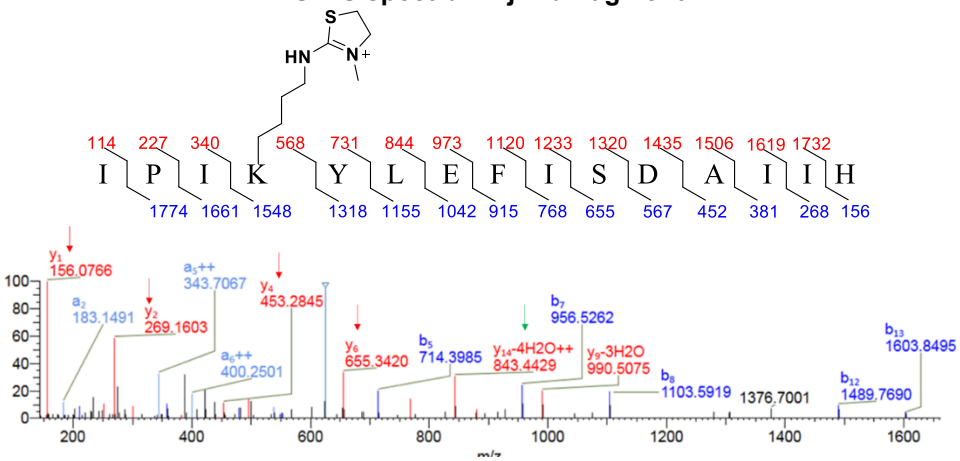


HRMS of 1j-Mb

Compound	Mass (m/z)
Expected unmodified myoglobin	16950
Observed unmodified myoglobin	16952.5000
Expected mono-modified myoglobin	17051
Observed mono-modified myoglobin	17052.0000



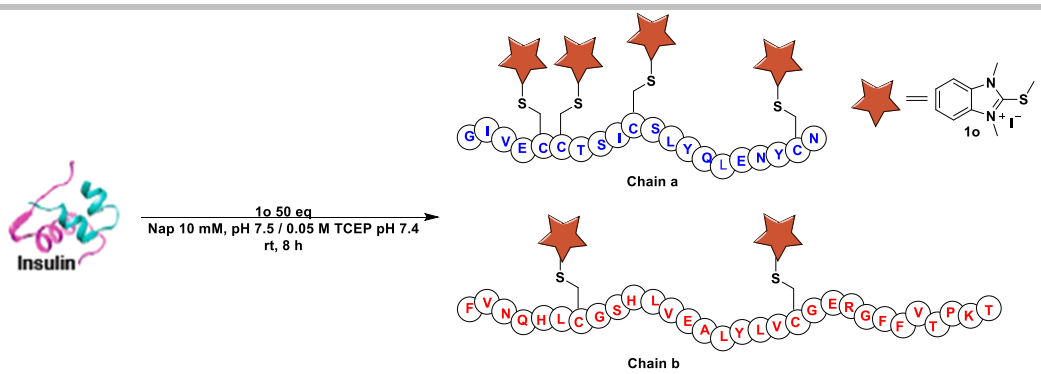
MS/MS spectrum 1j-Mb fragment



XXI. Supplementary Figure 14. Selective cysteine bioconjugation of Insulin a chain and b chain with compound 1o

Procedure for labeling of the reduced insulin.

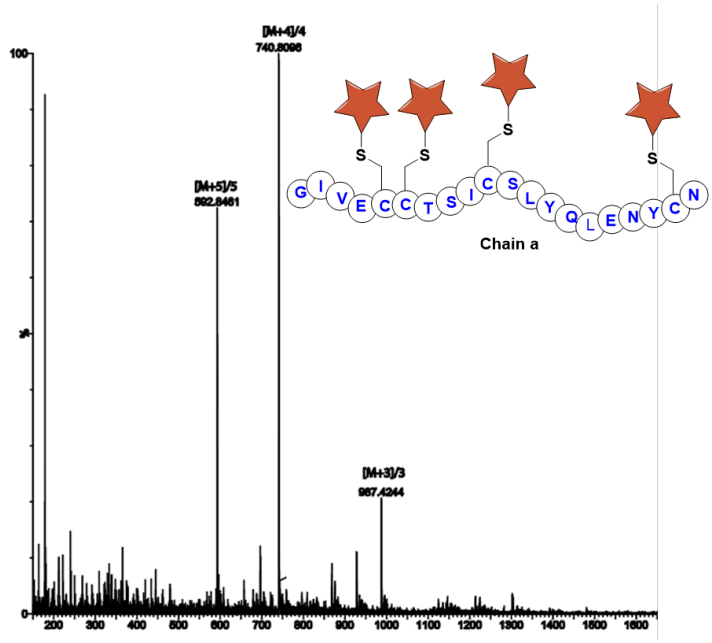
To 0.35 mg of Insulin (60 μmol, 0.15 mM) in 400 μL 10 mM Nap (pH 7.5), 20 μL of TCEP solution (pH 7.5, 50 mM) was added to reduce the insulin. The mixture was incubated at room temperature for 20 min. Probe **1o** (50 equiv.) was added into this mixture and the reaction was allowed to react at room temperature for 8 h. The **1o-chain a**, and **1o-chain b** bioconjugates were characterized by LCMS.



HRMS of modified chain a

Fragmentation of tetra-modified a chain	Expected mass (m/z)	Observed mass (m/z)
3	592	592.8461
4	740	740.8096
5	988	987.4244

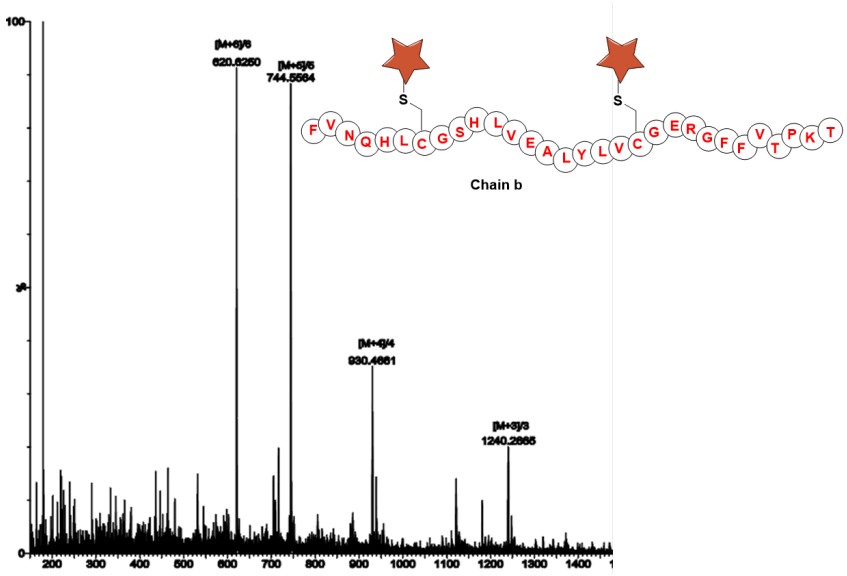
Modified chain a



HRMS of modified chain b

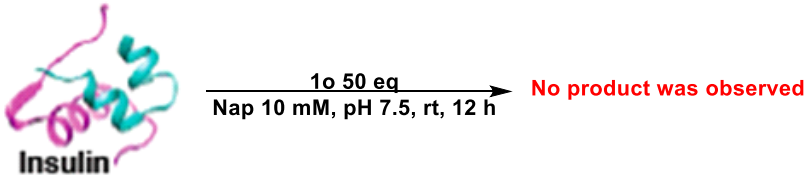
Fragmentation of double-modified chain b	Expected mass (m/z)	Observed mass (m/z)
3	1240	1240.2665
4	930	930.4661
5	744	744.5564
6	620	620.6260

Modified chain b



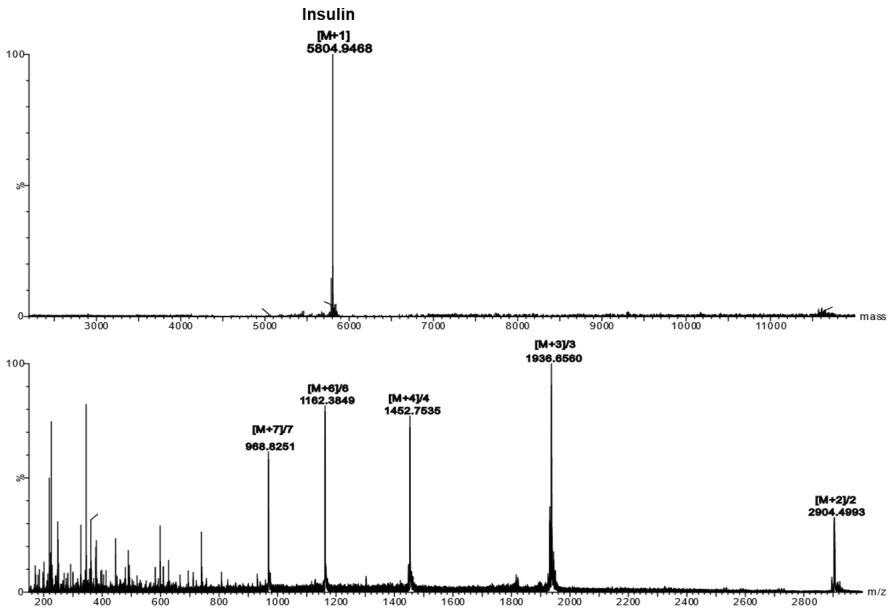
Procedure for the modification of intact insulin with compound 1o

To a mixture of Insulin (0.35 mg, 60 µmol, 0.15 mM) in 400 µL 10 mM Nap (pH 7.5), probe **1o** (0.96 mg, 3 mmol) was added. The reaction mixture was incubated at room temperature for 12 h. The crude was analyzed by LCMS. No modification of intact insulin was observed under the reaction conditions confirming high selectivity of probe for cysteine.



Compound	Mass (m/z)
Expected unmodified insulin	5804
Observed unmodified insulin	5804.9468

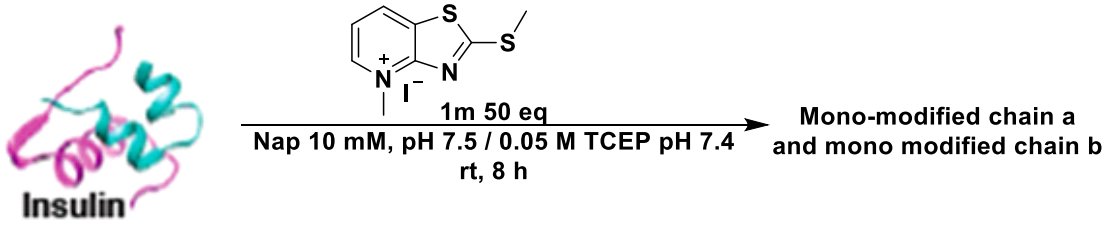
.



Procedure for labeling of the reduced insulin with 1m

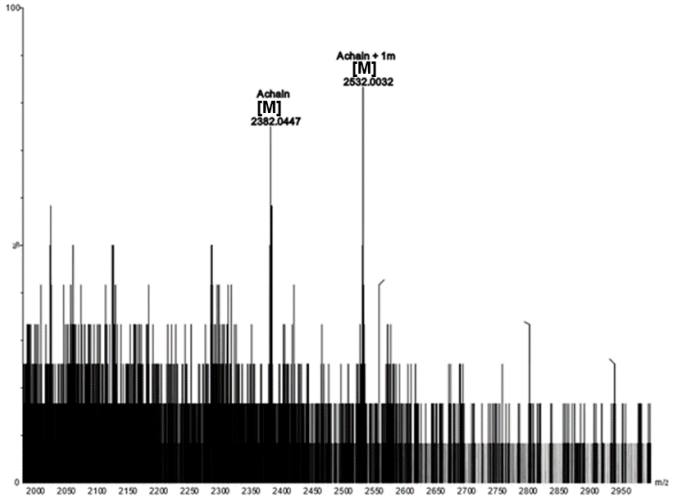
To 0.35 mg of Insulin (60 µmol, 0.15 mM) in 400 µL 10 mM Nap (pH 7.5), 20 µL of TCEP solution (pH 7.5, 50 mM) was added to reduce the insulin. The mixture was incubated at room temperature for 20 min. Probe **1m** (50 equiv.) was added into this mixture and the reaction was allowed to react at room temperature for 8 h. The **1m**-

chain a, and **1m-chain b** bioconjugates were characterized by LCMS.



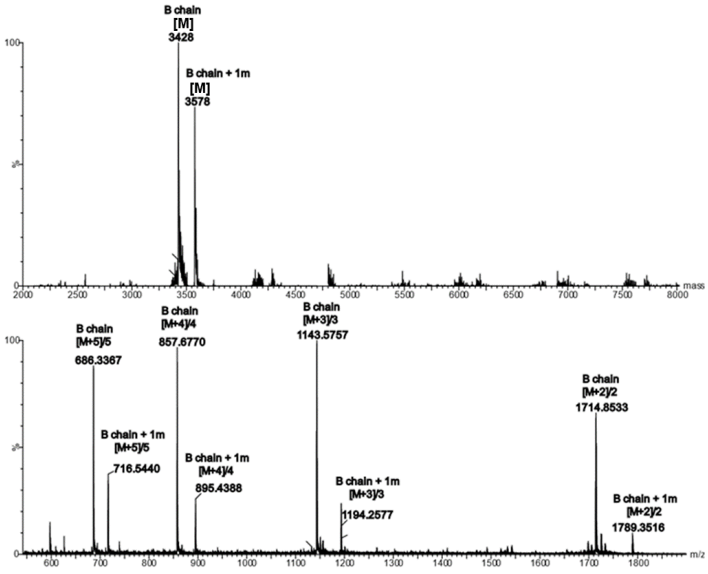
HRMS of 1m-chain a

Compound	Mass (m/z)
Expected unmodified insulin chain a	2383
Observed unmodified insulin chain a	2382.0447
Expected mono-modified insulin chain a	2532
Observed mono-modified insulin chain a	2532.0032



HRMS of 1m-chain b

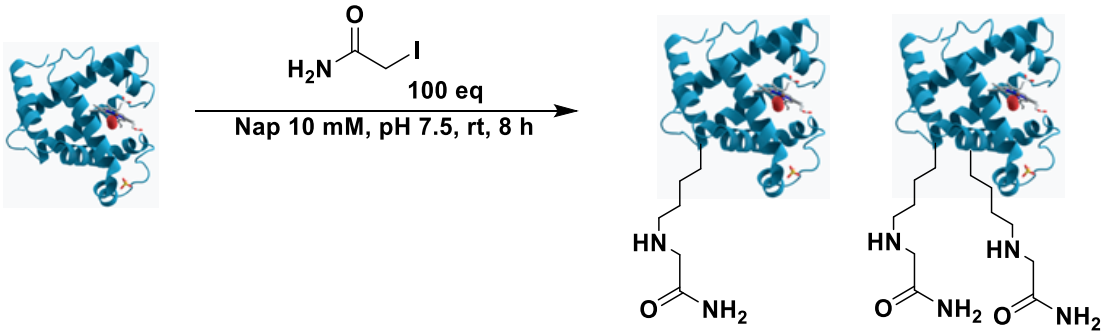
Compound	Mass (m/z)
Expected unmodified insulin chain b	3428
Observed unmodified insulin chain b	3428
Expected Mono-modified insulin chain b	3578
Observed mono-modified insulin chain b	3578



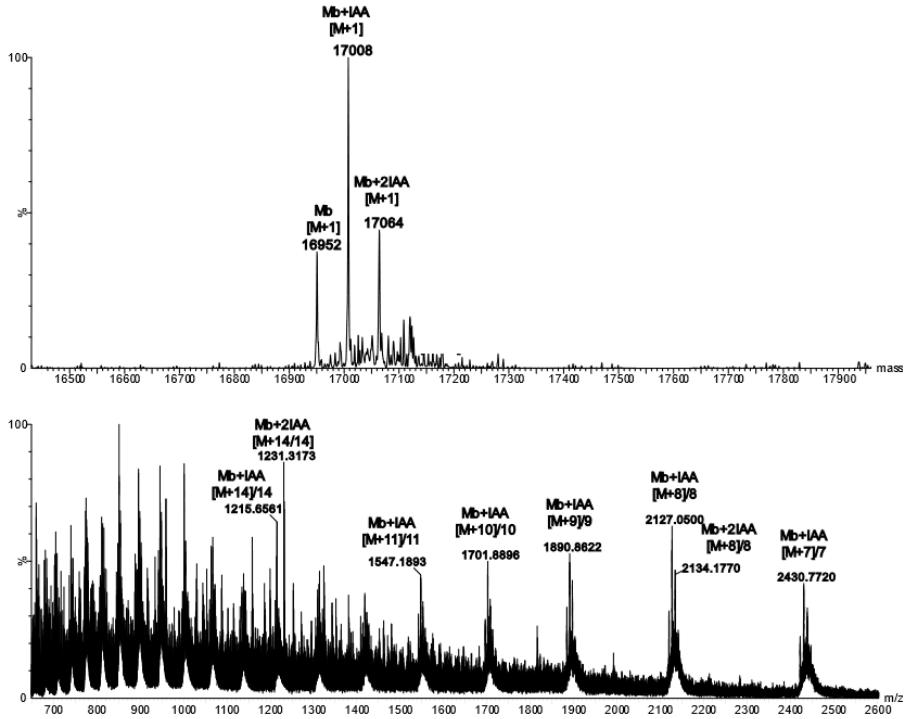
XXII. Supplementary Figure 15. Study of IAA chemical selectivity with myoglobin Mb

General procedure of labeling of myoglobin with IAA

To Myoglobin Mb (60 µmol, 0.15 mM) in 400 µL 10 mM NaP (pH 7.5), IAA (100 equiv.) was added. The reaction was incubated at room temperature for 8 h. The IAA-Mb bioconjugates were purified by molecular weight cut off and characterized by LCMS.



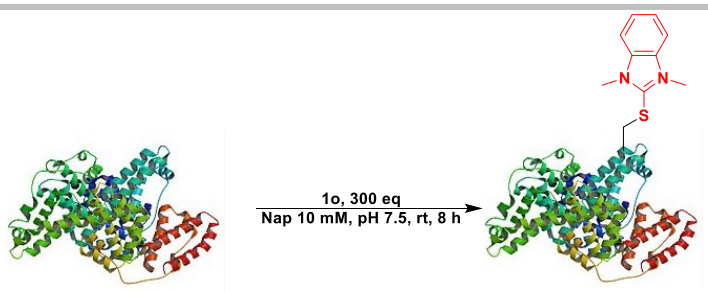
Number of modifications	Expected mass (m/z)	Observed mass (m/z)
1	17007	17008
2	17063	17064



XXIII.Supplementary Figure 16. Modification of bovine serum albumin (BSA) and lysozyme with HAT probe 1o

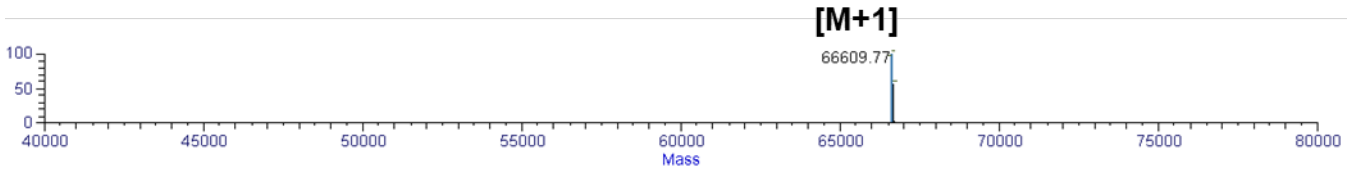
Procedure for the labeling of commercial proteins with compound 1o

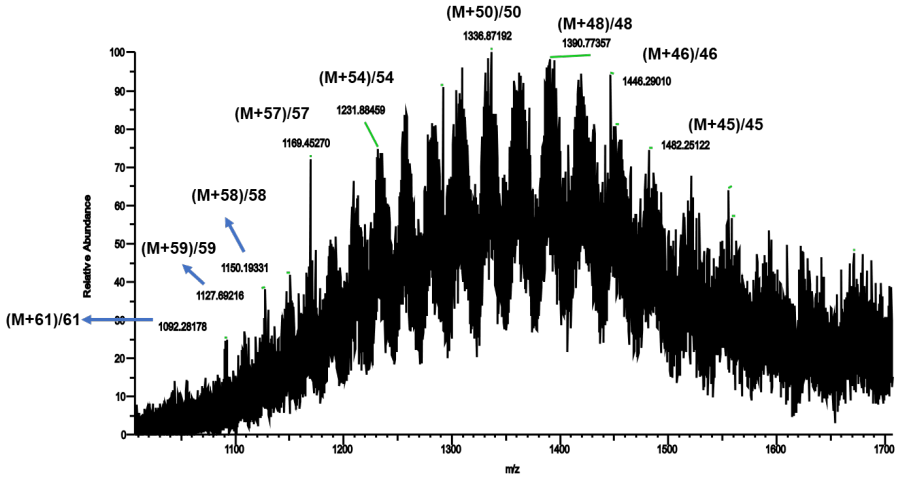
To a mixture of protein (60 µmol, 0.15 mM) in 400 µL 10 mM Nap (pH 7.5), probe **1o** (5.76 mg, 18 mmol, 300 equiv.) was added. The reaction mixture was incubated at room temperature for 8 h. The crude was analyzed by LCMS.

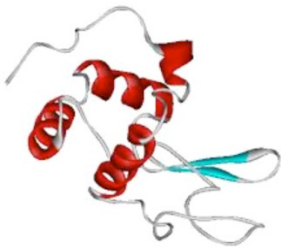


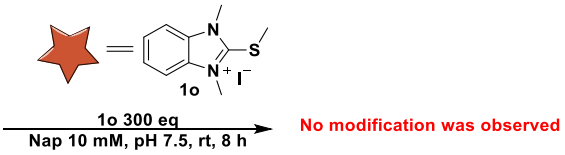
HRMS of 1o-BSA

Compound	Mass (m/z)
Expected 1o-BSA	66608
Observed 1o-BSA	66609.77



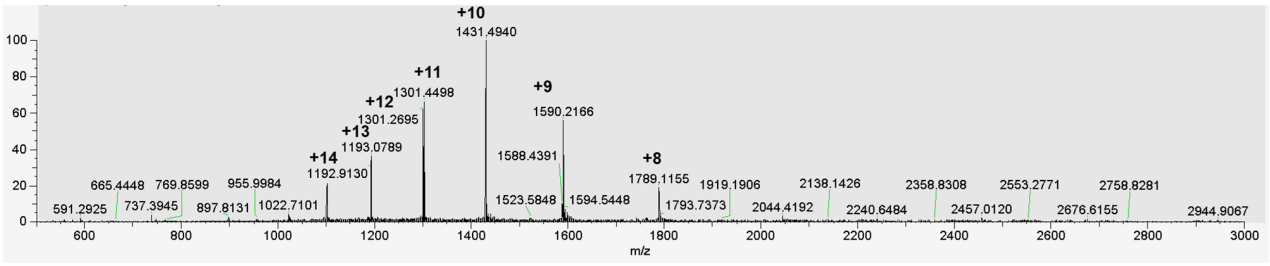


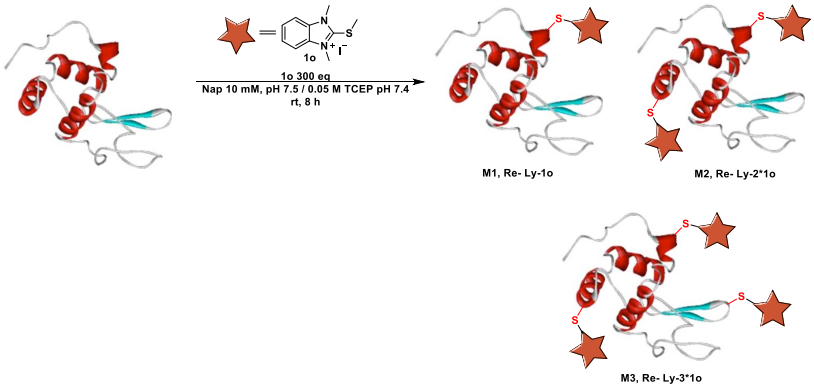




HRMS of unreduced lysozyme example

Fragmentation of unreduced lysozyme	Expected mass (m/z)	Observed mass (m/z)
8	1789	1789.1155
9	1590	1590.2166
10	1431	1431.4940
11	1301	1301.4498
12	1193	1193.0789
13	1101	1101.4863
14	1022	1022.7101



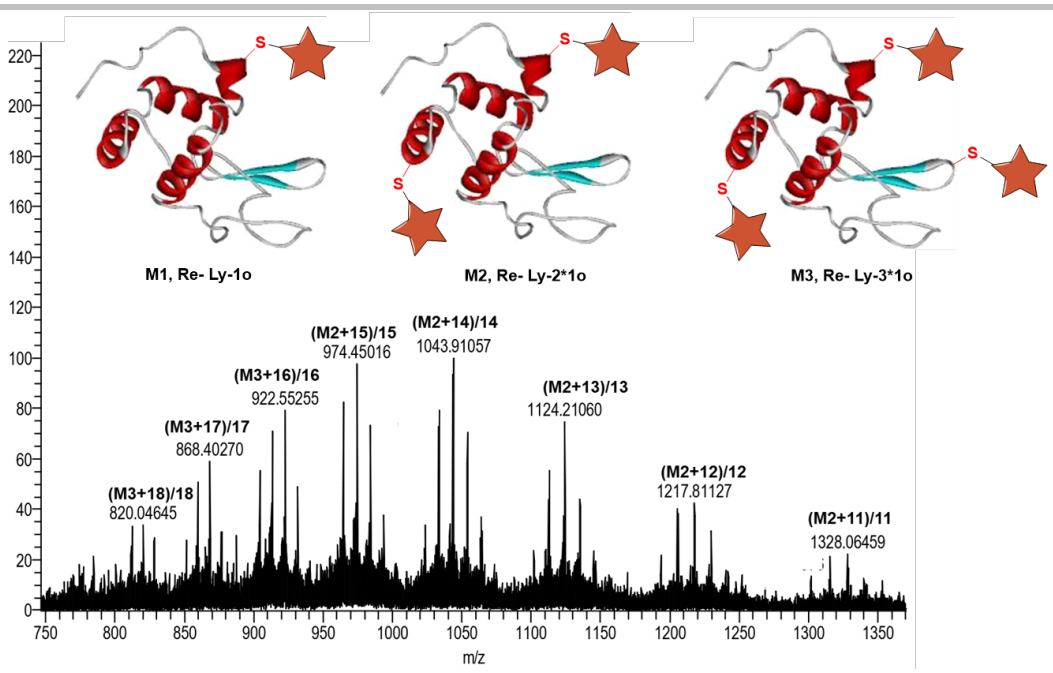


HRMS of modified lysozyme

Fragmentation of doubled-modified reduced lysozyme	Expected mass (m/z)	Observed mass (m/z)
11	1328	1328.0645
12	1217	1217.8112
13	1123	1124.2106
14	1043	1043.9105
15	974	974.4501

Fragmentation of triple-modified reduced lysozyme

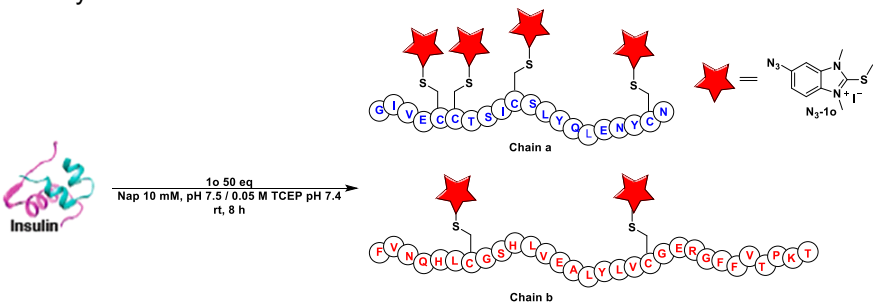
Fragmentation of triple-modified reduced lysozyme	Expected mass (m/z)	Observed mass (m/z)
16	922	922.5525
17	868	868.4027
18	820	820.0464



XXIV. Supplementary Figure 17. Reaction of N_3-1o with various protein substrates.

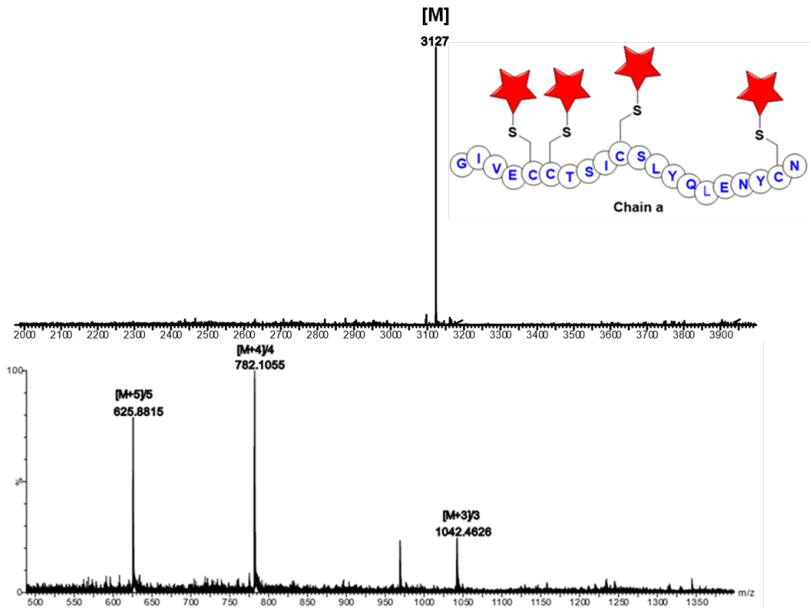
Procedure for labeling of reduced insulin with probe N_3-1o.

To 0.34 mg of Insulin (60 µmol, 0.15 mM) in 400 µL 10 mM Nap (pH 7.5), 20 µL of TCEP solution (pH 7.4, 50 mM) was added to reduce the insulin. The mixture was incubated at room temperature for 20 min. The **N_3-1o** (50 equiv.) was added into this mixture and the reaction was incubated at room temperature for 8 h. The **N_3-1o-chain a**, and **N_3-1o-chain b** bioconjugates were characterized by LCMS. All the four free cysteines on chain a and two cysteines on chain b is fully modified with N3-1o.



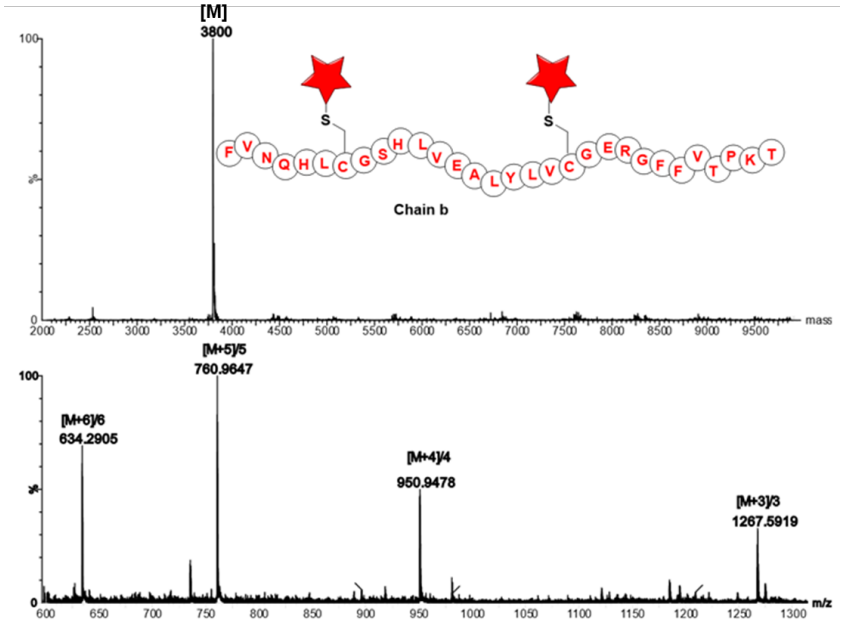
HRMS of N3-1o-insulin a chain

Compound	Mass (m/z)
Expected 1o tetra-modified insulin chain a	3127
Observed 1o tetra-modified insulin chain a	3127

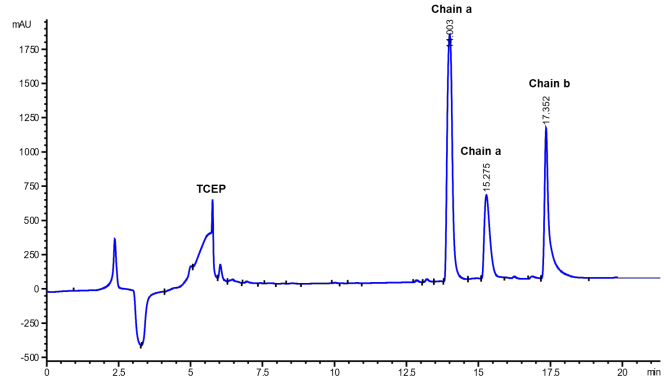


HRMS of N3-1o-insulin b chain

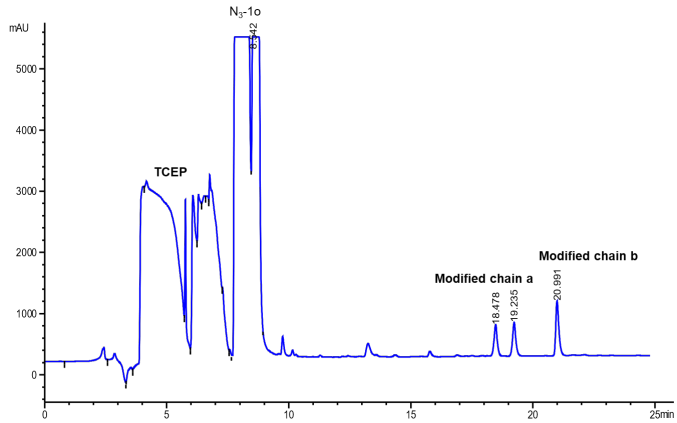
Compound	Mass (m/z)
Expected double-modified insulin chain b	3800
Observed double-modified insulin chain b	3800



HPLC trace of unmodified reduced insulin



HPLC trace of N3-1o modified reduced insulin

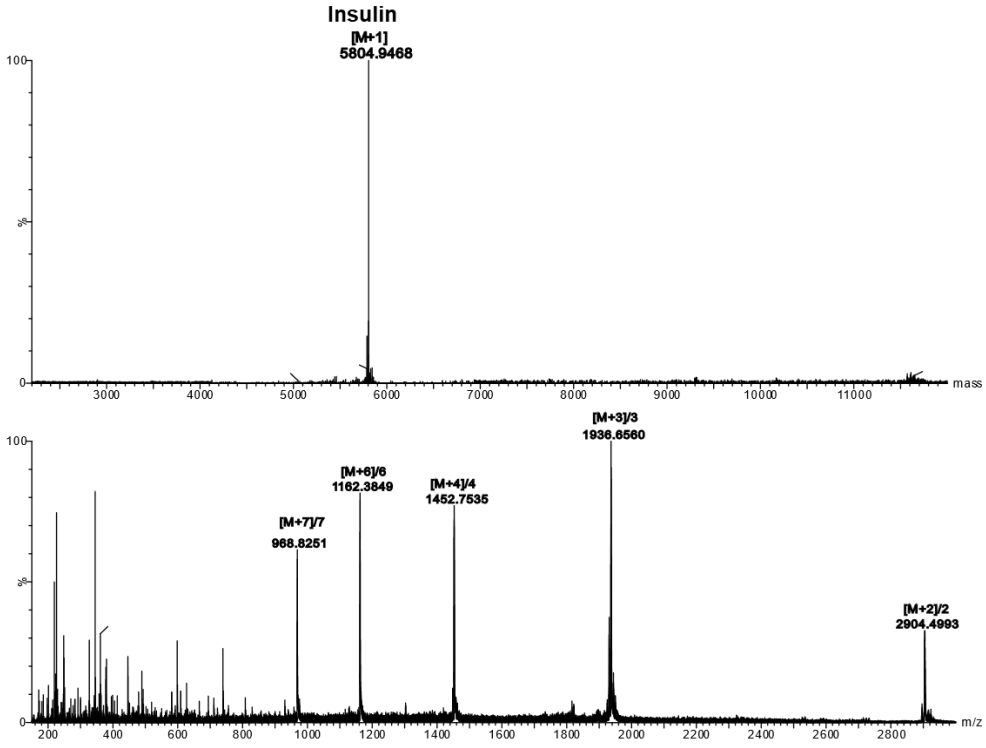


Procedure of intact insulin bioconjugation reaction with N_3-1o

To a mixture of Insulin (0.35 mg, 60 µmol, 0.15 mM) in 10 mM Nap (400 µL, pH 7.5) was added N_3-1o (1.08 mg, 3 mmol, 50 equiv.). The reaction mixture was incubated at room temperature for 12 h. The crude was analyzed by LCMS. No modification of insulin was observed under the reaction conditions confirming the high selectivity of **N_3-1o** probe for cysteine.

HRMS of N_3-1o with intact insulin

Compound	Mass (m/z)
Expected unmodified insulin	5804
Observed unmodified insulin	5805

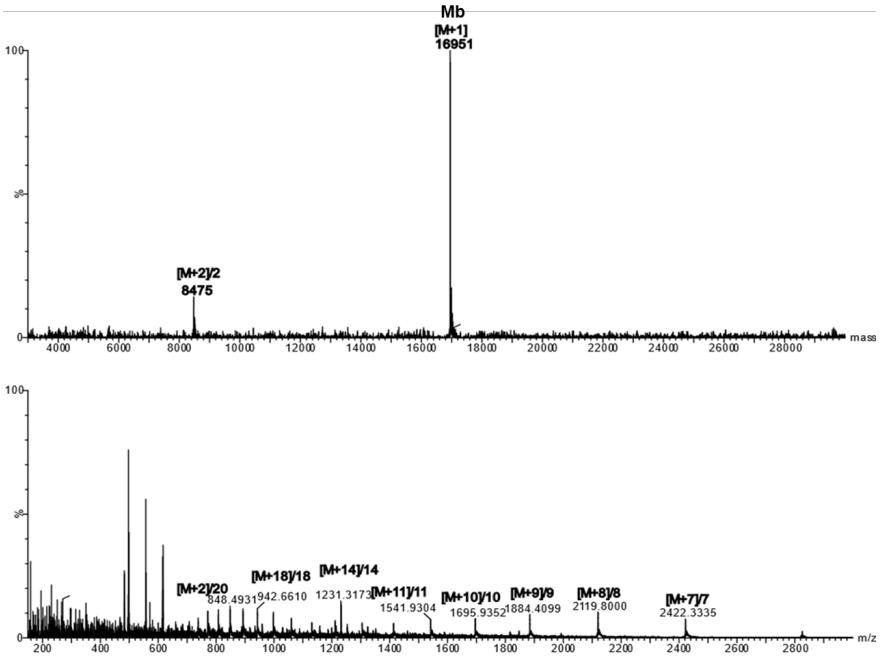


Procedure of N_3-1o Mb bioconjugation reaction

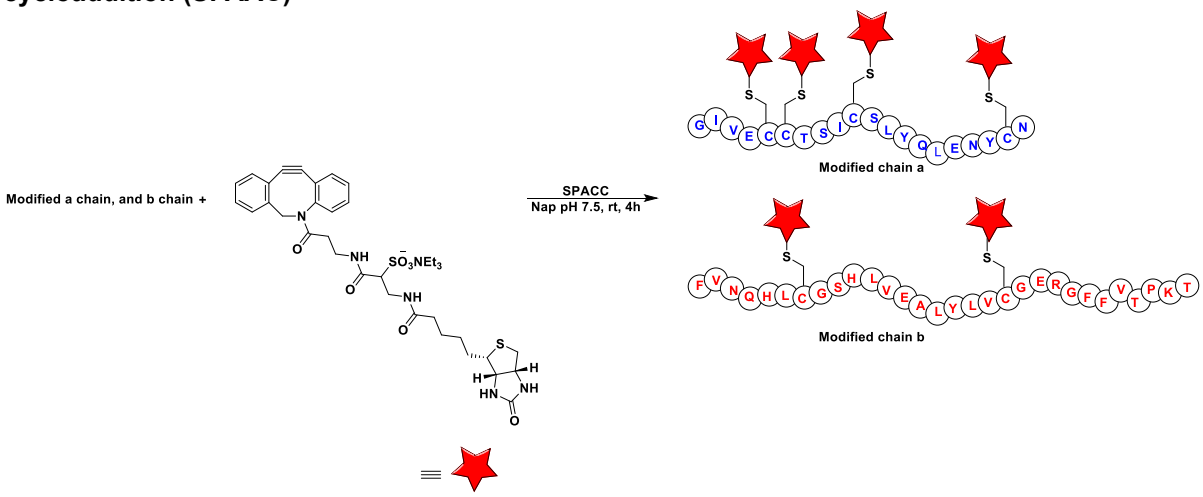
To Myoglobin Mb (60 µmol, 0.15 mM) in 400 µL 10 mM NaP (pH 7.5), **N3-1o** (100 equiv.) was added. The reaction was incubated at room temperature for 8 h. The mixture was purified by molecular weight cut off and analyzed by LCMS. No modification of Mb was observed under the reaction conditions confirming the high selectivity of probe for cysteine.

HRMS of N_3-1o with myoglobin

Compound	Mass (m/z)
Expected unmodified myoglobin	16950
Observed unmodified myoglobin mass	16951



Procedure for enrichment of N_3-1o-insulin a chain, N_3-1o-insulin b chain by strain-promoted alkyne-azide cycloaddition (SPAAC)



To a mixture of modified insulin a chain and b chain (60 µmol, 0.15 mM) in 10 mM Nap (400 µL, pH 7.5), 10 eq of DBCO biotin analog was added. The reaction mixture was incubated at room temperature for 4 h. The crude was analyzed by LCMS.

HRMS of DBCO-biotin a chain

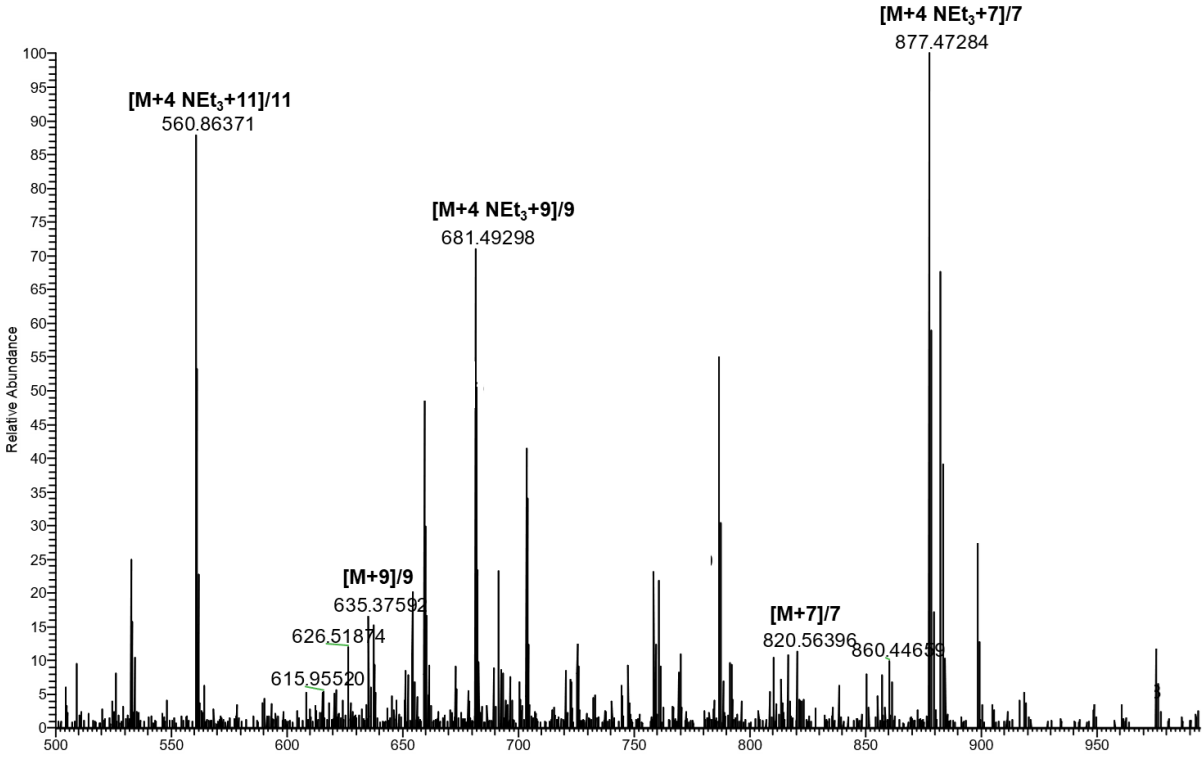
Compound	Mass (m/z)
Expected DBCO-biotin a chain	5731 [M+1], 6135 [M+4Net$_3$]

Fragment of DBCO-biotin a chain [M+1]

Fragmentation of DBCO-biotin a chain	Expected mass (m/z)	Observed mass (m/z)
7	820	820.5639
9	635	635.3759

Fragment of DBCO-biotin a chain [M+4Net$_3$]

Fragmentation of DBCO-biotin a chain	Expected mass (m/z)	Observed mass (m/z)
7	877	877.4728
9	681	681.4929
11	559	560.8637



HRMS of DBCO-biotin b chain

Compound	Mass (m/z)
Expected DBCO-biotin b chain	5103 [M+1], 5305 [M+2Net$_3$], 5149 [M+2Na]

Fragment of DBCO-biotin b chain [M+1]

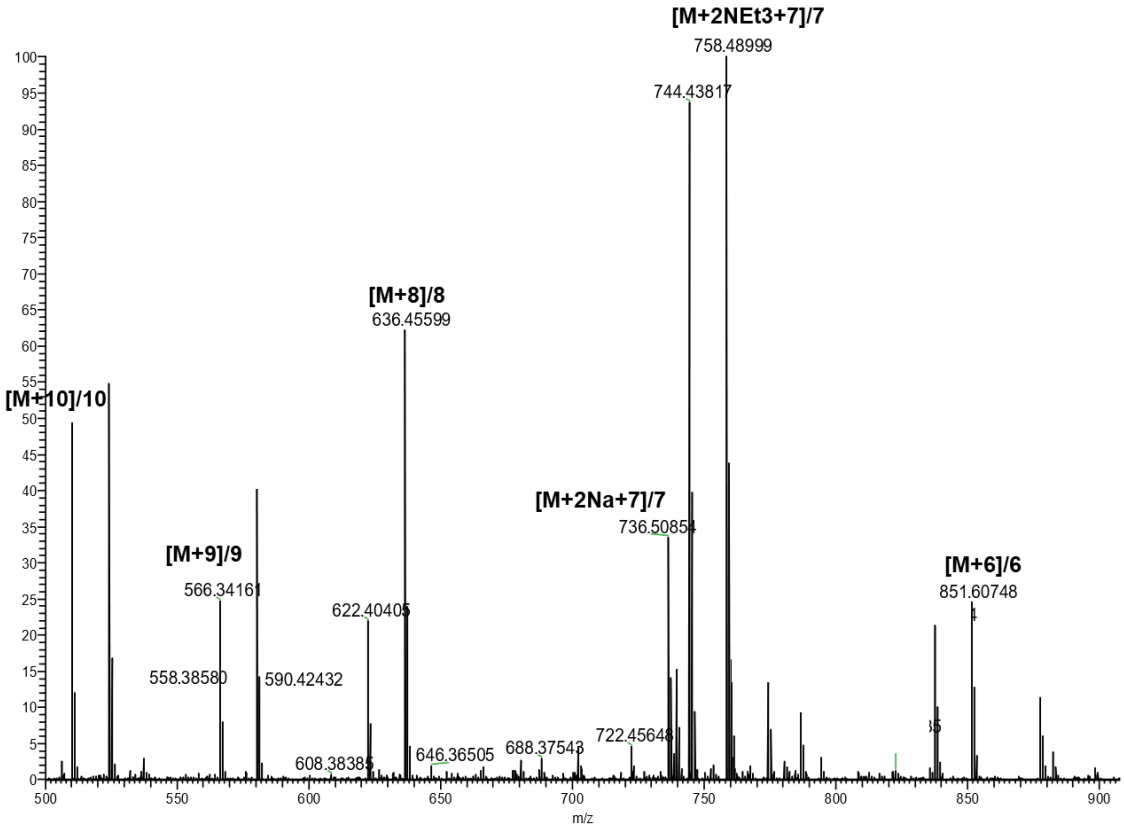
Fragmentation of DBCO-biotin b chain	Expected mass (m/z)	Observed mass (m/z)
6	851	851.6074
8	637	636.4559
9	567	5663.416
10	511	511.3021

Fragment of DBCO-biotin b chain [M+2Net$_3$]

Fragmentation of DBCO-biotin b chain	Expected mass (m/z)	Observed mass (m/z)
7	758	758.4899

Fragment of DBCO-biotin b chain [M+2Na]

Fragmentation of DBCO-biotin b chain	Expected mass (m/z)	Observed mass (m/z)
7	736	736.5085



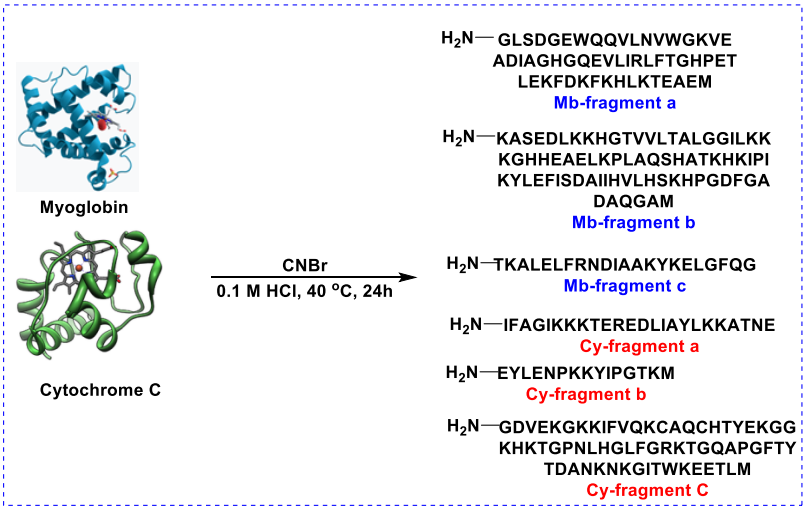
XXV. Supplementary Figure 18. Enrichment of cysteine containing peptides with 1o in mixture of proteolytic fragments.

Procedure for the digestion of cytochrome C and Myoglobin by CNBr[4]

Proteins (81 µmol, 0.25 mM) and 0.3 mg of CNBr (0.1875 mM) were mixed in 324 µL 0.1 M HCl, the reaction mixture was incubated at 40 ^{o}C for 24 h. The reaction mixture was quenched by freezing the sample at -80 °C. The frozen samples were then lyophilized to afford the dry peptide fragments.

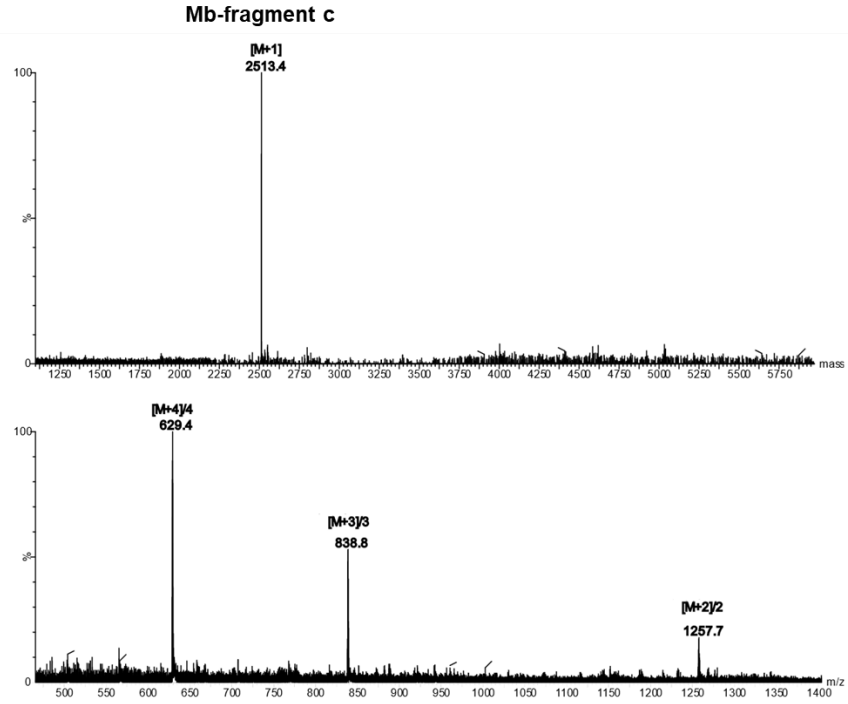
Procedure of enrichment of cysteine fragments by compound 1o:

To the mixture of proteolytic fragments (0.81 µmol of cytochrome C and myoglobin) in 0.4 mL of 10 mM sodium phosphate buffer (Nap, pH 7.5), insulin (81 µmol, 0.25 mM) in 10 mM Nap (400 µl, pH 7.5) and 20 µL of TCEP solution (pH 7.4, 50 mM) was added to generate reduced insulin, the mixture was incubated at room temperature for 20 min. Probe **1o** (100 eq) was added in the mixture of proteolytic fragments at room temperature and reaction was left for 12 h. The tagged proteolytic fragments were analyzed by LCMS without purification.



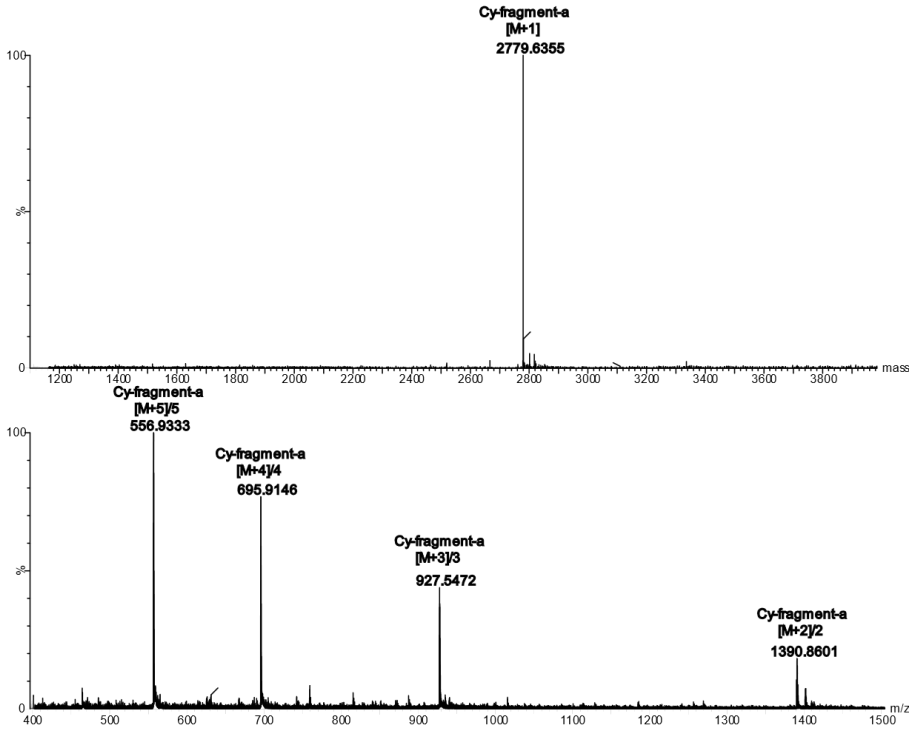
HRMS of Mb-fragment-c

Compound	Mass (m/z)
Unmodified fragment-c	2512.86
Observed unmodified Mb-fragment-c	2513.4



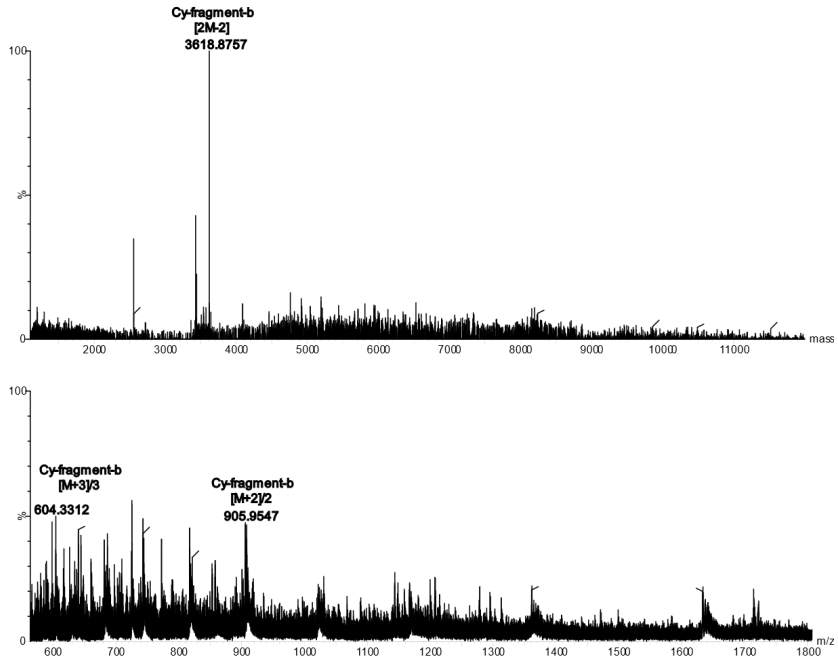
HRMS of Cy-fragment-a

Compound	Mass (m/z)
Expected unmodified Cy-fragment-a	2780.22
Observed unmodified Cy-fragment-a	2779.6355



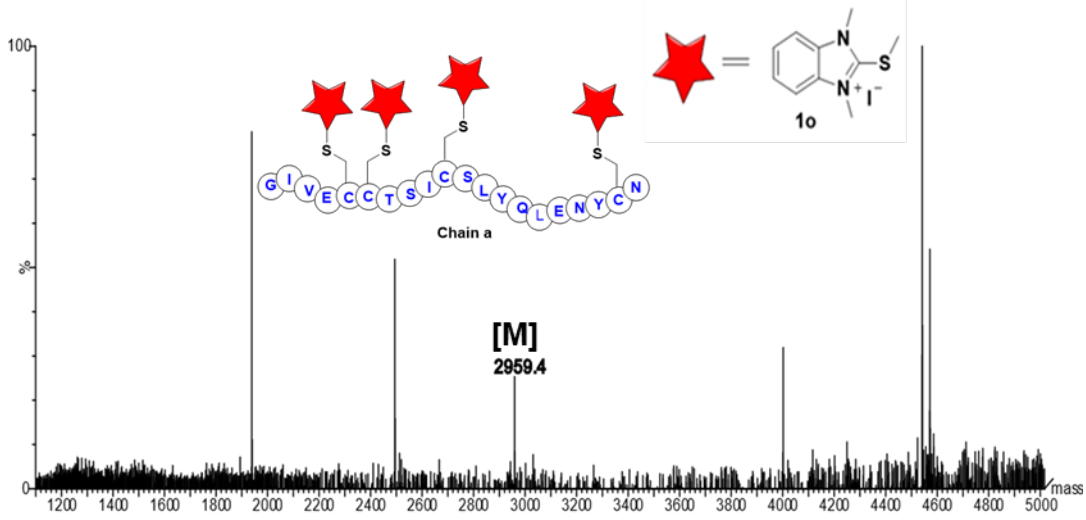
HRMS of Cy-fragment-b

Compound	Mass (m/z)
Expected unmodified Cy-fragment-b	1810.11
Observed unmodified Cy-fragment-b	3618.8757



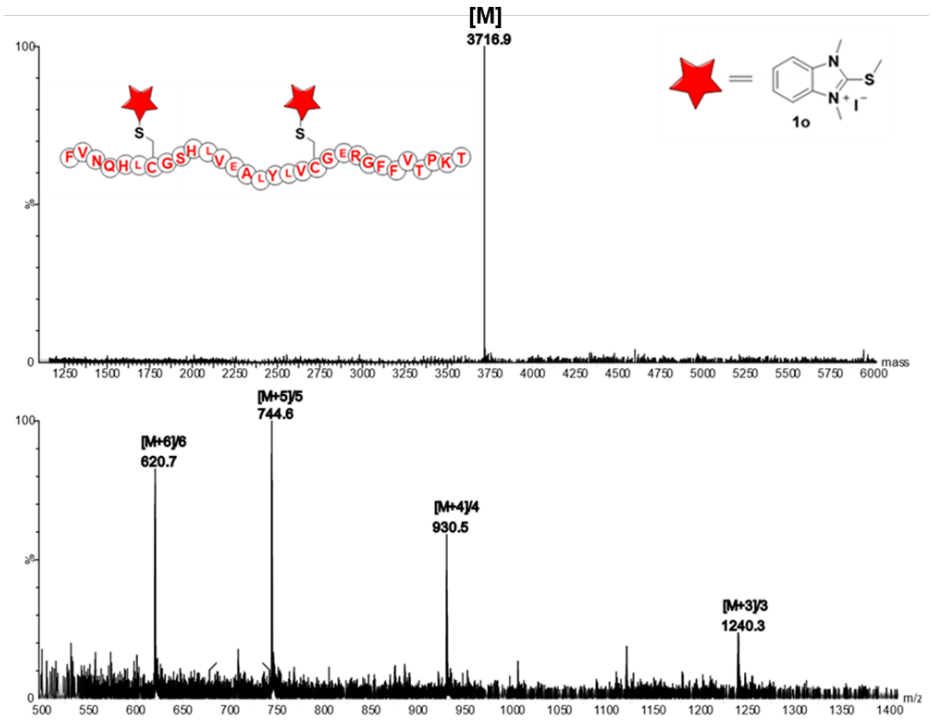
HRMS of 1o-insulin chain a

Compound	Mass (m/z)
Expected modified insulin a chain	2959
Observed modified insulin a chain	2959.4

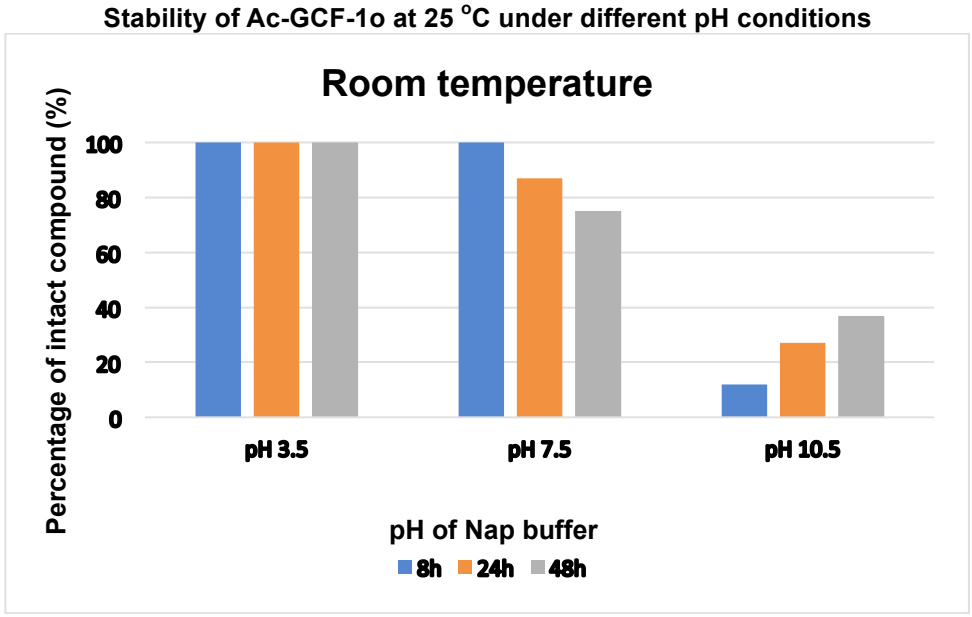


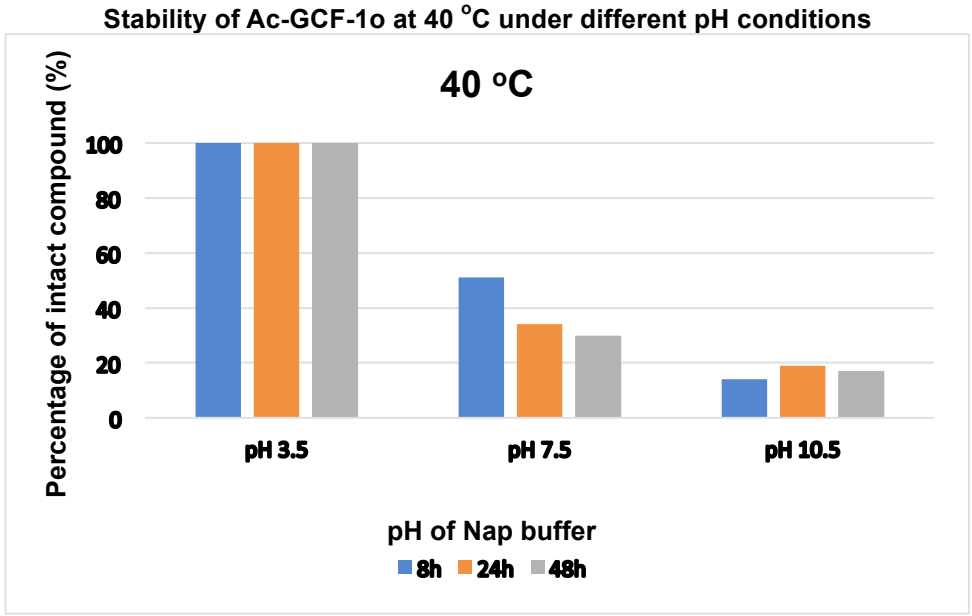
HRMS of 1o-insulin chain b

Compound	Mass (m/z)
Expected modified insulin b chain	3716
Observed modified insulin b chain	3716.9

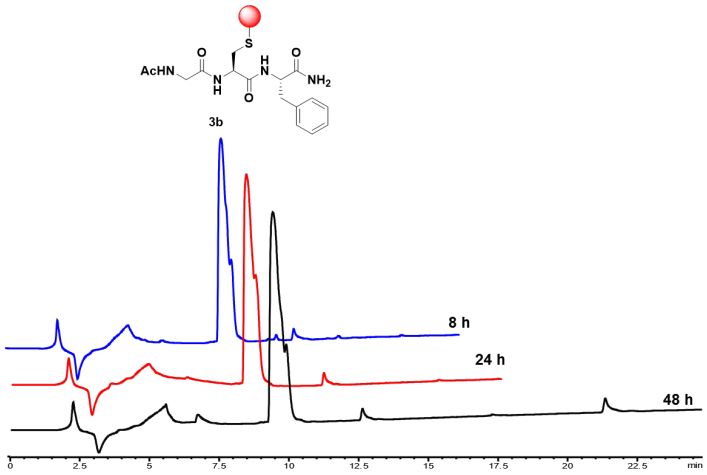


XXVI. Supplementary Figure 19. Stability study of Ac-GCF-1o under different pH conditions.
Ac-GCF-1o conjugate **3b** (3.75 mM) was incubated in 10 mM Nap at different pH ranging from 3.5 to 10.5 at room temperature and at 40 °C. The samples (50 µL) were taken from the reaction mixtures and directly injected into the HPLC. The reactions were monitored by injecting samples in HPLC after regular intervals of time 8h, 24h and 48 h. The bioconjugate product showed high stability under pH 3.5 at both room temperature and 40 °C. We observed high stability of the conjugate for 24h under physiological conditions (pH 7.5) at room temperature.

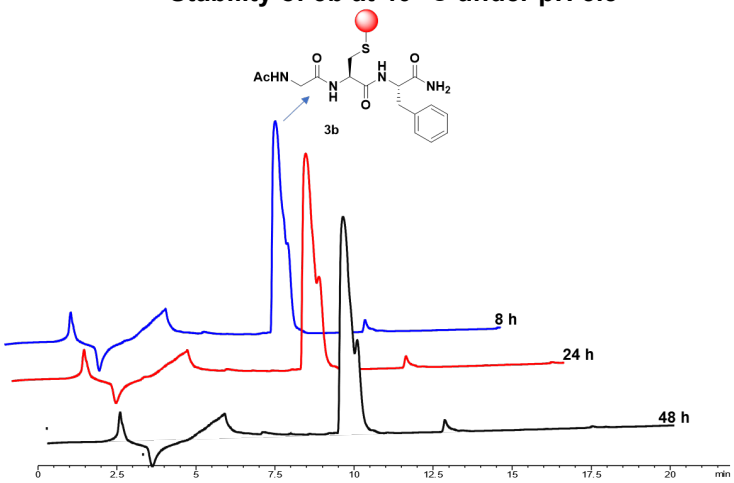




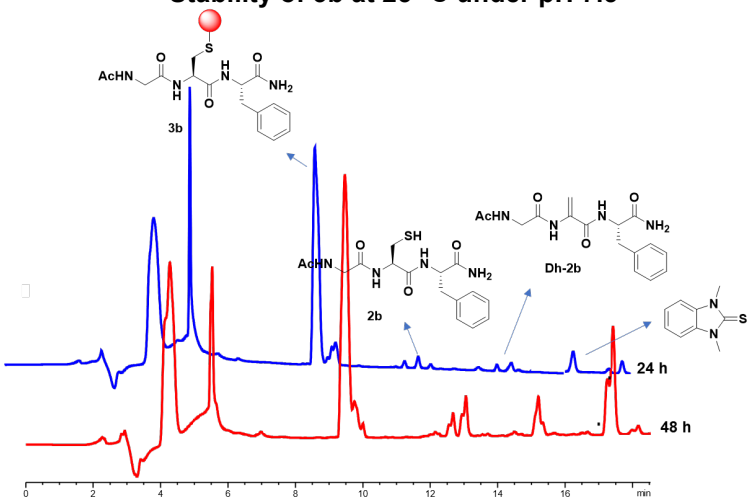
Stability of 3b at 25 °C under pH 3.5



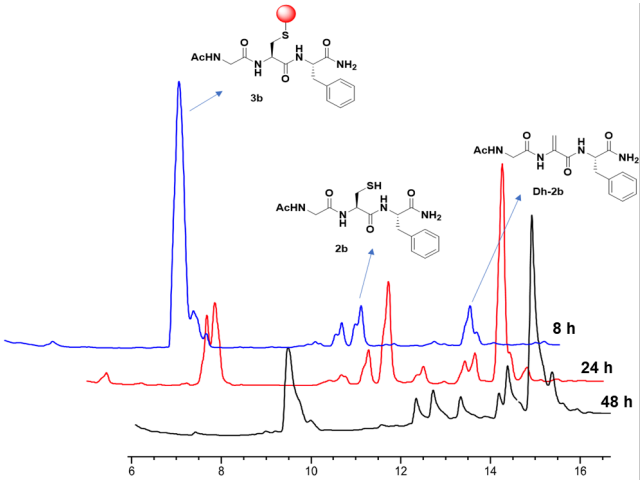
Stability of 3b at 40 °C under pH 3.5



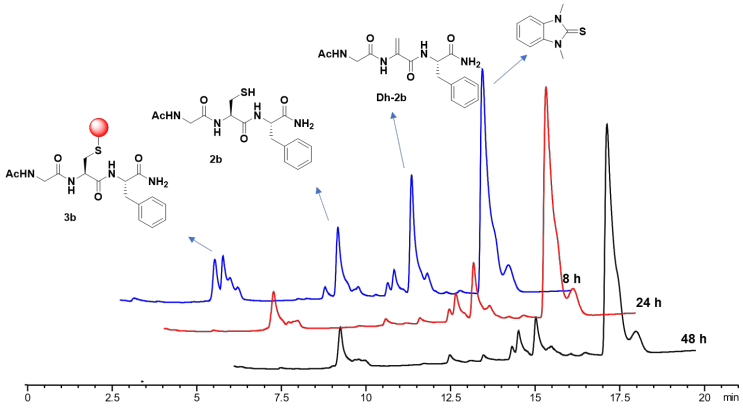
Stability of 3b at 25 °C under pH 7.5



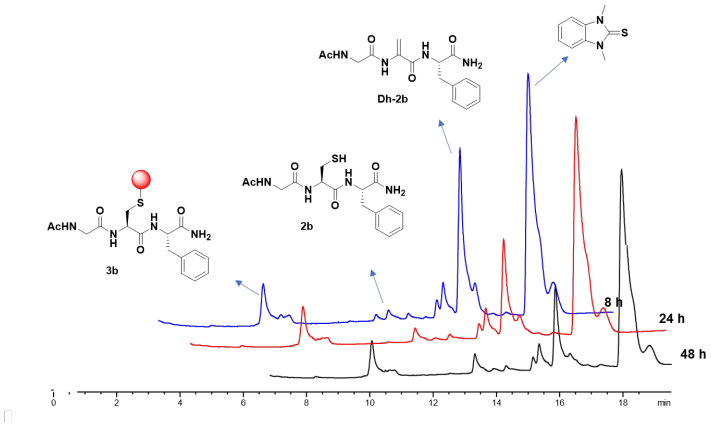
Stability of 3b at 40 °C under pH 7.5



Stability of 3b at 25 °C under pH 10.5



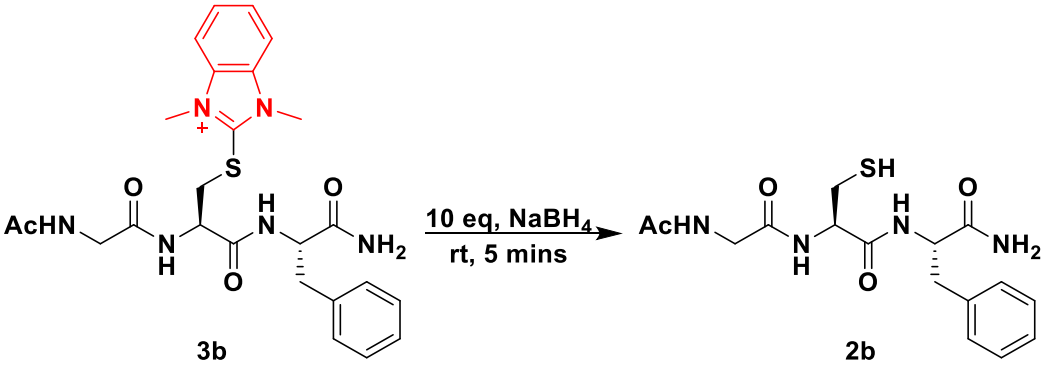
Stability of 3b at 40 °C under pH 10.5



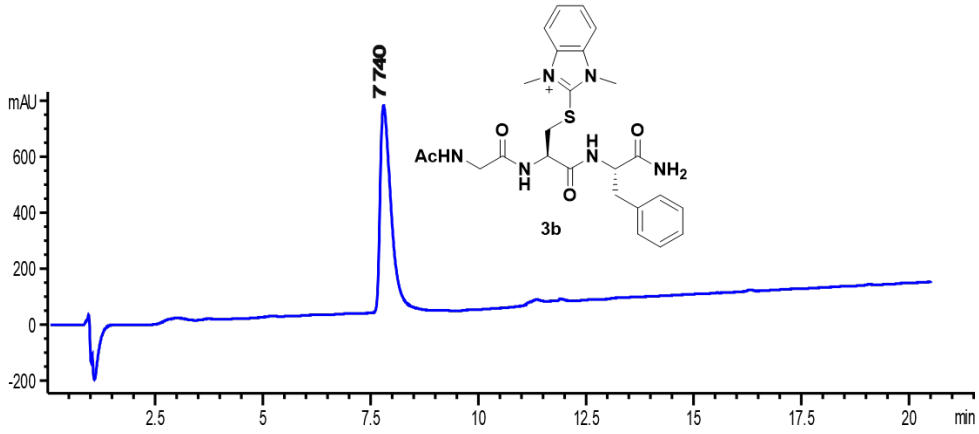
XXVII. Supplementary Figure 20. Reversible study of Cys-HAT biconjugate with $NaBH_4$

Procedure for reversible study of 3b by $NaBH_4$

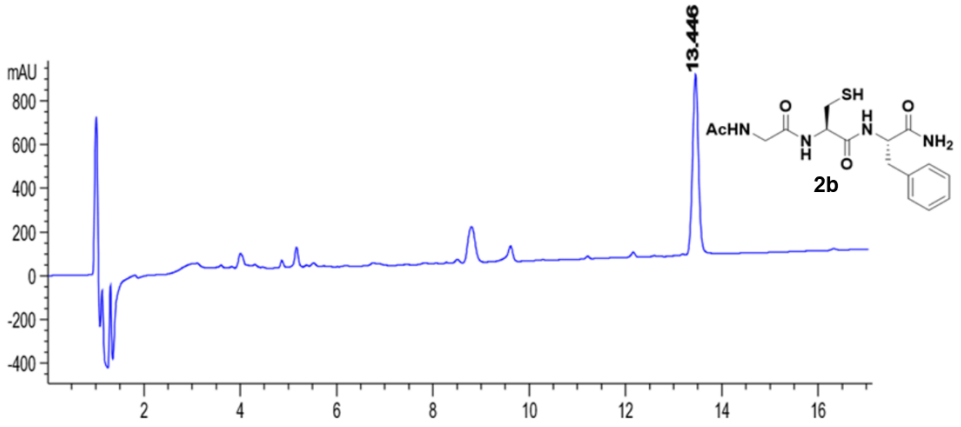
In a solution of peptide conjugate **3b** (6.3 mM) in 0.4 mL of 10 mM Nap (pH 7.5) was added 10 equiv. of $NaBH_4$ (63 mM). The mixture was stirred at room temperature for 5 min. The reaction was analyzed by HPLC and ESI-MS. HPLC was carried out with 1 % formic acid: water (solvent A): acetonitrile (solvent B); 0-80 % in 30 min, flow rate = 1.0 mL/min, detection wavelength 220 nm.



HPLC trace of 3b

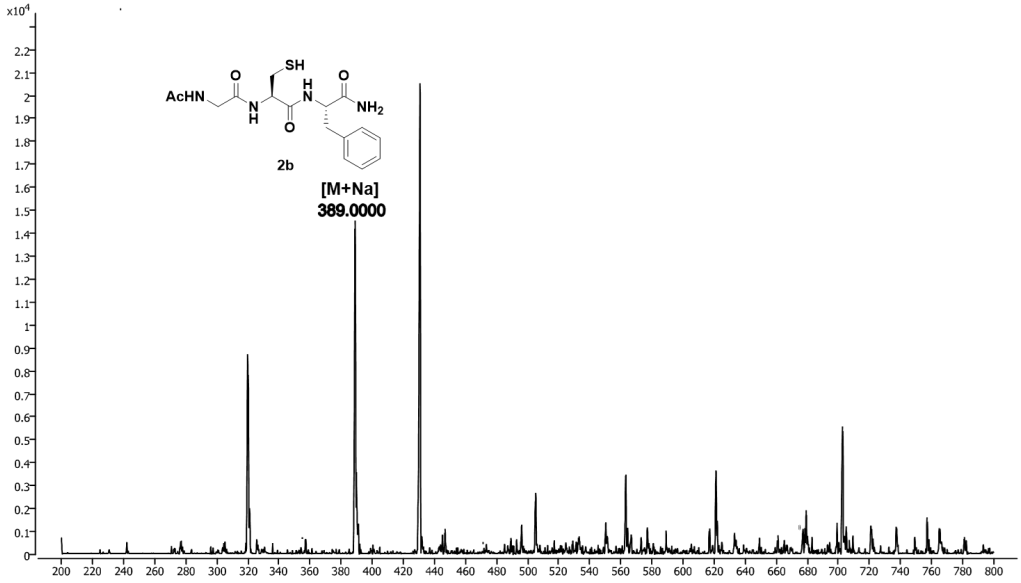


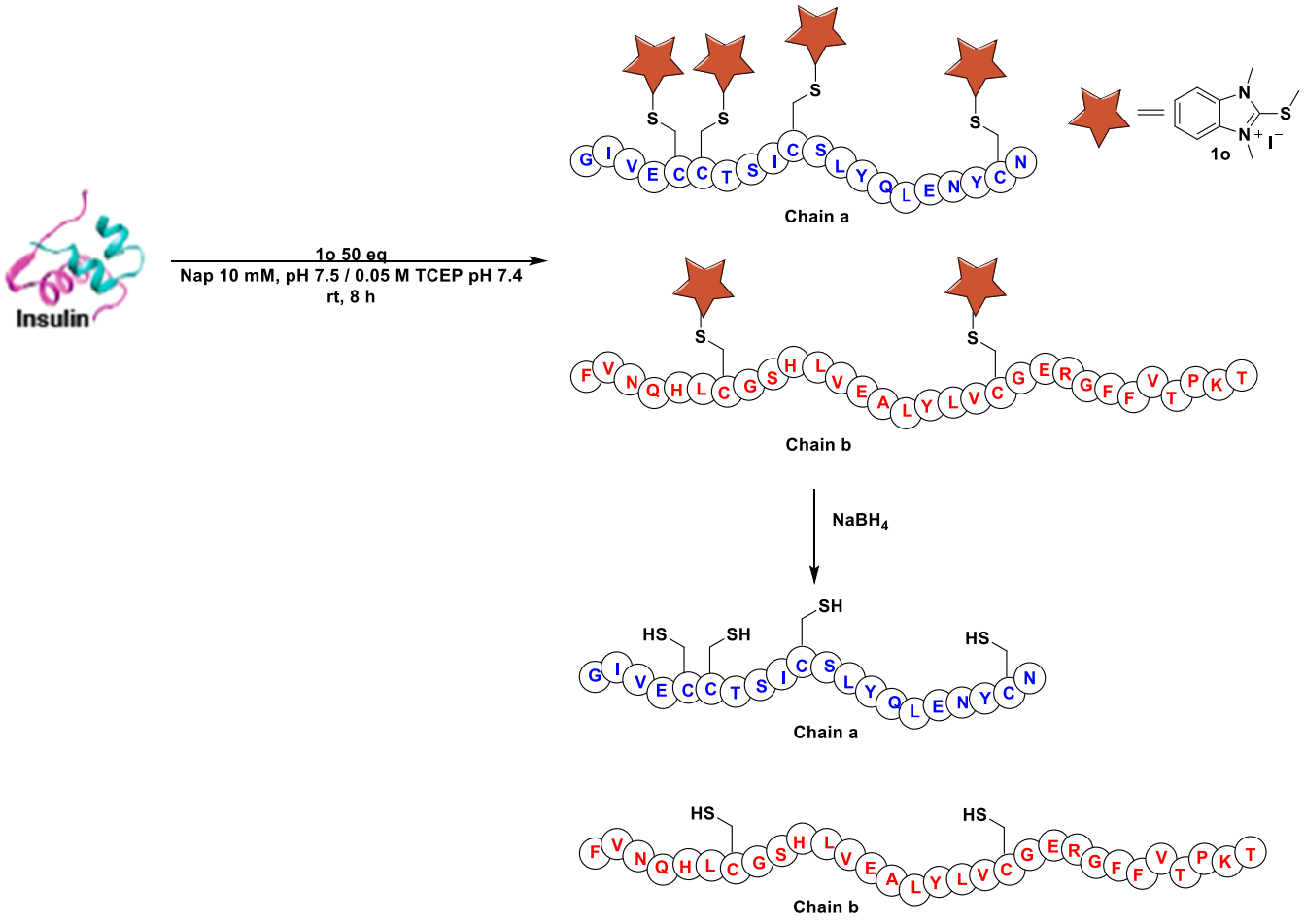
HPLC trace after addition of $NaBH_4$ for 5 min



ESI-MS after addition of $NaBH_4$

Compound	Mass (m/z)
Expected mass of 2b	366
Observed mass of 2b	389 [M+Na]

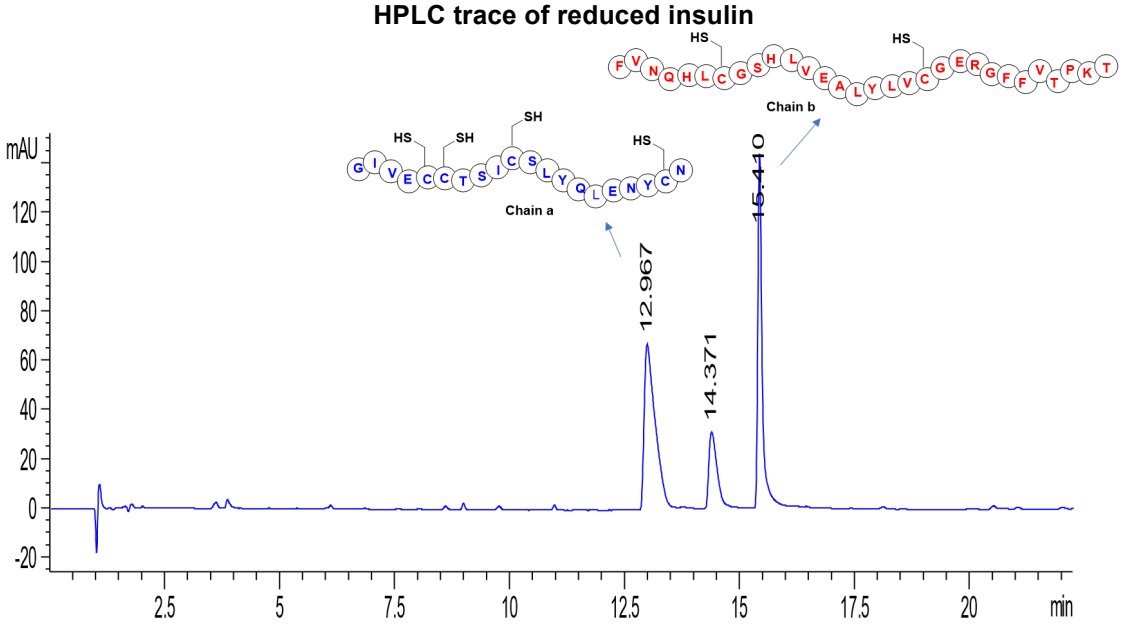




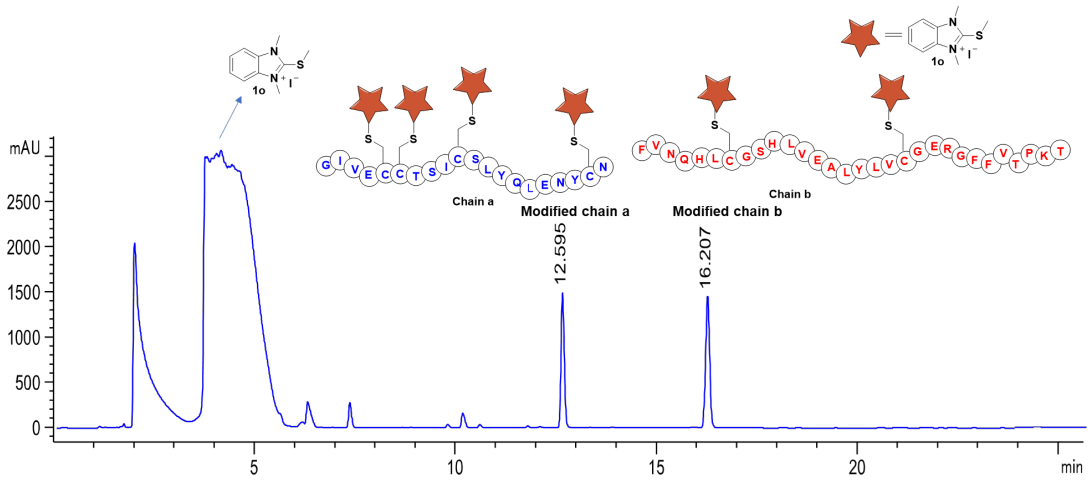
Procedure for reversible study of modified reduced insulin by $NaBH_4$

In a solution of modified reduced insulin (0.15 mM) in 400 µL of 10 mM Nap (pH 7.5) was added 10 equiv. of $NaBH_4$ (1.5 mM). The mixture was stirred at room temperature for 5 min. The reaction was analyzed by HPLC and ESI-MS. HPLC was carried out with 1 % formic acid: water (solvent A): acetonitrile (solvent B); 0-80 % in 30 min, flow rate = 1.0 mL/min, detection wavelength 220 nm.

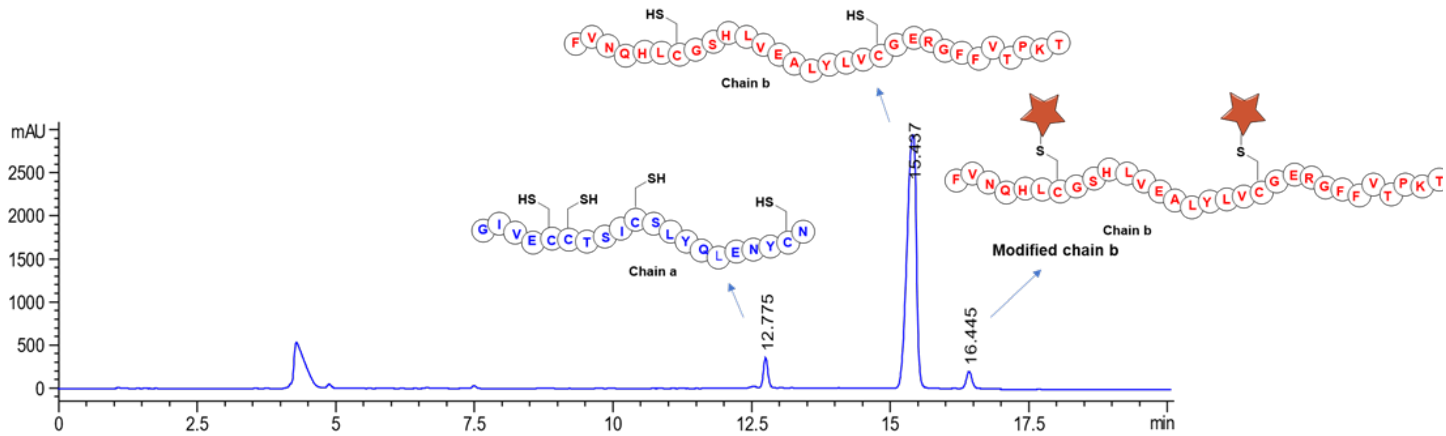
HPLC trace of reduced insulin



HPLC trace of modified chain a and chain b



HPLC trace after addition of $NaBH_4$

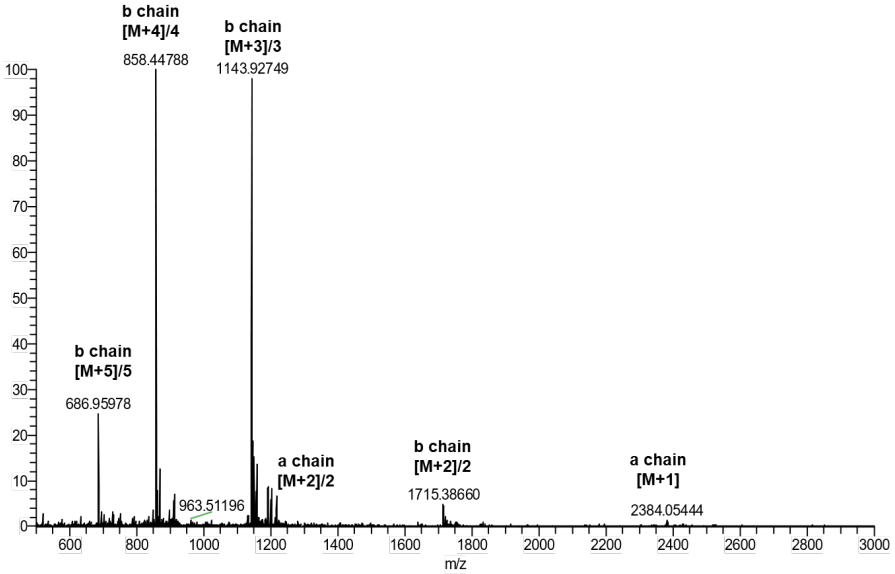


HRMS after addition of $NaBH_4$

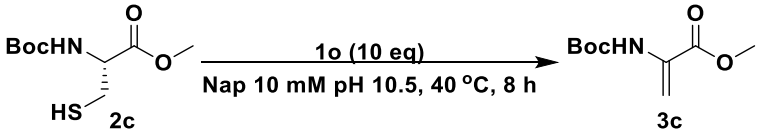
Compound	Mass (m/z)
Expected mass of chain a	2383
Observed mass of chain a	2384.0544

HRMS of insulin chain b

Fragmentation of double-modified chain b	Expected mass (m/z)	Observed mass (m/z)
2	1715	1715.3866
3	1143	1143.9274
4	858	858.4478
5	686	686.9597

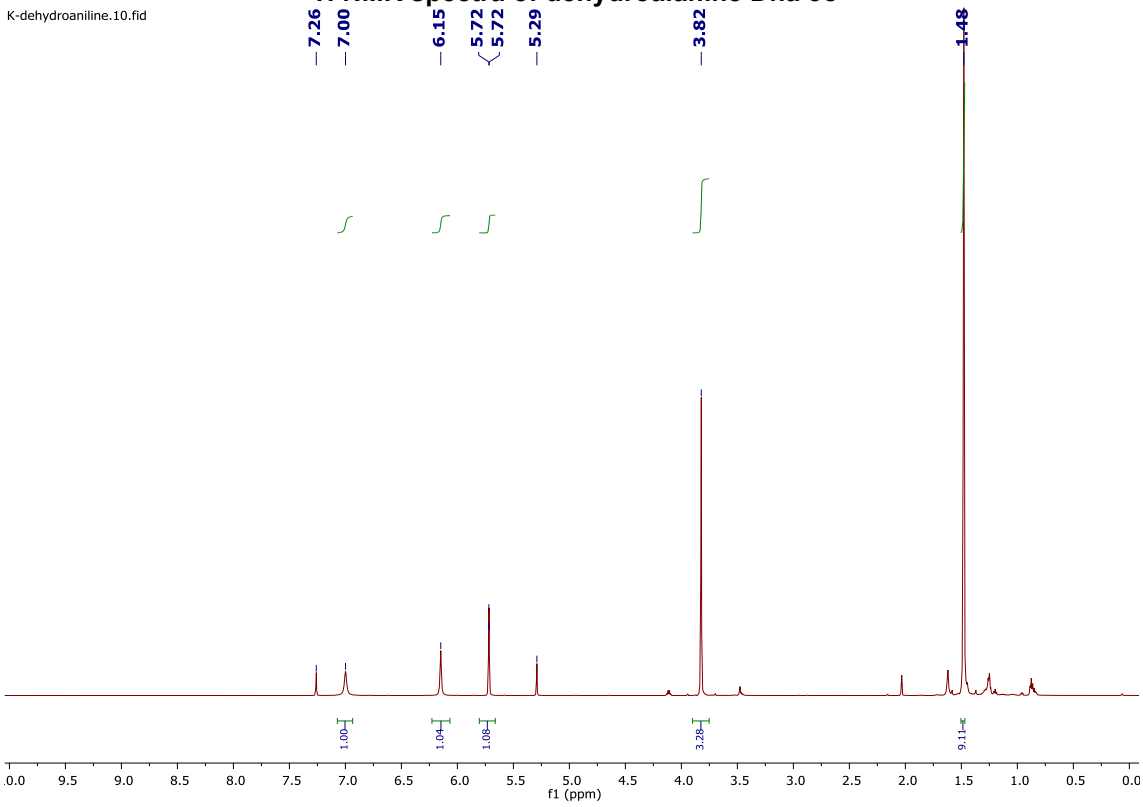


XXVIII. Supplementary Figure 21. Dehydroalanine synthesis from cysteine 2c using 1o at high pH
Procedure for synthesis of dehydroalanine from cysteine 2c

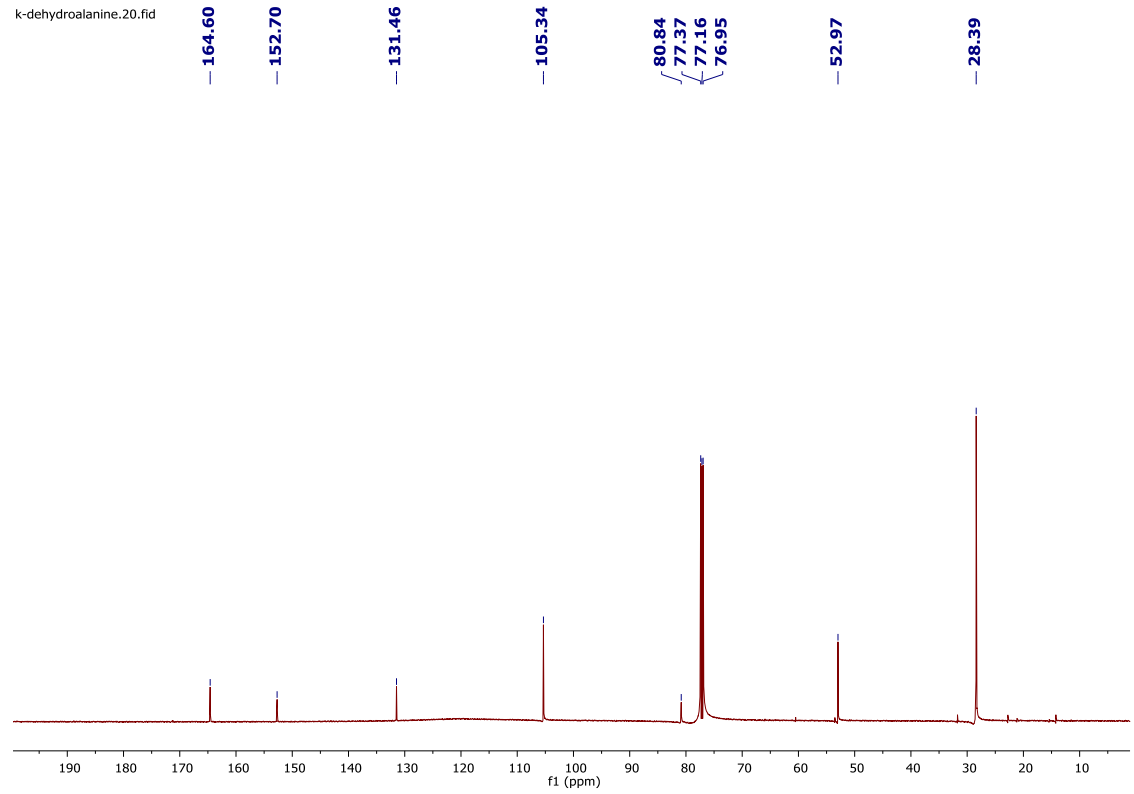


To 500 mg (2.12 mmol) of Boc-Cys-OMe **2c** in 1 mL of 10 mM Nap (pH 10.5), probe **1o** (50 equiv.) was added and the reaction was allowed to react at 40 ^{o}C for 8 h. The reaction solution was washed with ethyl acetate and brine for 3 times. The residue was concentrated under reduced pressure and purified by the column chromatography (hexane: ethyl acetate 3:1) to obtain dehydroalanine **3c** as colorless oil (312.2 mg, 73 %). NMR. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.00 (br, 1H), 6.15 (s, 1H), 5.72 (d, 1H, J = 1.5 Hz), 3.82 (s, 3H), 1.49 (s, 9H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 164.6, 152.7, 131.7, 105.3, 80.8, 53.0, 28.4.

^{1}H NMR spectra of dehydroalanine Dha 3c



^{13}C NMR spectra of dehydroalanine Dha 3c

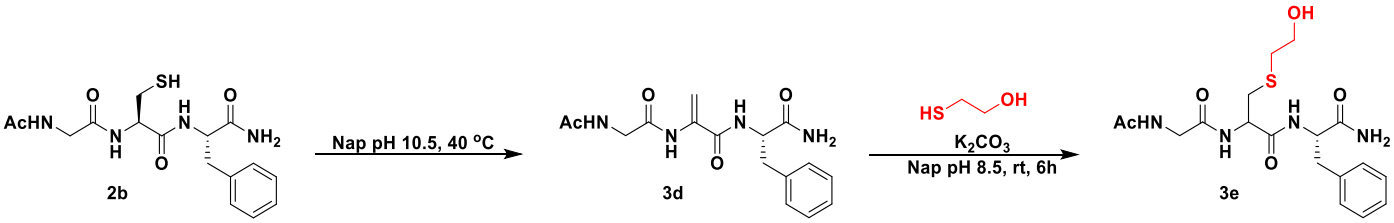


XXIX. Supplementary Figure 22. Aza-Michael addition and thiol-ene reaction of dehydroalanine.
General procedure for synthesis of Dha peptide 3d
To a solution of Ac-GCF **2b** (0.57 mg, 0.0015 mmol) in 400 µL of 10 mM Nap (pH 10.5) was added **1o** (25 equiv.,

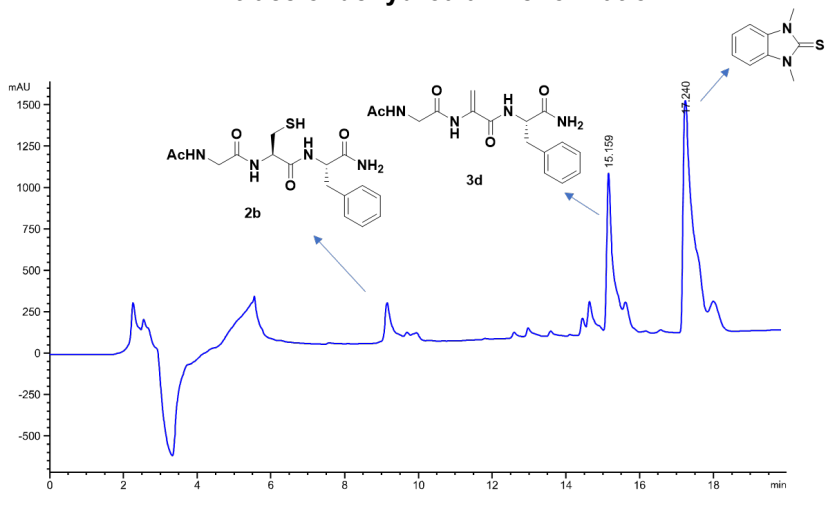
0.0375 mmol). The reaction mixture was incubated at 40 °C for 12 h. The crude compound was purified by HPLC and lyophilized to afford dehydroalanine (88 % conversion). The reaction was analyzed by HPLC and ESI-MS. HPLC was carried out with 1 % formic acid: water (solvent A): acetonitrile (solvent B); 0-80 % in 30 min, flow rate = 1.0 mL/min, detection wavelength 220 nm.

Procedure for Dha peptide thiol-ene reaction

To a solution of Dha peptide **3d** (0.0015 mmol) in 400 µL of 10 mM Nap (pH 8.5) was added K_2CO_3 (4 equiv., 0.006 mmol) and 2-mercaptoethanol (10 eq, 0.015 mmol). The reaction mixture was incubated at rt for 6 h to generate **3e** (>99 % conversion). The reaction was analyzed by HPLC and ESI-MS. HPLC was carried out with 1 % formic acid: water (solvent A): acetonitrile (solvent B); 0-80 % in 30 min, flow rate = 1.0 mL/min, detection wavelength 220 nm.

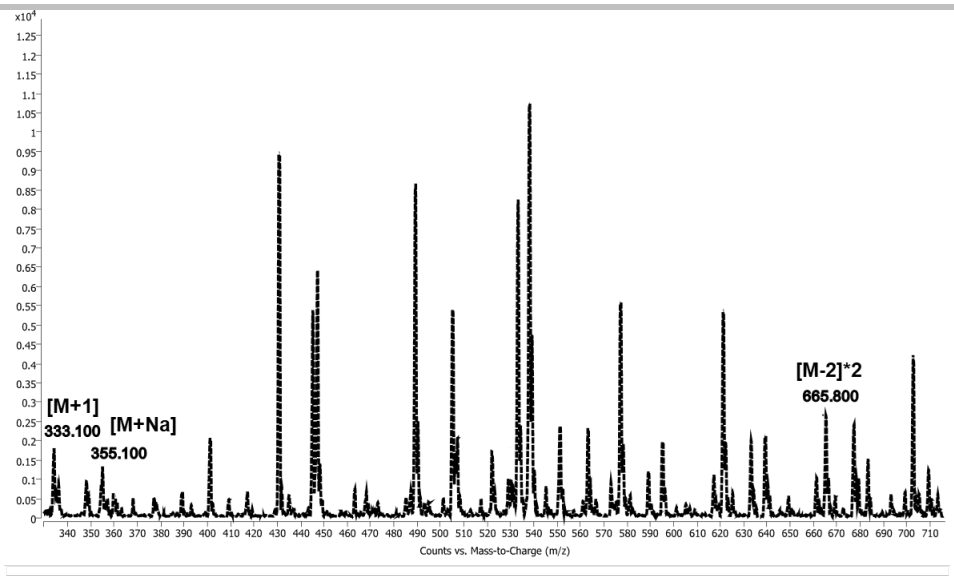


HPLC trace of dehydroalanine formation



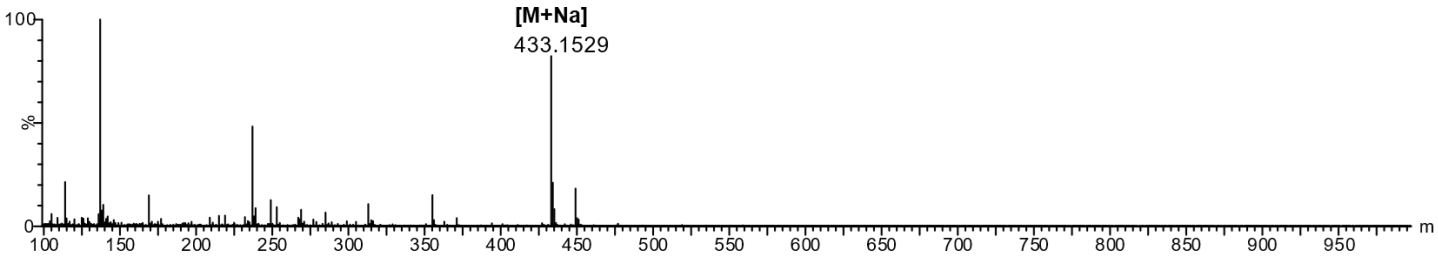
LC-MS of 3d

Compound	Mass (m/z)
Expected mass of 3d	332
Observed mass of 3d	333.1 [M+1], 335.1 [M+Na], 665.8 [2M-2]

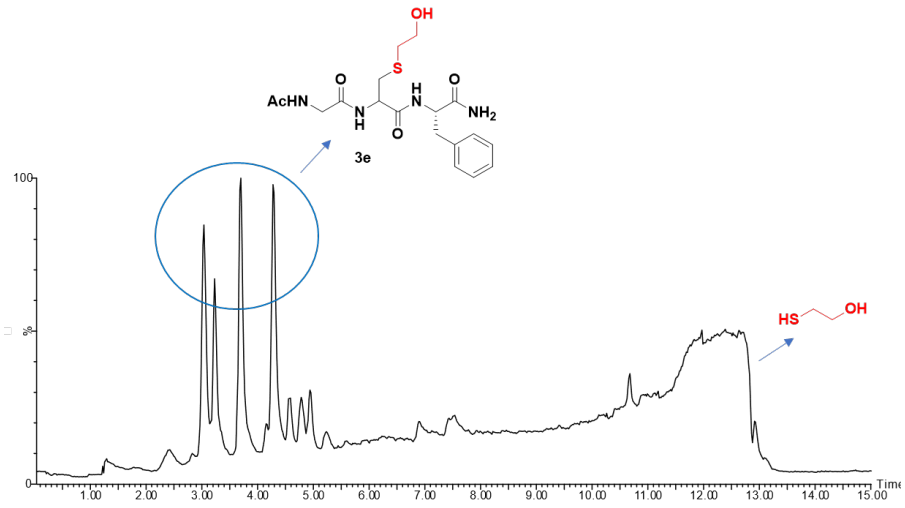


HRMS of 3e

Compound	Mass (m/z)
Expected mass of 3e	410.16
Observed mass of 3e	433.1529 [M+Na]

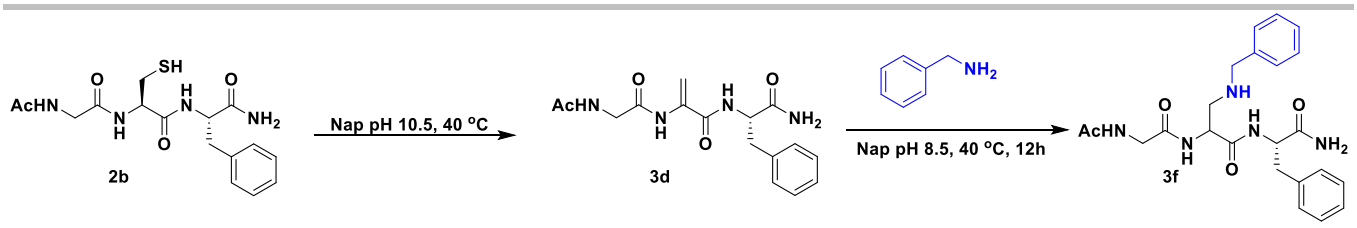


HPLC trace of thio-ene reaction



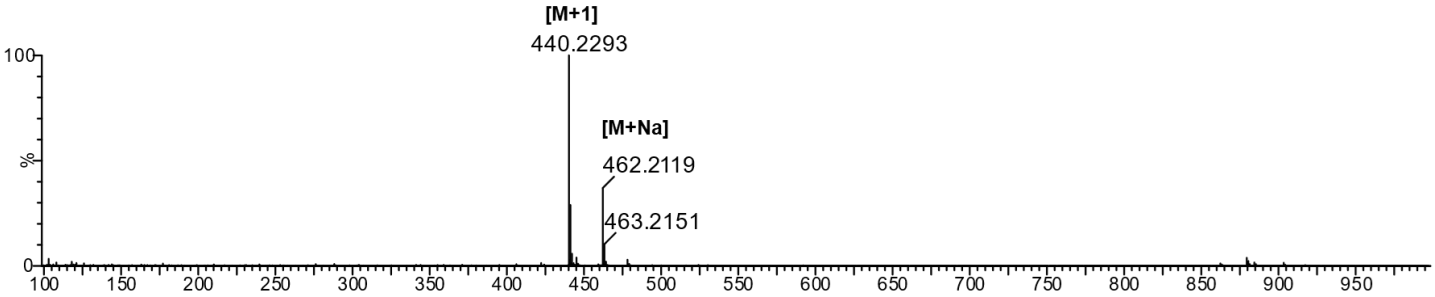
Procedure for dehydroalanine aza-Michael addition

Dha peptide **3d** (0.0015 mmol) was dissolved in 0.4 mL of 10 mM Nap (pH 8.5), 4 equiv. of benzylamine was added and the reaction mixture was stirred at 40 ^{o}C for 12 h to generate 3f (> 99% conversion). The reaction was analyzed by HPLC and ESI-MS. HPLC was carried out with 1 % formic acid: water (solvent A): acetonitrile (solvent B); 0-80 % in 30 min, flow rate = 1.0 mL/min, detection wavelength 220 nm.

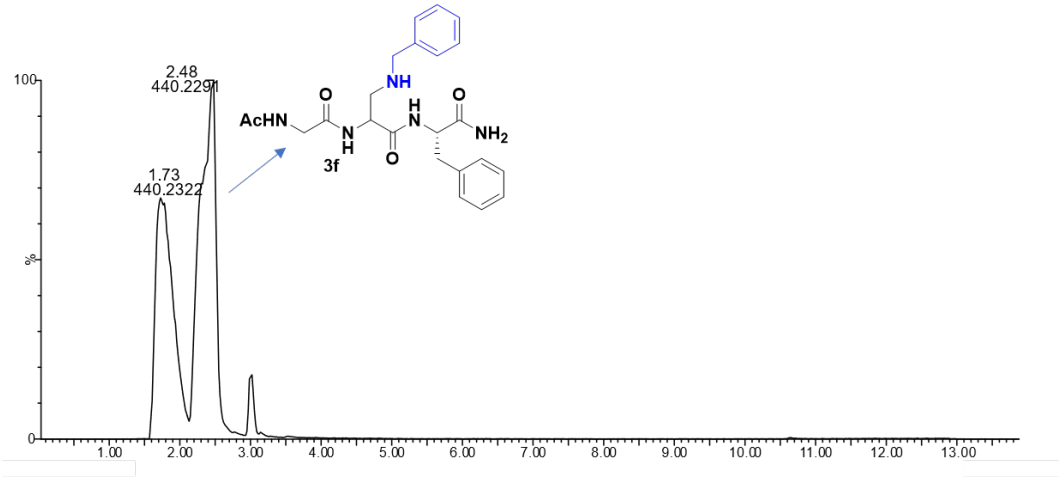


HRMS of 3f

Compound	Mass (m/z)
Expected mass of 3f	439.22
Observed mass of 3f	440.2293 [M+1], 462.2119 [M+Na]

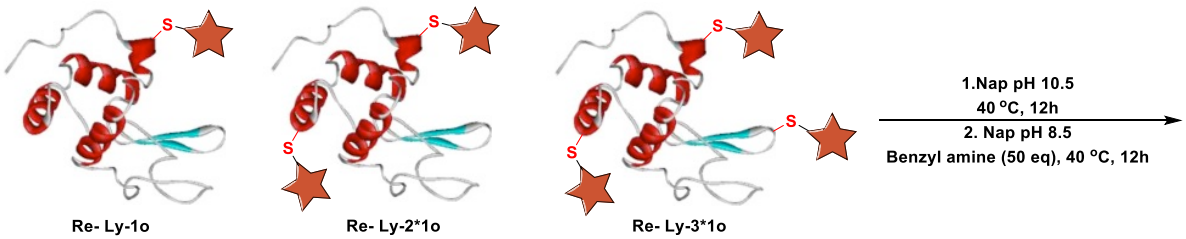


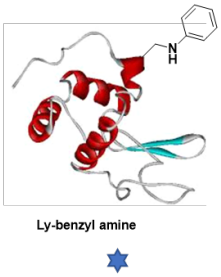
HPLC trace of aza-Michael addition

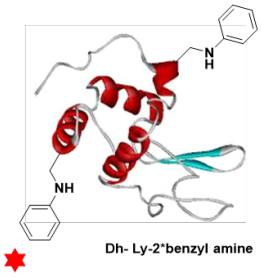


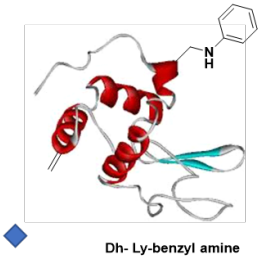
XXX. Supplementary Figure 23. Aza-Michael addition of dehydroalanine lysozyme

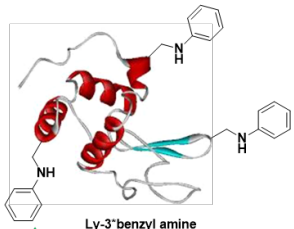
To a mixture of lysozyme (60 µmol, 0.15 mM) in 400 µL 10 mM Nap (pH 7.5), probe **1o** (5.76 mg, 18 mmol, 300 equiv.) was added. The reaction mixture was inubated at room temperature for 8 h and lyophilized to obtain modified lysozyme. To a solution of modified lysozyme (0.15 mM) in 400 µL of 10 mM Nap (pH 10.5) was incubated at 40 °C for 12 h. The crude compound was purified by molecular weight-cutoff and lyophilized to afford dh-lysozyme. In a solution of dh-lysozyme (0.15 mM) in 400 µL of 10 mM Nap (pH 8.5) was incubated with benzyl amine (7.5 mM) at 40 °C for 12 h. The reaction was analyzed by ESI-MS.

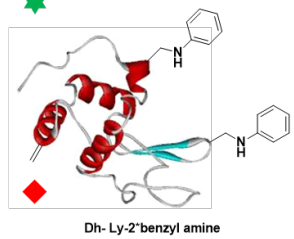


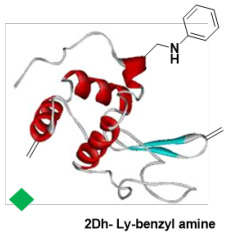


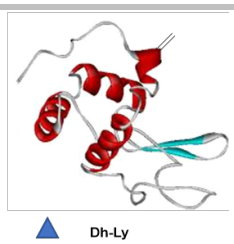


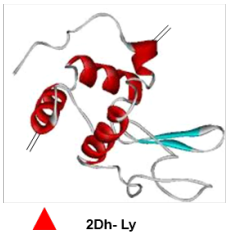


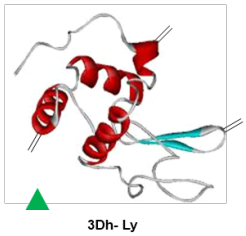












MS fragment of Ly-benzyl amine

Fragmentation of Ly-benzyl amine	Expected mass (m/z)	Observed mass (m/z)
27	533	533.3822
28	514	514.2238

MS fragment of Dh-Ly-2*benzyl amine

Fragmentation of Dh-Ly-2*benzyl amine	Expected mass (m/z)	Observed mass (m/z)
14	1033	1032.1148
21	689	689.2380

MS fragment of Ly-3*benzyl amine

Fragmentation of Ly-3*benzyl amine	Expected mass (m/z)	Observed mass (m/z)
15	969	970.6966
22	662	662.5040
27	539	539.1148

MS fragment of Dh-Ly-benzyl amine

Fragmentation of Dh-Ly-benzyl amine	Expected mass (m/z)	Observed mass (m/z)
24	598	598.7516
25	574	574.4489

MS fragment of Dh-Ly-2*benzyl amine

Fragmentation of Dh-Ly-2*benzyl amine	Expected mass (m/z)	Observed mass (m/z)
17	849	849.1764
22	656	656.4021
25	577	578.7610

MS fragment of 2Dh-Ly

Fragmentation of 2Dh-Ly	Expected mass (m/z)	Observed mass (m/z)
17	838	838.6134

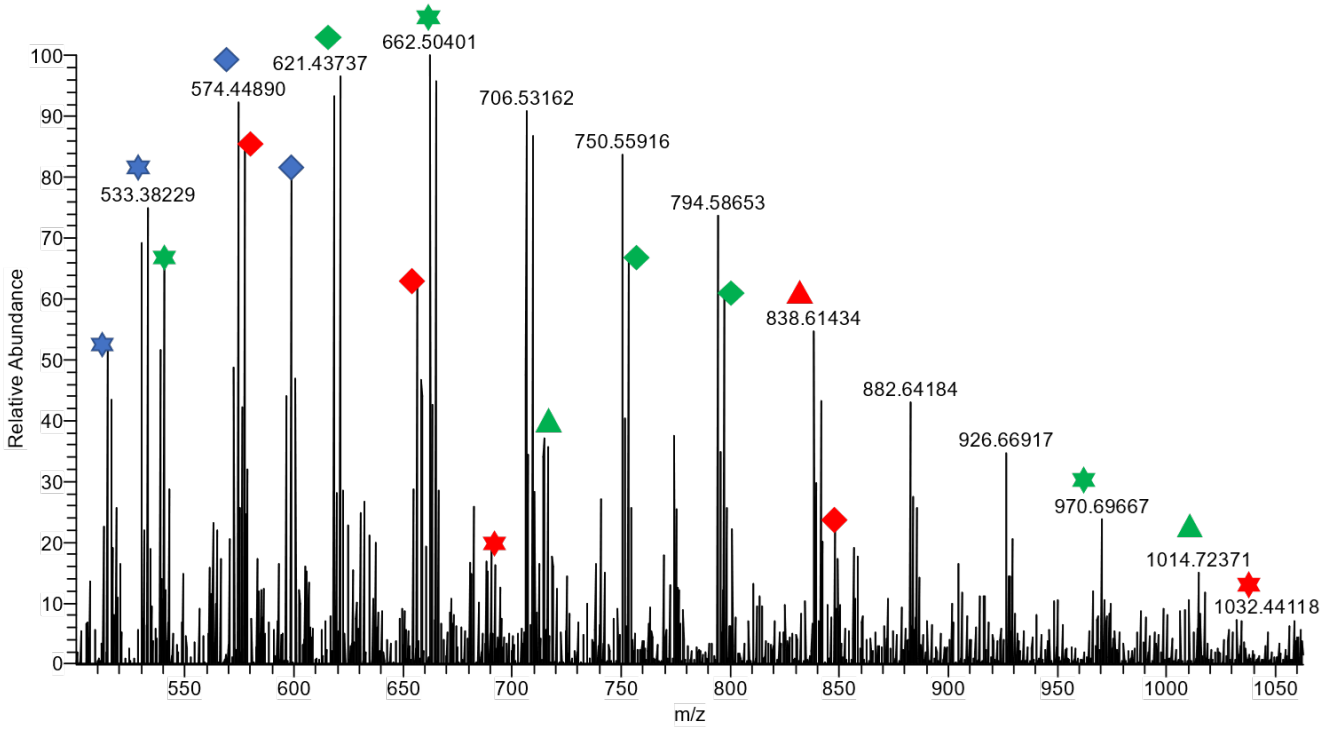
MS fragment of 2Dh-Ly-benzyl amine

Fragmentation of 2Dh-Ly-benzyl amine	Expected mass (m/z)	Observed mass (m/z)
18	796	796.1213
19	754	754.2631
23	622	621.4373

MS fragment of 3Dh-Ly

Fragmentation of 3Dh-Ly	Expected mass (m/z)	Observed mass (m/z)
14	1014	1014.7237
20	711	712.2375

HRMS of dehydroalanine lysozyme Aza-Michael addition



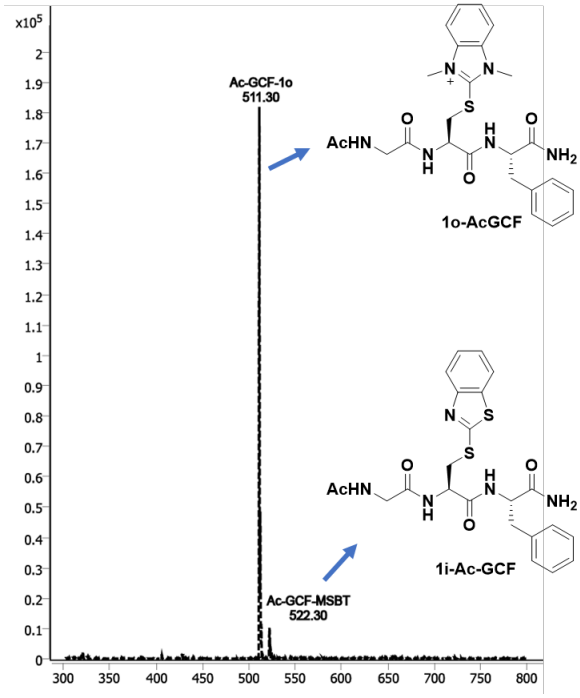
XXXI. Supplementary Figure 24. Mass sensitivity booster capability of 1o-modified peptides.

Procedure for checking intensity ratios of IAA-Ac-GCF, 1i-Ac-GCF and 1o-AcGCF

The peptides 5 µmol (**IAA-Ac-GCF, 1i-Ac-GCF and Ac-GCF**) were taken in an eppendorf tube containing acetonitrile (100 µL). The **1o-AcGCF** 5 µmol were taken in another eppendorf tube containing acetonitrile (100 µL). Equal volumes (50 µL) of each solution were taken from the stock solution in another eppendorf tube. The mixture was vortexed, and 50 µL was transferred to the HPLC vial for ESI-MS. Subsequently, the intensity ratios were analyzed by MS.

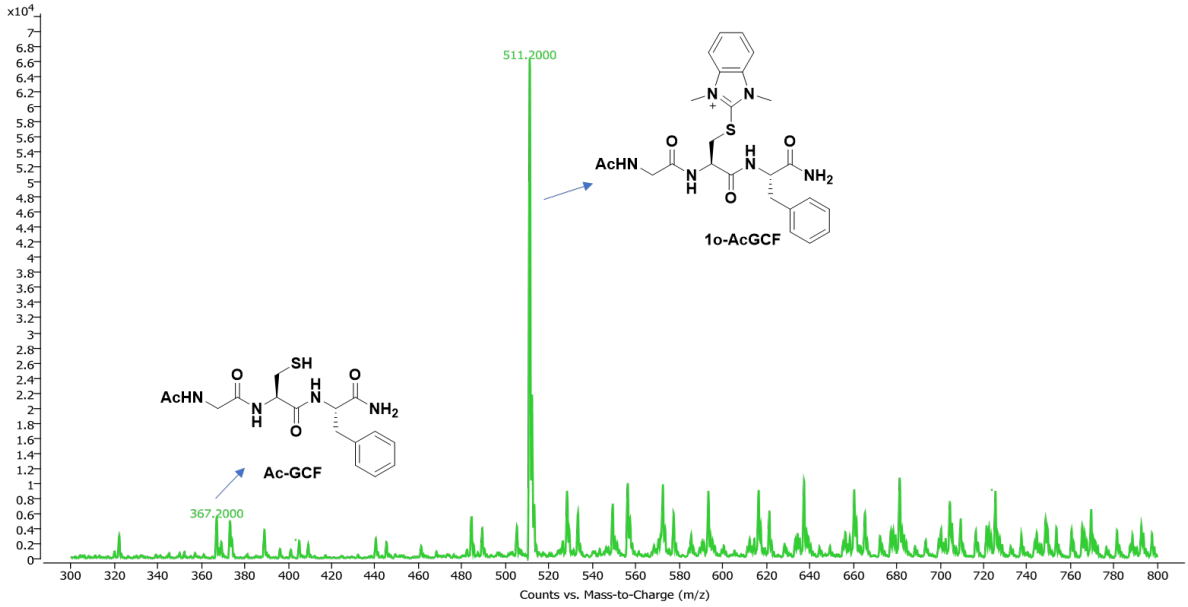
ESI-MS of 1i-Ac-GCF and 1o-AcGCF (5 µM each)

Compound	Mass (m/z)
Expected mass of 1i-Ac-GCF	499.13
Observed mass of 1i-Ac-GCF	522.30 [M+Na]
Expected mass of 1o-Ac-GCF	511.21
Observed mass of 1o-Ac-GCF	511.30



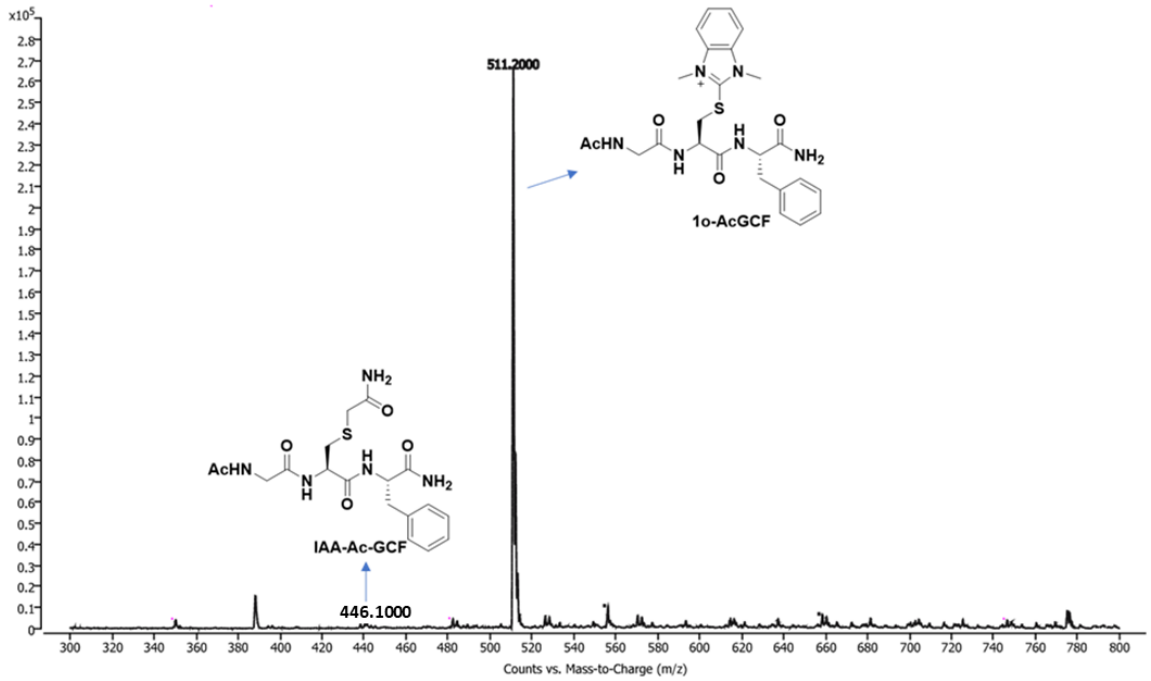
ESI-MS of Ac-GCF and 1o-AcGCF (5 µM each)

Compound	Mass (m/z)
Expected mass of Ac-GCF	366.21
Observed mass of Ac-GCF	367.20
Expected mass of 1o-Ac-GCF	511.21
Observed mass of 1o-Ac-GCF	511.20



ESI-MS of IAA-Ac-GCF and 1o-AcGCF (5 µM each)

Compound	Mass (m/z)
Expected mass of IAA-Ac-GCF	423.16
Observed mass of IAA-Ac-GCF	446.10 [M+Na]
Expected mass of 1o-Ac-GCF	511.21
Observed mass of 1o-Ac-GCF	511.20



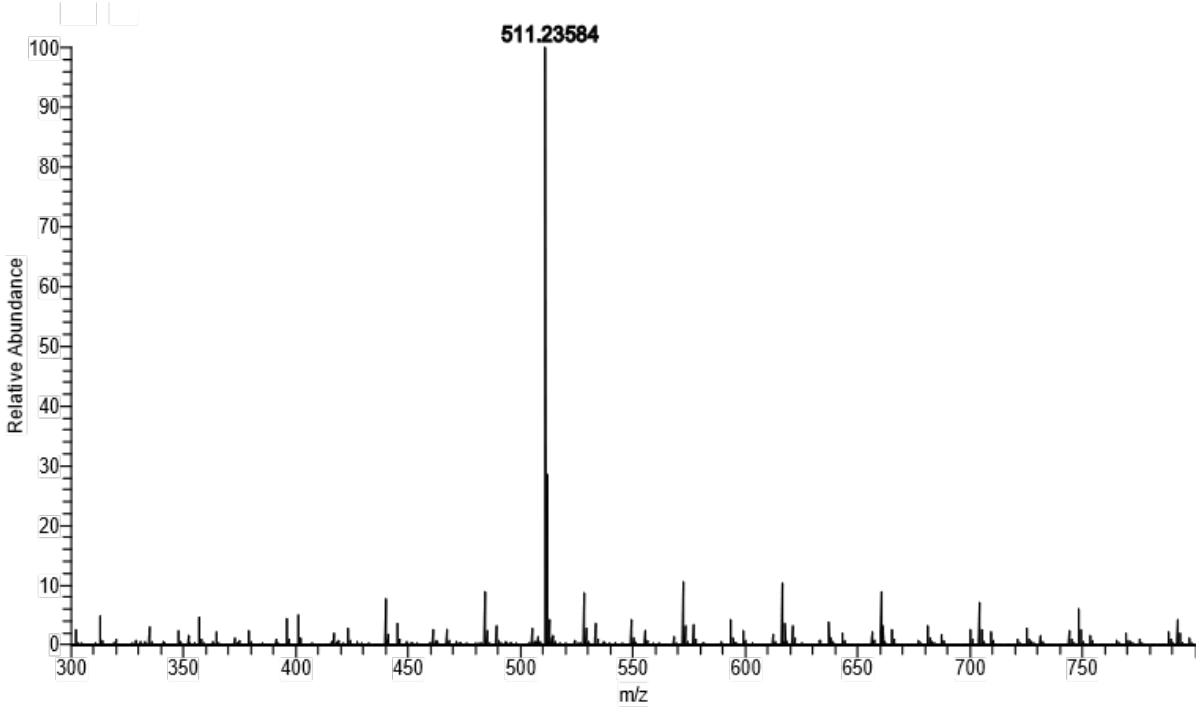
Mass sensitivity of 1o-Ac-GCF (low concentration)

General procedure for checking mass intensity of 3b at low concentration

The modified peptide 3b (5 μmol) in 100 μL Nap pH 7.5 was diluted to 5 nM, 0.5 nM with DI water. The mixture was vortexed, and 50 μL was transferred to the HPLC vial for ESI-MS. Subsequently, the mass intensity of each concentration were analyzed by MS.

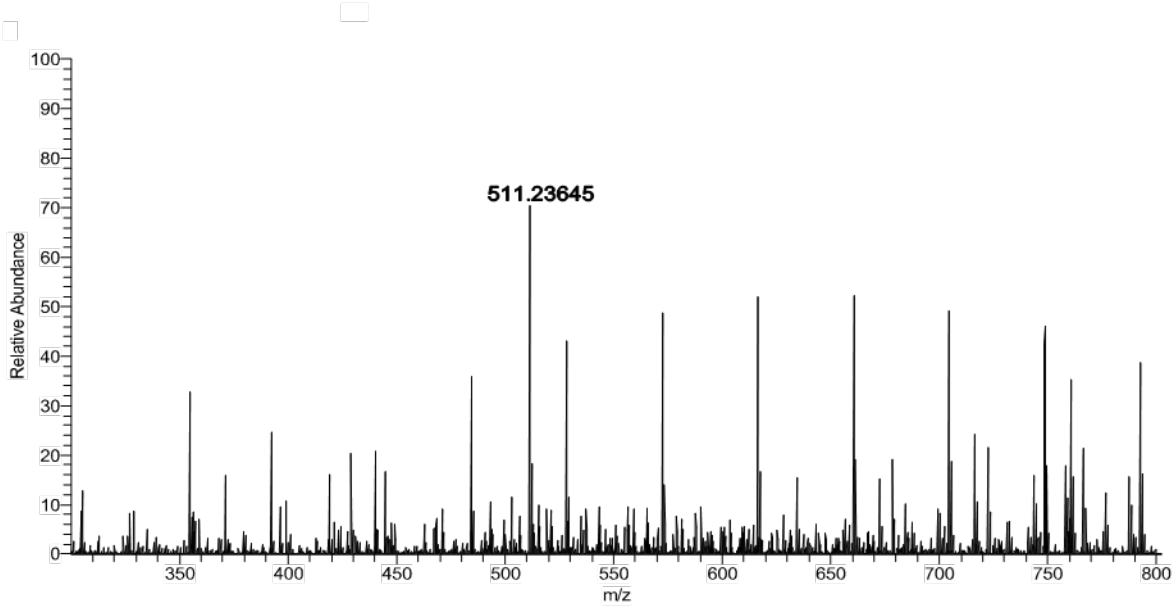
Mass spectra of 3b at 0.05 μM

Compound	Mass (m/z)
Expected mass of 3b	511.21
Observed mass of 3b	511.2358



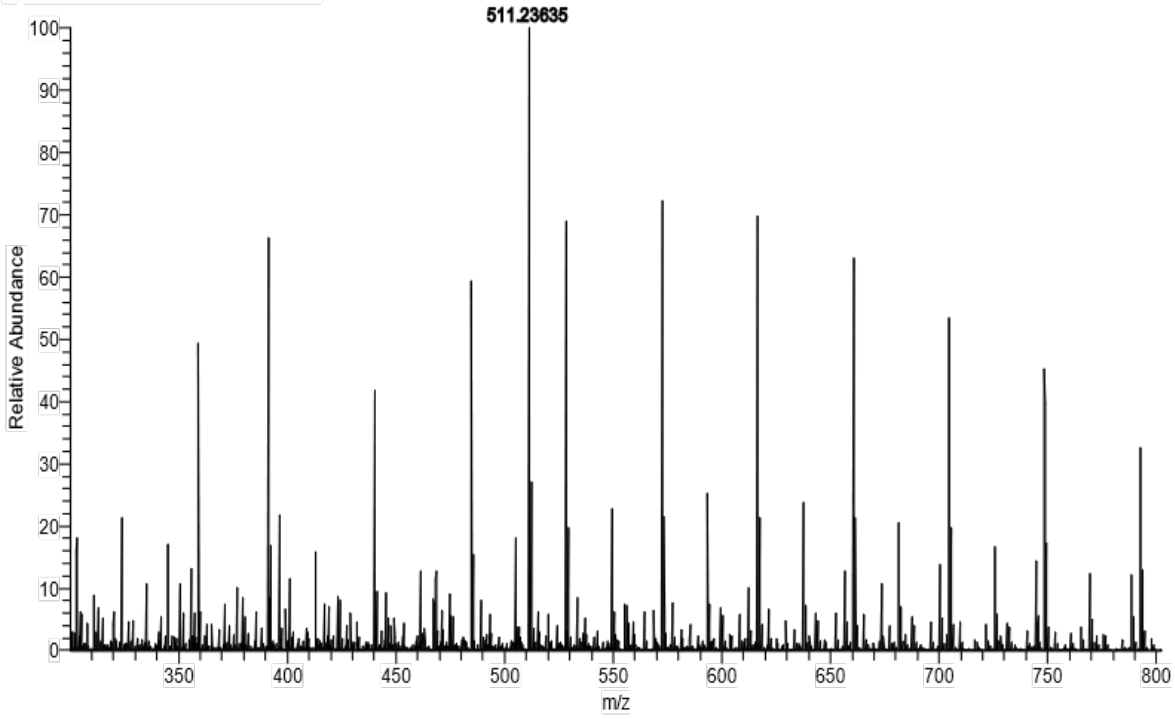
Mass spectra of 3b at 5 nM

Compound	Mass (m/z)
Expected mass of 3b	511.21
Observed mass of 3b	511.2364



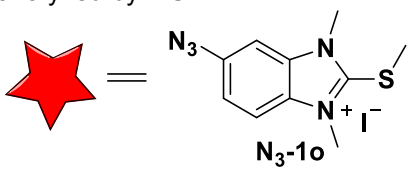
Mass spectra of 3b at 0.5 nM

Compound	Mass (m/z)
Expected mass of 3b	511.21
Observed mass of 3b	511.2363



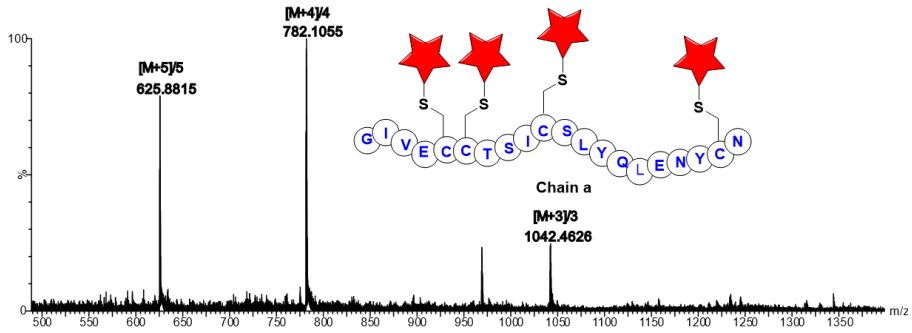
XXXII. Supplementary Figure 25. Mass intensity enhancement of N_3-1o -reduced insulin bioconjugate products.

Procedure for checking intensity ratios of N_3-1o-reduced insulin and IAA-reduced insulin. To 0.35 mg of insulin (60 μmol, 0.15 mM) in 400μL 10 mM Nap (pH 7.5), 20 μL of TCEP solution (pH 7.4, 50 mM) was added to reduce the insulin. The mixture was incubated at room temperature for 20 min. Probe N_3-1o or IAA (50 equiv.) was added into this mixture and the reaction was allowed to react at room temperature for 8 h. Equal volume (50 μL) of each solution was taken from the reaction mixture then transferred into the HPLC vial for ESI-MS. Subsequently, the intensity ratios were analyzed by MS.



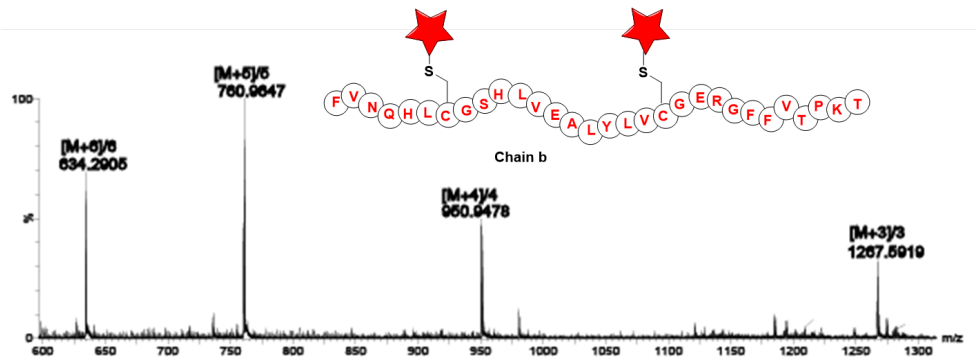
HRMS of insulin chain a-N3-1o

Fragmentation of tetra-modified chain a	Expected mass (m/z)	Observed mass (m/z)
3	1042	1042.4626
4	781	782.1055
5	625	625.8815



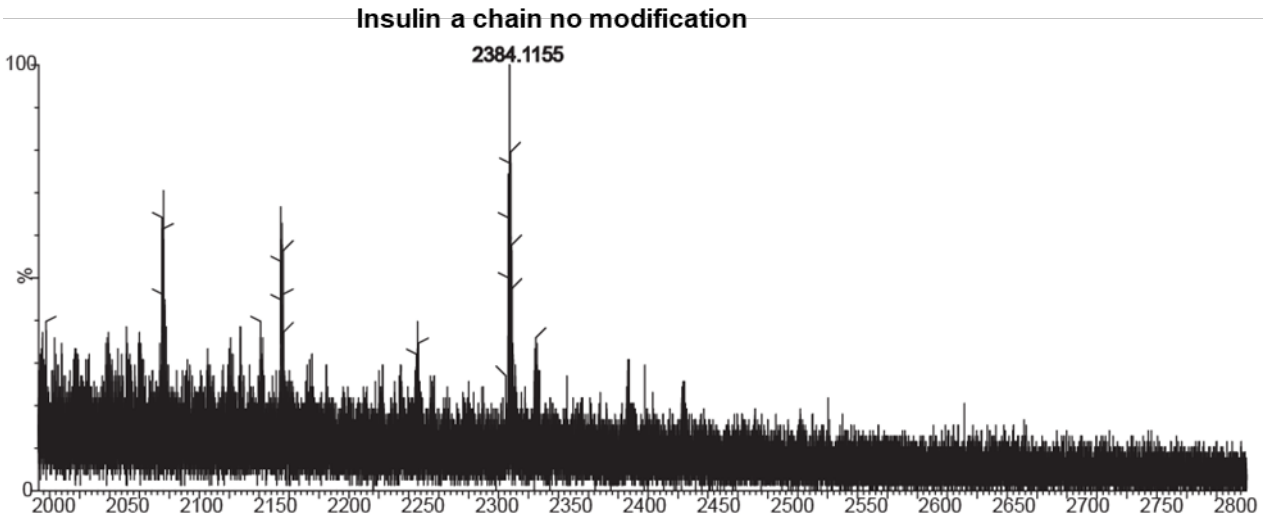
HRMS of insulin chain b-N3-1o

Fragmentation of double-modified chain b	Expected mass (m/z)	Observed mass (m/z)
3	1267	1267.5919
4	950	950.9478
5	760	760.9647
6	634	634.2905



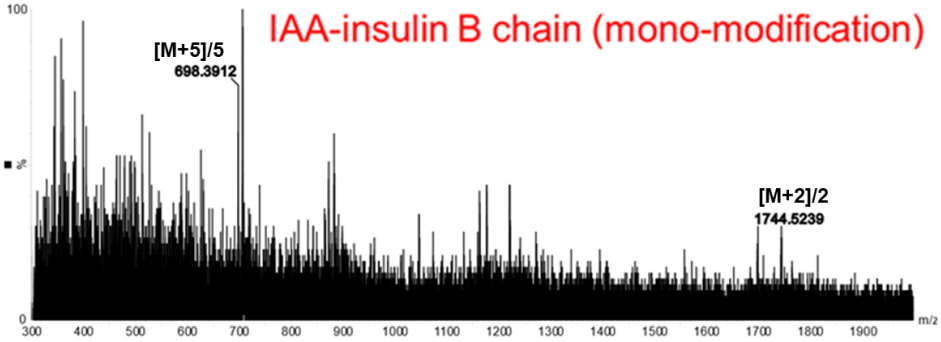
HRMS of insulin chain a

Compound	Mass (m/z)
Expected mass of chain a	2383
Observed mass of chain a	2384.1155



HRMS of insulin chain b-IAA

Fragmentation of IAA-chain b	Expected mass (m/z)	Observed mass (m/z)
2	1744	1744.5239
5	698	698.3912



Mixture of N3-1o-reduced insulin and IAA-reduced insulin:

Procedure for checking intensity ratios of N_3-1o-reduced insulin and IAA-reduced insulin in a mixture. The equal amounts (7.5 µmol) of IAA-labeled and HAT-labeled insulin protein fragments (both chain A and B) in water (50 µL) are evaluated in a single MS spectrum. We did not observe the modified IAA-labeled chain A and chain B of insulin in the presence of HAT-labeled chain A and Chain B of insulin in the MS spectra.

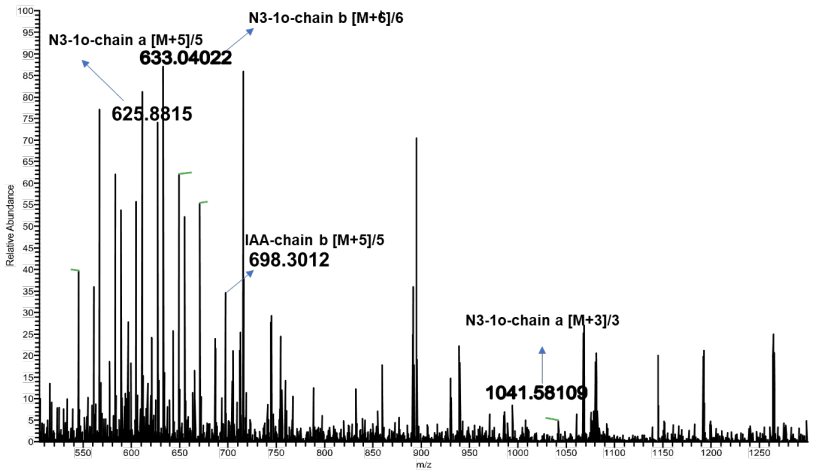
Fragmentation of 1o tetra-modified chain a	Expected mass (m/z)	Observed mass (m/z)
3	1042	1041.5810
5	625	625.5815

Fragmentation of 1o-chain b

Fragmentation of 1o double-modified chain b	Expected mass (m/z)	Observed mass (m/z)
6	634	633.0402

Fragmentation of IAA-chain b

Fragmentation of IAA-chain b	Expected mass (m/z)	Observed mass (m/z)
5	698	698.3012



XXXIII. Supplementary Figure 26. HAT probes for gel-based ABPPCell culture and preparation of cell lysates. Cell culture reagents including Dulbecco's phosphate-buffered saline (DPBS), Dulbecco's modified Eagle's medium (DMEM)/high glucose media, trypsin-EDTA and penicillin/streptomycin (Pen/Strep) were purchased from Fisher Scientific. Fetal Bovine Serum (FBS) were purchased from Avantor Seradigm (lot # 214B17).

HEK293T (ATCC: CRL-3216) cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics (Penn/Strep, 100 U/mL). Media was filtered (0.22 µm) prior to use. Cells were maintained in a humidified incubator at 37 °C with 5% CO_2. Cell lines were validated prior to use and tested regularly for myoplasma.

HEK293T cells were harvested once cells were grown to 90 – 95% confluence by centrifugation (4500g, 5 min, 4 °C), washed twice with cold DPBS, resuspended in 300 µL DPBS, sonicated, and clarified by centrifuging (21,000 g, 10 min, 4 °C). The lysates were then transferred to an eppendorf tube. Protein concentrations were determined using a Bio-Rad DC protein assay kit using reagents from Bio-Rad Life Science (Hercules, CA) and the lysate diluted to the working concentrations indicated below.

Gel-based ABPP with N3-1o. HEK293T proteome (50 µL of 1.5 mg/mL, prepared as described above) was labeled with various concentration of **N3-1o** (stock solutions in DMSO, final concentration as indicated), IAA (1 µL of 5 mM stock solution in DMSO, final concentrations = 1-200 µM) or DMSO for vehicle control for 1h at ambient temperature followed by adding 1 µM IA-Rh. Samples were allowed to react for another hour at ambient temperature at which point the reactions were quenched with 4× Laemmli buffer (20 µL). Samples were then denatured (5 min, 95 °C) and then resolved by SDS-PAGE. SDS-PAGE gels were imaged on the Bio-Rad ChemiDoc™ Imager using rhodamine channel.

Gel-based ABPP with N3-1o. HEK293T proteome (50 µL of 1.5 mg/mL) was labeled with different amount of **N3-1o** (stock solutions in DMSO, final concentration as indicated), STP-alkyne (1 mM) or DMSO for vehicle control for 1h at ambient temperature followed by adding 1 µM NHS-Rh. Samples were allowed to react for another hour at ambient temperature at which point the reactions were quenched with 4× Laemmli buffer (20 µL). Samples were then denatured (5 min, 95 °C) and then resolved by SDS-PAGE. SDS-PAGE gels were imaged on the Bio-Rad ChemiDoc™ Imager using rhodamine channel.

XXXIV. References.

[1] W. C. Chan, P. D. White, Fmoc solid phase peptide synthesis: a practical approach; Oxford University Press: New York, 2000.

[2] Tang, K. C.; Raj, M., One-Step Azolation Strategy for Site- and Chemo-Selective Labeling of Proteins with Mass-Sensitive Probes. *Angew Chem Int Edit* **2021,** *60* (4), 1797-1805.

[3] Fukuda, N.; Ikemoto, T., Imide-Catalyzed Oxidation System: Sulfides to Sulfoxides and Sulfones. *J Org Chem* **2010,** *75* (13), 4629-4631.

[4] McNamara, Y. M.; Cloonan, S. M.; Knox, A. J. S.; Keating, J. J.; Butler, S. G.; Peters, G. H.; Meegan, M. J.; Williams, D. C., Synthesis and serotonin transporter activity of 1,3-bis(aryl)-2-nitro-1-propenes as a new class of anticancer agents. *Bioorgan Med Chem* **2011,** *19* (3), 1328-1348.