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Supporting Methods

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 reactions were 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′-iisopropylcarbodiimide (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), N,N-dimethylformamide (DMF), dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), tetrahydrofuran (THF) were obtained from VWR (100 Matsonford Road Radnor, Pennsylvania).

III. Analytical Methods.

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).

LC/MS. 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) or an high-performance LC system (Agilent, 1100 series) coupled with triple quadrupole lc-ms (Agilent technologies 6460) with electrospray ionization (ESI) in positive mode using Agilent mass hunter (10.0). 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 a 400 µL/min flow rate of mobile phase of solution A (90 % H_2O, 10 % acetonitrile and 0.1 % formic acid (FA)) and solution B (95 % acetonitrile, 5 % H_2O, and 0.1 % formic acid) 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 (90 % H_2O, 10 % acetonitrile and 0.1 % formic acid) and solution B (90 % acetonitrile, 10 % H_2O, and 0.1 % formic acid) beginning gradient- Time- 1 min 0% B; 1-10 min 100% B for chromatography analysis (or) directly injected with mobile phase 90 % H_2O: 10 % ACN, 0.1% formic acid at 400 µL/min flow rate in ESI positive mode.

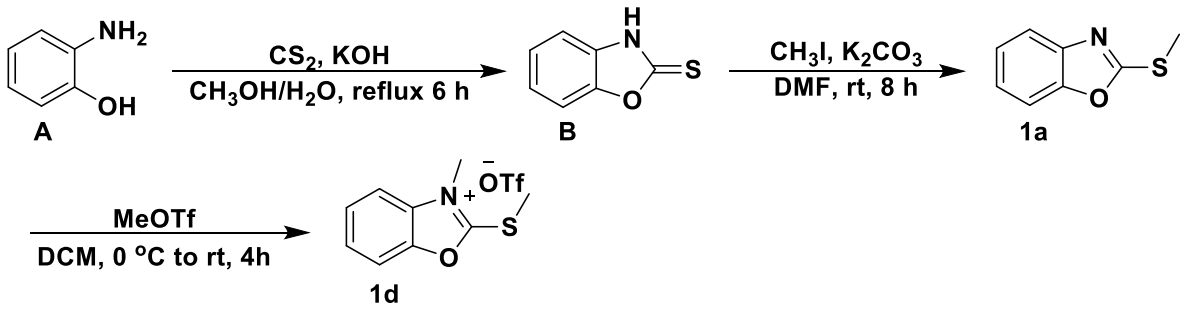
HRMS and MS/MS. High resolution MS data were acquired on a Q-TOF mass spectrometer using positive polarity electrospray ionization (+ESI). Tandem MS experiments were performed using collision-induced dissociation (CID) with N_2 as the collision gas.

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 220 nm unless otherwise noted.

IV. 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. Compounds **1a**[2a], **1b**[2b], **1c**[2c], **1d**[2c] compounds are known in literature for different applications.

V. Supplementary Figure 1: Synthesis of TARE probes (1a-1f)



Synthesis of compound B

2-aminophenol **A** (1 g, 18.3 mmol) was dissolved in methanol / water (9:1, 90 mL) and KOH (1 g, 18.3 mmol) and CS_2 (3.3 mL, 55.5 mmol) were added sequentially. The reaction mixture was refluxed for 6 h. The reaction solution was washed with ethyl acetate and brine thrice. The residue was concentrated under reduced pressure and purified by the column chromatography (hexane: ethyl acetate 2:1) to obtain compound **B** as off-white powder (91 %, 2.1 g). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 13.8 (br s, 1H), 7.45 (d, J = 7.6 Hz, 1H), 7.27-7.20 (m, 3H).**^{13}C NMR** (151 MHz, DMSO-d_6) δ 180.11, 148.10, 131.15, 125.06, 123.69, 110.42, 109.91.

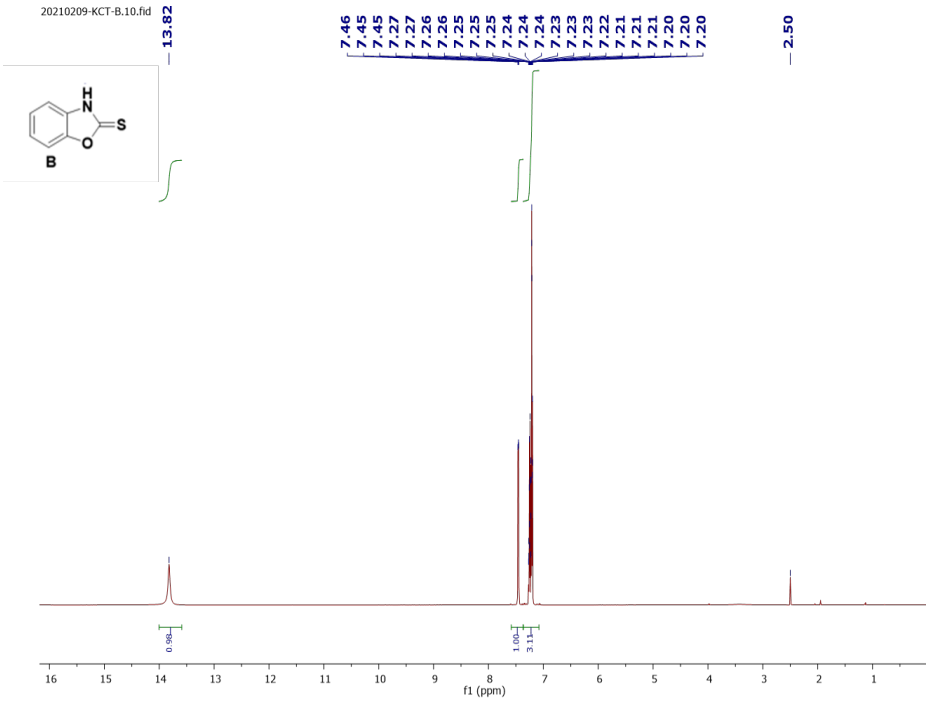
Synthesis of compound 1a

2-Mercaptobenzoxazole **B** (1 g, 6.62 mmol), potassium carbonate (914 mg, 6.62 mmol), and methyl iodide (412 µL, 6.62 mmol) were sequentially added into dry DMF (13.5 mL) at 0 °C. The mixture was warmed to room temperature and stirred for 8 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 5:1) to yield compound **1a** as yellow oil (895.7 mg, 82 %). **^{1}H NMR** (600 MHz, $CDCl_3$ δ 7.58 (dd, *J* = 8.0, 4.4 Hz, 1H), 7.37 (dd, *J* = 9.0, 4.3 Hz, 1H), 7.23 – 7.16 (m, 2H), 2.70 (s, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 165.62, 151.93, 141.93, 124.18, 123.70, 118.25, 109.74, 14.46.

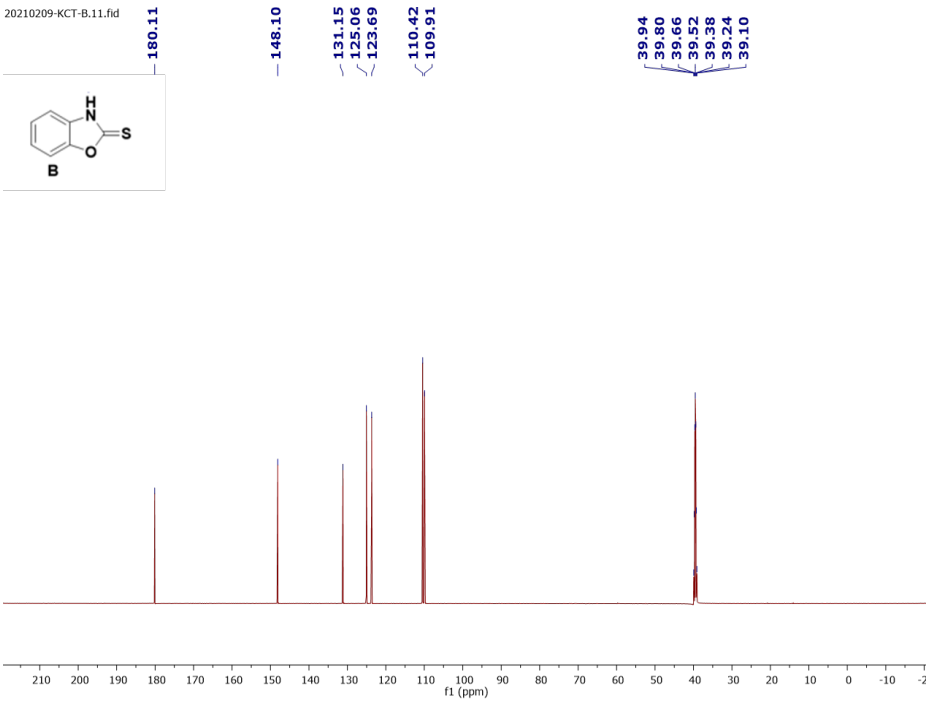
Synthesis of compound 1d

To a mixture of 2-(methylthio)benzoxazole **1a** (86 mg, 0.52 mmol) in dry DCM (10 mL), MeOTf (70 µL, 0.625 mmol) was added at 0 °C. The reaction mixture was warmed to room temperature. After 4 h, solvent was removed by rotary evaporation to afford crude compound. The crude sample was purified by recrystallization in hexane to obtain pure compound **1d** as white powder (214.6 mg, 45 %). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.14 – 8.06 (m, 1H), 8.03 (dd, *J* = 8.1, 1.2 Hz, 1H), 7.76 – 7.60 (m, 2H), 3.94 (s, 3H), 3.05 (s, 3H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 172.04, 131.57, 127.52, 123.85, 122.16, 113.44, 112.27, 32.51, 14.68.

^{1}H NMR of compound B



^{13}C NMR of compound B



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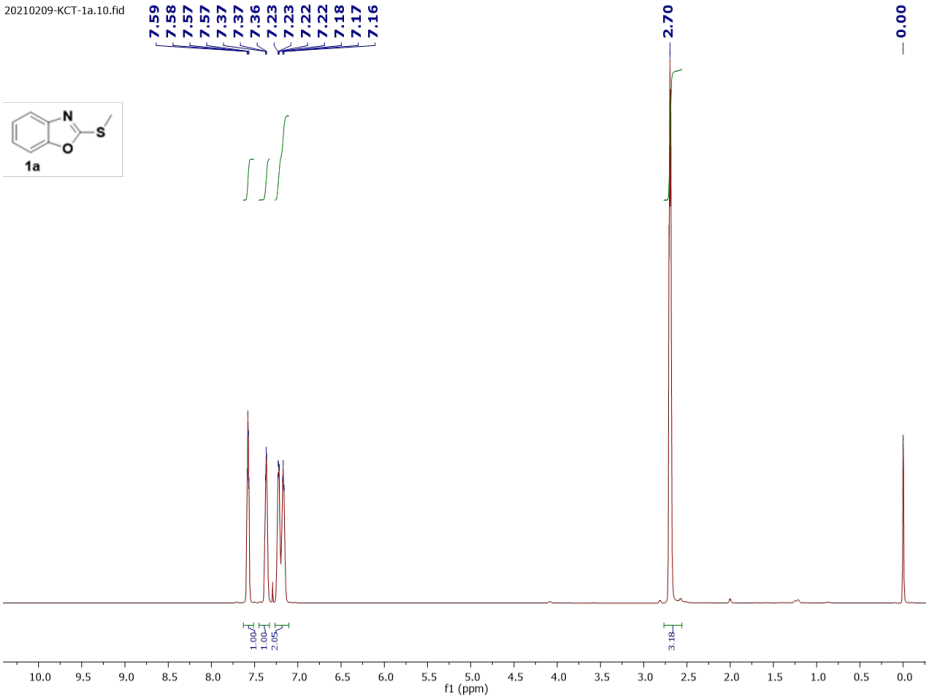

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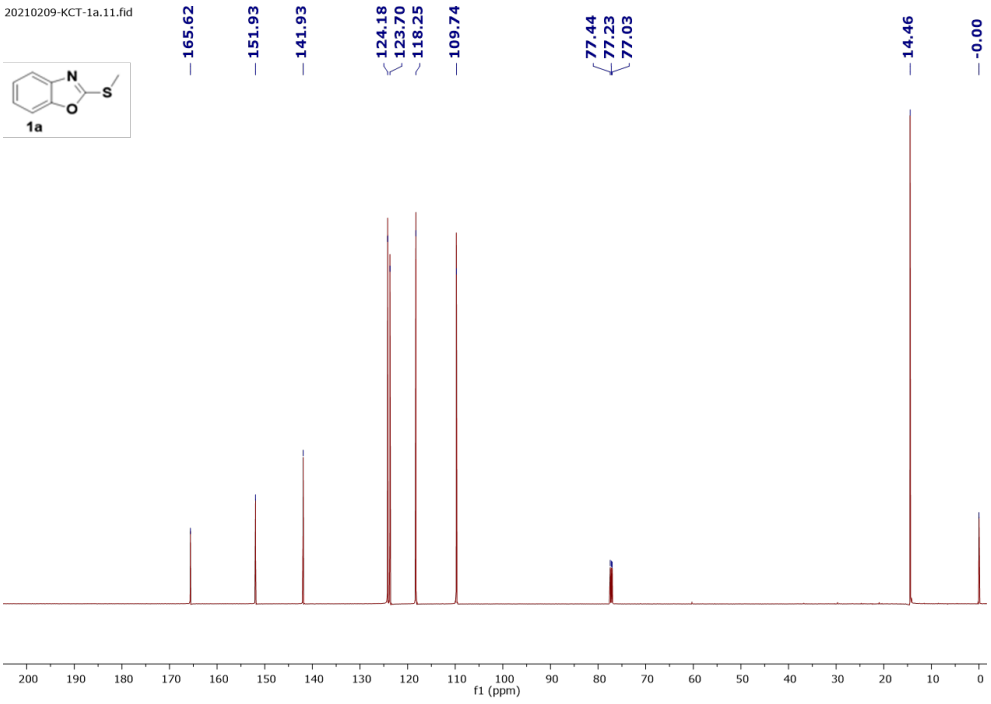

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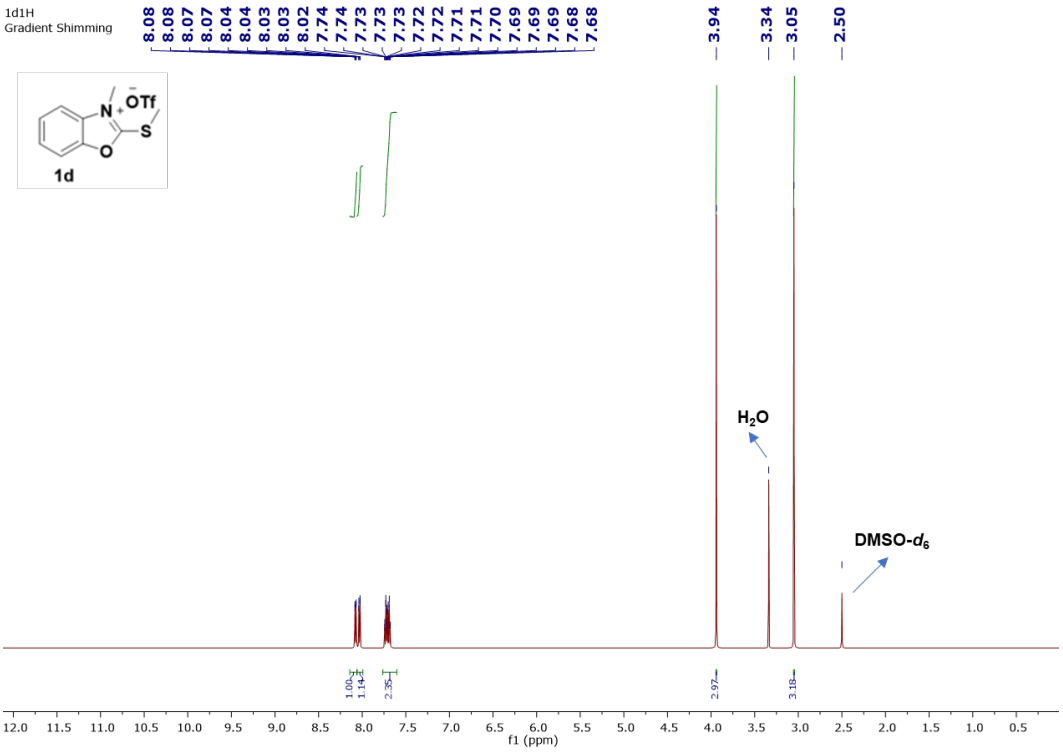

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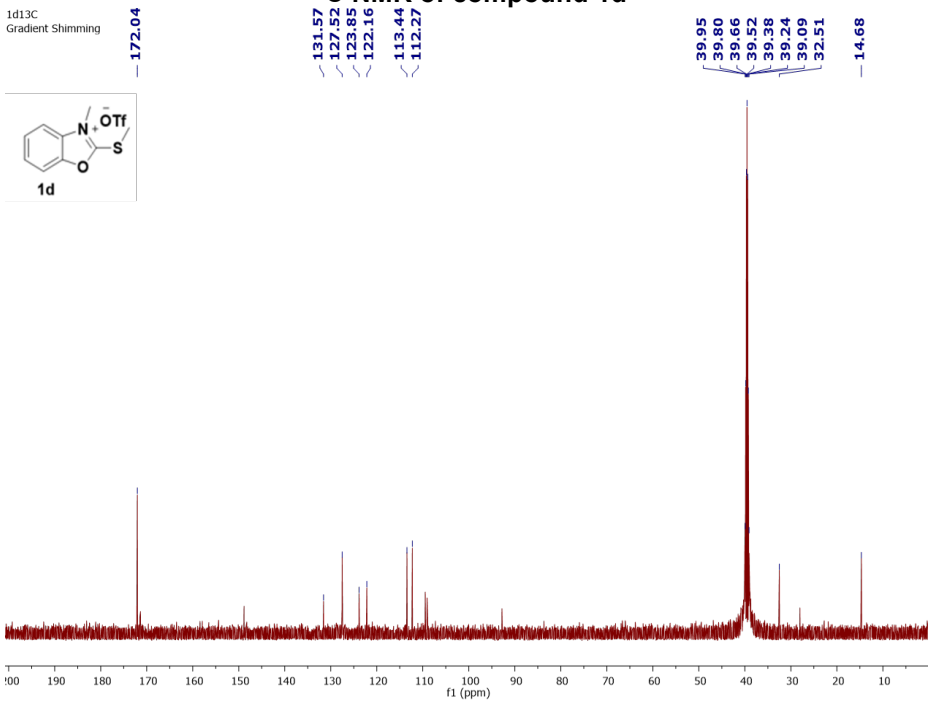
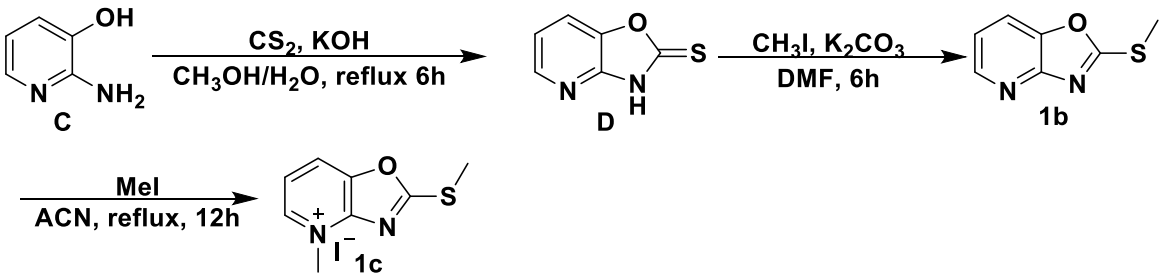




Synthesis of compound D

2-Amino-3-hydroxypyridine **C** (1.1 g, 10 mmol) was dissolved in methanol / water (10:1, 81 mL) and KOH (1.38 g, 10 mmol) and CS_2 (1.8 mL, 30 mmol) were added sequentially. The reaction mixture was refluxed for 6 h. The solution was then cooled to RT and treated with glacial acetic acid (5 mL) and diluted with water (150 mL). The resulting precipitate was filtered off and washed with hexane for three times to obtain compound **D** as off-white powder (82 %, 1.24 g). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 14.47 (s, 1H), 8.20 (d, *J* = 5.0 Hz, 1H), 7.86 – 7.83 (m, 1H), 7.25 (d, *J* = 5.3 Hz, 1H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 181.33, 146.97, 144.16, 141.59, 119.09, 117.01.

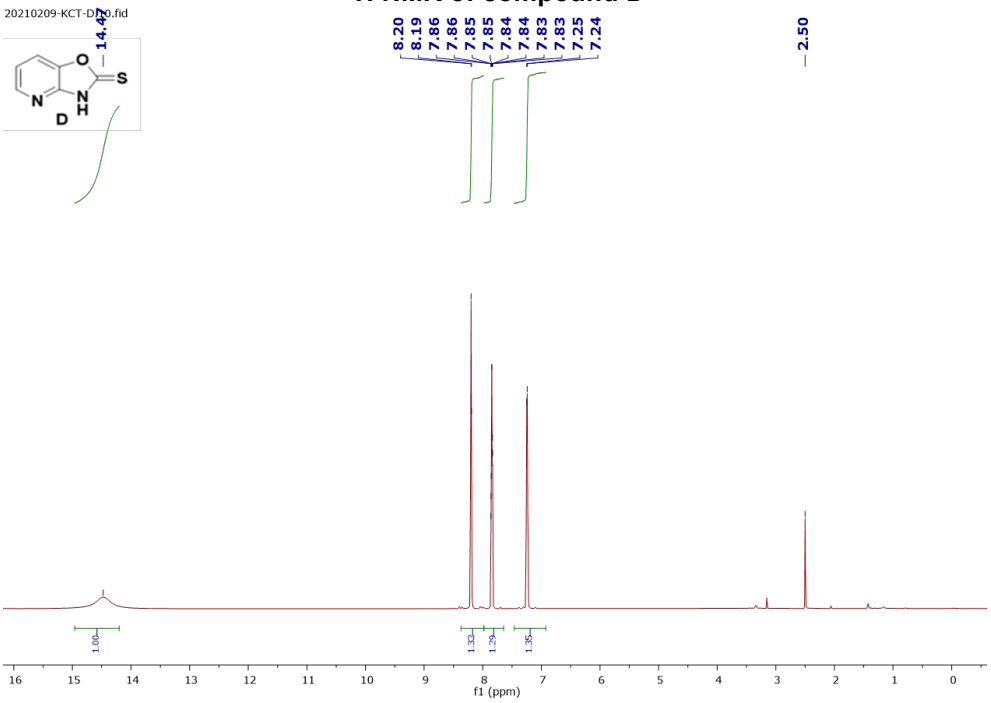
Synthesis of compound 1b

To solution of oxazolo[4,5-b]pyridine-2(3H)-thione **D** (500 mg, 3.29 mmol) in dry DMF (12 mL) was added K_2CO_3 (454 mg, 3.29 mmol), MeI (206 µL, 3.29 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 5:1) to afford compound **1b** as white powder (79 %, 431 mg). **^{1}H NMR** (600 MHz, $CDCl_3$) δ) δ 8.46 – 8.44 (m, 1H), 7.70 – 7.69 (m, 1H), 7.18 (dd, *J* = 8.0, 5.0 Hz, 1H), 2.81 (s, 3H).**^{13}C NMR** (151 MHz, $CDCl_3$) δ 170.35, 156.22, 145.89, 144.25, 118.86, 117.08, 14.82.

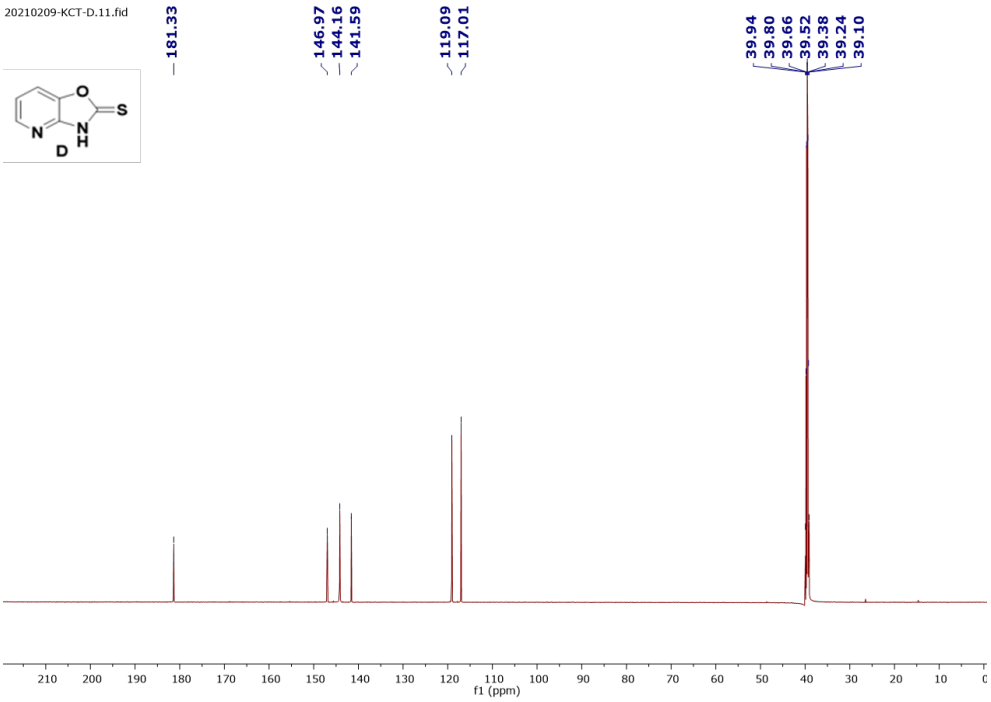
Synthesis of compound 1c

2-(Methylthio)oxazolo[4,5-b]pyridine **1b** (166 mg, 1 mmol) was dissolved in dry ACN (10 mL), and treated with methyl iodide (313 µL, 5 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 (1:15) to obtain compound **1c** as bright yellow powder (80 %, 246 mg). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.87 – 8.84 (m, 1H), 8.83 – 8.80 (m, 1H), 7.93 – 7.85 (m, 1H), 4.37 (s, 3H), 2.94 (s, 3H).**^{13}C NMR** (151 MHz, DMSO-d_6) 177.23, 171.99, 150.63, 147.54, 139.75, 125.25, 120.51, 41.69, 15.01.

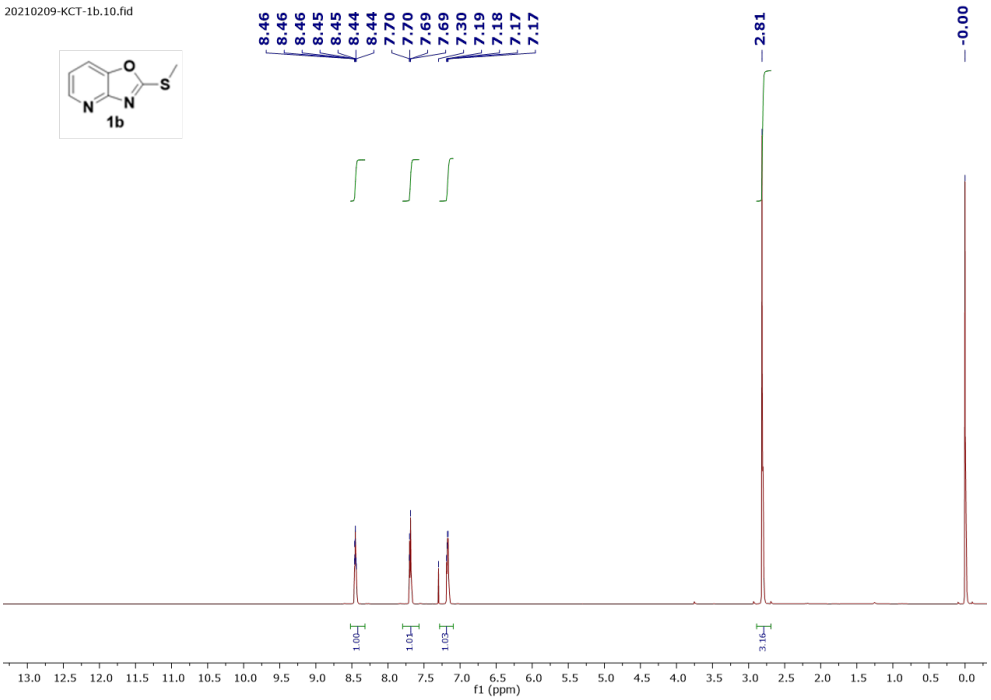
^{1}H NMR of compound D



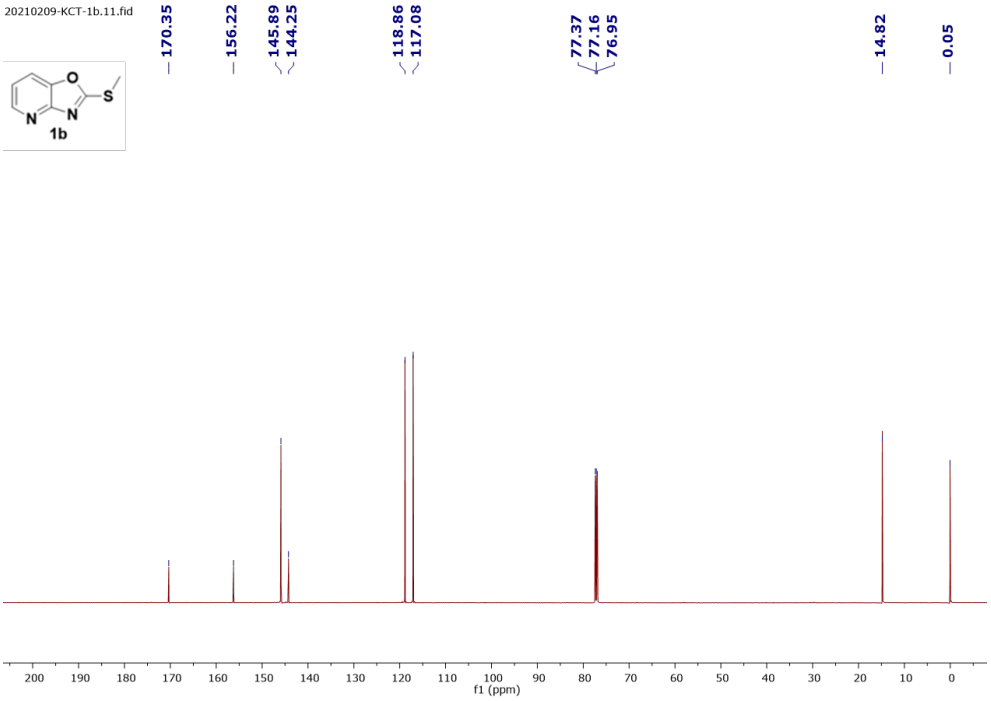
^{13}C NMR of compound D



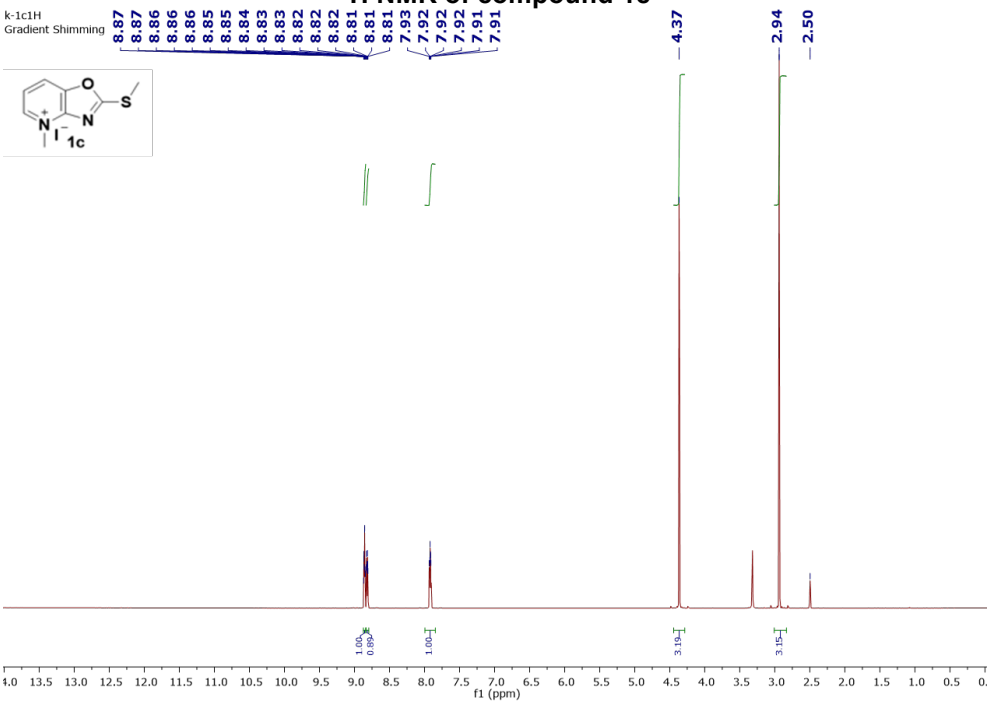
^{1}H NMR of compound 1b



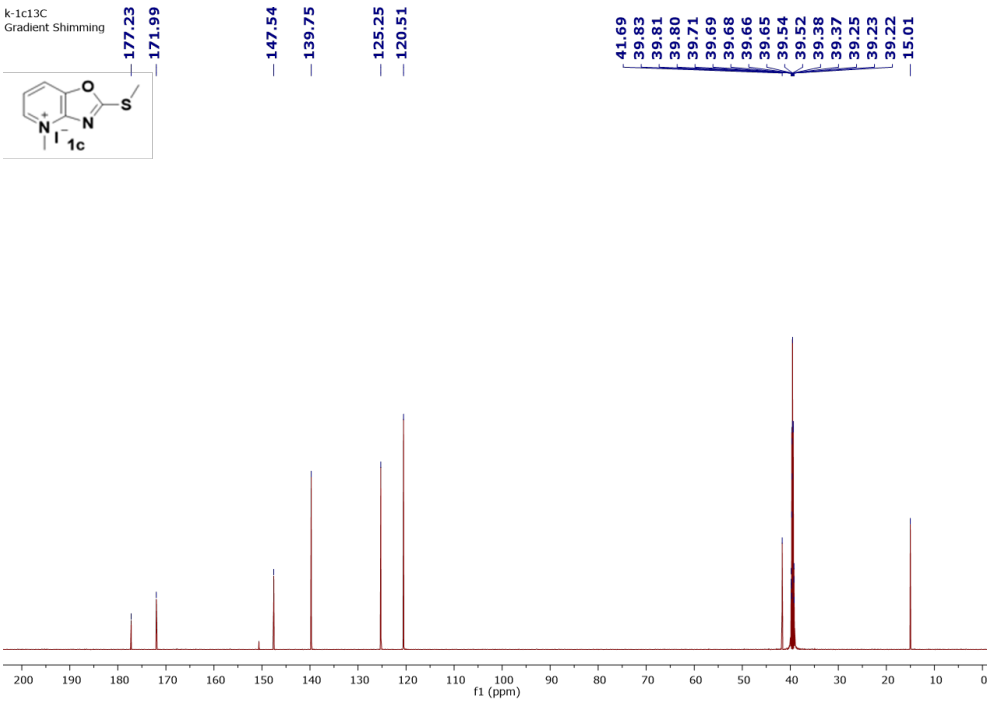
^{13}C NMR of compound 1b

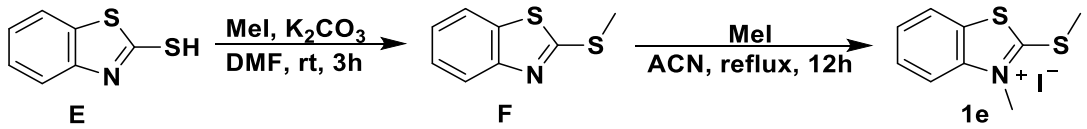


^{1}H NMR of compound 1c



^{13}C NMR of compound 1c





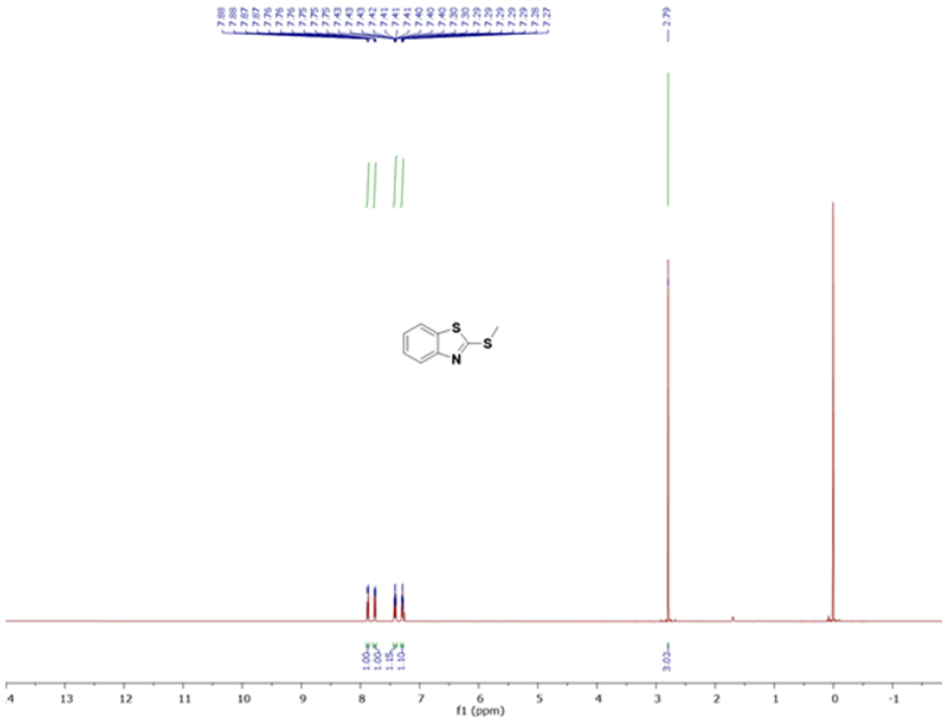
Synthesis of compound F

Mercaptobenzothiazole **E** (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 (20 mL) 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 **F** 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), 2.79 (s, 3 H)**. ^{13}C NMR** (151 MHz, $CDCl_3$) δ 167.93, 153.29, 135.09, 125.97, 124.00, 121.30, 120.88, 15.85.

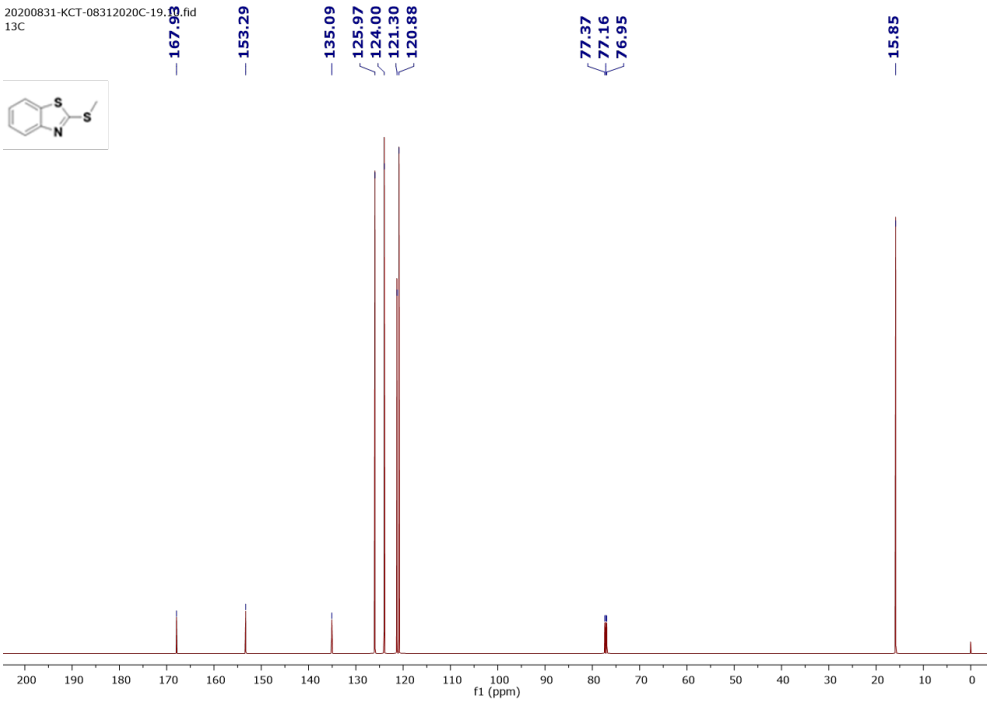
Synthesis of compound 1e

The mixture of 2-(Methylthio)benzothiazole **F** (1.5 g, 8.28 mmol) and MeI (2.6 mL, 41.43 mmol) in dry ACN (10 mL) was refluxed for 12 hours. The reaction mixture was cooled to room temperature and solvent was removed by rotary evaporation. The recrystallization was carried out in EtOH : Hexane (1:20) to obtain pure compound **1e** as yellow powder (92 %, 1.49 g). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.41 (d, *J* = 8.1 Hz, 1H), 8.20 (d, *J* = 8.5 Hz, 1H), 7.84 (t, *J* = 8.0 Hz, 1H), 7.73 (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, 128.17, 126.93, 123.93,115.66, 36.53, 18.20.

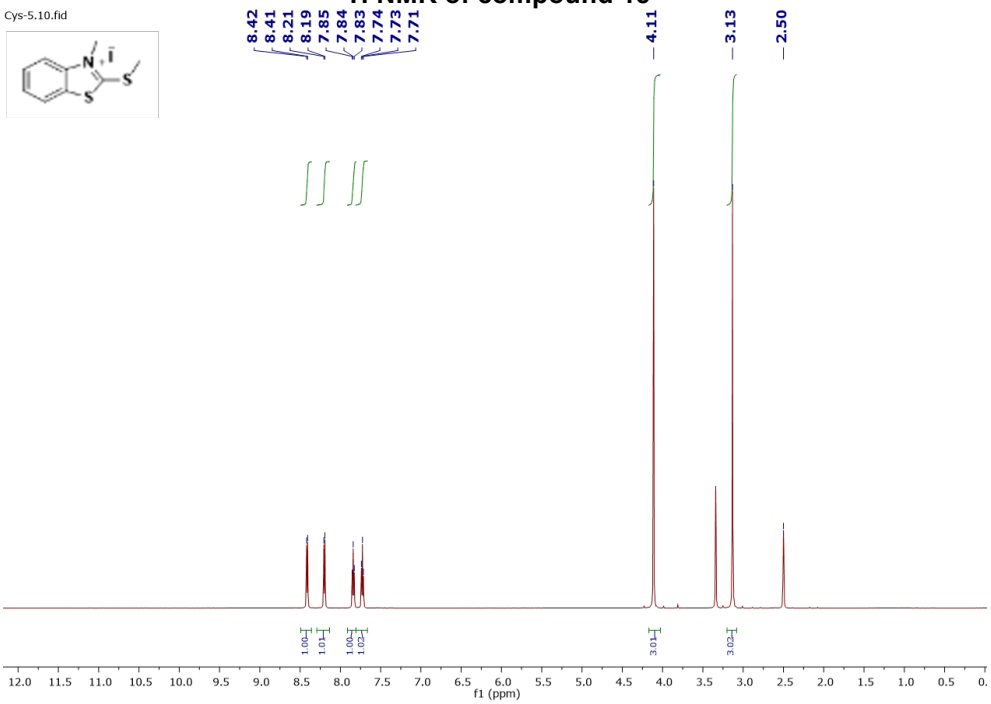
^{1}H NMR of compound F

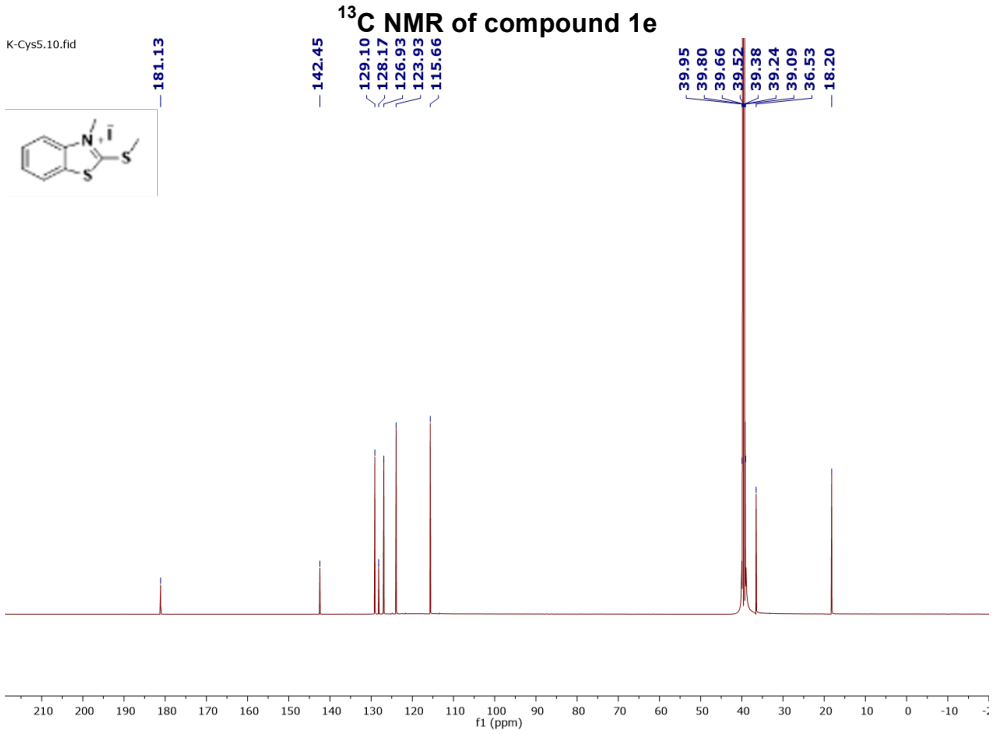


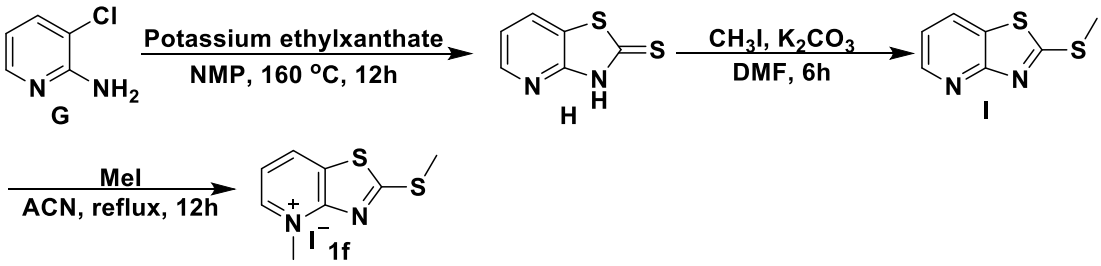
^{13}C NMR of compound F



^{1}H NMR of compound 1e







Synthesis of compound H

2-Amino-3-chloro-pyridine **G** (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 **H** as off-white powder (65 %, 850 mg). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 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 191.48, 154.21, 147.27, 130.99, 124.65, 120.06.

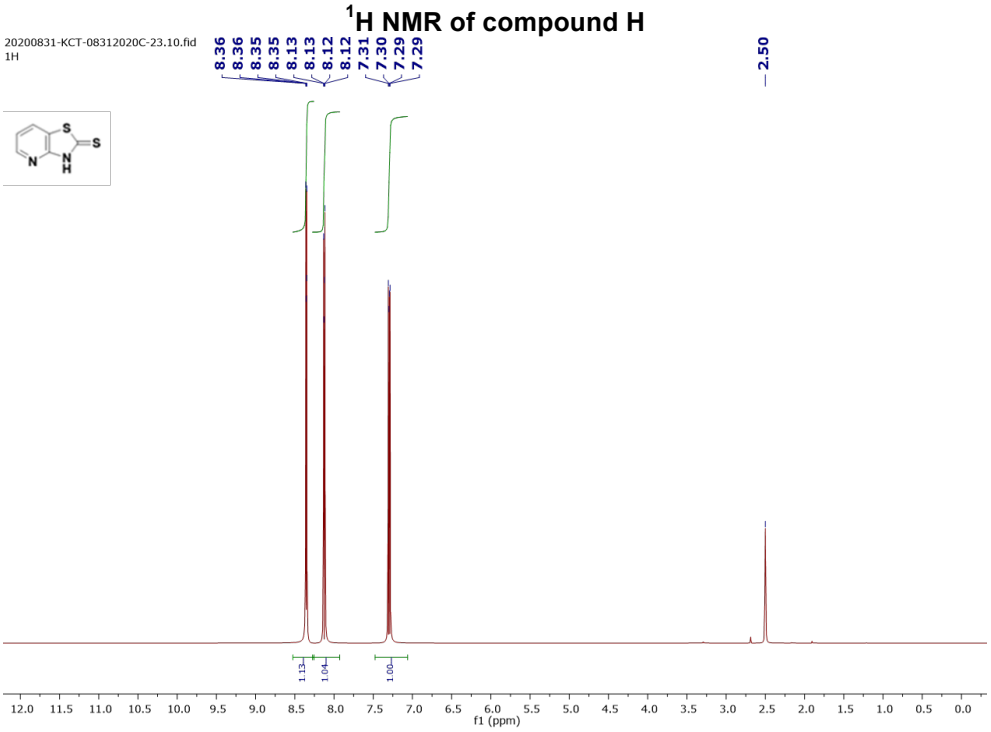
Synthesis of compound I

To solution of thiazolo[4,5-b]pyridine-2(3H)-thione **H** (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 °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 **I** as colorless crystal (73 %, 312 mg). **^{1}H NMR** (600 MHz, $CDCl_3$) δ 8.66 (d, J = 2.2 Hz, 1H), 8.28 (dd, J = 8.9,

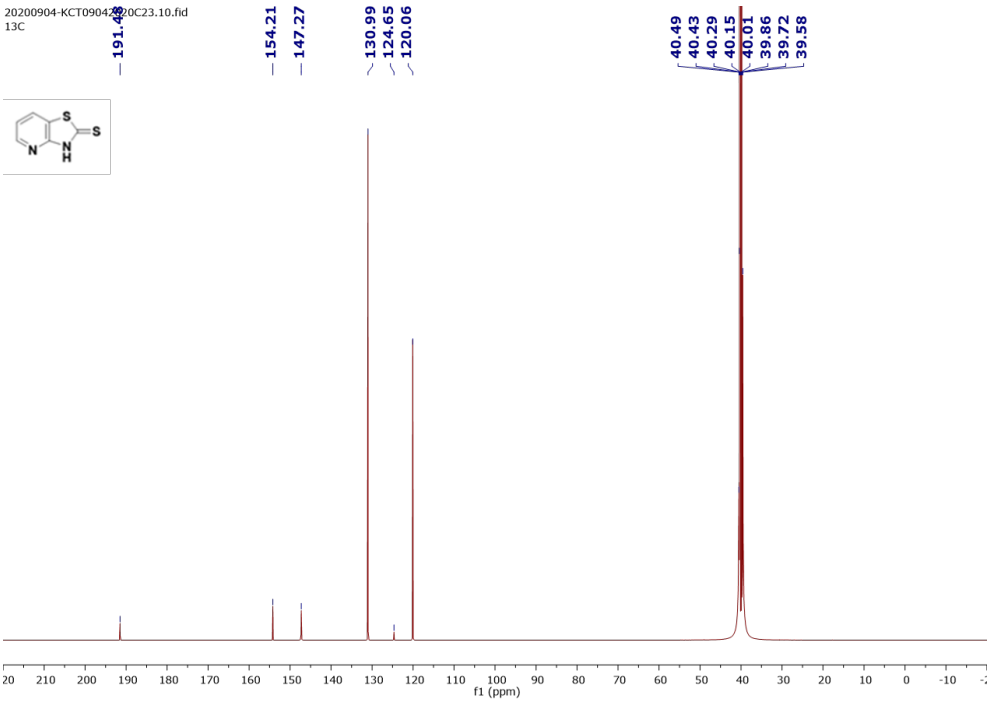
2.2 Hz, 1H), 7.88 (d, *J* = 8.9 Hz, 1H), 2.84 (s, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 172.38, 163.77, 147.41, 129.59, 128.67, 118.74, 15.79.

Synthesis of compound 1f

2-(Methylthio)thiazolo[4,5-b]pyridine **I** (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 **1f** as bright yellow powder (91 %, 242 mg). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 9.16 (d, *J* = 8.0 Hz, 1H), 8.98 (d, *J* = 6.1 Hz, 1H), 7.91 (dd, *J* = 8.0, 6.2 Hz, 1H), 4.46 (s, 3H), 2.97 (s, 3H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 177.18, 171.97, 147.51, 139.72, 125.23, 120.50, 41.69, 15.03.



^{13}C NMR of compound H



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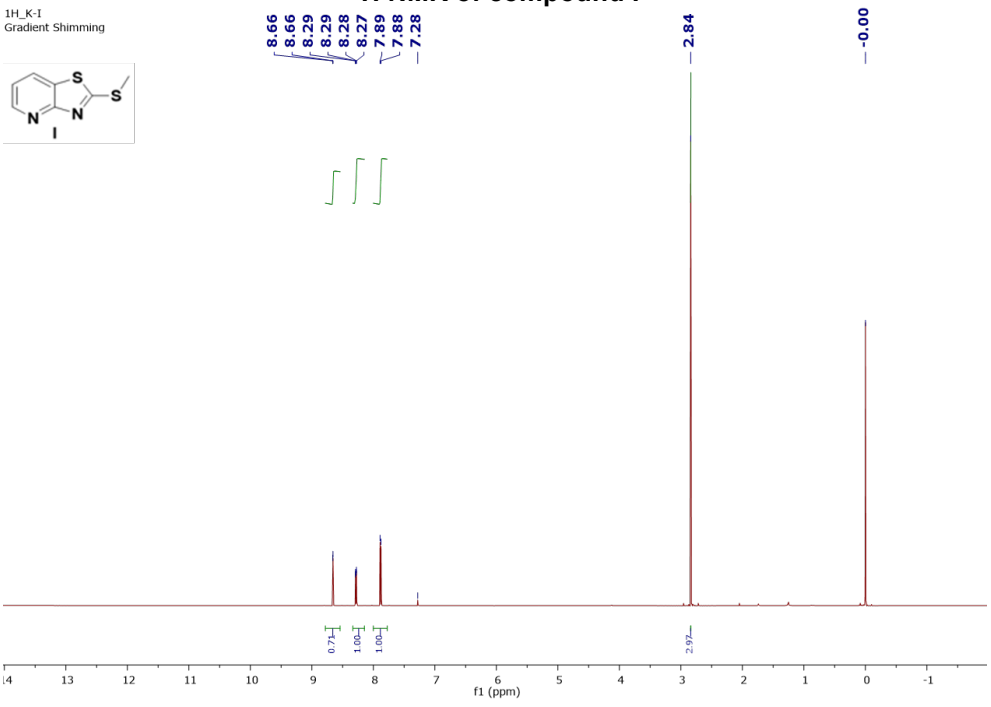

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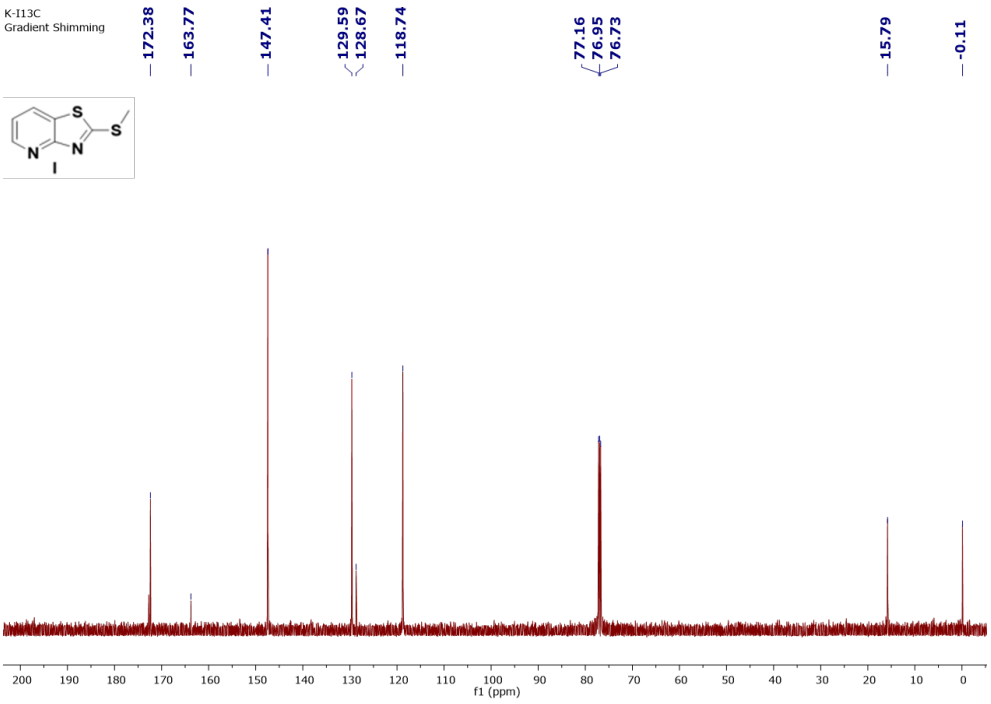
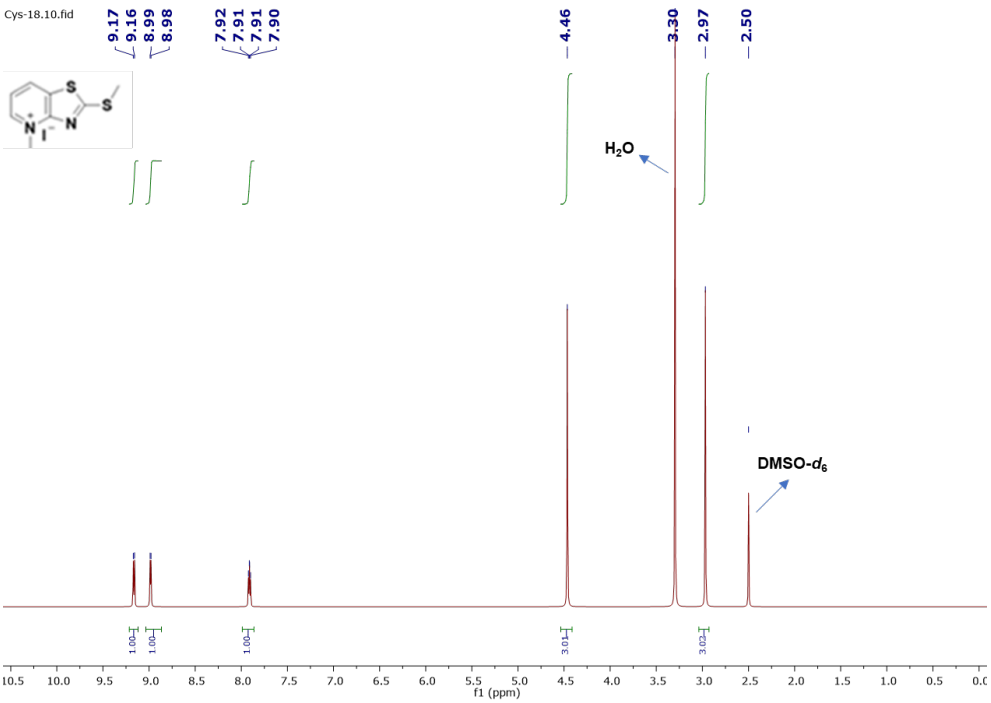
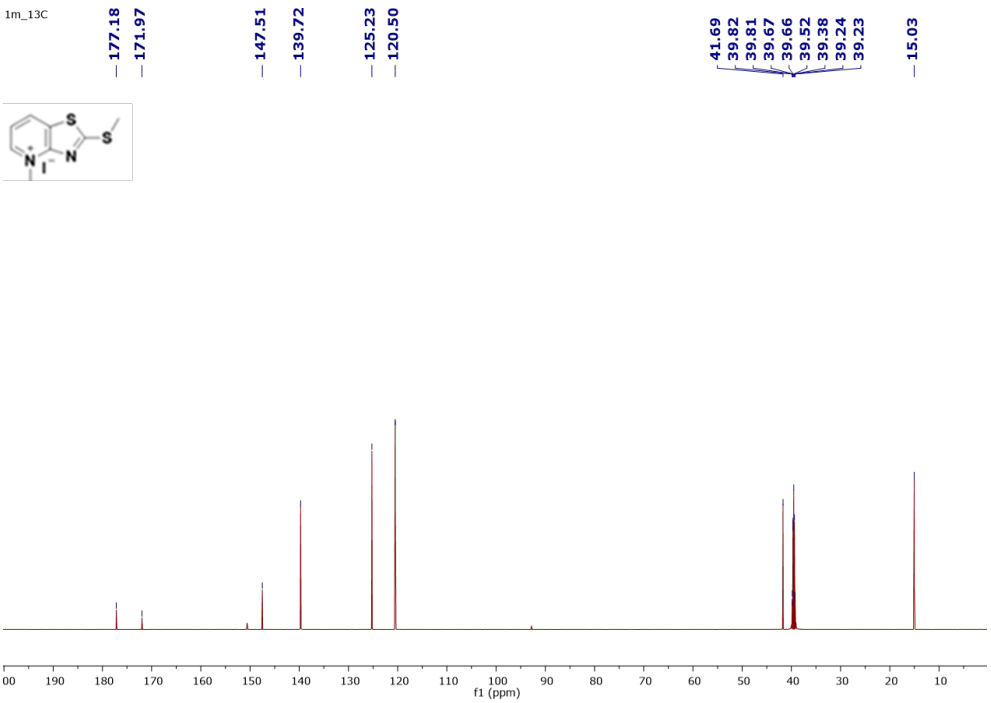


^{1}H NMR of compound 1f



^{13}C NMR of compound 1f

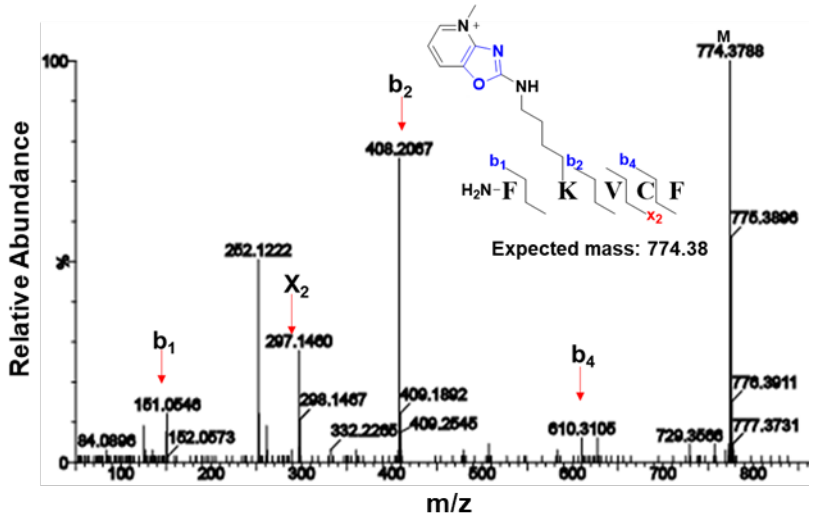


VI. Supplementary Figure 2: Screening of TARE probes 1a-1f, NHS ester, and STP alkyne with peptide FKVCF 2a.

To a 1 mg of FKVCF **2a** (4 mM) in 0.4 mL of 10 mM phosphate buffer (Nap, pH 7.5) was added TARE probe (**1a-1f**), NHS-yne, and STP-yne (10 equiv., 40 mM). The reaction was stirred at room temperature for 3 h. 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.

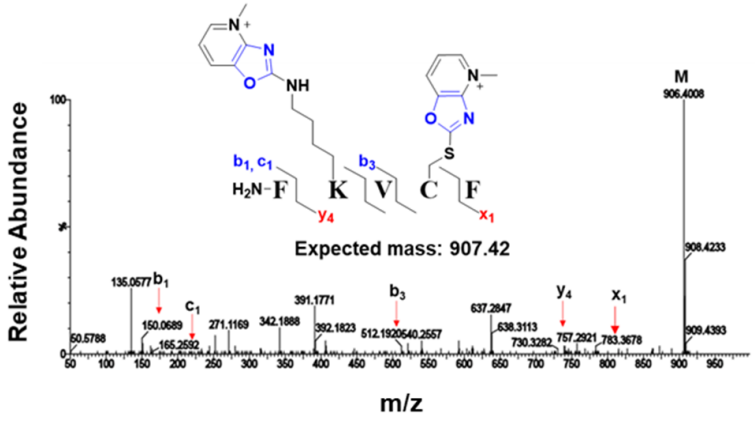
MS/MS of 1c-FKVCF (Lysine-mono-modification)

Compound	Mass (m/z)
Unmodified peptide	641.34
Modified peptide	774.38



MS/MS of 1c-FKVCF (Lysine and Cysteine double-modification)

Compound	Mass (m/z)
Unmodified peptide	641.34
Modified peptide	907.42

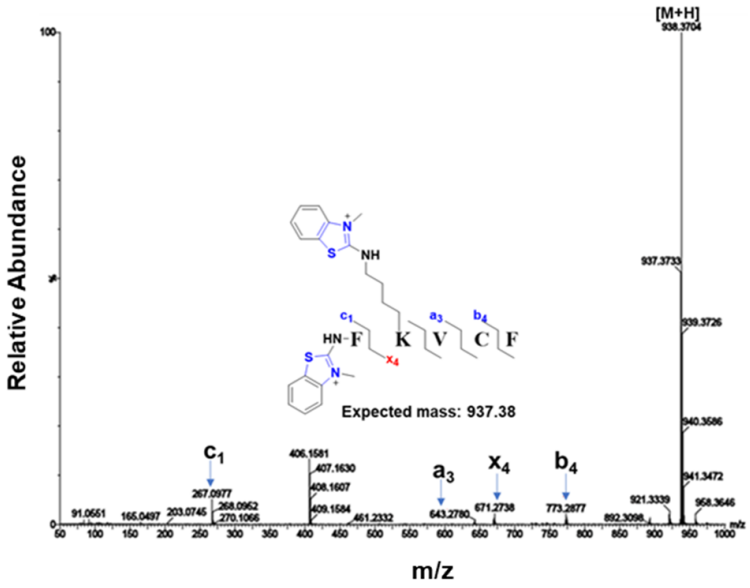


MS/MS of 1d-FKVCF (Lysine modification)

Compound	Mass (m/z)
Unmodified peptide	641.34
Modified peptide	773.83

MS/MS of 1e-FKVCF (Lysine and N-terminus double modification)

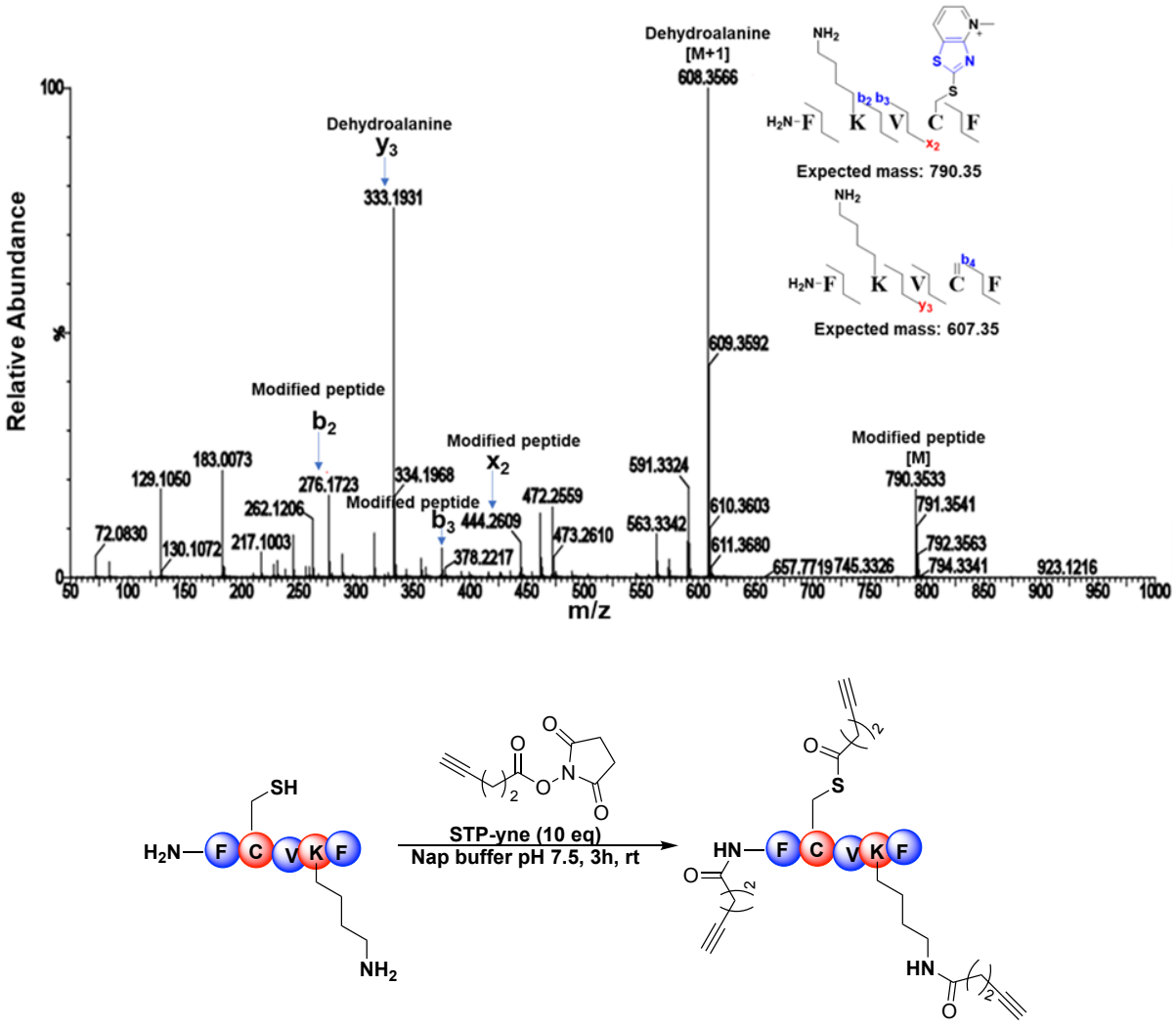
Compound	Mass (m/z)
Unmodified peptide	641.34
Modified peptide	937.38



MS/MS of 1f-FKVCF (Cysteine modification)

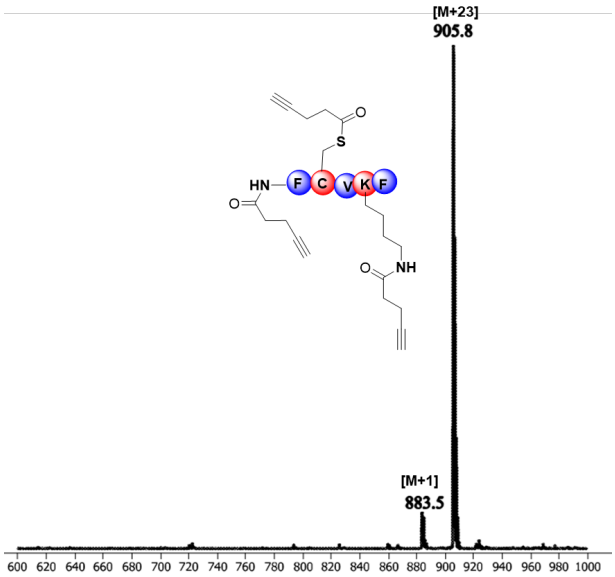
Compound	Mass (m/z)
Unmodified peptide	641.34
Modified peptide	790.35
Dehydroalanine[a]	607.35

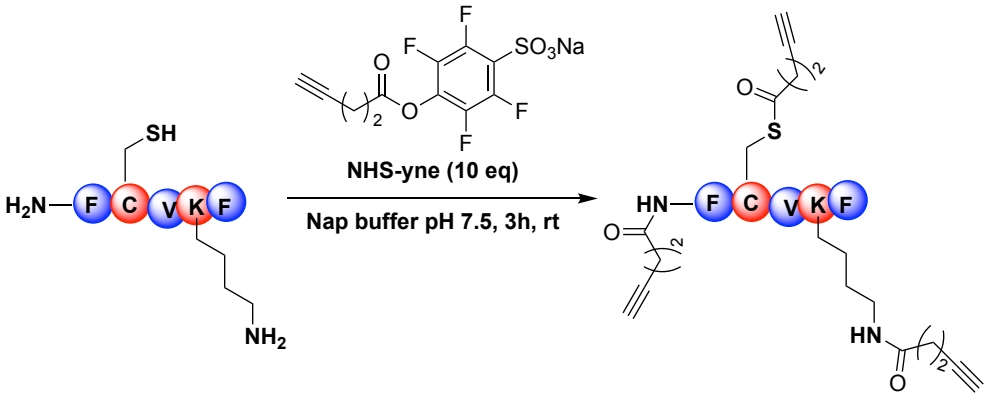
[a] The corresponding dehydroalanine compound was observed during MS/MS analysis.



LC-MS of modified FKVCF with NHS-yne (Lys, Cys, and N-terminus modifications)

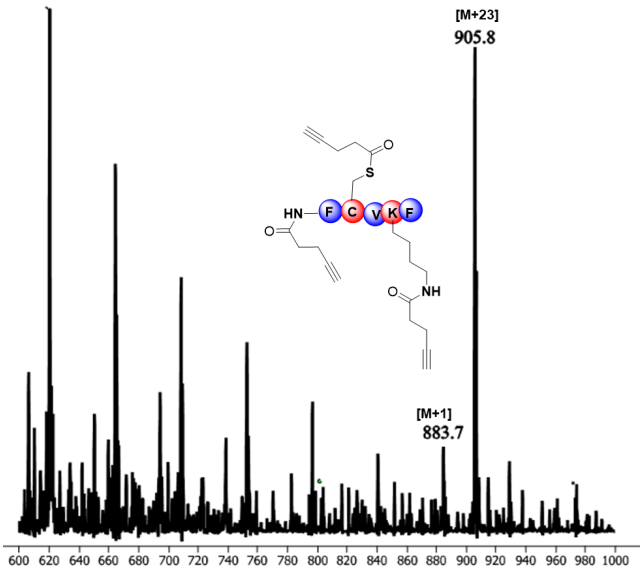
Compound	Mass (m/z)
Unmodified peptide	641.34
Modified peptide	882.40





LC-MS of modified FKVCF with STP-yne (Lys, Cys, and N-terminus modifications)

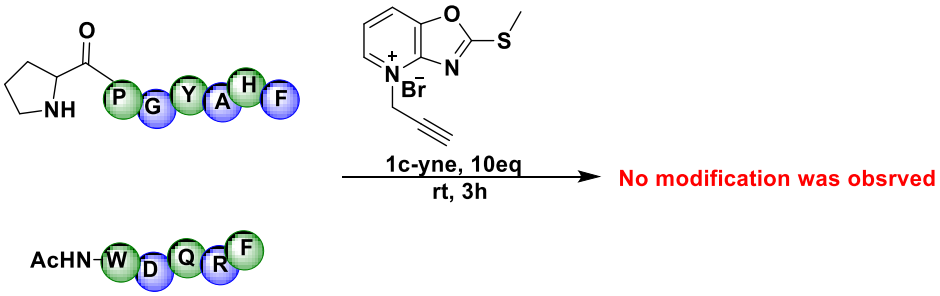
Compound	Mass (m/z)
Unmodified peptide	641.34
Modified peptide	882.40



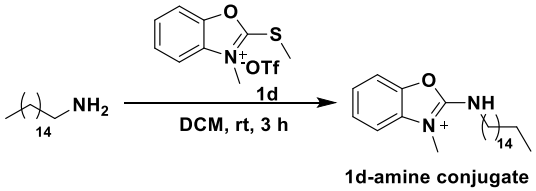
Confirmation of the high chemo- and site-selectivity of probe 1c-yne towards lysine

Procedure for chemo-selectivity study of 1c-yne

To peptide (4 mM) in 0.4 mL Nap buffer pH 7.5 10 mM, 1-cyne (10 equiv.) was added and the reaction was allowed to react at room temperature for 3 h. The reaction mixture was analyzed by HPLC and LCMS. No modified peptides were observed.

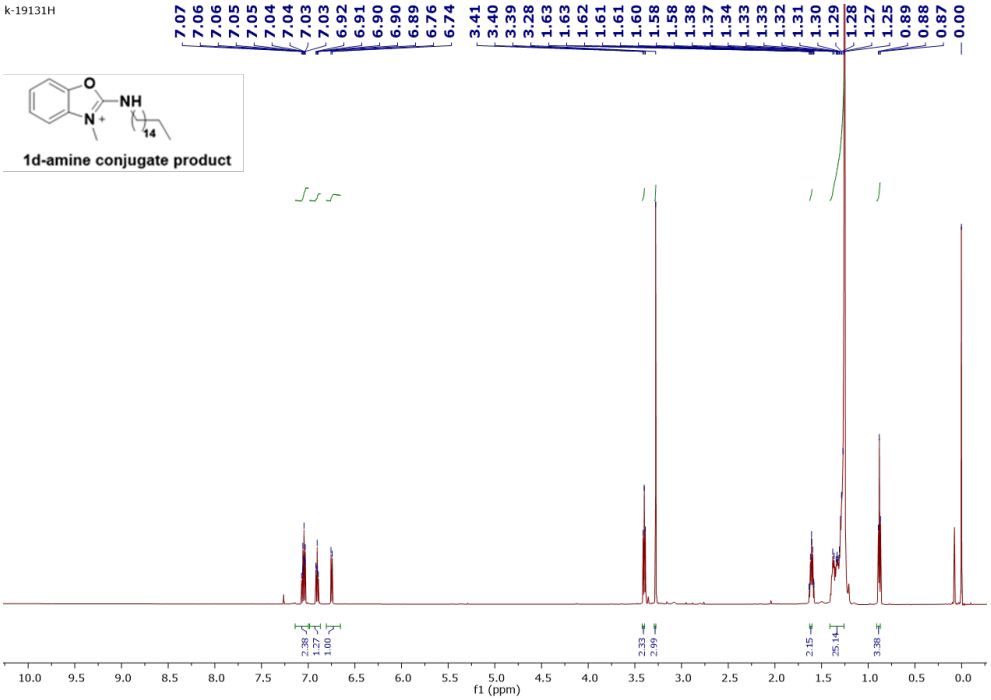


VII. Supplementary Figure 3. Procedure for large scale synthesis of 1d-amine conjugated product

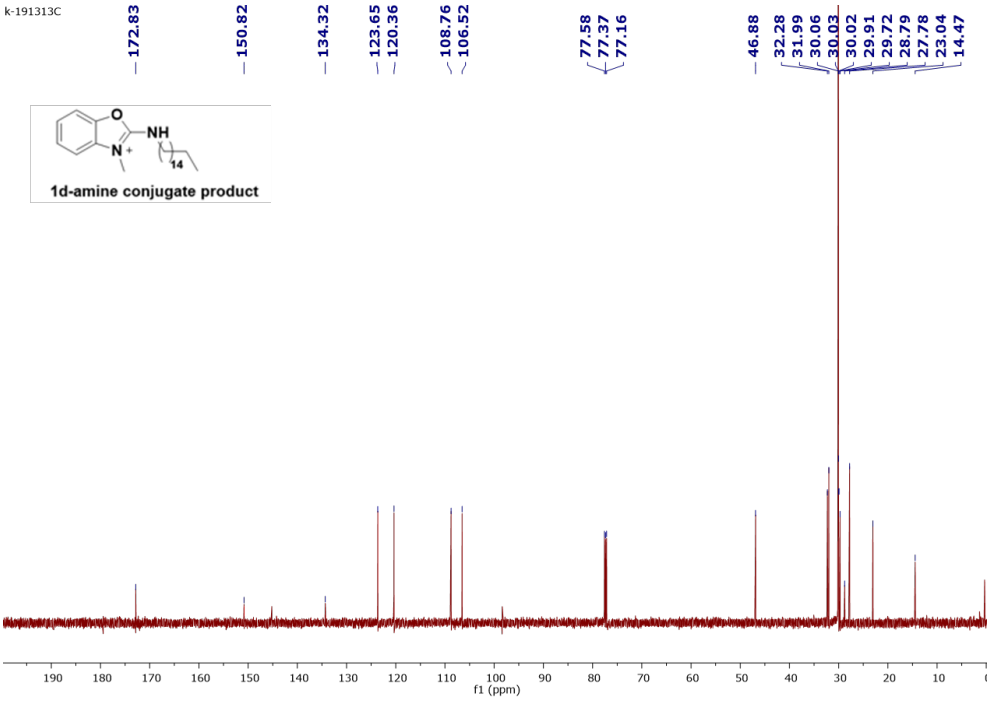


To a mixture of hexadecylamine (90 mg, 0.372 mmol) in DCM (1.5 mL), **1d** (60 mg, 0.183 mmol) was added. The reaction mixture was stirred at room temperature for 3 h. The mixture was dried under reduced pressure and purified by HPLC and characterized by NMR. **^{1}H NMR** (600 MHz, $CDCl_3$) δ 7.07 – 7.00 (m, 2H), 6.98 – 6.87 (m, 1H), 6.75 (d, *J* = 7.7 Hz, 1H), 3.41 (d, *J* = 7.3 Hz, 2H), 3.28 (s, 3H), 1.63 – 1.60 (m, 2H), 1.41 – 1.26 (m, 25H), 0.89 (d, *J* = 6.4 Hz, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 172.83, 150.82, 134.32, 123.65, 120.36, 108.76, 106.52, 46.88, 32.28, 31.99, 30.06, 30.03, 30.02, 29.91, 29.72, 28.79, 27.78, 23.04, 14.47.

^{1}H for 1d-amine conjugate product

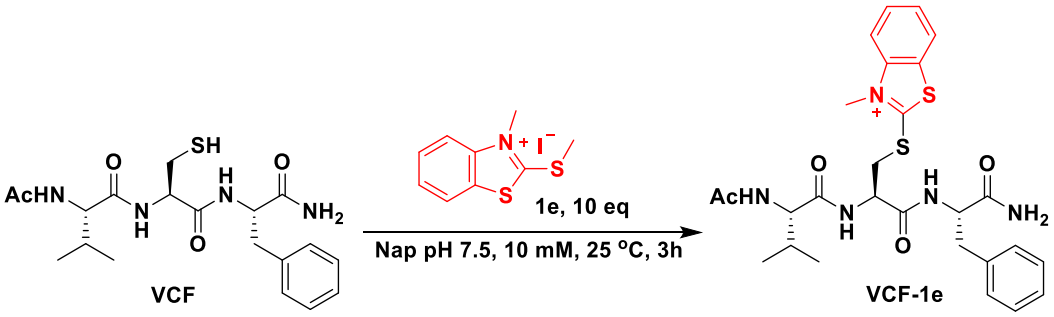


^{13}C for 1d-amine conjugate product

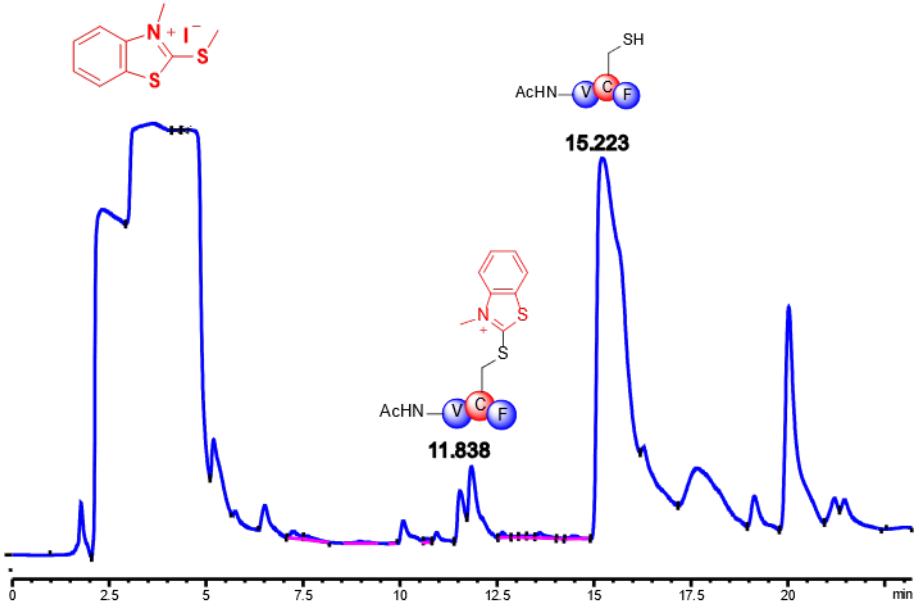


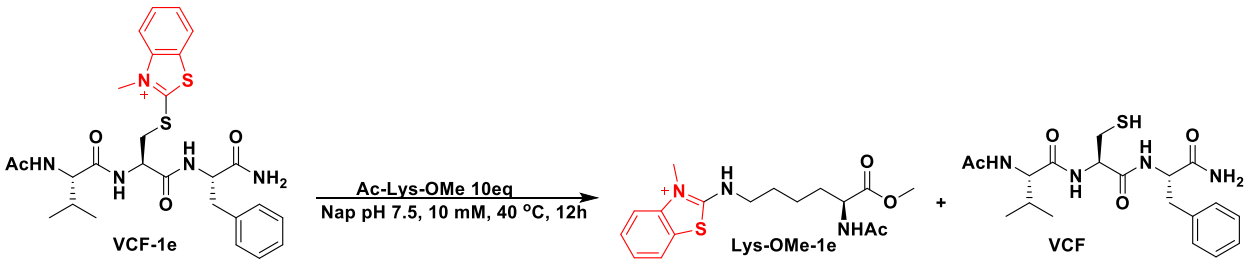
VIII. Supplementary Figure 4. Reversibility study of 1e-Cys-conjugate (VCF-1e) and 1d-thio-conjugate with lysine methylester.

Ac-VCF-NH_2 (6.3 mM) was dissolved in 0.4 mL of 10 mM phosphate buffer (Nap, pH 7.5), and then 10 equiv. of **1e** was added. The mixture was stirred at room temperature for 3 hours. The reaction mixture was purified by HPLC then dried by lyophilized to afford pure VCF-1e. To a solution of pure VCF-1e (6.3 mM) in Nap buffer pH 7.5, 400 µL, 10 eq of lysine methyl ester was added. The reaction was incubated at 40 ^{o}C for 12 h. 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.

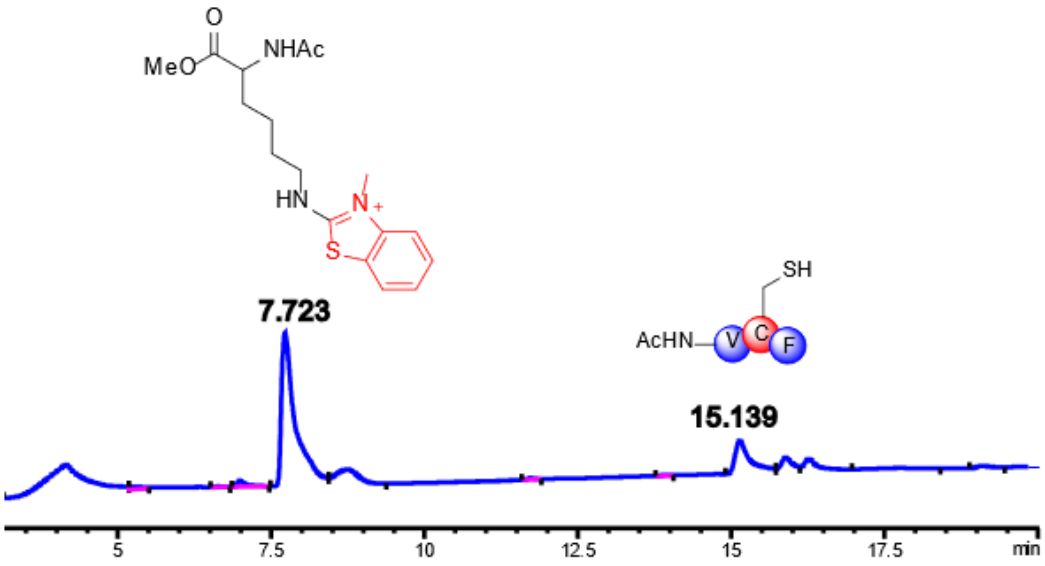


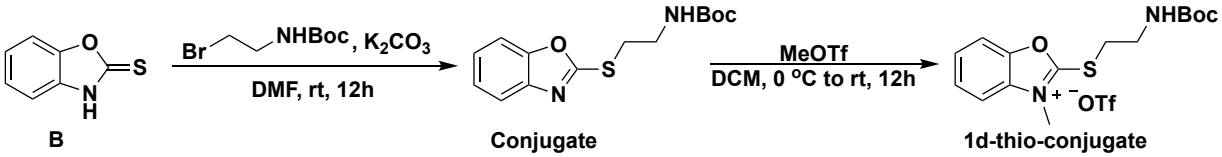
HPLC trace of modified VCF by probe 1e





HPLC trace after incubating with Ac-Lys-OMe for 12 h at 40 °C



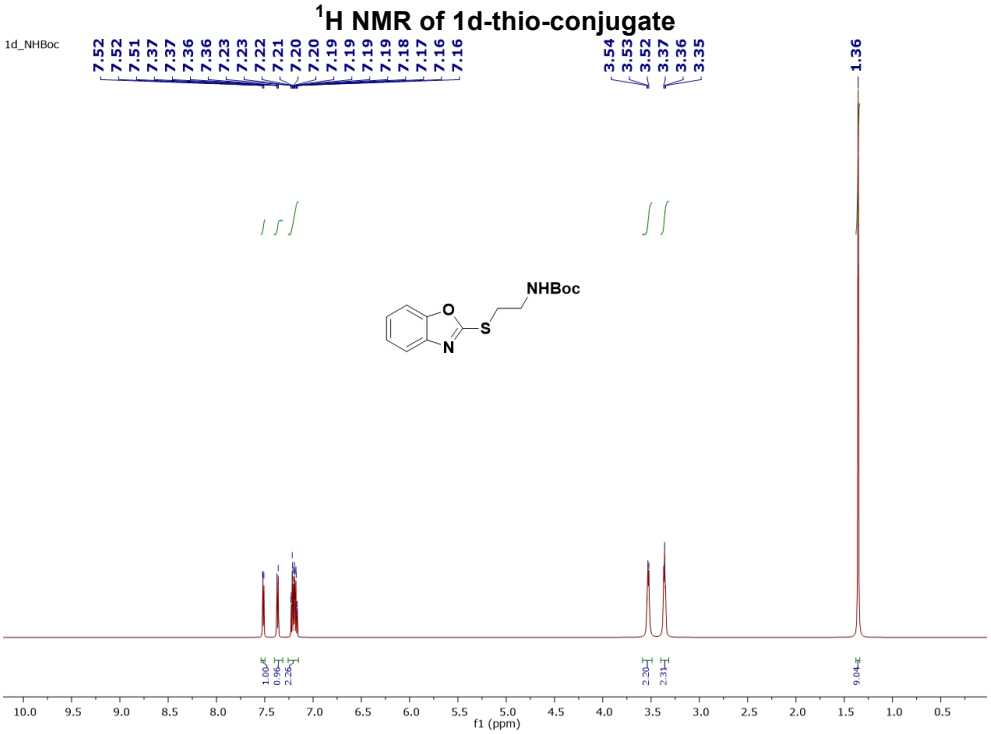


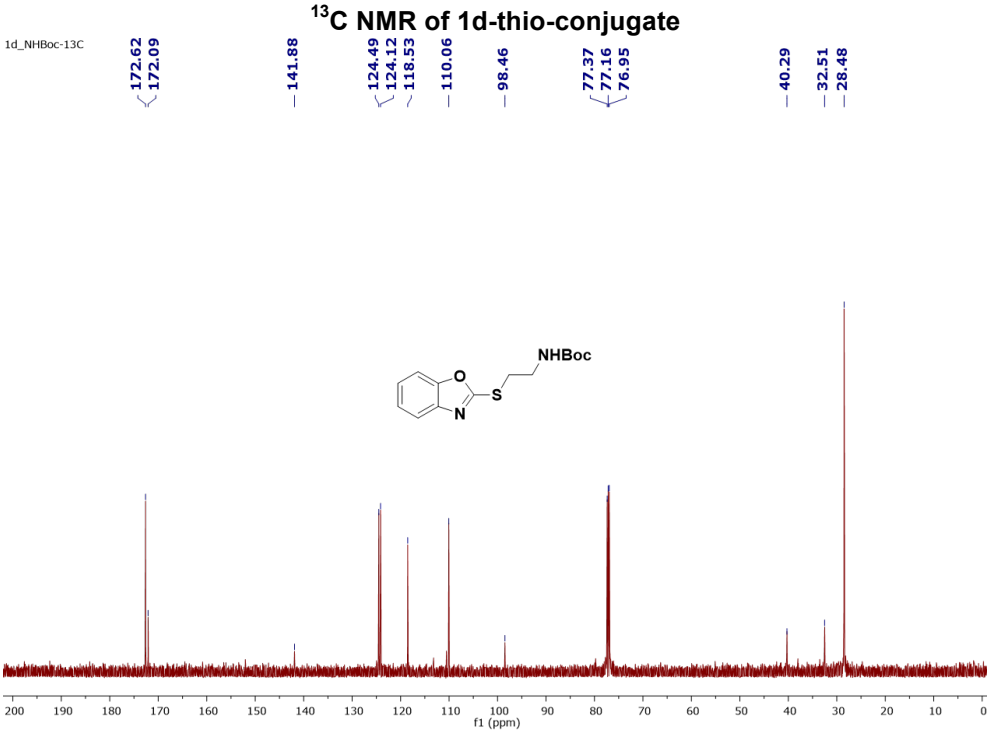
Synthesis of 1d-thio-conjugate

2-Mercaptobenzoxazole **B** (500 mg, 3.31 mmol), potassium carbonate (456 mg, 3.31 mmol), and tert-butyl (2-bromoethyl)carbamate (738.4mg, 3.31 mmol) were sequentially added into dry DMF (10 mL) at 0 °C. The mixture was warmed to room temperature and stirred for 12 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 conjugate as colorless crystal (739.6 mg, 76 %). **^{1}H NMR** (600 MHz, $CDCl_3$ δ 7.54 – 7.50 (m, 1H), 7.37 (dd, *J* = 7.9, 1.1 Hz, 1H), 7.26 – 7.15 (m, 2H), 3.59 – 3.49 (m, 2H), 3.36 (t, *J* = 6.2 Hz, 2H), 1.36 (s, 9H). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 172.62, 172.09, 141.88, 124.49, 124.12, 118.53, 110.06, 98.46, 40.29, 32.51, 28.48.

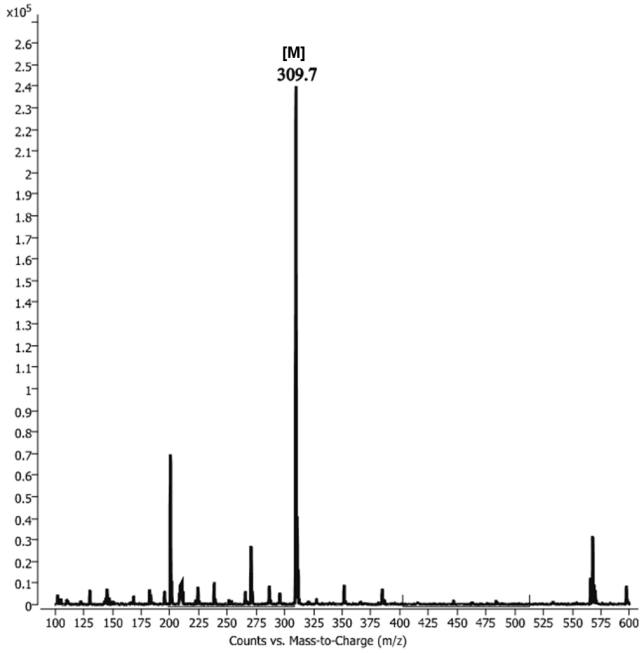
To a mixture of **conjugate** (93 mg, 0.316 mmol) in dry DCM (10 mL), MeOTf (41.8 µL, 0.379 mmol) was added at 0 °C. The reaction mixture was warmed to room temperature. After 12 h, solvent was removed

by rotary evaporation to afford brown oil product. The **1d-thio-conjugate** was directly used without purification.



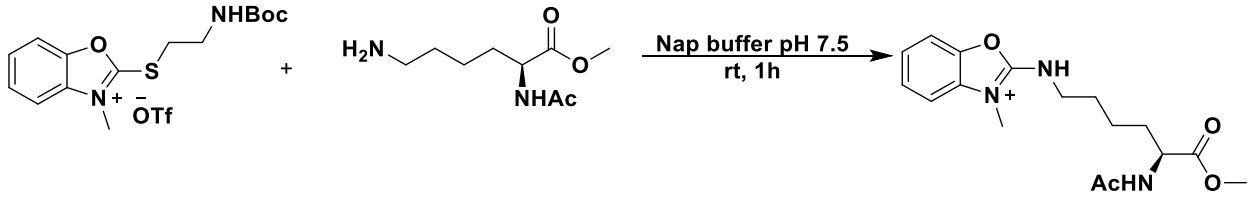


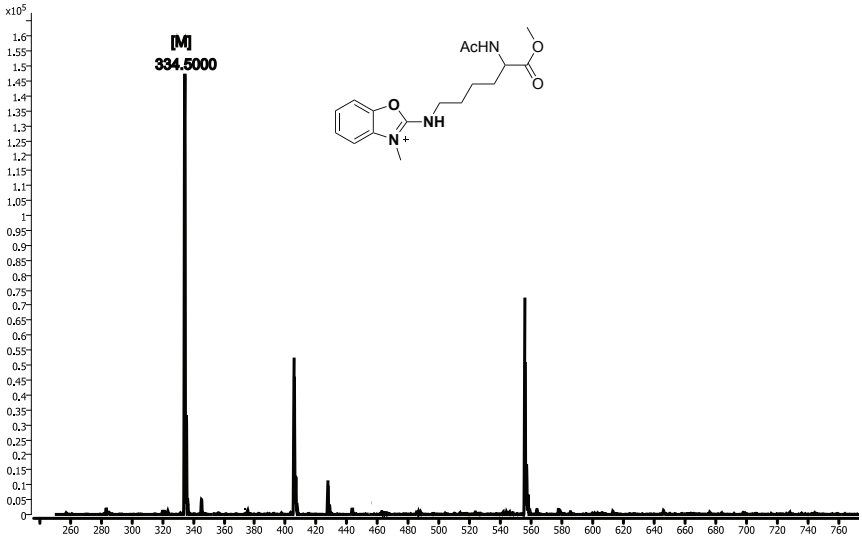
LC-MS spectrum of 1d-thio-conjugate

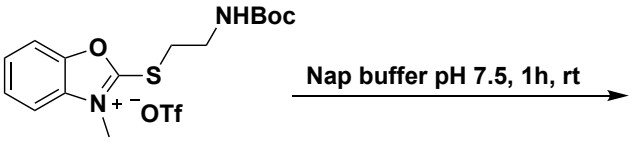


Reversibility study of 1d-thio-conjugate with lysine methylester.

General procedure for reversibility study of 1d-thio-conjugate: Ac-Lys-OMe (7.23 mg, 0.030 mmol, 10 eq) was dissolved in 0.4 mL of 10 mM phosphate buffer (Nap, pH 7.5), and then 1 equiv. of **1d-thio-conjugate** was added. The mixture was stirred at room temperature for 1 hour. The reaction was analyzed by LC-MS.





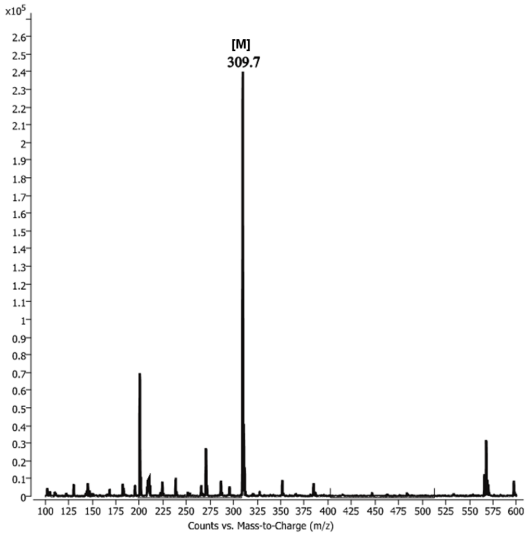


1d-thio-conjugate

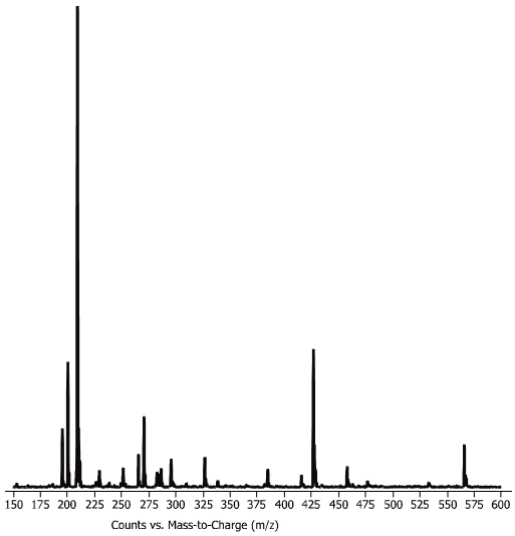
Procedure for stability study of 1d-thio-conjugate in sodium phosphate buffer.

1d-thio-conjugate (0.035 mmol) was incubated in 400 µL of 10 mM Nap (pH 7.5) at room temperature. A sample (10 µL) was taken from the mixture and directly injected into LC-MS. The stability study was monitored by LC-MS.

LC-MS of pure 1d-thio-conjugate at 0 min



LC-MS of pure 1d-thiol conjugate after 1 hour (Compound was completely decomposed in one hour)



IX. Supplementary Figure 5. Modification of myoglobin Mb by 1d and 1e

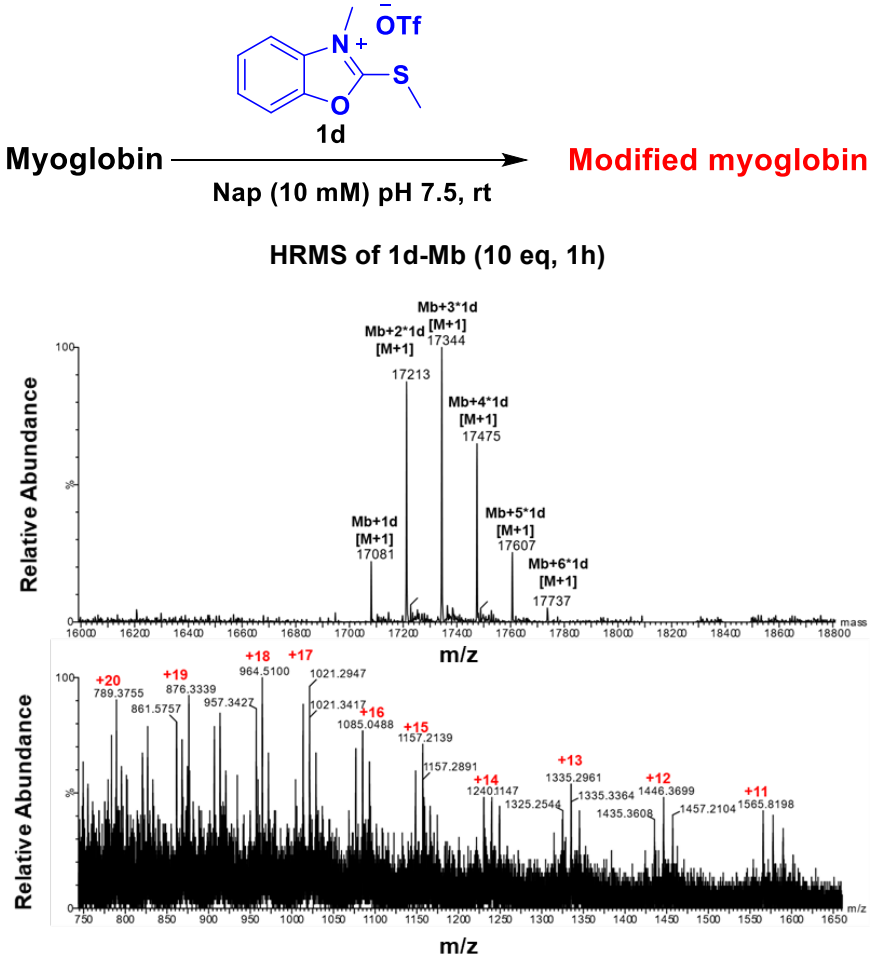
To a 1 mg of myoglobin (3 mM) in 0.2 mL of 10 mM phosphate buffer (Nap, pH 7.5), compound 1d (1 or 10 equiv.) was added. The reaction was stirred at room temperature for 1 h. The Mb-1d protein bioconjugate was purified by molecular weight cut off and characterized by LC-MS/MS.

Procedure for in-solution digestion and analysis of modified myoglobin

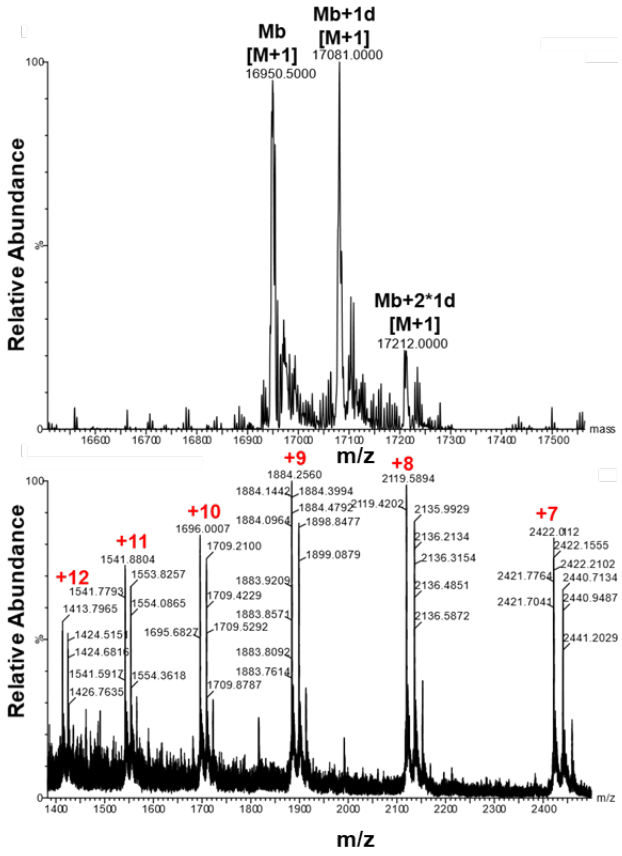
Protein (1 mg) in 10 mM tris (100 µL, pH 7.5) with urea (6 M) was incubated for 30 minutes at 37 °C. To reduce the urea concentration to 0.6 M, the sample was diluted with grade I water. To this solution, 100 µL of enzyme (αchymotrypsin/trypsin) solution (0.1 mg, enzyme/protein (1:20); enzyme in 1 mM HCl was dissolved in 0.1 M tris and 0.01 M $CaCl_2$) was added and the mixture was incubated at 37 °C for 18 h. The pH of digested mixture was adjusted to < 6 (confirmed by pH paper) with trifluoroacetic acid (0.5%). Subsequently, the sample was used for peptide mapping by MS and sequencing by MS/MS**.** The protein fragments were analyzed by Thermo Ultimate 3000 nanoLC/Orbitrap Q-Exactive Plus MS with positive mode. The identification of protein sequences was achieved by Thermo BioPharma Finder.

Myoglobin from equine heart amino acid sequence:

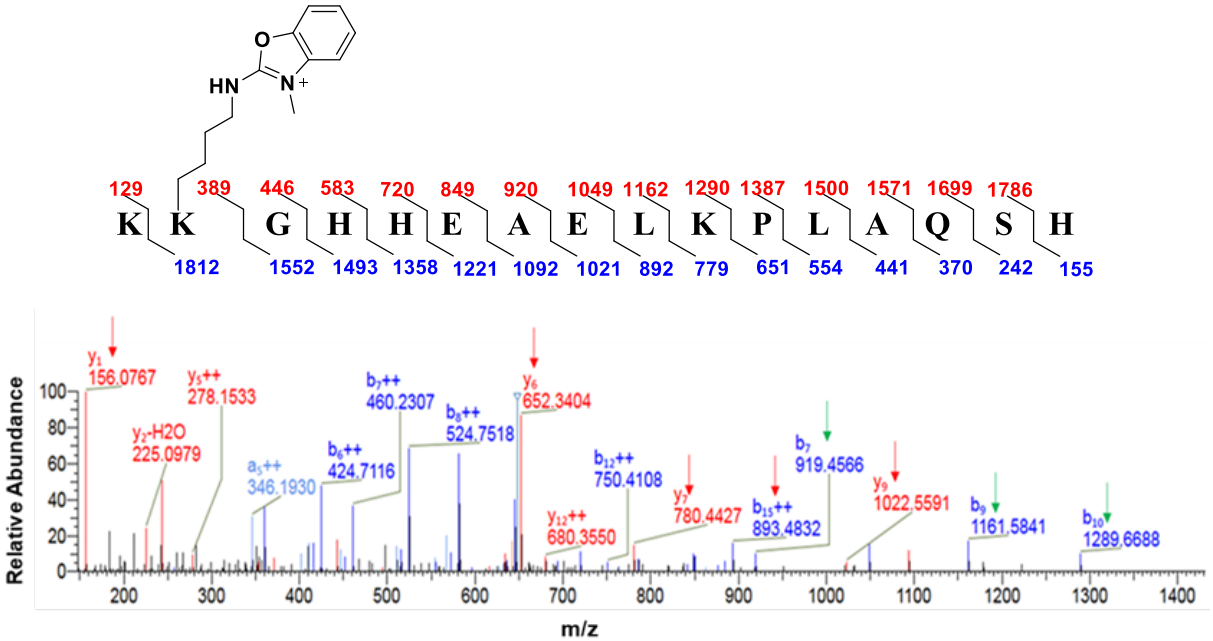
GLSDGEWQQVLNVWGKVEADIAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGTVVL TALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSKHPGDFGADAQGAMTKALELFRNDI AAKYKELGFQG (no cysteine or disulfide)



HRMS of 1d-Mb (1 eq, 1h)



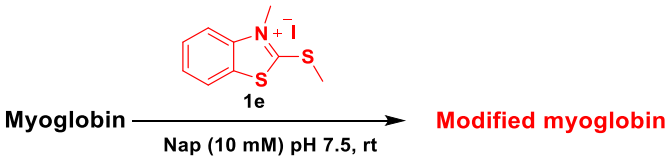
MS/MS spectrum Mb-1d fragment (1eq, 1h)



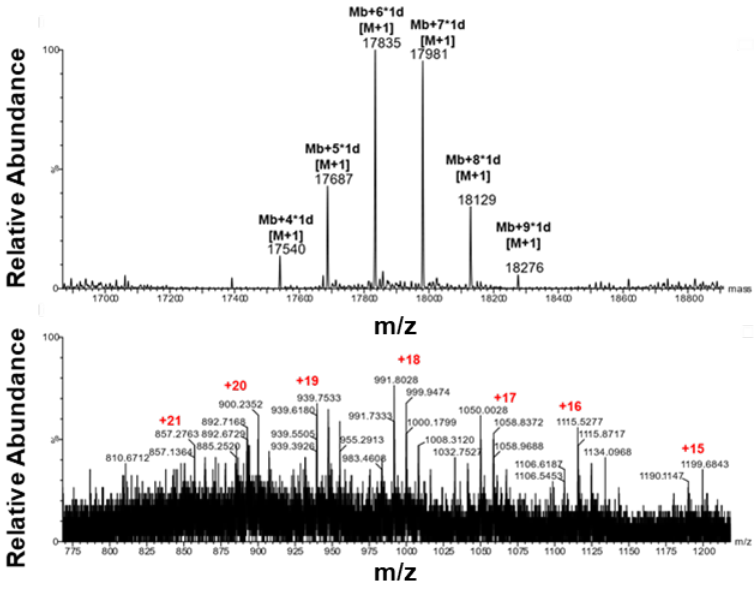
Protein	Peptide Sequence	Modification	Site	Confidence Score	ID Type	M/Z	Charge State	Mono Mass Exp.	Avg Mass Exp.
Modified Myoglobin	KKGHHEAELK PLAQSH	131.0337	~K79	100	MS2	486.259	4	1940.004	1941.15

General procedure of tagging multiple lysines with compound 1e

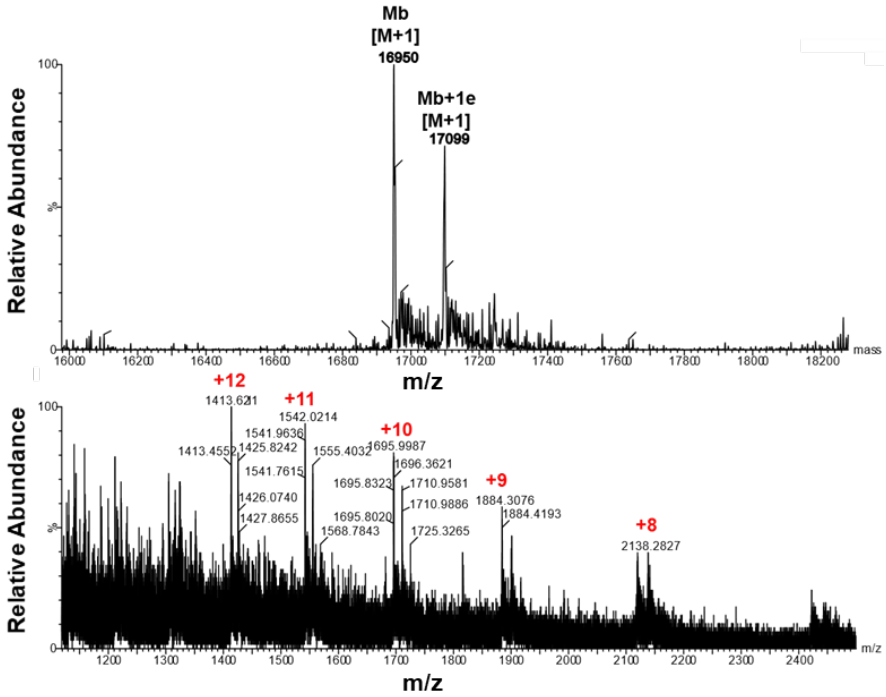
To a 1 mg of myoglobin (3 mM) in 0.2 mL of 10 mM phosphate buffer (Nap, pH 7.5), compound 1e (10 or 100 equiv.) was added. The reaction was stirred at room temperature for 1 or 12 h. The Mb-1e protein bioconjugate was purified by molecular weight cut off and characterized by LC-MS/MS



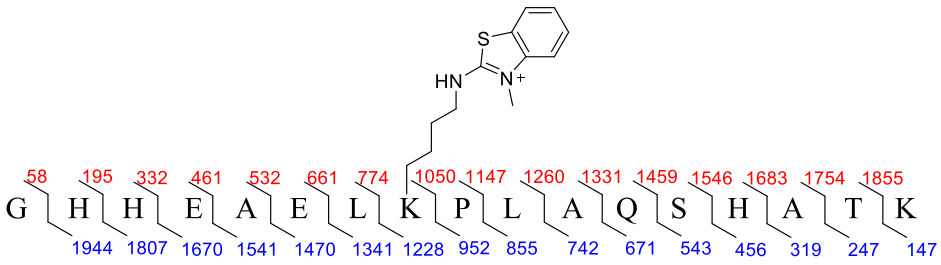
HRMS of 1e-Mb (100 eq, 12h)



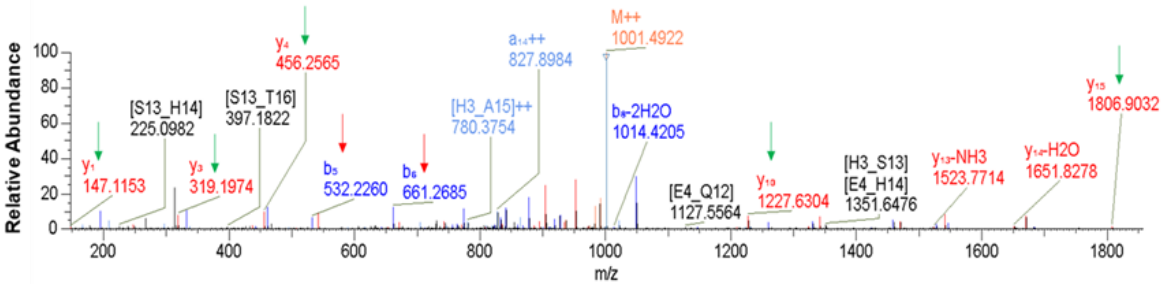
HRMS of 1e-Mb (10 eq, 1h)



MS/MS spectrum Mb-1e fragment (10 eq, 1h)



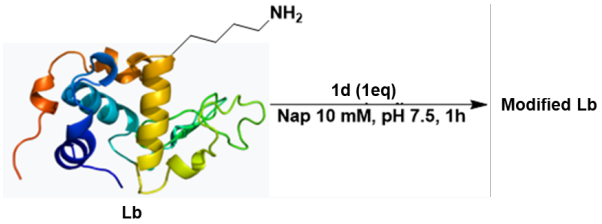
Protein	Peptide Sequence	Modification	Site	Confidence Score	ID Type	m/z	Charge State	Mono Mass Exp.	Avg Mass Exp.
Modified Myoglobin	GHHEAELKPLAQSHATK	147.0146	~K87	100	MS2	1000.992	2	1999.9698	2001.03

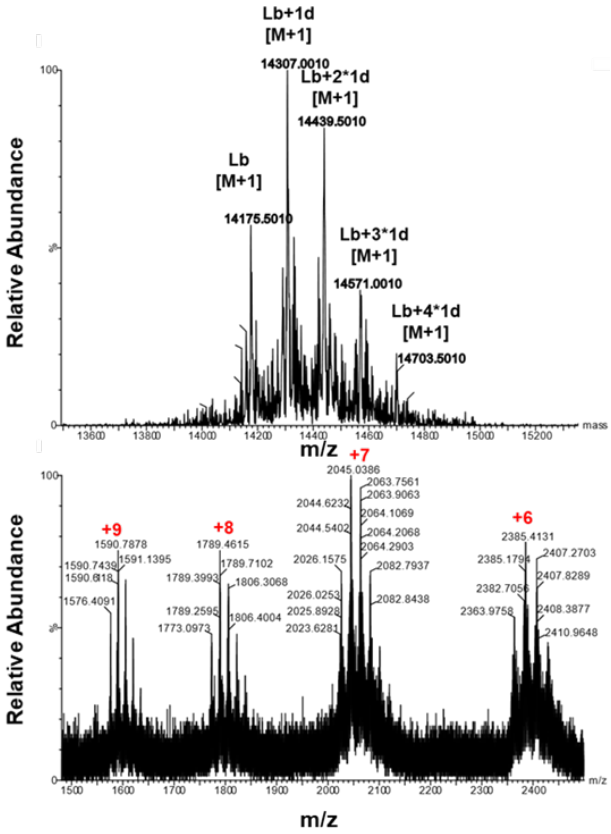


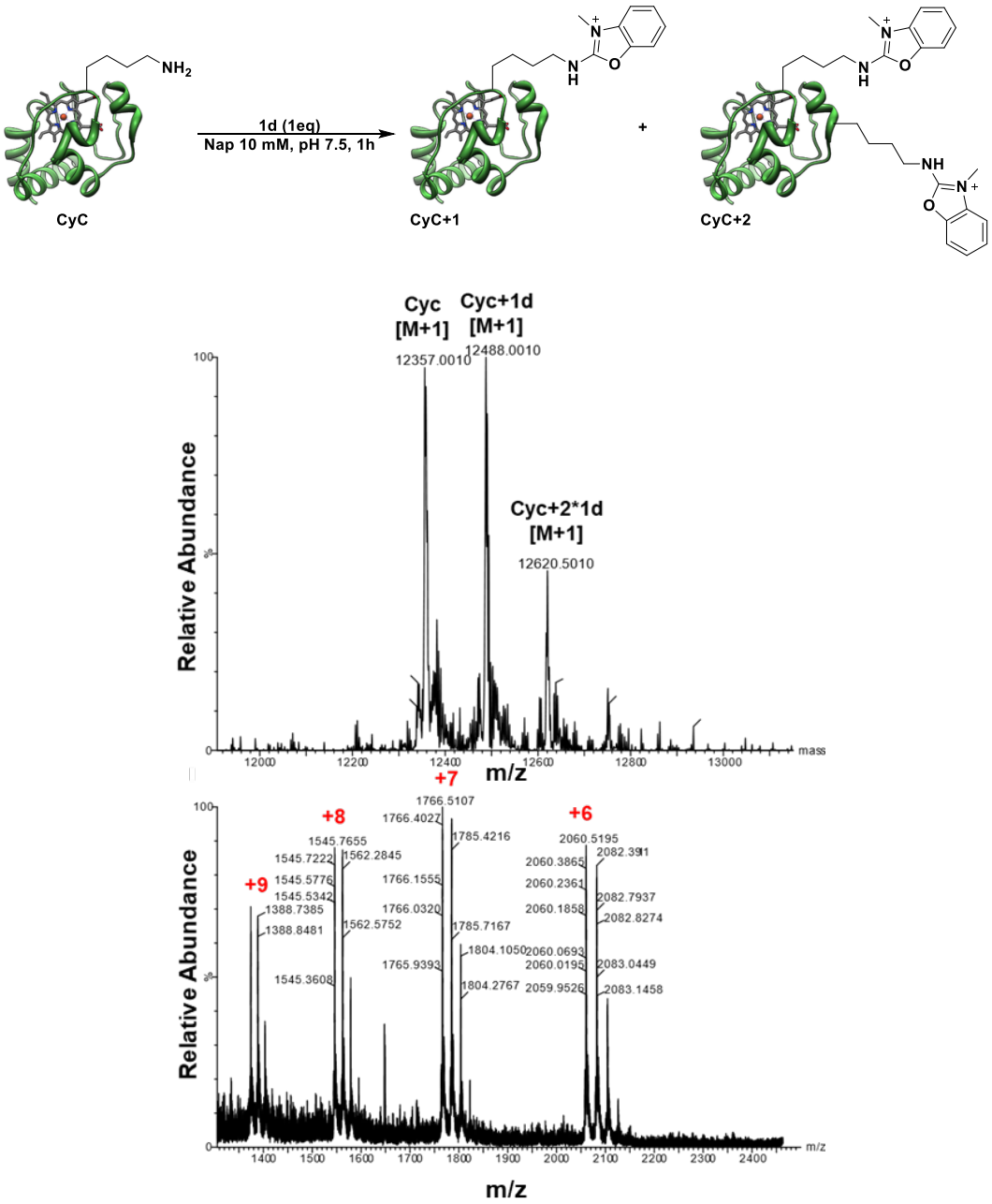
X. Supplementary Figure 6. Lysine-tagging of α-lactalbumin (lb) and cytochrome C (CyC) with probe 1d. To protein (Lb or CyC) (3 mM) in 0.2 mL 10 mM phosphate buffer (pH 7.5), 1d (1 equiv.) was added. The reaction was stirred at room temperature for 1 h. The Lb-1d and CyC-1d protein bioconjugates were purified by molecular weight cut off and characterized by LC-MS.

Cytochrome C from equine heart amino acid sequence:
GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFTYTDANKNK
GITWKEETLMEYLENPKKYIPGTKMIFAGIKKKTEREDLIAYLKKATNE (1-disulfide bond)
α-Lactalbumin from bovine milk amino acid sequence:

EQLTKCEVFRELKDLKGYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNKIWCKNDQDPHS
SNICNISCDKFLNNDLTNNIMCVKKILDKVGINYWLAHKALCSEKLDQ
WLCEKL (8-disulfide bonds)







XI. Supplementary Figure 7. Mass intensity enhancement by labeling with 1d for easy analysis by mass spectrometer.

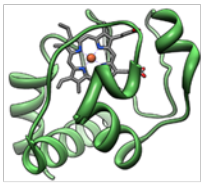
Procedure for the digestion of cytochrome C by CNBr[3].

1 mg (5 nM) of cytochrome C 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 in the incubator 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.

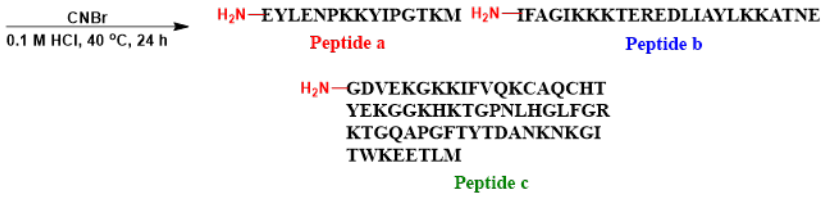
Procedure for labeling of digested cytochrome C by 1d.

To the 1 mg mixture of proteolytic fragments in 0.4 mL of 10 mM sodium phosphate buffer (Nap, pH 7.5), 10 equiv. of 1d or STPyne or NHSyne was added. The reaction was allowed to react at 25 ^{o}C in the incubator for 1 h. Equal volume (50 µL) of each solution was taken from the reaction mixture then

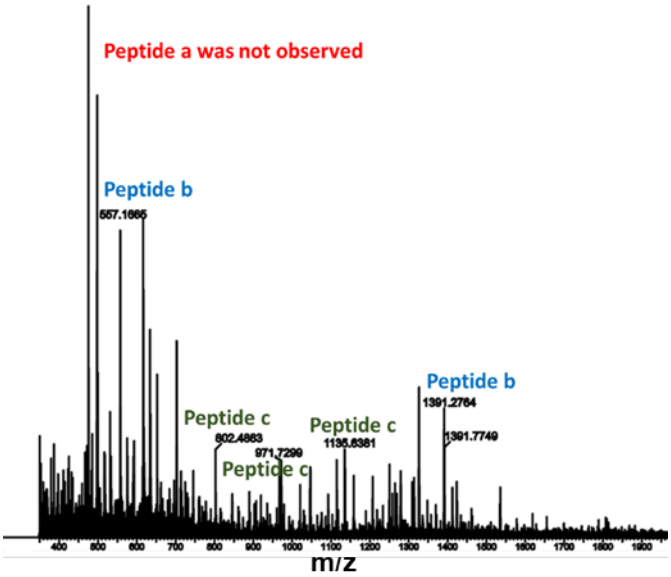
transferred into the HPLC vial for ESI-MS. The modified solution was separated on HPLC and intensity ratios of the modified fragments were analyzed by MS.

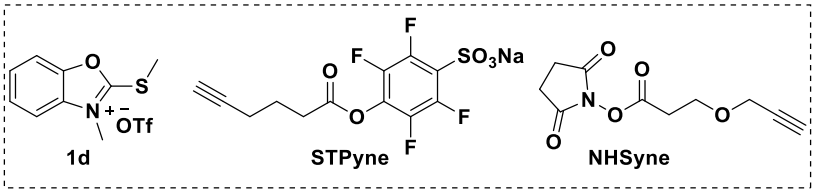


Cytochrome C



MS spectra of unmodified cytochrome C proteolytic fragments





Peptide a

H_2N—IFAGIKKKTEREDLIAYLKKATNE

Peptide b

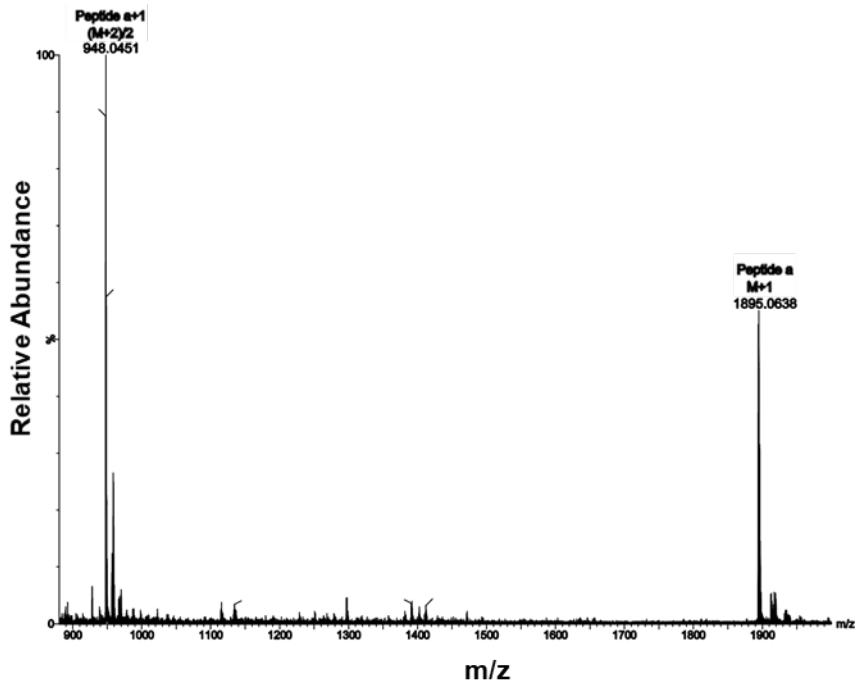
H_2N—EYLENPKKYIPGTKM

Probe (10 eq)
Nap pH 7.5, rt, 1h
→ Modified fragments

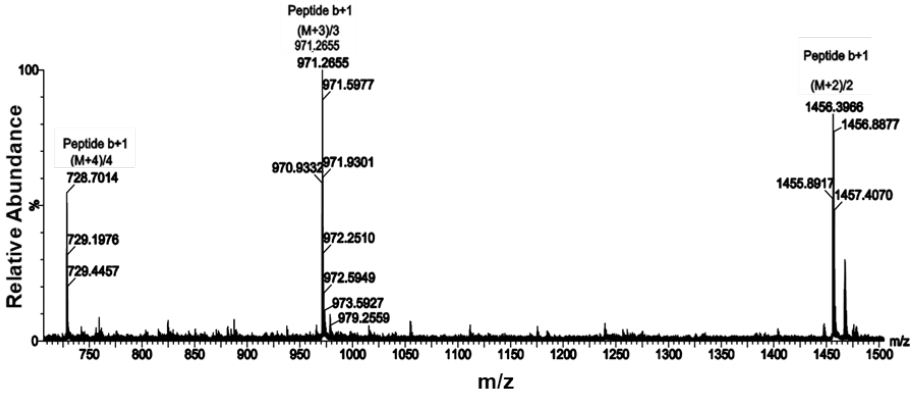
Peptide c

H_2N—GDVEKGKKIFVQKCAQCHTYEKGG
KHKTGPNLHGLFGRKTGQAPGFTY
TDANKNKGITWKEETLM

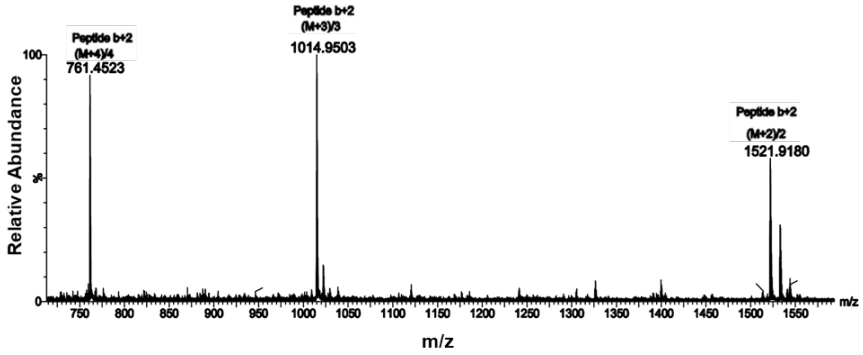
MS spectra of 1d-modified peptide fragment a



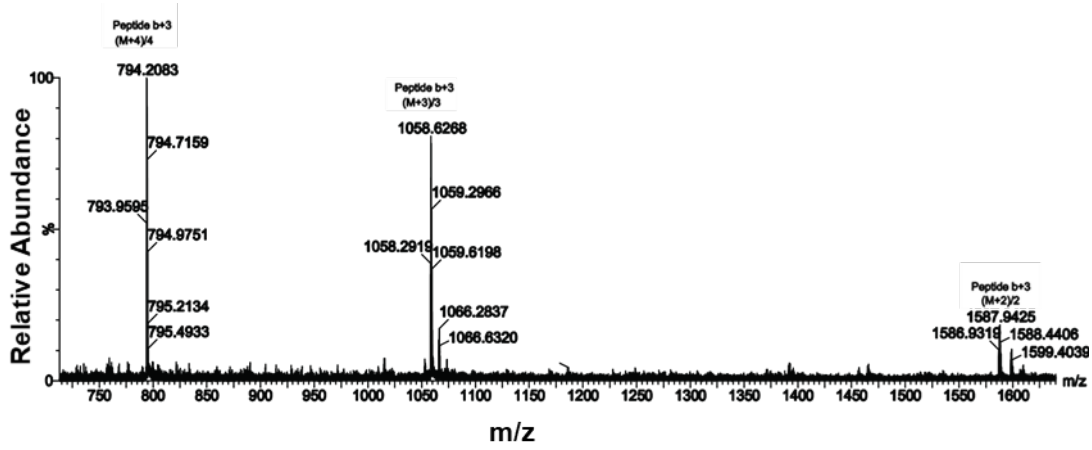
MS spectra of 1d-mono-modified peptide fragment b



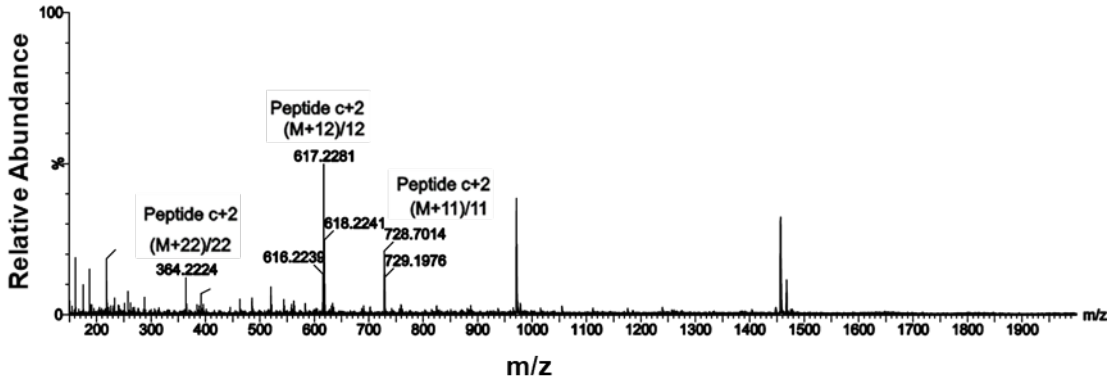
MS spectra of 1d-double-modified peptide fragment b



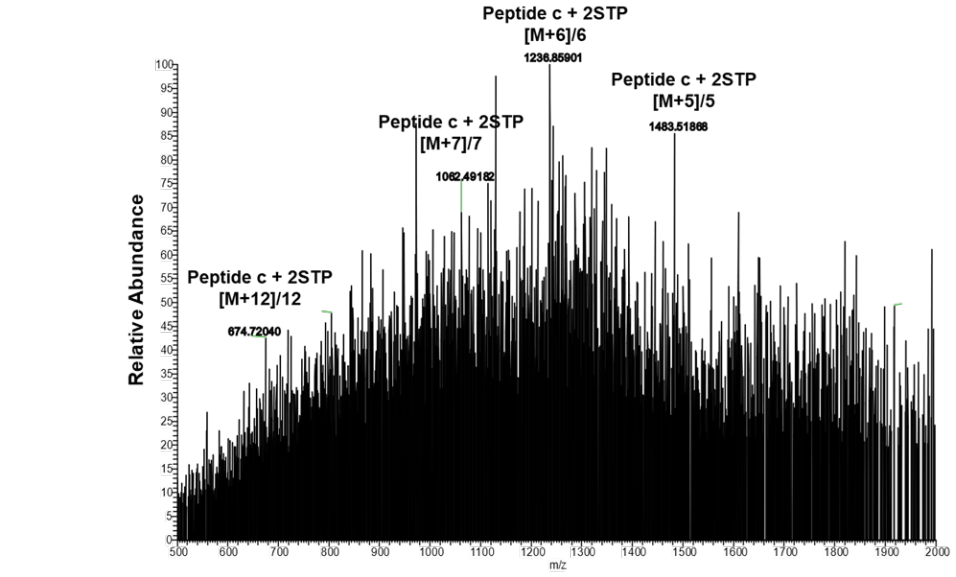
MS spectra of 1d-triple-modified peptide fragment b

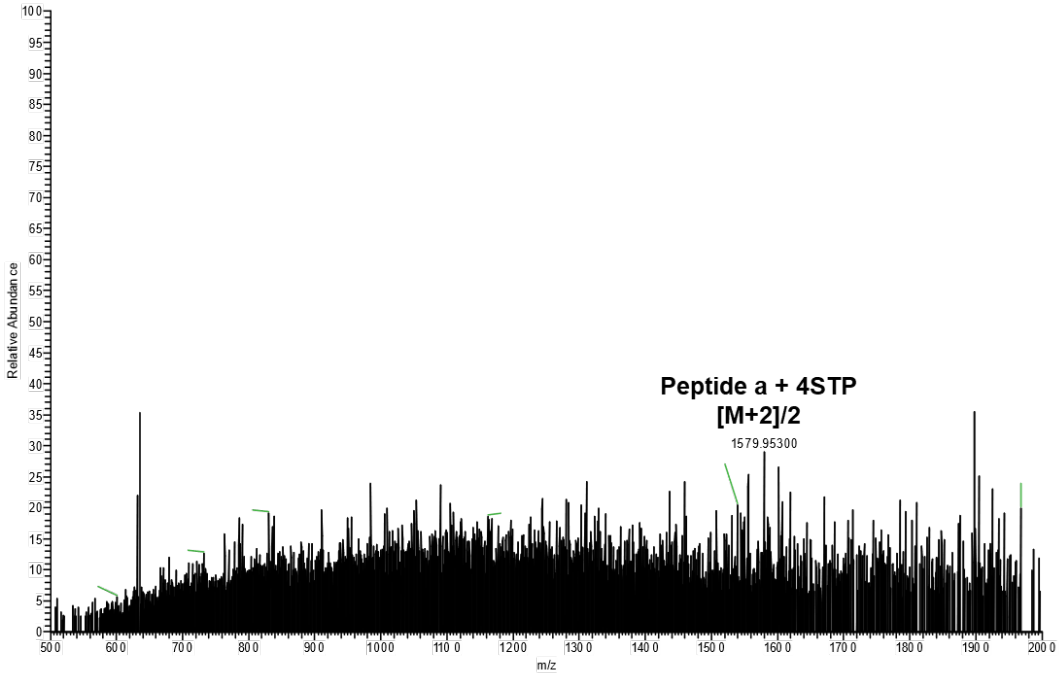


MS spectra of 1d-double-modified peptide fragment c

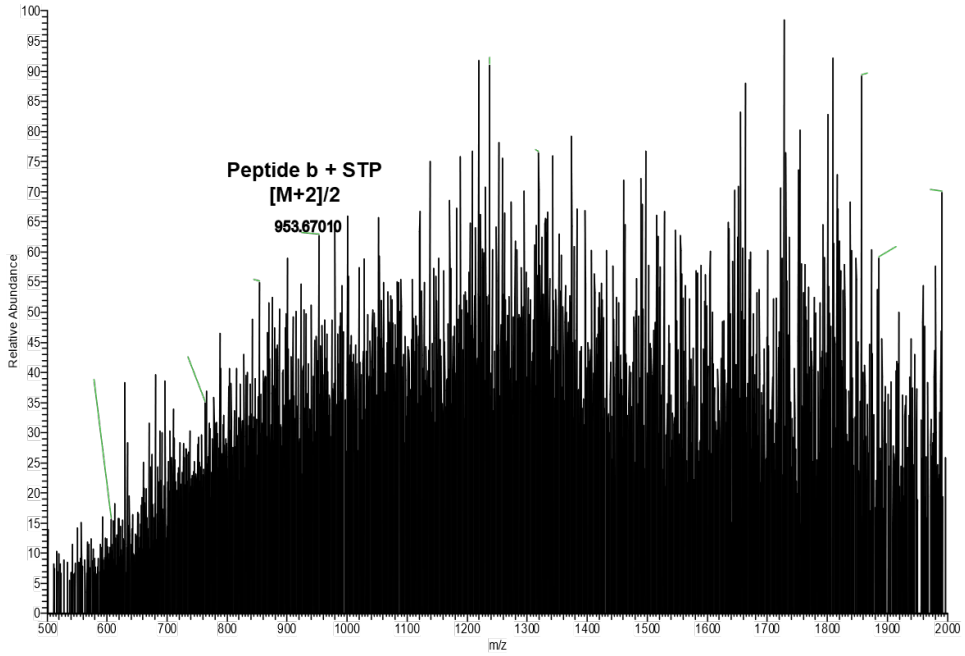


MS spectra of STP-modified peptide fragment a: Very poor mass intensity

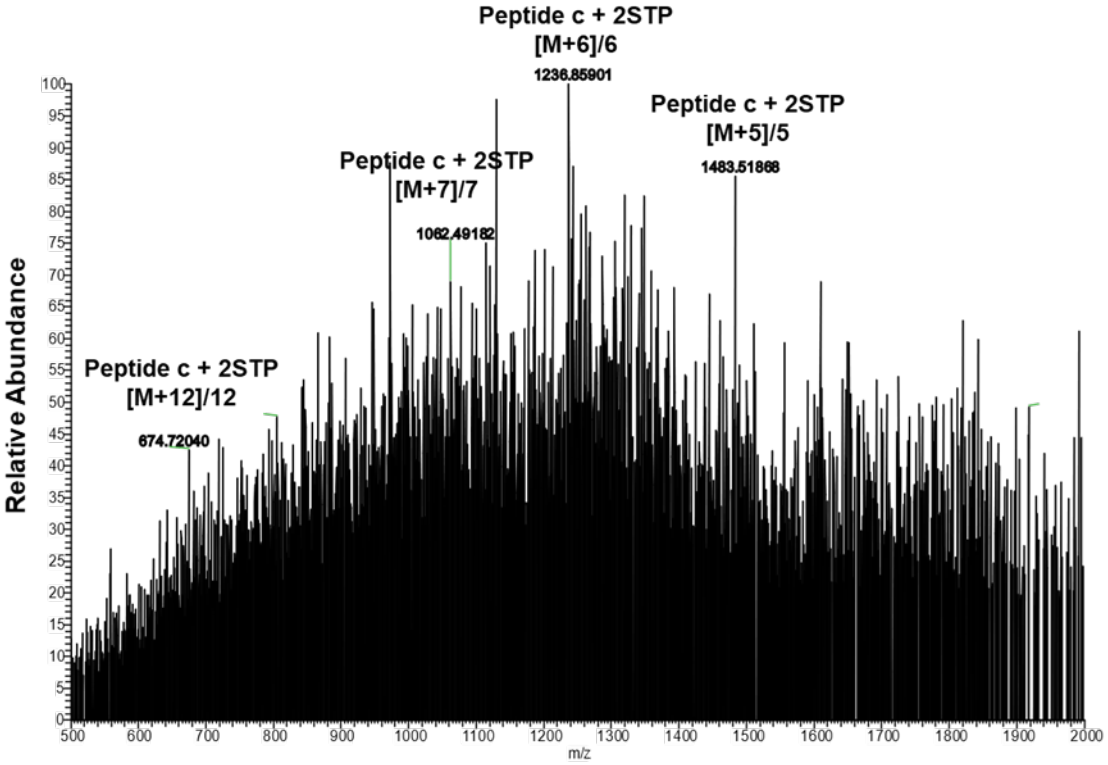




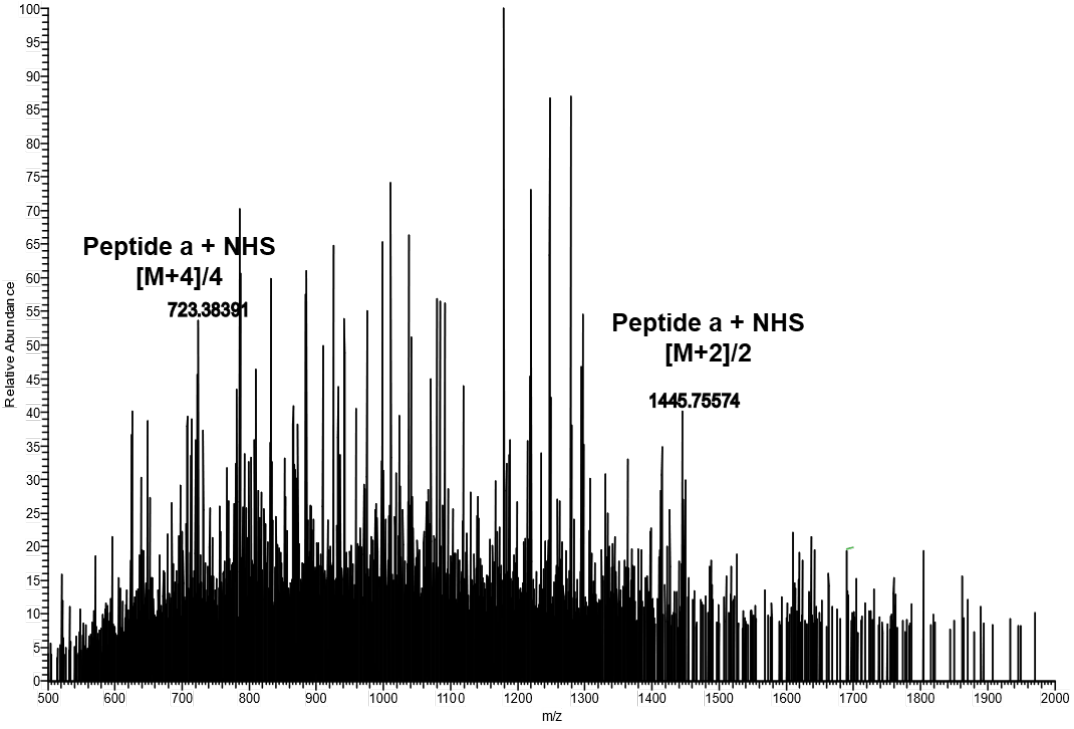
MS spectra of STP-modified peptide fragment b: Very poor mass intensity



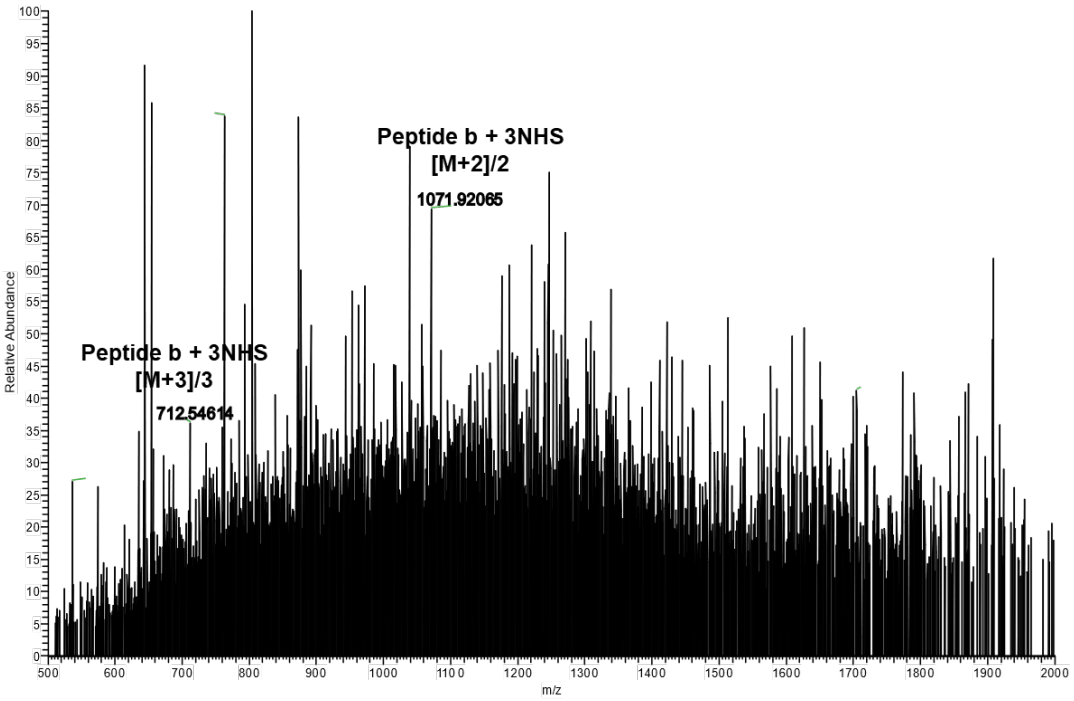
MS spectra of STP-modified peptide fragment c: Very poor mass intensity



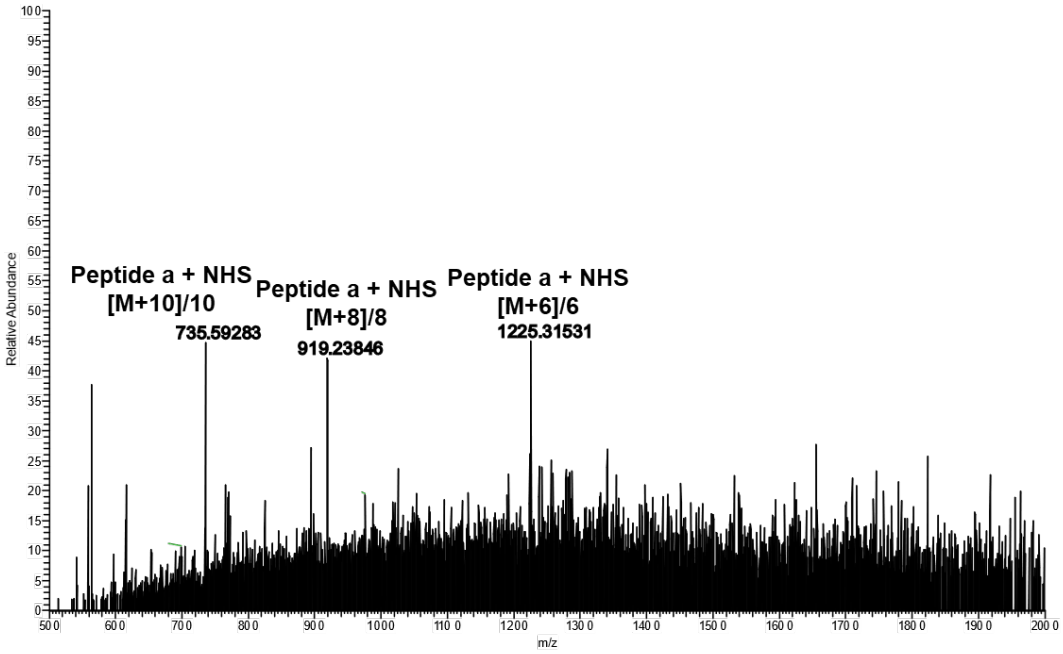
MS spectra of NHS-modified peptide fragment a: Very poor mass intensity



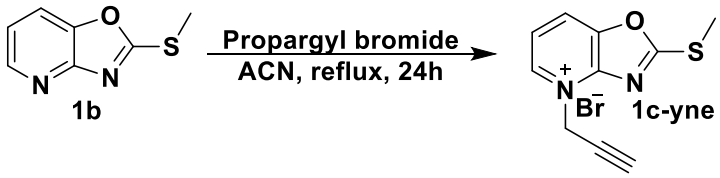
MS spectra of NHS-modified peptide fragment b: Very poor mass intensity



MS spectra of NHS-modified peptide fragment c: Very poor mass intensity



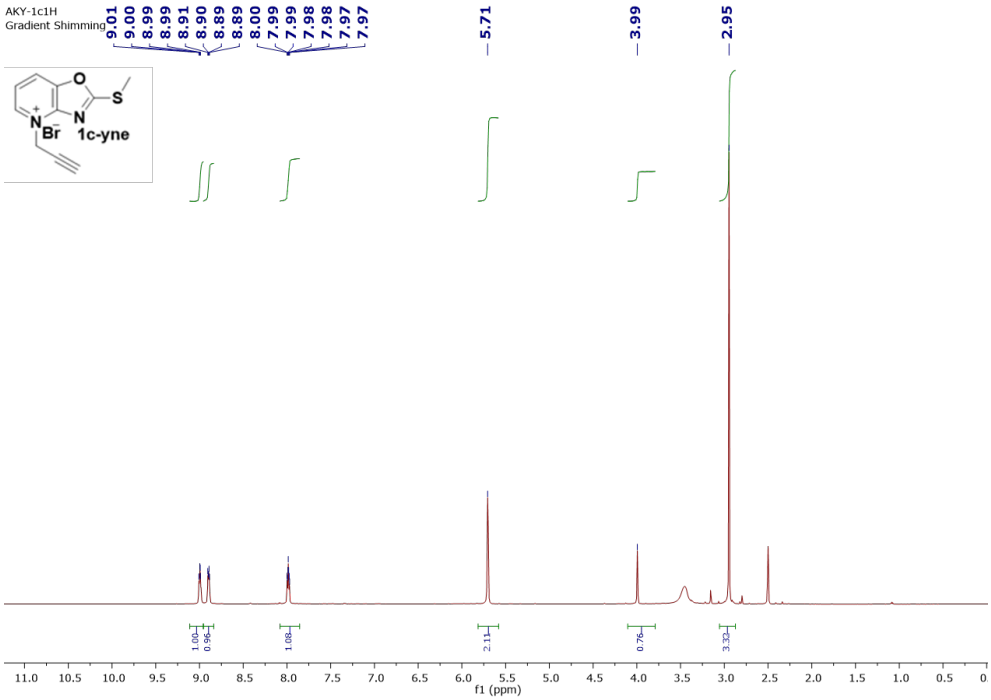
XII. Supplementary Figure 8: Synthesis of functionalized TAREs 1c-yne, 1d-yne and N_3-1e



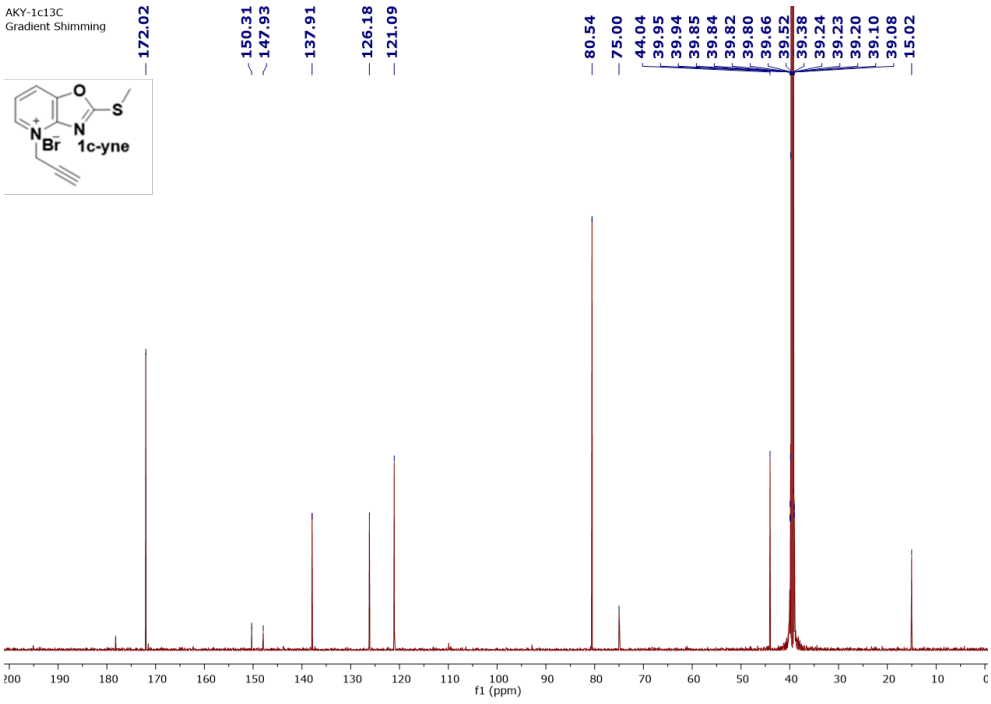
Synthesis of compound 1c-yne

2-(Methylthio)oxazolo[4,5-b]pyridine **1b** (83 mg, 0.5 mmol) was dissolved in dry ACN (3 mL), and treated with propargyl bromide (600 µL, 5 mmol). The reaction mixture was refluxed for 24 h. The mixture was cooled to room temperature and ACN was removed by rotary evaporation. The crude was purified by recrystallization with ether to afford compound **1c-yne** as brown powder (80%, 246 mg). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 9.01 – 8.99 (m, 1H), 8.89 (dd, J = 8.8, 4.5 Hz, 1H), 8.00 – 7.97 (m, 1H), 5.71 (s, 2H), 3.99 (s, 1H), 2.95 (s, 3H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 172.02, 150.31, 147.93, 137.91, 126.18, 121.09, 80.54, 75.00, 44.04, 15.02.

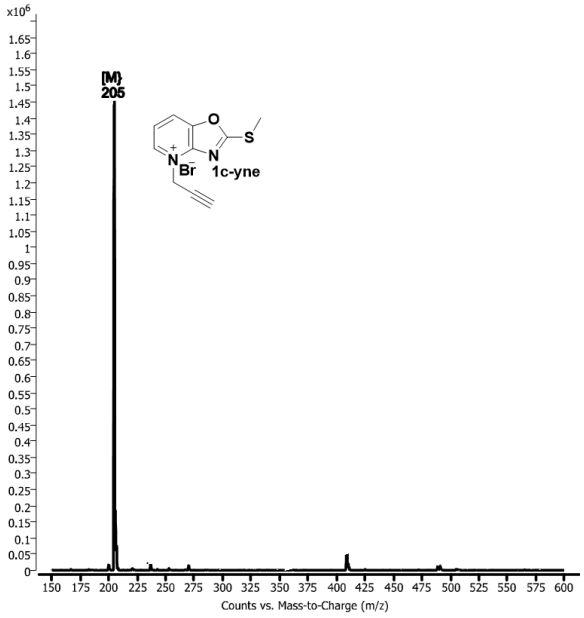
^{1}H NMR of compound 1c-yne

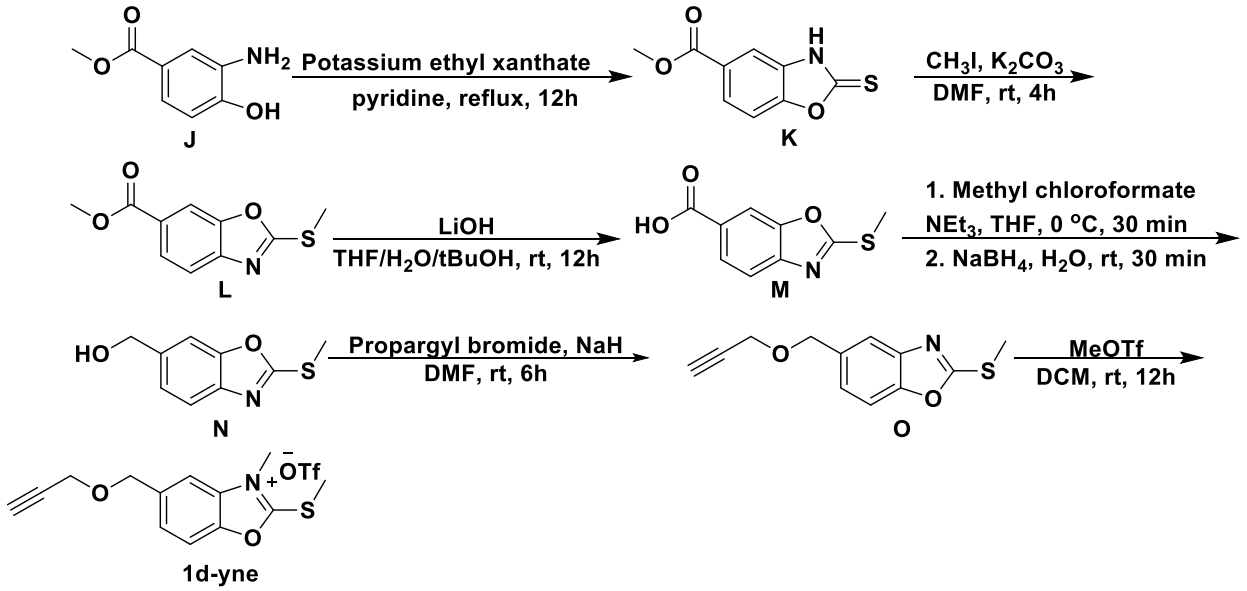


^{13}C NMR of compound 1c-yne



LC-MS of 1c-yne





Synthesis of compound K

Methyl 3-amino-4-hydroxybenzoate **J** (4 g, 24 mmol) was dissolved in dry pyridine (30 mL) and potassium ethyl xanthate (4.05 g, 25.27 mmol) was added. The solution was refluxed for 12 h. The solution was then cooled down to RT and treated with DI water (500 mL) and acetic acid (50 mL). The precipitate was filtered off and washed with hexane for three times. The off-white precipitate was dried under vacuum to obtain compound **K** as off-white powder (75 %, 3.8 g). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 7.96 (d, J = 1.4 Hz, 1H), 7.90 (dd, J = 8.2, 1.6 Hz, 1H), 7.31 (d, J = 8.2 Hz, 1H), 3.85 (s, 3H).**^{13}C NMR** (151 MHz, DMSO-d_6) δ 181.27, 165.54, 147.97, 135.43, 126.96, 124.98, 110.47, 110.31, 52.43.

Synthesis of compound L

Methyl 2-thioxo-2,3-dihydrobenzo[d]oxazole-5-carboxylate **K** (2.35 g, 11.2 mmol), potassium carbonate (1.54 g, 11.2 mmol), and methyl iodide (700 µL, 11.2 mmol) were added sequentially into dry DMF (11.2 mL) at 0 ^{o}C. The mixture was warmed to room temperature and stirred for 4 hours. The reaction mixture was washed with ethyl acetate and brine for 3 times. The organic portion was collected, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by the column chromatography (hexane: ethyl acetate 7:1) to yield compound **L** as colorless oil (2.17 g, 87 %). **^{1}H NMR** (600 MHz, $CDCl_3$) δ 8.12 (d, *J* = 1.5 Hz, 1H), 8.03 (dd, *J* = 8.3, 1.5 Hz, 1H), 7.61 (d, *J* = 8.3 Hz, 1H), 3.94 (s, 3H), 2.78 (s, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 172.64, 169.30, 166.78, 151.83, 146.03, 126.49, 117.93, 111.50, 52.48, 14.73.

Synthesis of compound M

To a stirred solution of methyl 2-(methylthio)benzo[d]oxazole-6-carboxylate **L** (160 mg, 0.71 mmol) in THF (3.55 mL) were added t-BuOH (1.42 mL) and a solution of $LiOH{\cdot}H_2O$ (120 mg, 2.86 mmol) in water (1.42 mL), followed by stirring at rt for 12 h. The solvent was evaporated under reduced pressure. The mixture was extracted with ethyl acetate. The organic layer was washed with 0.5-N HCl solution, water, and brine, and dried over anhydrous $MgSO_4$. After filtration, the solvent was evaporated under reduced pressure to afford compound **M** as white solid (141 mg, 95 %). Compound M was directly used without further purification.

Synthesis of compound N

To a solution of 2-(methylthio)benzo[d]oxazole-6-carboxylic acid **M** (150 mg, 0.71 mmol) in THF (10 mL) was added Et_3N (130 µL, 0.93 mmol), followed by dropwise addition of methyl chloroformate (66 µL, 0.86 mmol) at 0 °C for 30 min. The appeared salt was filtered off. To the resulting filtrate, $NaBH_4$ (108 mg, 2.86 mmol) in water (800 µL) was added dropwise. The reaction mixture was stirred at rt for 30 min, diluted with water and washed with ethyl acetate and brine, and dried over $MgSO_4$. After filtration, the solvent was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane: ethyl acetate 3:1) to obtain compound **N** as white solid (92.2 mg, 66 %)**^{1}H NMR** (600 MHz, $CDCl_3$) δ 7.53 (d, *J* = 0.6 Hz, 1H), 7.46 (dd, *J* = 1.6, 0.7 Hz, 1H), 7.26 (d, *J* = 8.9 Hz, 1H), 4.78 (s, 2H), 2.76 (s, 3H).**^{13}C NMR** (151 MHz, $CDCl_3$) δ 172.63, 152.34, 141.54, 137.44, 123.42, 118.19, 108.62, 65.29, 14.66.

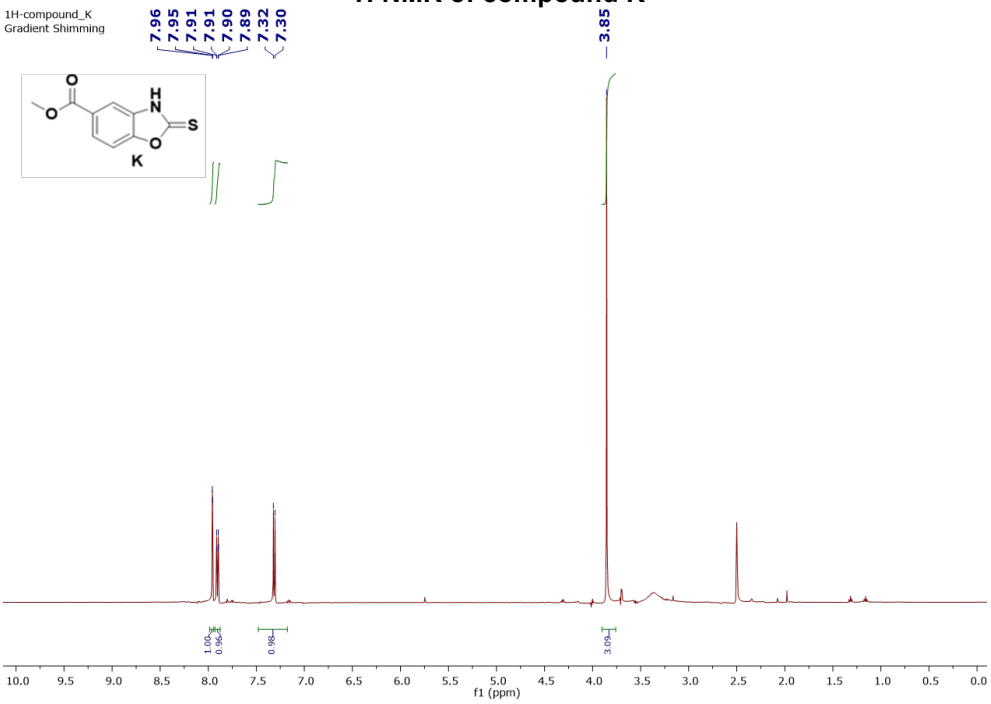
Synthesis of compound O

To a solution of (2-(methylthio)benzo[d]oxazol-6-yl)methanol **N** (36 mg, 0.184 mmol) in dry DMF (5 mL) at 0 ^{o}C, NaH (5.31 mg, 0.22 mmol) was added. After 10 mins propargyl bromide (20 µL, 0.22 mmol) was added dropwise and the reaction mixture was allowed to warm to room temperature. After 6 h reaction mixture was washed with ethyl acetate and brine. The organic part was dried over anhydrous $MgSO_4$, filtered, and concentrated. The crude was purified by flash column (hexane: ethyl acetate 5:1) to afford compound **O** as yellow solid (28 mg, 67 %)**. ^{1}H NMR (**400 MHz, $CDCl_3$) δ 7.59 (d, *J* = 8.1 Hz, 1H), 7.49 (dd, *J* = 1.5, 0.7 Hz, 1H), 7.31 (s, 1H), 4.72 (s, 2H), 4.21 (d, *J* = 2.4 Hz, 2H), 2.78 (s, 3H), 2.51 (s, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 166.36, 152.29, 141.90, 133.67, 124.60, 118.14, 109.78, 79.59, 74.96, 71.47, 57.19, 53.57, 14.67.

Synthesis of compound 1d-yne

2-(methylthio)-5-((prop-2-yn-1-yloxy)methyl)benzo[d]oxazole **O** (86 mg, 0.52 mmol) and methyl trifluoromethanesulfonate (70 µL, 0.625mmol) were mixed in 10 mL of DCM in a nitrogen environment at 0 ^{o}C. The reaction mixture was allowed to warm to 25 ^{o}C for 12 h. Precipitation with hexane was followed by filtration and yielded compound **1d-yne** as white powder (192 mg, 93 %). **^{1}H NMR** (600 MHz, Methanol-d_4) δ 7.92 (s, 1H), 7.83 (d, *J* = 8.4 Hz, 1H), 7.68 (d, *J* = 8.4 Hz, 1H), 5.46 (dd, *J* = 2.8, 1.4 Hz, 2H), 4.77 (s, 2H), 3.95 (s, 3H), 3.31 (s, 1H), 3.08 (s, 3H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 172.04, 131.98, 123.62, 113.21, 111.03, 109.18, 108.70, 80.16, 77.47, 70.59, 56.71, 28.11, 14.71.

^{1}H NMR of compound K



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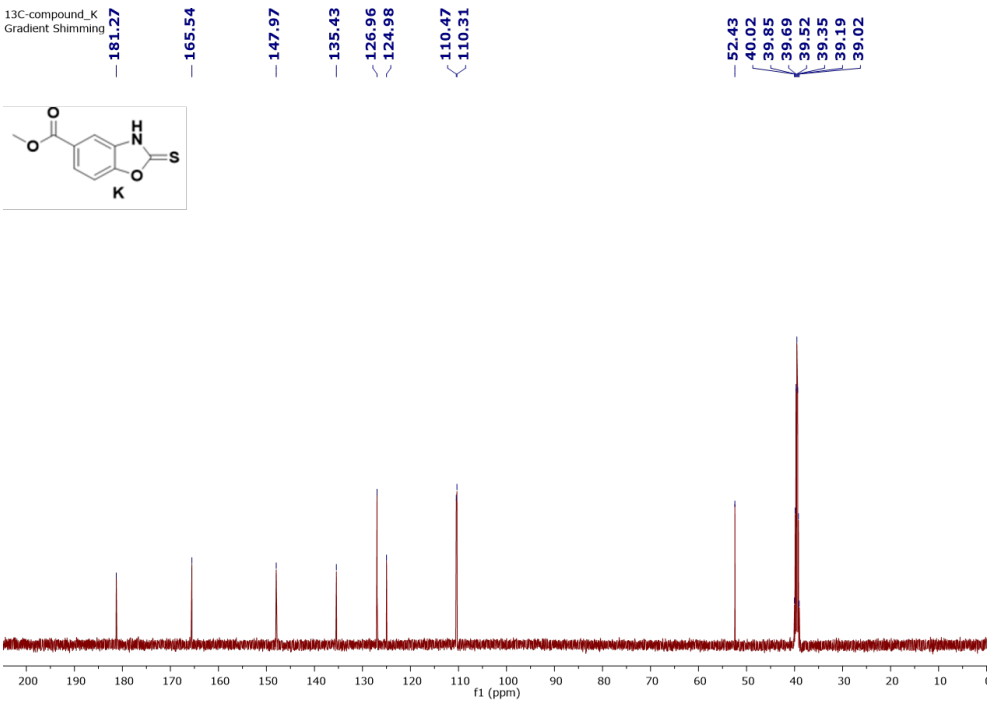

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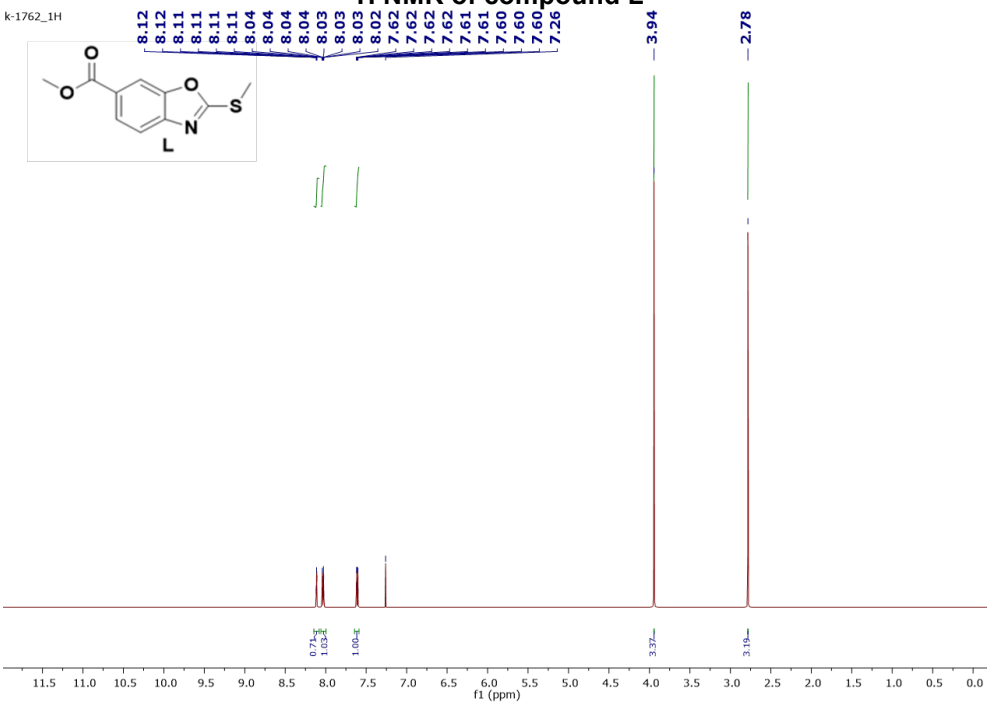
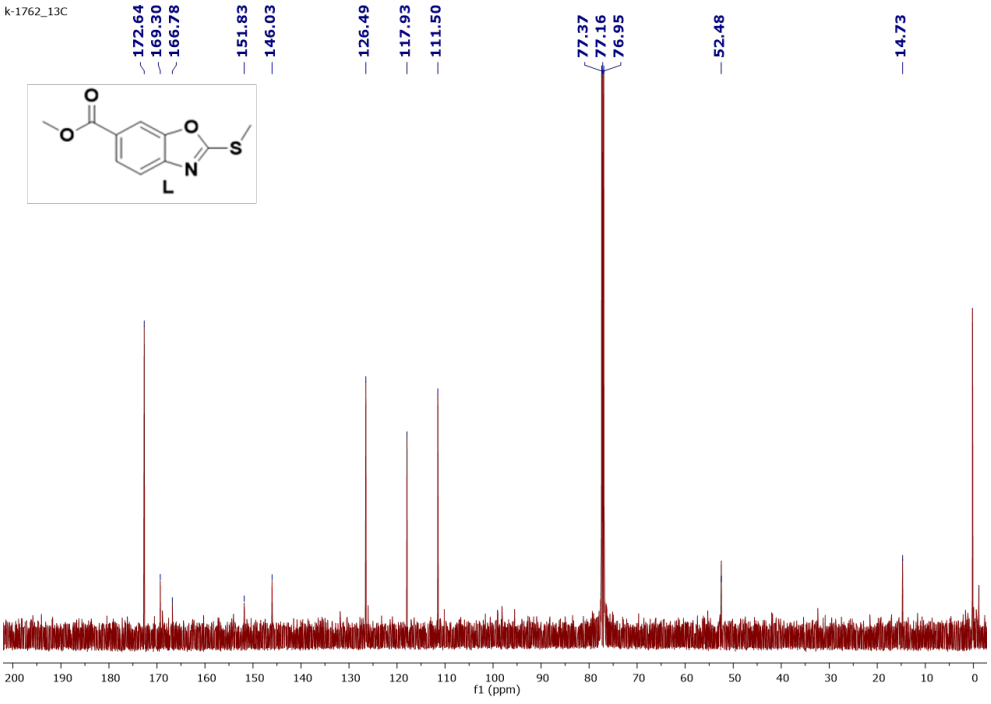
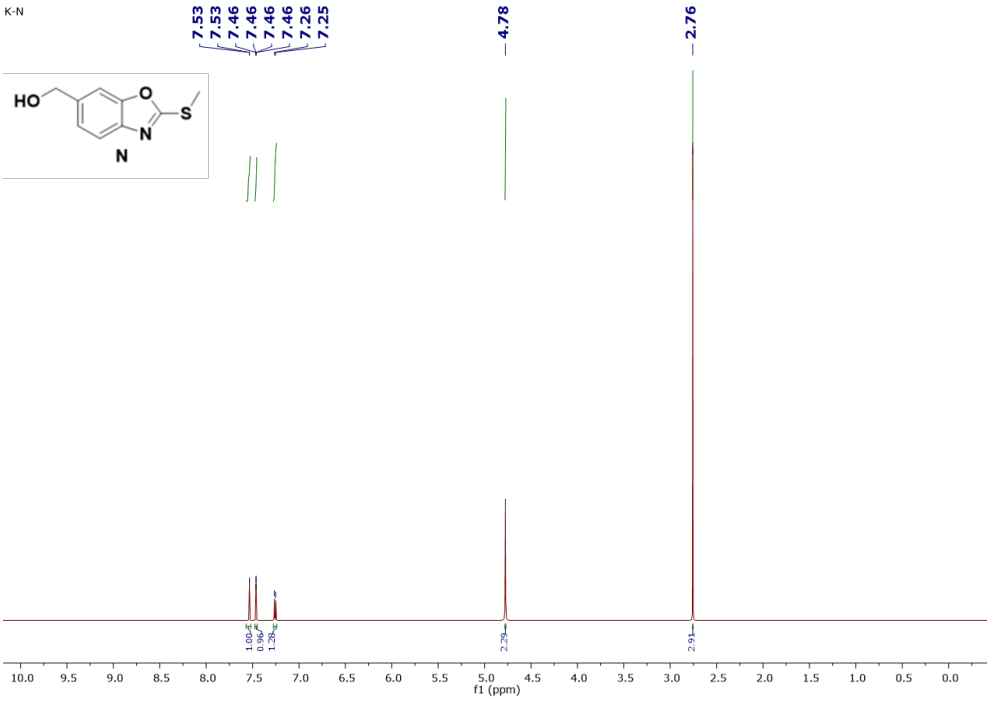


^{13}C NMR of compound L



^{1}H NMR of compound N



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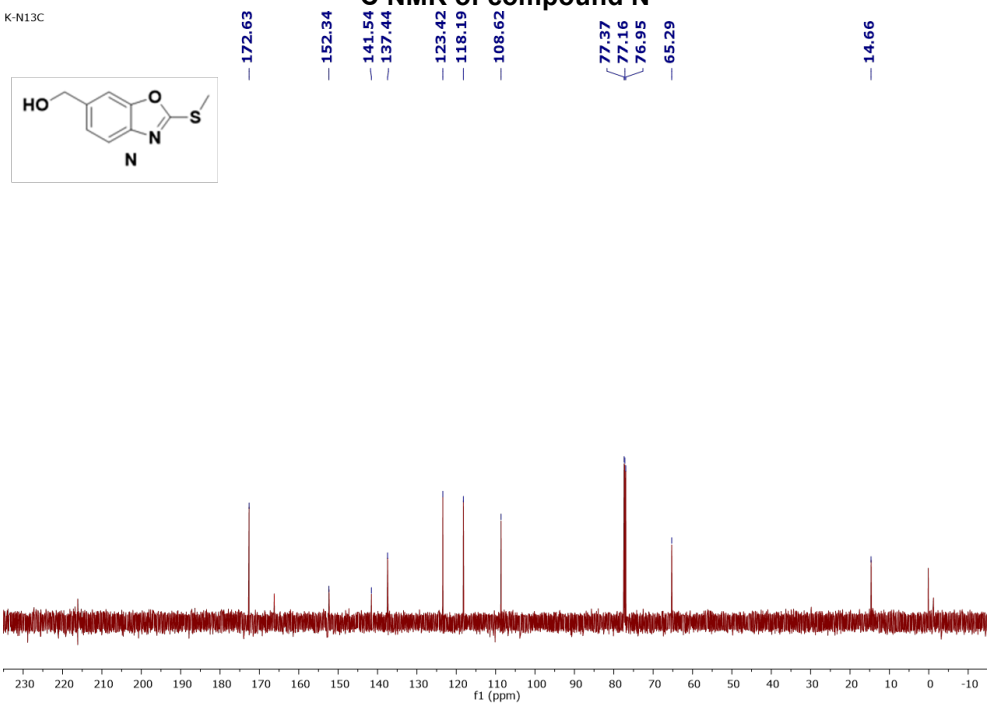
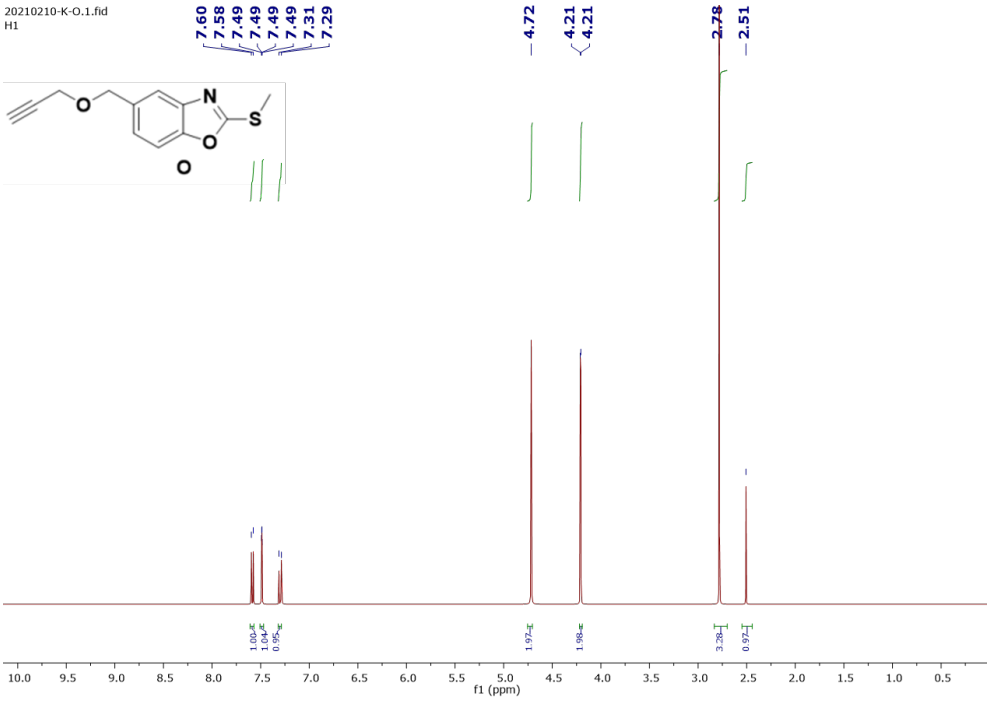
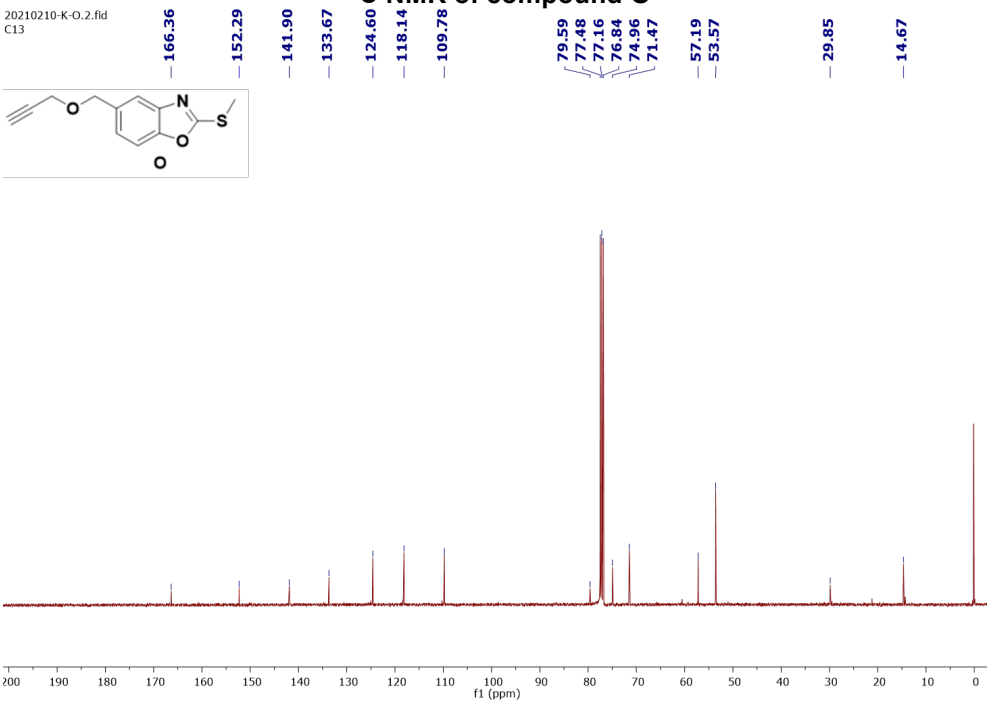


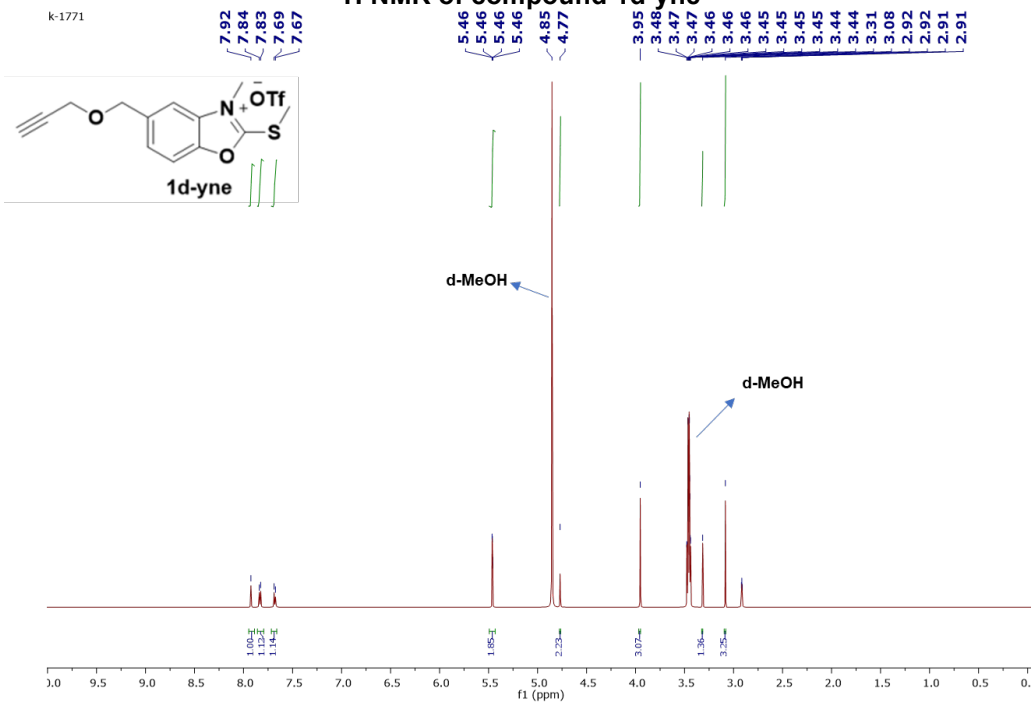
^{1}H NMR of compound O



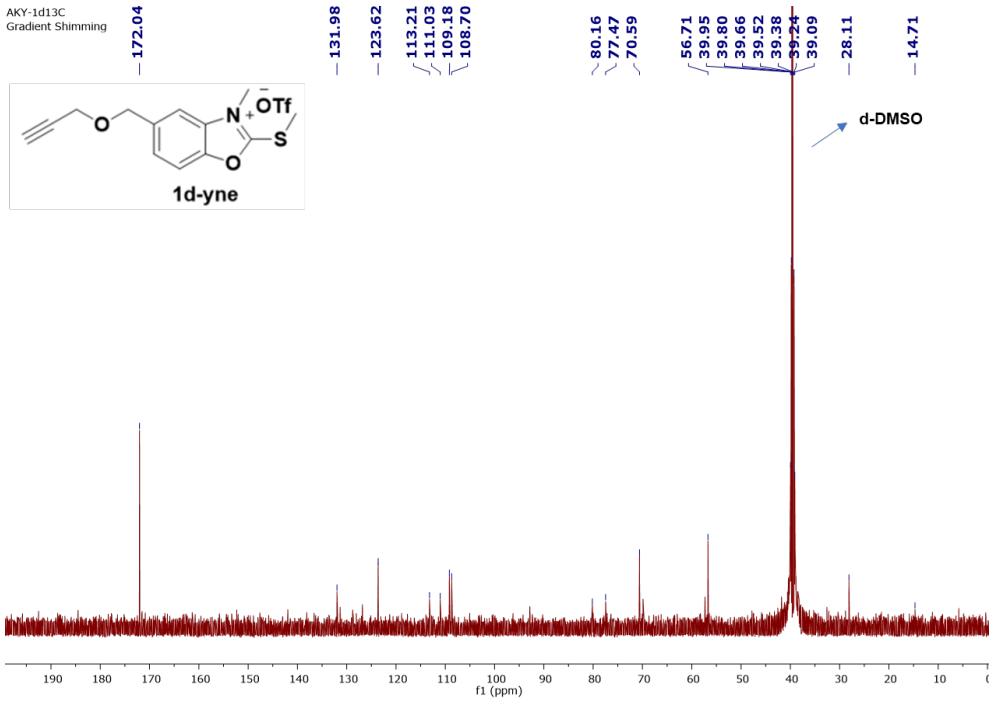
^{13}C NMR of compound O



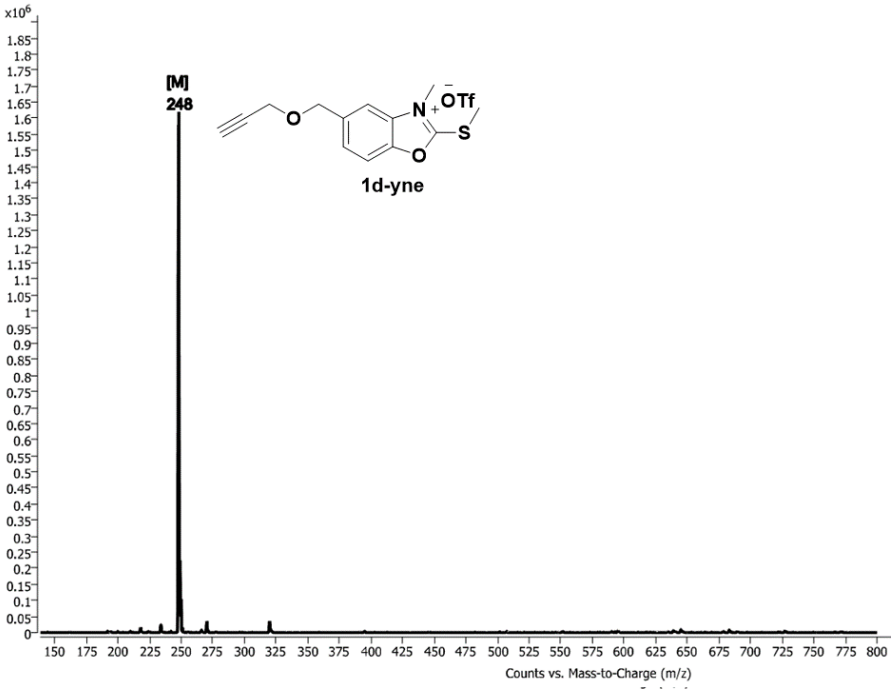
^{1}H NMR of compound 1d-yne

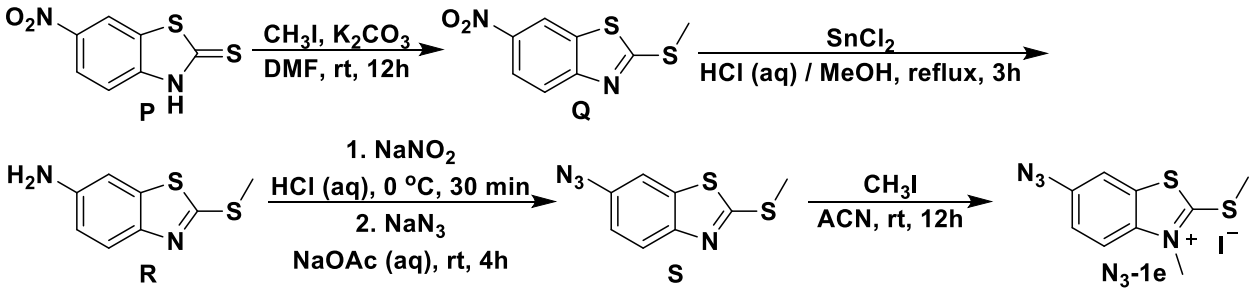


^{13}C NMR of compound 1d-yne



LC-MS of 1d-yne





Synthesis of compound Q

To a stirred solution of 6-nitrobenzo[d]thiazole-2(3H)-thione **P** (1.2 g, 5.66 mmol) in dry DMF (15 mL), K_2CO_3 (782.5 mg, 5.66 mmol) and MeI (354 µL, 5.66 mmol) were added consecutively at room temperature, then the reaction was stirred at room temperature 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 5:1) to afford compound **Q** as yellow powder (72 %, 921 mg). **^{1}H NMR** (600 MHz, $CDCl_3$) δ 8.64 (s, 1H), 8.26 (dd, J = 8.9, 2.3 Hz, 1H), 7.86 (d, J = 8.9 Hz, 1H), 2.82 (s, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 175.16, 172.59, 157.15, 135.63, 121.98, 121.24, 117.44, 16.15.

Synthesis of compound R

A mixture of 2-(methylthio)-6-nitrobenzo[d]thiazole **Q** (500 mg, 2.21 mmol), $SnCl_2.2H_2O$ (12.0 g, 8.84 mmol) in concentrated HCl (5 mL) and MeOH (5 mL) was refluxed for 3 h. After the reaction was completed, 30% NaOH solution (20 mL) was added and extracted with ethyl acetate. The organic phase was washed with saturated brine for three times, dried over $MgSO_4$, filtered and the solvent was removed

under reduced pressure to obtain compound **R** as off-white powder (77%, 333.2 mg). The product **R** was used without further purification.

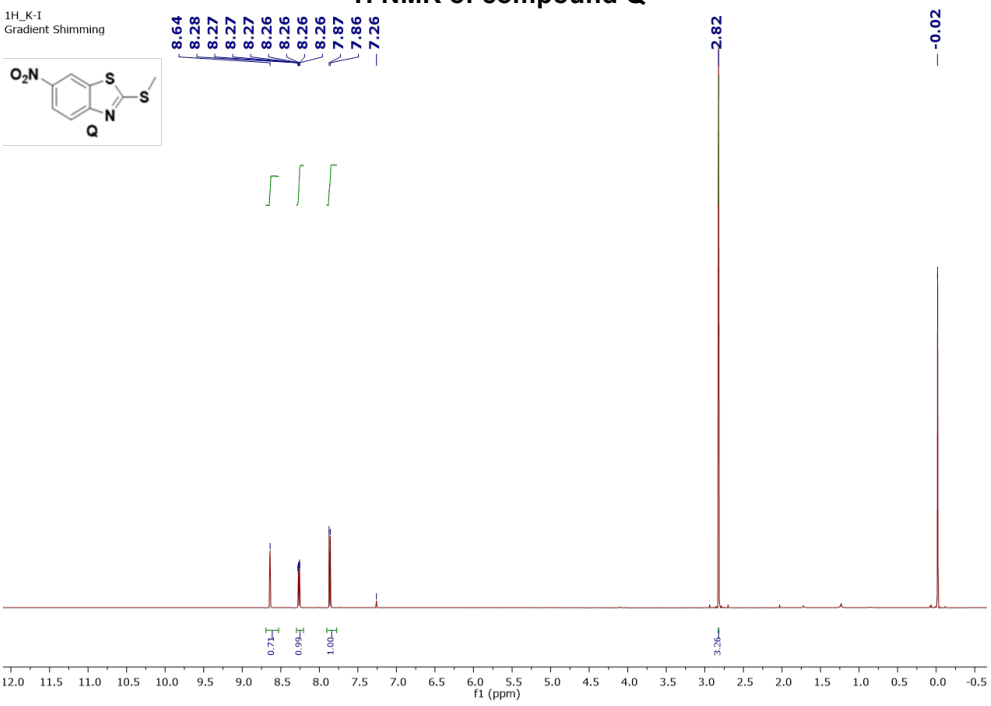
Synthesis of compound S

A solution of sodium nitrite (84.2 mg, 1.22 mmol) in water (600 µL) was added to a stirred solution of 6-amino-2-methylbenzothiazole **R** (1.0 g 200 mg, 6.1 mmol 1.22 mmol) in a mixture of concentrated hydrochloric acid (600 µL) and water (1.8 mL) and the mixture was stirred for 30 min at 0 ^{o}C. Then a solution of sodium azide (78 mg, 1.22 mmol) in water (800 µL) saturated with sodium acetate was added dropwise at 0 ^{o}C, and the mixture was stirred at this temperature for 4 h. The reaction residue was washed with $NaHCO_3$ (aq) and brine for three times. The organic layer was collected and dried over $MgSO_4$. After filtration, the crude mixture was purified by flash chromatography (hexane: ethyl acetate 6:1) to afford compound **S** as yellow solid (170 mg, 75 %). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 8.7 Hz, 1H), 7.36 (d, *J* = 2.2 Hz, 1H), 7.07 (dd, *J* = 8.7, 2.3 Hz, 1H), 2.78 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 167.73, 150.97, 136.79, 136.49, 122.26, 117.84, 111.05, 16.06.

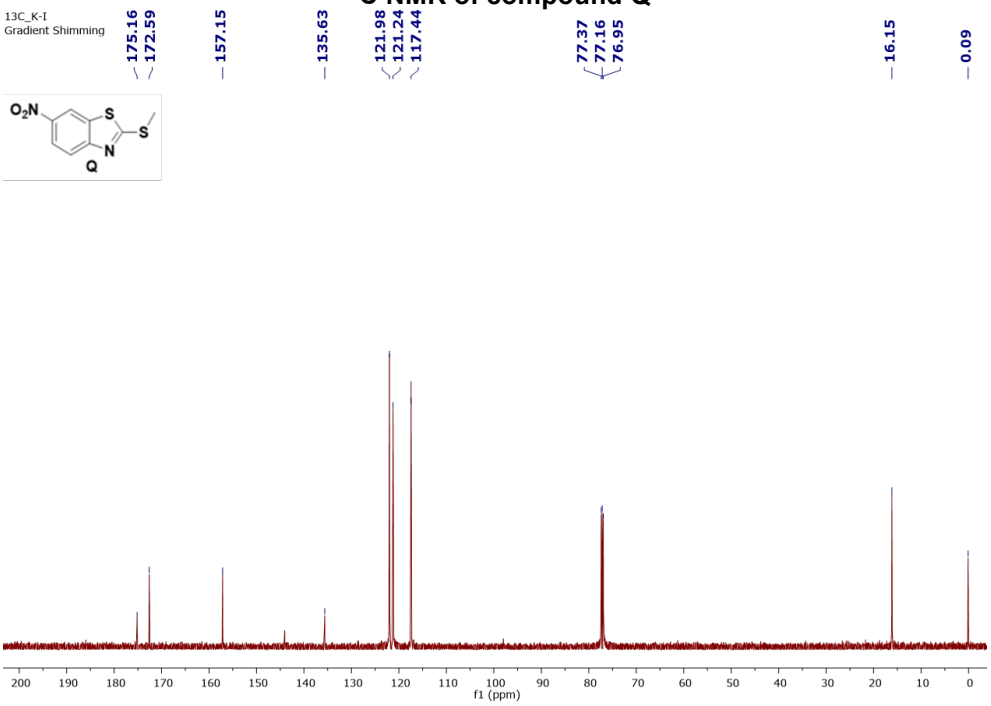
Synthesis of compound N_3-1e

To the 6-azido-2-(methylthio)benzo[d]thiazole **S** (50 mg, 0.225 mmol) in the dry acetonitrile (7 mL), was added MeI (71.3 µL, 1.14 mmol) at room temperature under nitrogen. The reaction was stirred for 12 h. The solvent was removed by rotary evaporation to afford crude powder. The crude mixture was washed with hexane three times, than dried under vacuum to obtain pure compound **N_3-1e** as bright yellow powder (80 %, 65.5 mg). **^{1}H NMR** (600 MHz, DMSO-d_6) δ 7.87 – 7.83 (m, 2H), 7.18 (d, J = 8.6 Hz, 1H), 2.85 (s, 3H), 2.78 (s, 3H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 167.87, 150.48, 136.22, 135.81, 121.94, 118.20, 112.17, 26.19, 15.55.

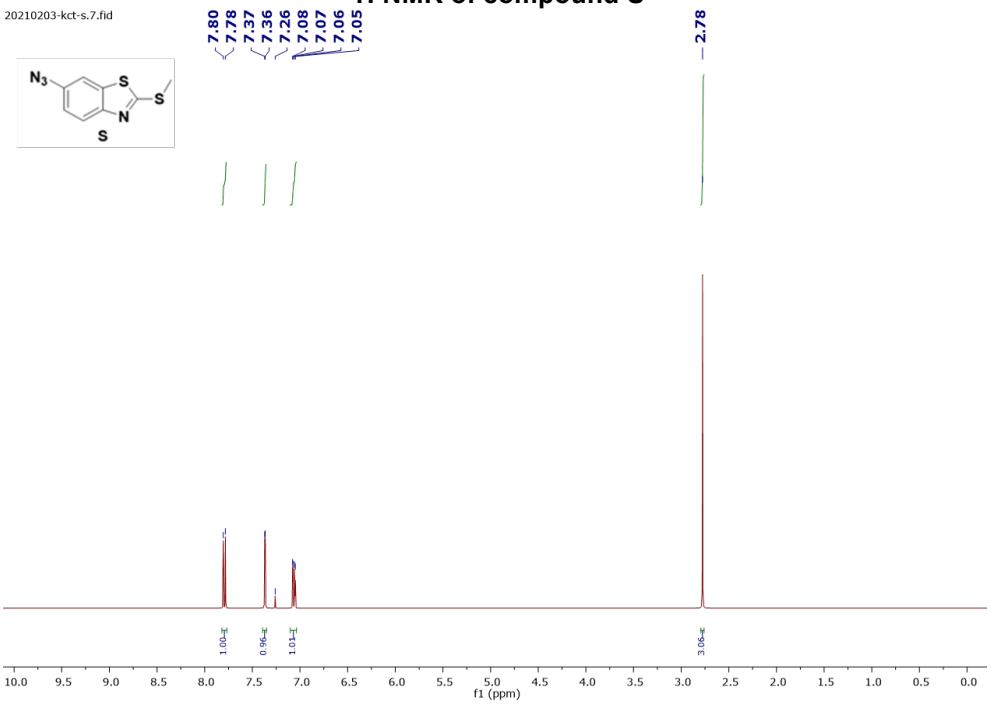
^{1}H NMR of compound Q



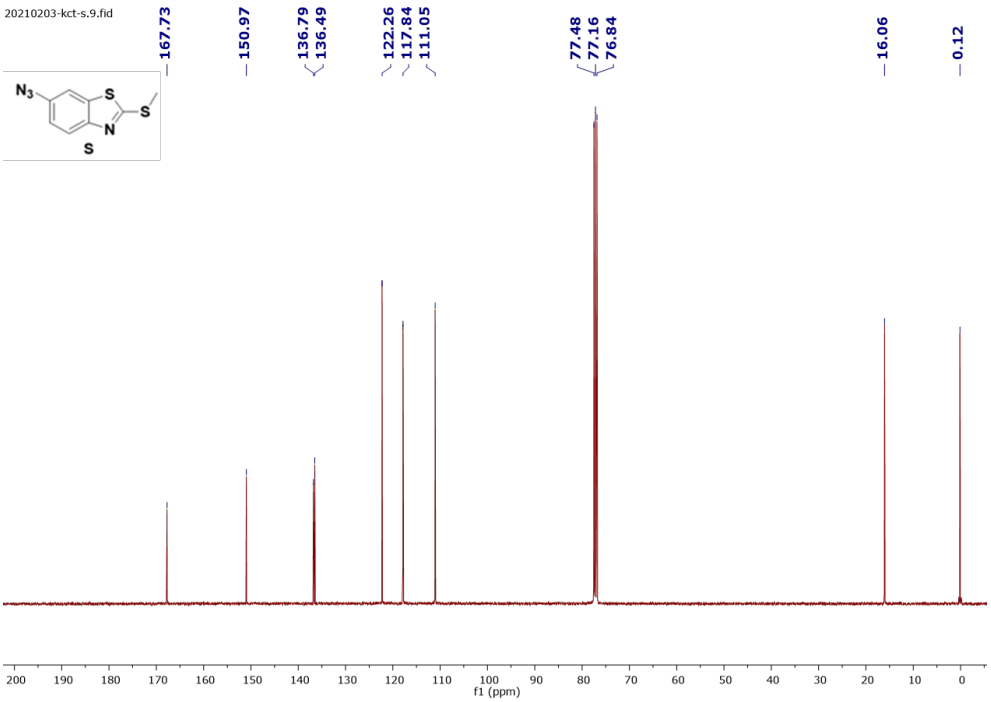
^{13}C NMR of compound Q



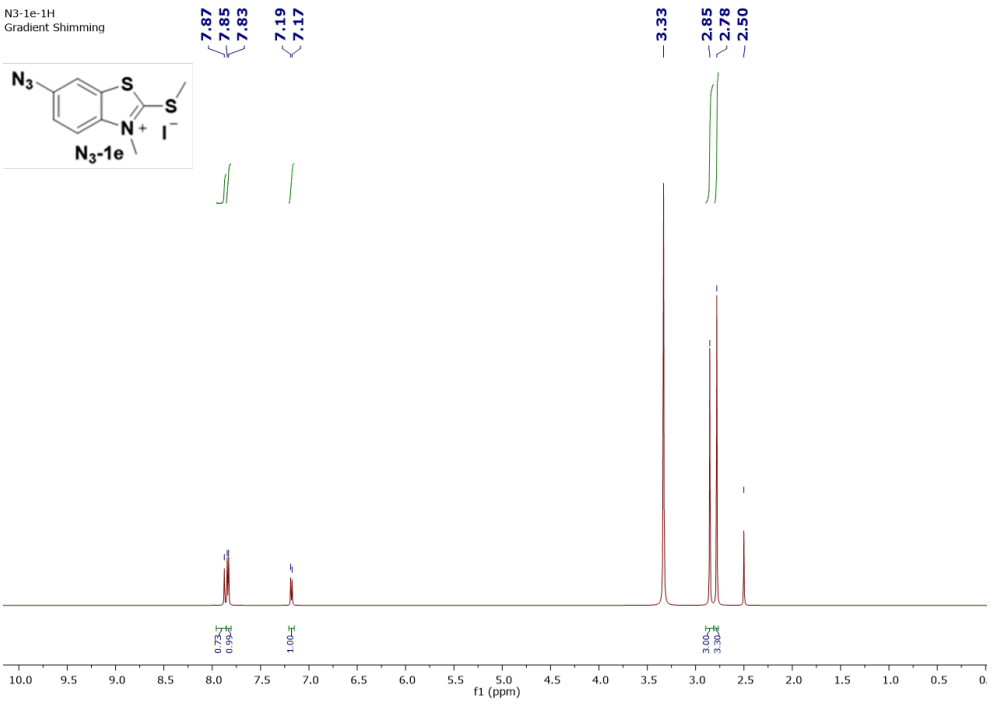
^{1}H NMR of compound S



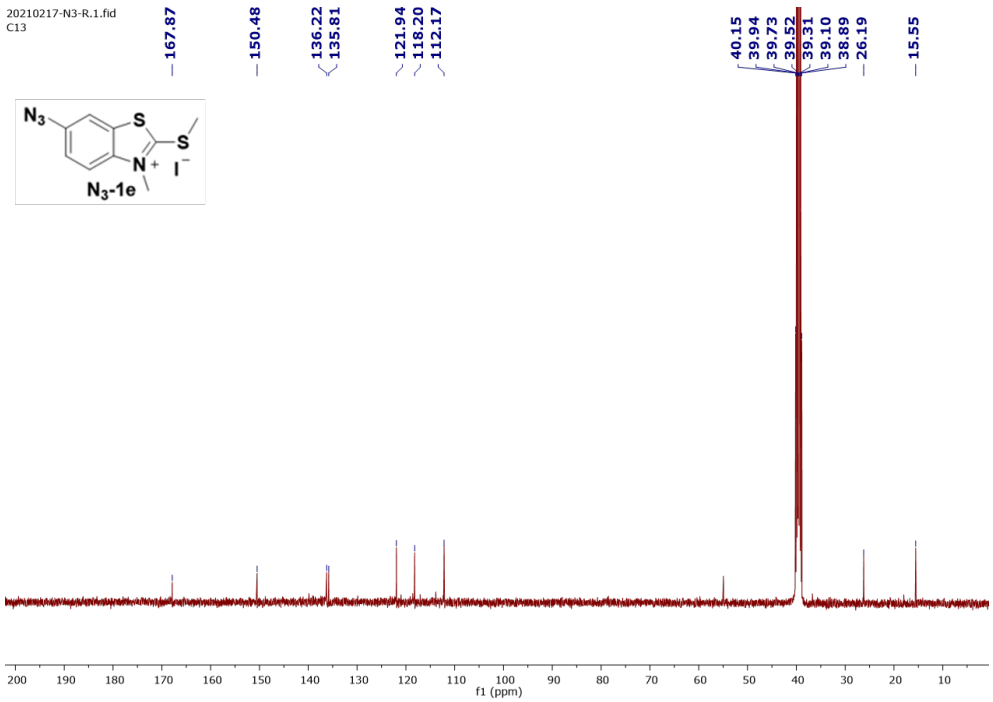
^{13}C NMR of compound S



^{1}H NMR of compound N_3-1e



^{13}C NMR of compound N_3-1e



XIII. Supplementary Figure 9. Bioconjugation of functioned-TARE probes with different proteins. To proteins (60 μmol) in 200 μL 10 mM phosphate buffer (pH 7.5), 1c-yne (1 eq.) or N_3-1e (25 eq) was added. The reactions were stirred at room temperature for 12 h. The TARE-protein bioconjugates were purified by molecular weight cut off and characterized by LCMS.

Reaction of proteins with high amounts of probes: To proteins (60 μmol) in 200 μL 10 mM phosphate buffer (pH 7.5), 1c-yne (50 eq.) was added. The reactions were stirred at room temperature for 1 h. The TARE-protein bioconjugates were purified by molecular weight cut off and characterized by LCMS.

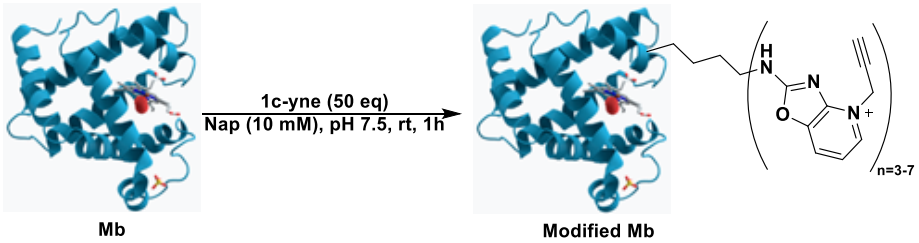
Procedure for in-solution digestion of modified myoglobin followed by MS/MS analysis

Protein (1 mg) in 10 mM tris (100 μL, pH 7.5) with urea (6 M) was incubated for 30 minutes at 37 °C. To reduce the urea concentration to 0.6 M, the sample was diluted with grade I water. To this solution, 100 μL of enzyme (αchymotrypsin/trypsin) solution [0.1 mg, enzyme/protein (1:20); enzyme in 1 mM HCl was dissolved in 0.1 M tris and 0.01 M $CaCl_2$] was added and the mixture was incubated at 37 °C for 18 h. The pH of digested mixture was adjusted to < 6 (confirmed by pH paper) with trifluoroacetic acid (0.5%). Subsequently, the sample was used for peptide mapping by MS and sequencing by MS/MS**.** The protein fragments were analyzed by Thermo Ultimate 3000 nanoLC/Orbitrap Q-Exactive Plus MS with positive mode. The identification of protein sequences was achieved by Thermo BioPharma Finder.

In myoglobin 7 lysine residues were modified out of 19 lysines with in 1 h.

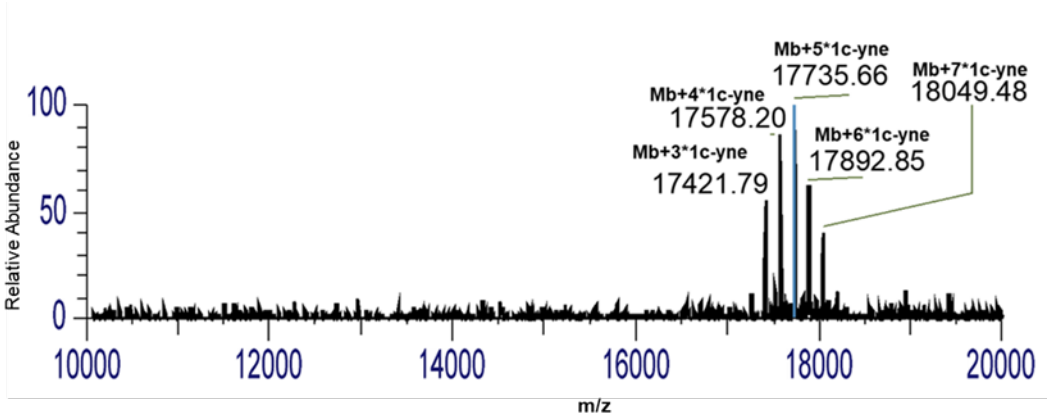
In lactalbumin, 7 lysine residues were modified out of 12 lysines with in 1 h.

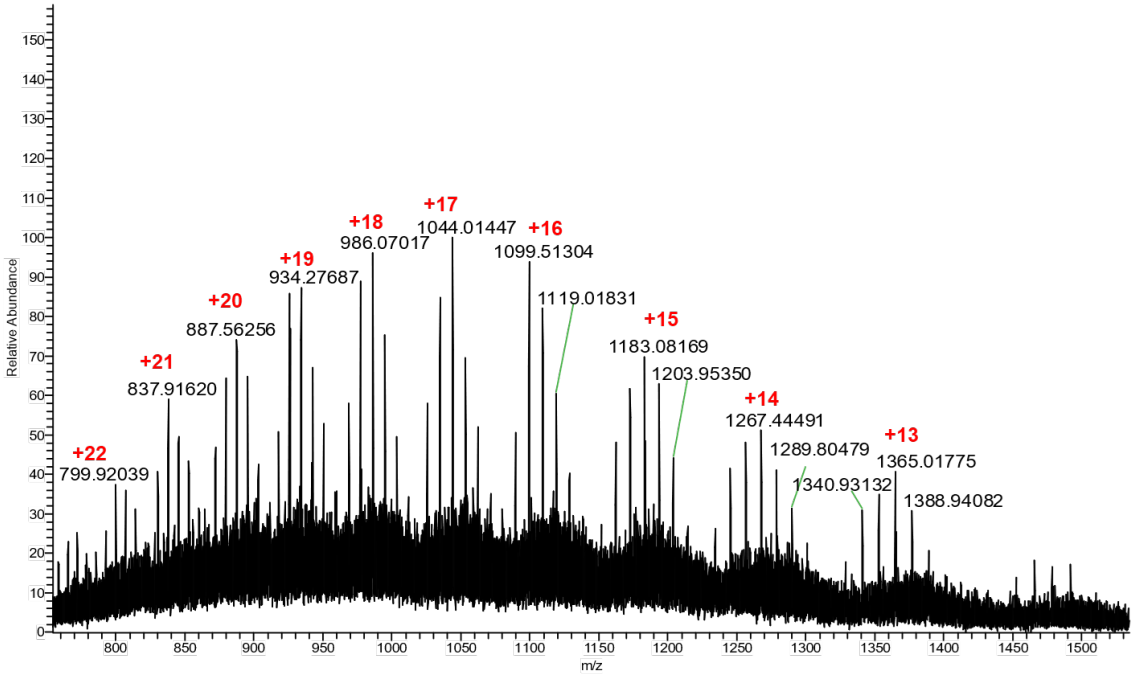
In cytochrome C, 6 lysine residues were modified out of 12 lysines with in 1 h.

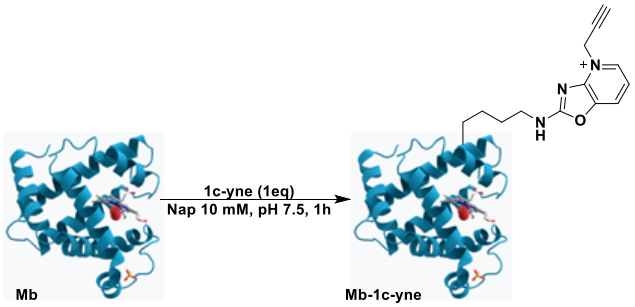


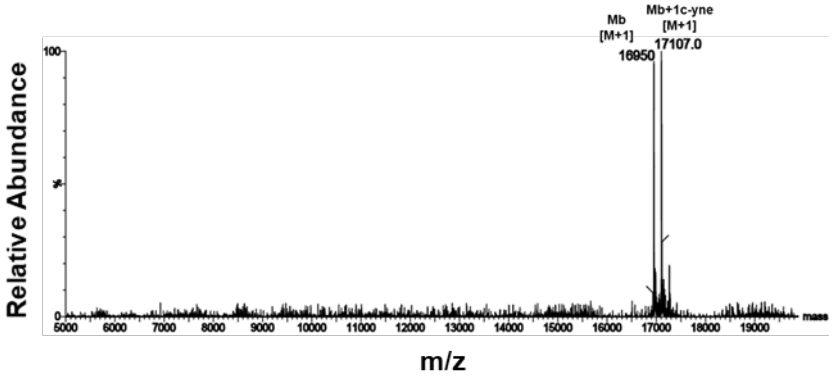
HRMS of 1cyne-modified Mb

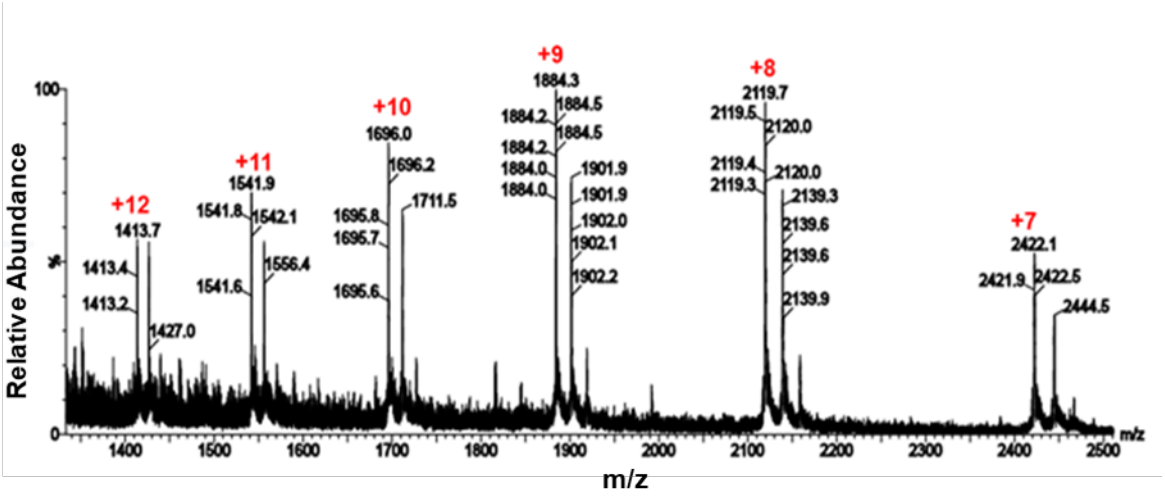
Number of modifications	Expected mass (m/z)	Observed mass (m/z)
3	17421	17421.79
4	17578	17578.20
5	17735	17735.66
6	17892	17892.58
7	18049	18049.48



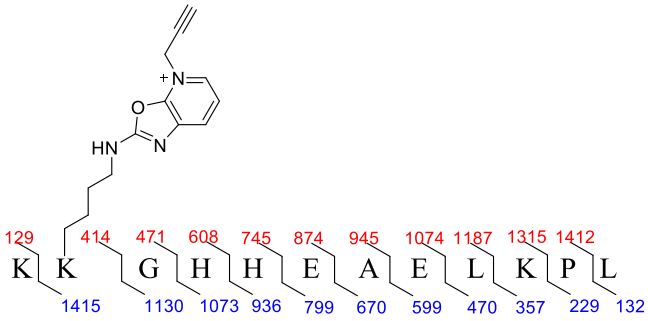

Relative Abundance
m/z
+22
799.92039
+21
837.91620
+20
887.56256
+19
934.27687
+18
986.07017
+17
1044.01447
+16
1099.51304
1119.01831
+15
1183.08169
1203.95350
+14
1267.44491
1289.80479
+13
1365.01775
1340.93132
1388.94082


1c-yne (1eq)
Nap 10 mM, pH 7.5, 1h
Mb
Mb-1c-yne

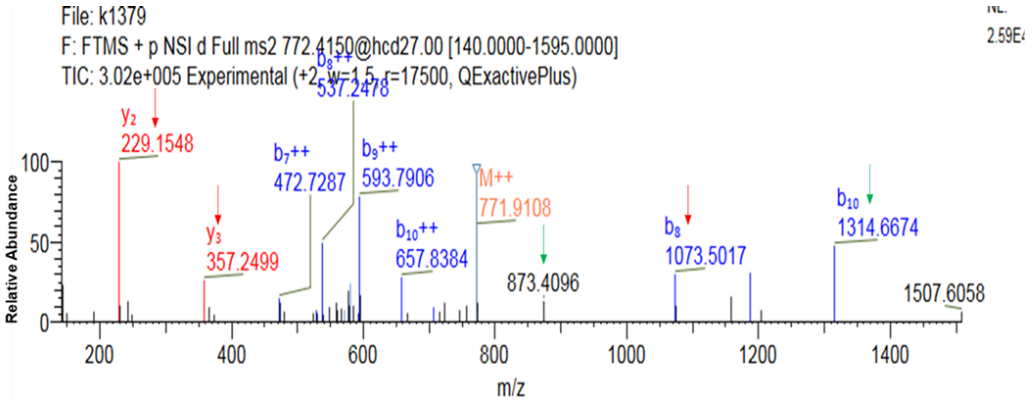

Mb
[M+1]
16950
Mb+1c-yne
[M+1]
17107.0
Relative Abundance
m/z
mass

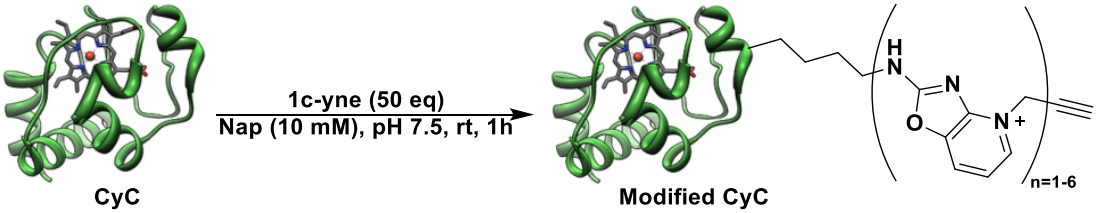


MS/MS spectrum Mb-1c-yne fragment (1 eq, 1h)



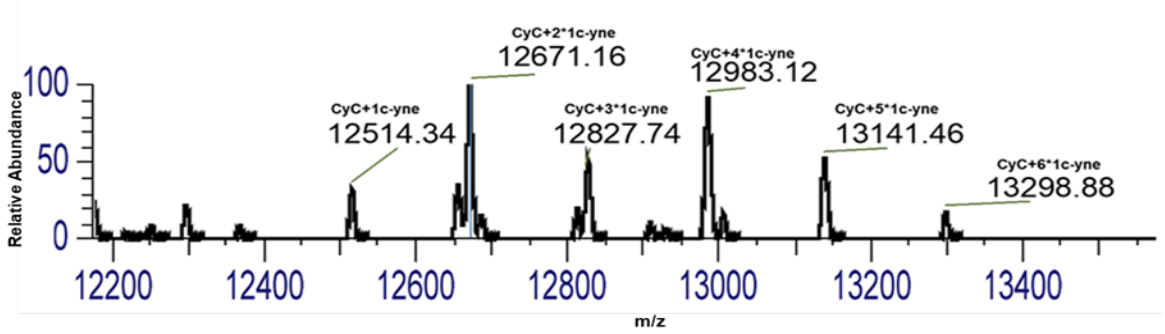
Protein	Peptide Sequence	Modification	Site	Confidence Score	ID Type	M/Z	Charge State	Mono Mass Exp.	Avg Mass Exp.
Modified Myoglobin	KKGHHEAELKPL	156.0328	~K87	99.8	MS2	386.46	4	1541.8127	1542.56

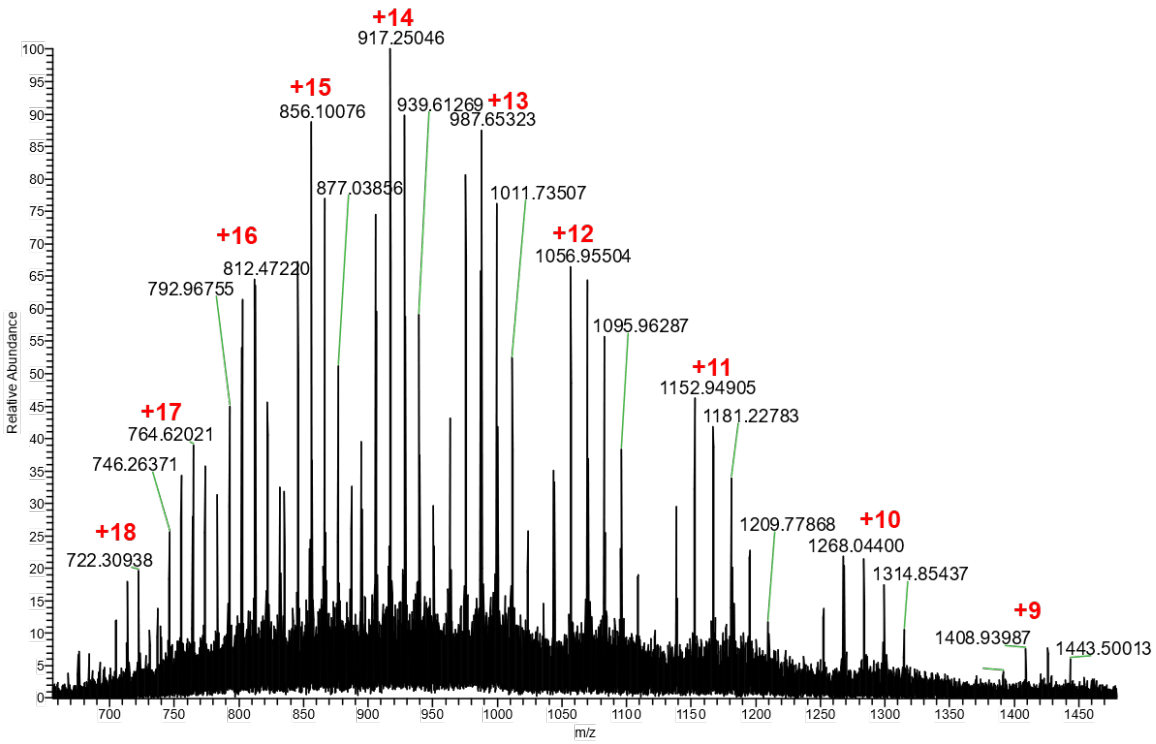


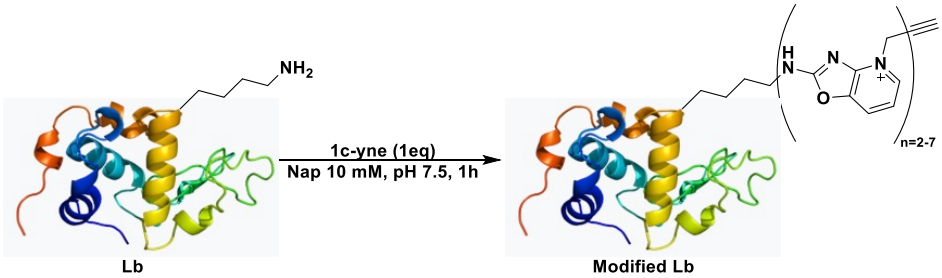


HRMS of Modified CyC

Number of modifications	Expected mass (m/z)	Observed mass (m/z)
1	12513	12514.34
2	12670	12671.16
3	12827	12827.74
4	12984	12983.12
5	13141	13141.46
6	13298	13298.88

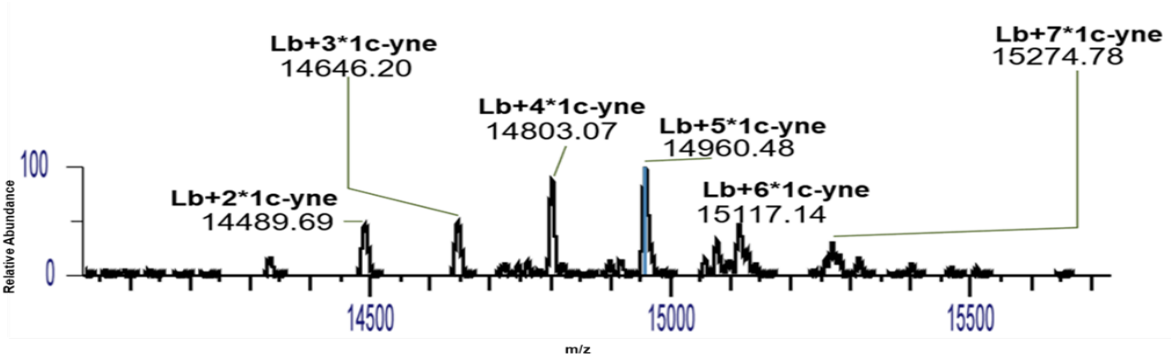


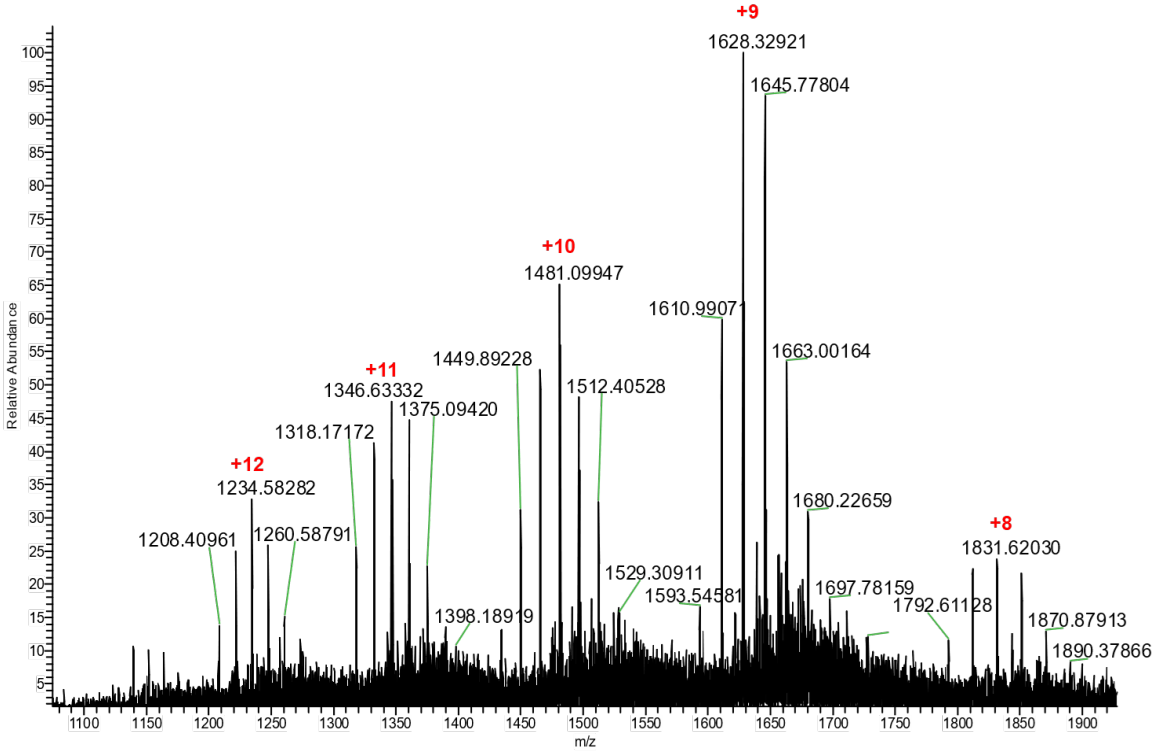


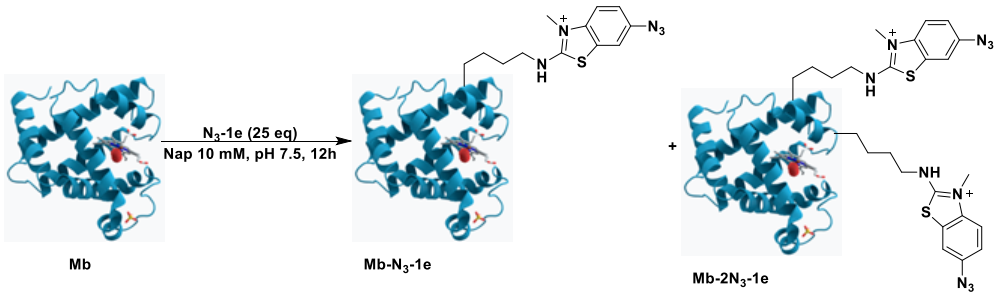


HRMS of modified Lb

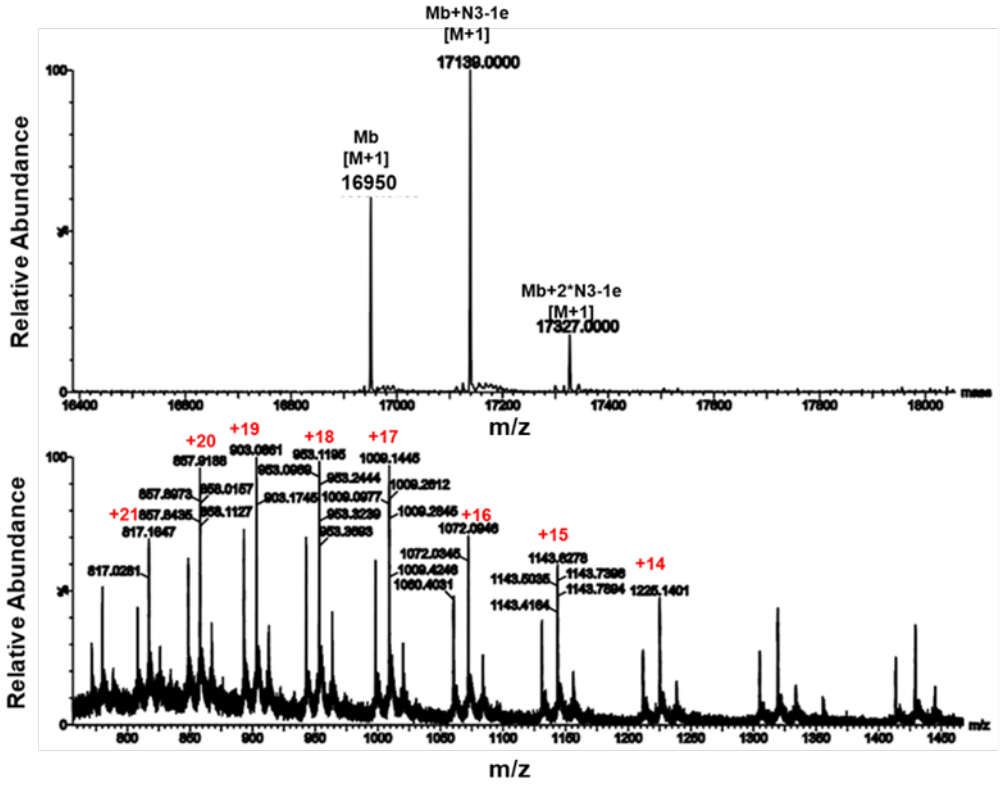
Number of modifications	Expected mass (m/z)	Observed mass (m/z)
2	14489	14489.69
3	14646	14646.20
4	14803	14803.07
5	14960	14960.48
6	15117	15117.14
7	15274	15274.78


Lb+3*1c-yne
14646.20
Lb+4*1c-yne
14803.07
Lb+5*1c-yne
14960.48
Lb+7*1c-yne
15274.78
Lb+2*1c-yne
14489.69
Lb+6*1c-yne
15117.14
Relative Abundance
100
0
14500
15000
15500
m/z

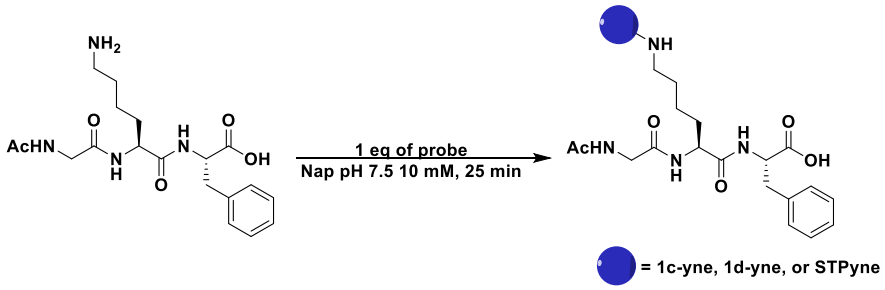

+9
1628.32921
1645.77804
+10
1481.09947
1610.99071
1663.00164
+11
1346.63332
1449.89228
1375.09420
1512.40528
1318.17172
+12
1234.58282
1208.40961
1260.58791
1680.22659
+8
1831.62030
1529.30911
1593.54581
1697.78159
1792.61128
1870.87913
1398.18919
1890.37866
Relative Abundance
m/z

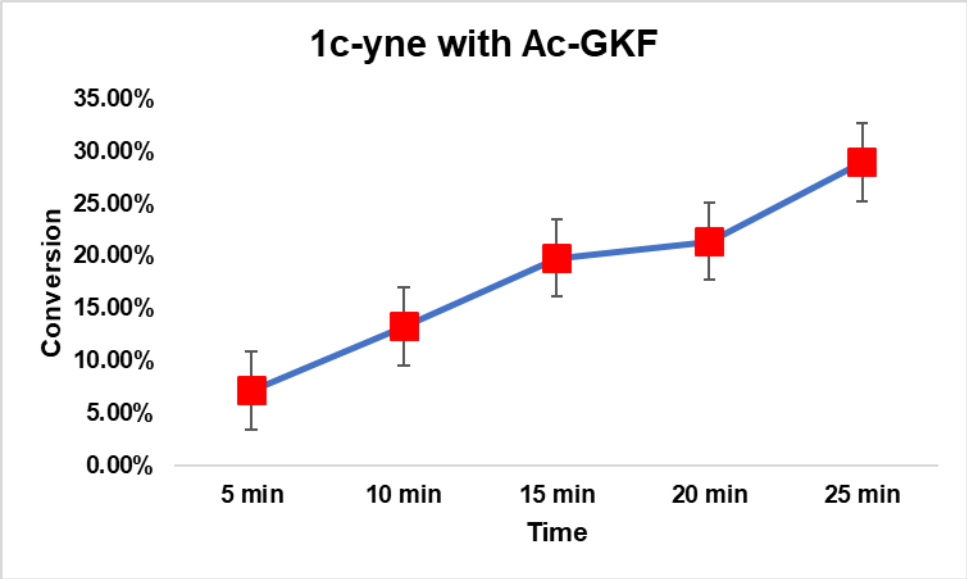

N3-1e (25 eq)
Nap 10 mM, pH 7.5, 12h
Mb
Mb-N3-1e
Mb-2N3-1e

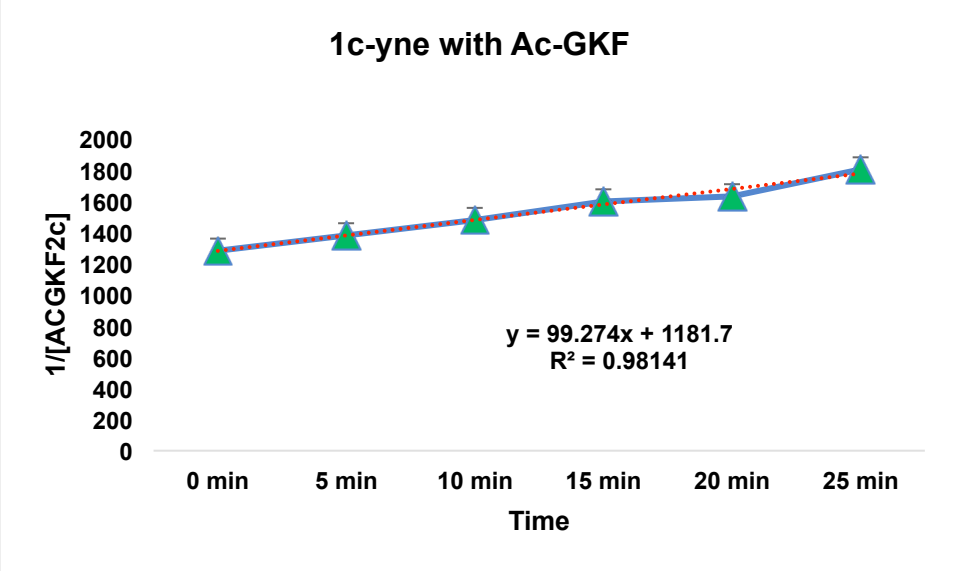
HRMS of Mb-N_3-1e and Mb-2*N_3-1e

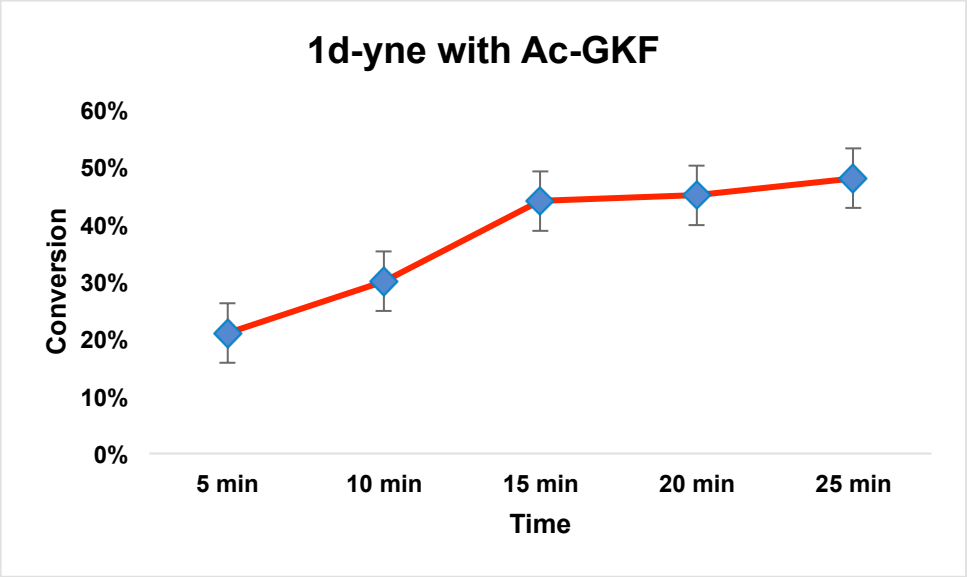


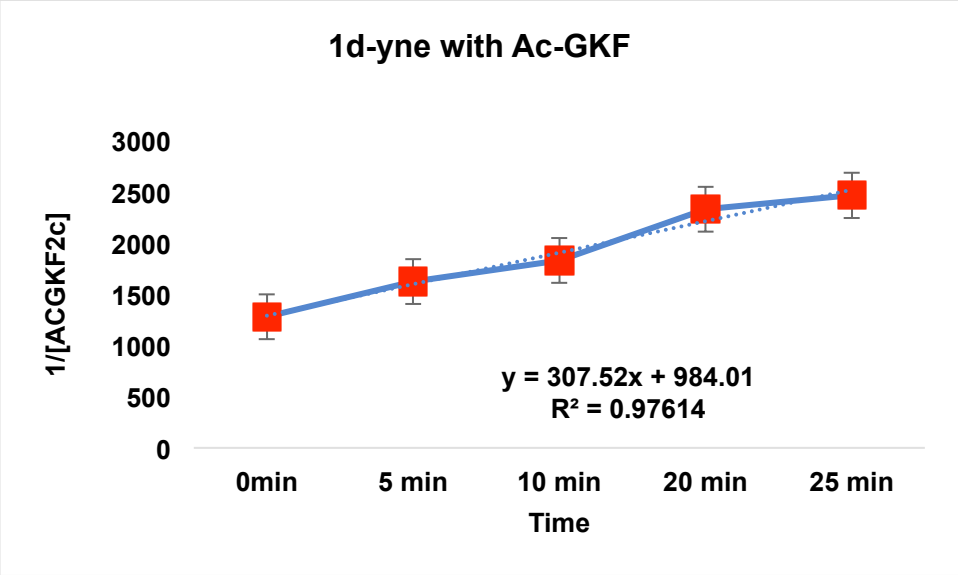
XIV. Supplementary Figure 10. Rate study of 1c-yne and 1d-yne with peptide Ac-GKF 2c.
To a solution of Ac-GKF **2c** (0.56 mg, 0.0014 mmol) in 2.0 mL of 10 mM phosphate buffer (Nap, pH 7.5) was added **1c-yne** or **1d-yne** (1 equiv., 0.0014mmol). For analysis, a sample (200 µL) was taken from the mixture 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 1c-yne, 1d-yne, STPyne bioconjugate reactions are second order reaction with **k = 99.27 $M^{-1}S^{-1}$**, **307.52 $M^{-1}S^{-1}$**, and **190.92 $M^{-1}S^{-1}$** respectively.

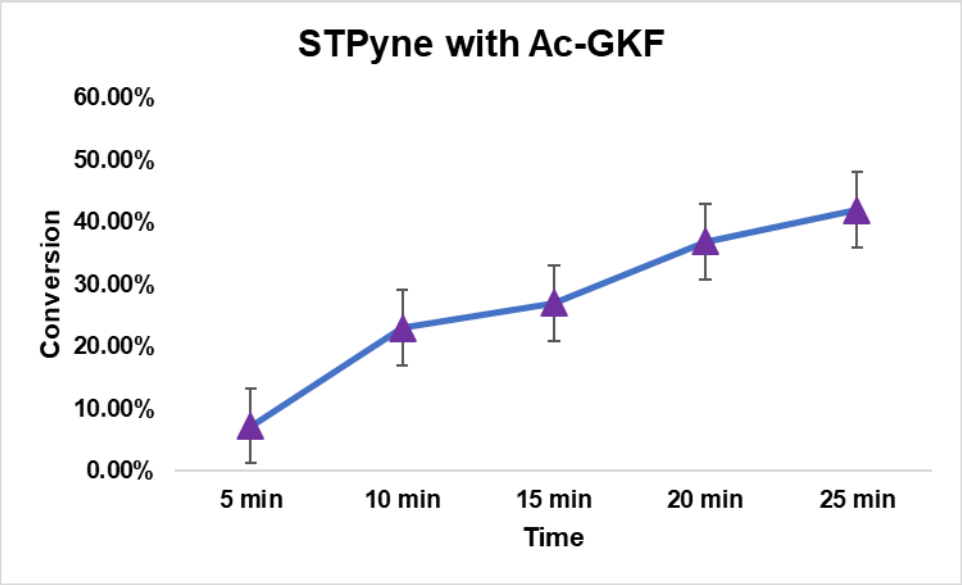


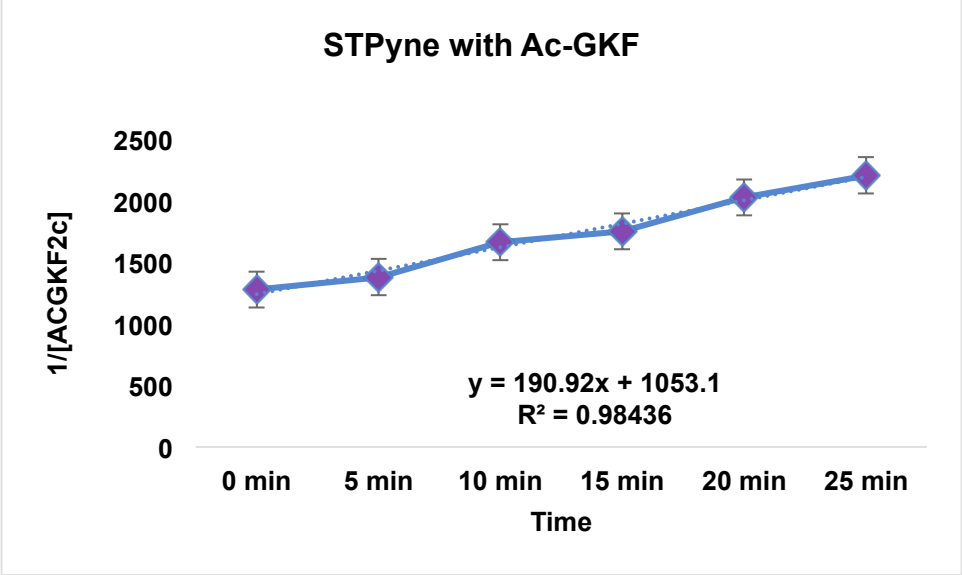

1c-yne with Ac-GKF
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
Conversion
5 min
10 min
15 min
20 min
25 min
Time


1c-yne with Ac-GKF
2000
1800
1600
1400
1200
1000
800
600
400
200
0
1/[ACGKF2c]
y = 99.274x + 1181.7
R² = 0.98141
0 min
5 min
10 min
15 min
20 min
25 min
Time

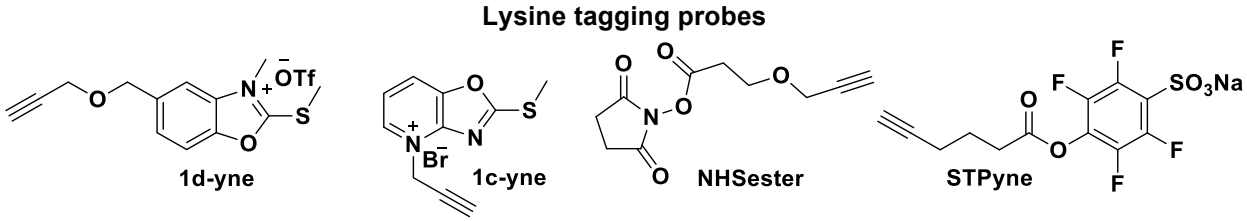

1d-yne with Ac-GKF
60%
50%
40%
30%
20%
10%
0%
Conversion
5 min
10 min
15 min
20 min
25 min
Time

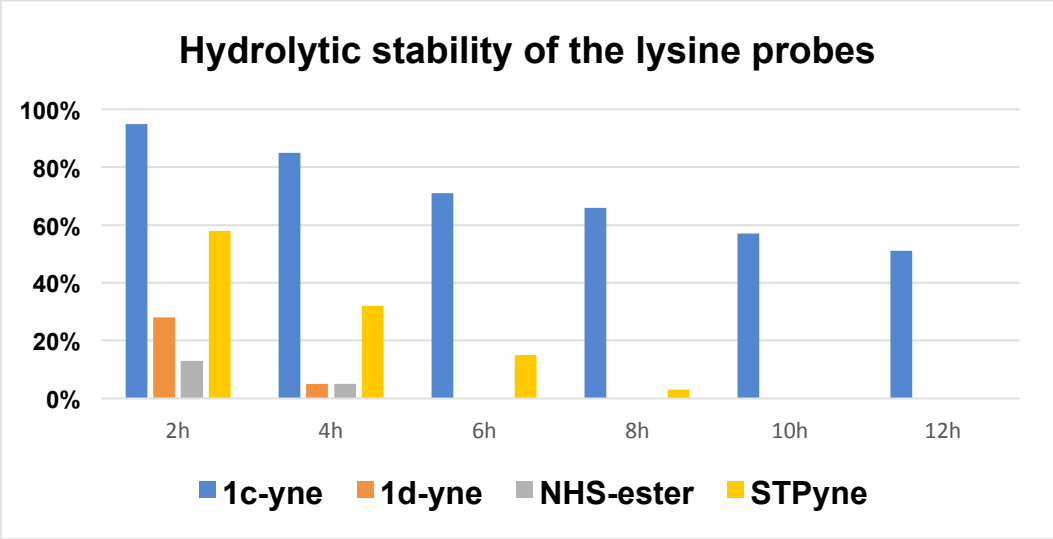

1d-yne with Ac-GKF
3000
2500
2000
1500
1000
500
0
1/[ACGKF2c]
y = 307.52x + 984.01
R² = 0.97614
0min
5 min
10 min
20 min
25 min
Time





XV. Supplementary Figure 11. Stability study of 1c-yne, 1d-yne, NHS ester and STPyne
1c-yne, 1d-yne, NHS ester, and STPyne (0.035 mmol) were 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 2h, 4h, 6 h, 8h, 10h, and 12h.





XVI. Supplementary Figure 12. TAREs for gel-based ABPP

Cell 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 1d. HEK293T proteome (50 µL of 1.5 mg/mL, prepared as described above) was labeled with various concentrations of **1d** (stock solutions in DMSO, final concentration as indicated), STPyne (1 µL of 5 mM stock solution in DMSO, final concentration = 100 µM) or DMSO for vehicle control for 1h at ambient temperature followed by adding 1 µM NHS-Rh or 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.

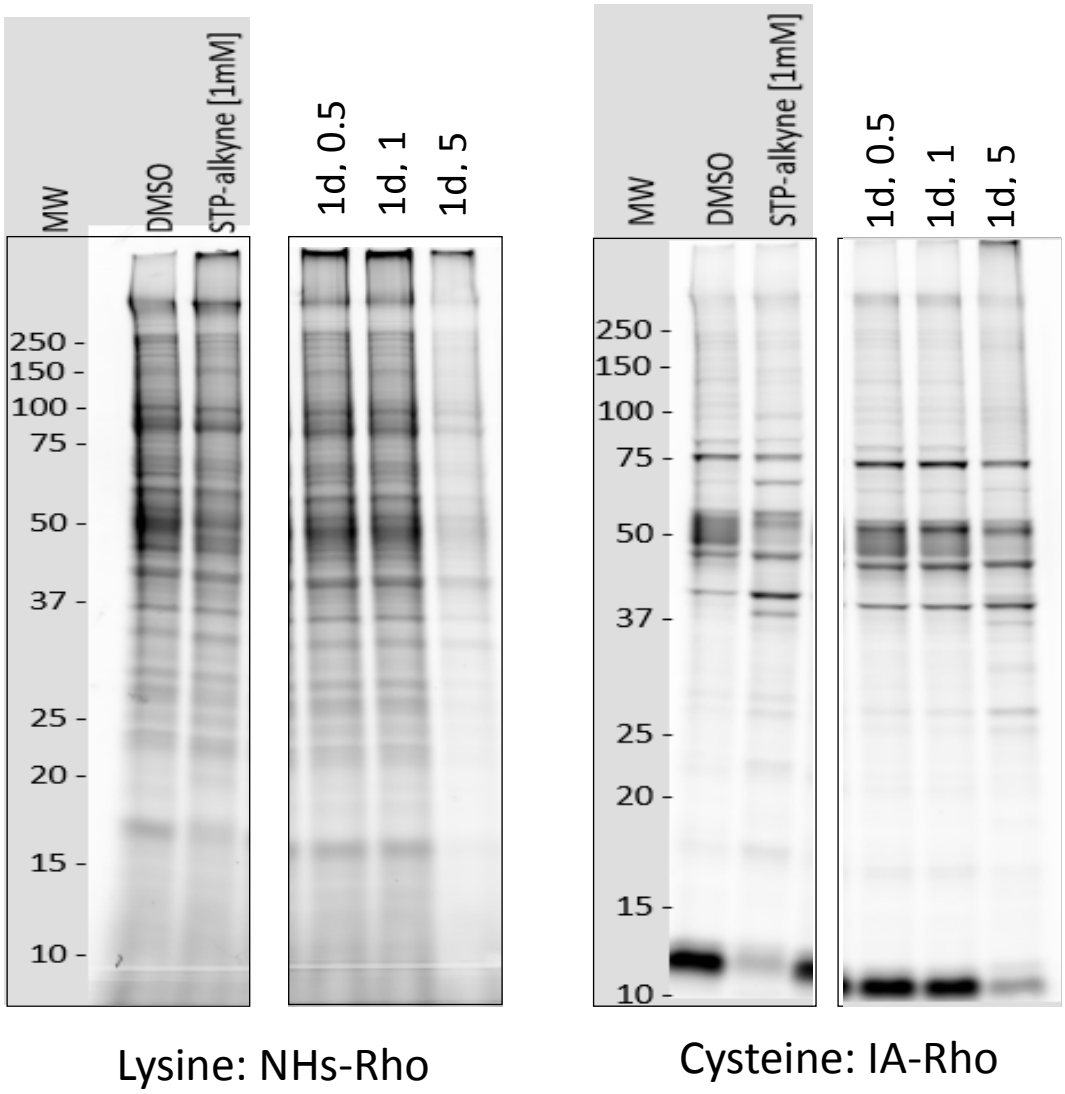
Gel-based ABPP with 1c-yne. HEK293T proteome (50 µL of 1.5 mg/mL) was labeled with different amounts of **1c-yne** (stock solutions in DMSO, final concentration as indicated) for 1h at ambient temperature followed by adding 1 µM NHS-Rh or IA-Rh. Samples were allowed to react for another hour at ambient 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 CuAAC using 1c-yne. HEK293T proteome (50 µL of 1.5 mg/mL) was labeled with IA-alkyne (**IA-aky**) (1 µL of 5 mM stock in DMSO, final concentration = 100 µM) or **1c-yne** (1 µL of 50 mM stock in DMSO, final concentration = 1 mM) for 1h at ambient temperature followed by copper-mediated azide-alkyne cycloaddition (CuAAC). CuAAC was performed with biotin-azide (200 µM for **IA-**

alkyne treated sample and 2 mM for **1c-yne** treated sample), tris(2-carboxyethyl)phosphine hydrochloride (TCEP; 1 µL of fresh 50 mM stock in water, final concentration = 1 mM), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 3 µL of 1.7 mM stock in DMSO/t-butanol 1:4, final concentration = 1 00 µM), and CuSO4 (1 µL of 50 mM stock in water, final concentration = 1 mM). Samples were allowed to react for 1h 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 analyzed by SDS-PAGE, using Criterion™ TGX Stain-Free™ gels obtained from Bio-Rad. Loading control images were obtained using the stain-free workflow on a Bio-Rad ChemiDoc™ Imager.

Gel-based ABPP with CuAAC using 1d-yne. HEK293T proteome (50 µL of 1.5 mg/mL) was labeled with different concentrations of **1d-yne** or **STPyne** (1 µL of 5 mM stock solution in DMSO, final concentration = 100 µM) for 1h at ambient temperature followed by copper-mediated azide-alkyne cycloaddition (CuAAC) as described above and the labeling was resolved by SDS-PAGE.

Streptavidin blot. Gels were transferred to either polyvinylidene difluoride (PVDF, Bio-Rad, 1620177) or nitrocellulose (Bio-Rad, 1704271) membranes using a Trans-Blot Turbo transfer system (Bio-Rad) following the manufacturer's instructions. After transfer, the membranes were then blocked (2% w/v of BSA in TBS-T, 30 min) and probed with streptavidin-IRDye® 800CW (Fisher, NC0883593, 1:4000) in TBS-T. Blots were incubated overnight at 4 °C with rocking and were then washed (3 × 5 min, TBS-T). The membranes were then imaged with a Bio-Rad ChemiDoc™ Imager using the 800 NIR channel.

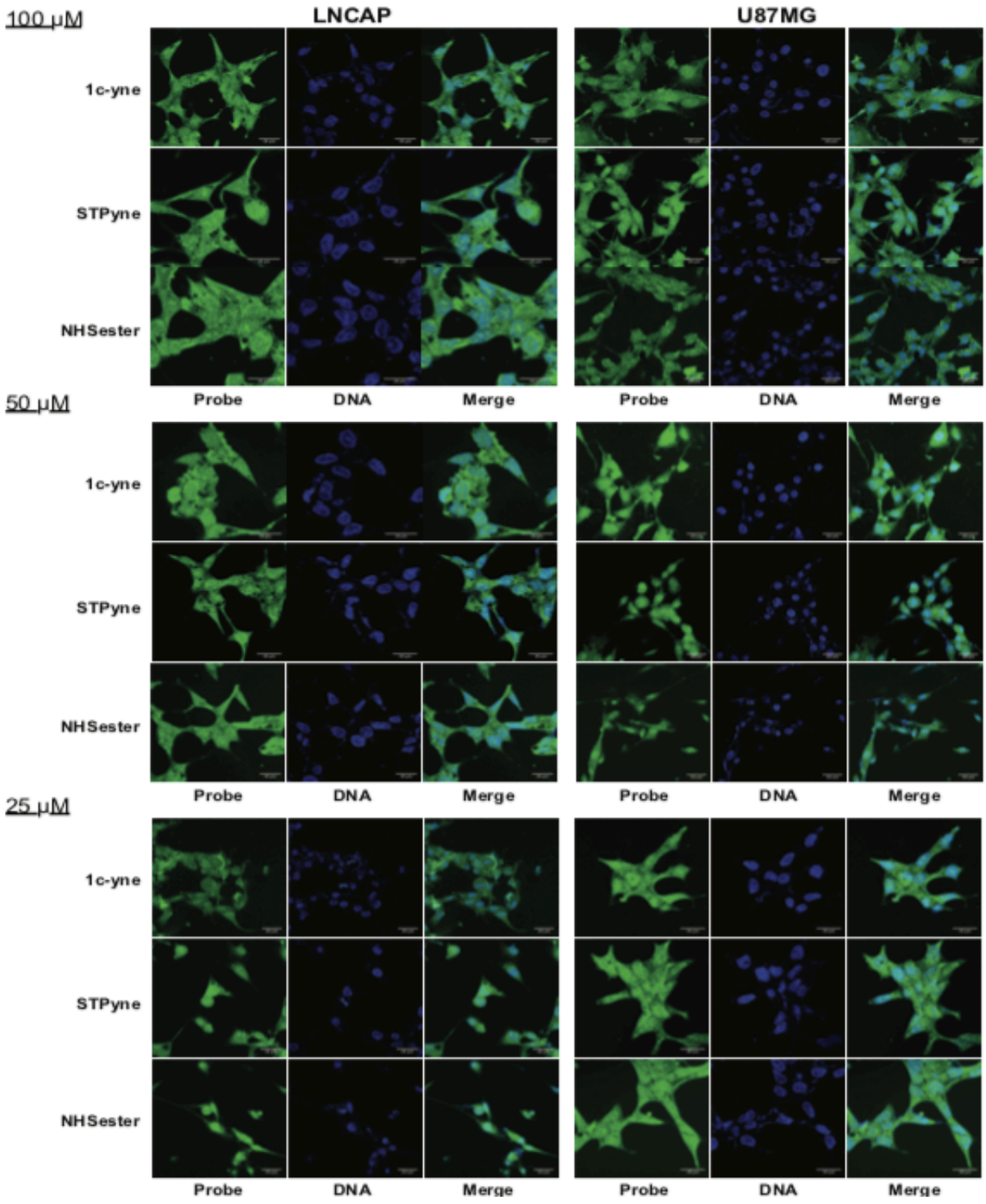


XVII. Supplementary Figure 13. Live cell labeling of proteins using different probes.

Cell Culture – Cells were maintained at 37° C and 5% CO_2. T47D and LNCaP cells were cultured in RPMI supplemented with 10% (V/V) fetal bovine serum (FBS) and 1% (V/V) penicillin/streptomycin (100 µg/mL). U87MG cells were cultured in DMEM supplemented with 10% (V/V) fetal bovine serum (FBS) and 1% (V/V) penicillin/streptomycin (100 µg/mL).

Copper Azide-Alkyne Cycloaddition (CuAAC) reaction – CuAAC on fixed cells was performed using 4 mM $CuSO_4$, 8 mM THPTA, 75 µM picolyl azide-conjugated fluorophore (ClickChemistryTools), and 10 mM sodium ascorbate. For confocal microscopy, cells were fixed in 3.7% PFA and permeabilized with 0.5% Triton-X. CuAAC reagents were added directly to cells, then incubated, rocking, for 45 min.

Confocal microscopy – LNCaP, T47D, and U87MG cells were plated on glass coverslips (Fisherbrand) in supplemented RPMI or DMEM media. After 16 h, cells were incubated with the indicated concentrations of **1c-yne, STPyne,** or **NHS-ester**. Prior to imaging, cells were fixed in 3.7% PFA and permeabilized with 0.5% Triton-X. CuAAC reaction was performed to attach a 488 nM picolyl azide-conjugated fluorophore (ClickChemistryTools) and Hoechst counterstain was used to image nuclei. Cells were imaged on Leica SP8 confocal microscope and images processed and analyzed using ImageJ.

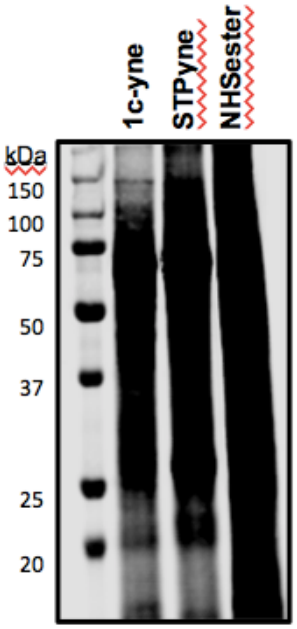


Live cell Labeling using TAREs and other probes at different concentrations. LNCAP and U87MG cells treated with 25-100 µM 1c-yne, STPyne, or NHSester for 2h show labeling in multiple cellular compartments.

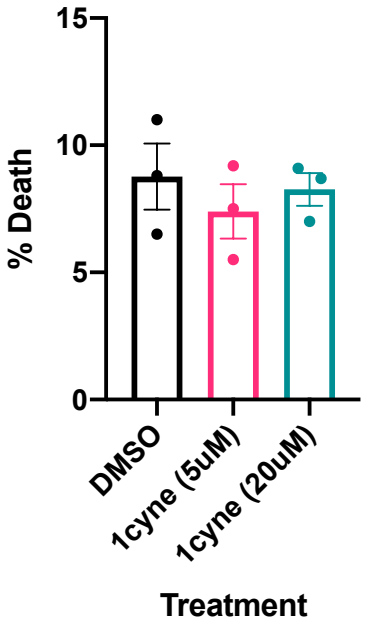
XVIII. Supplementary Figure 14. Intracellular labeling of proteins using different probes.

Cell Lysis and Western Blotting – Whole cell lysate was generated by lysing cells in RIPA buffer (50 mM TrisHCl [pH 8], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors. Lysates were centrifuged 6,500 x g, 10 min, soluble lysate collected, and CuAAC reaction was performed to attach a 680 nm picolyl azide-conjugated fluorophore (ClickChemistryTools). Whole cell lysate proteins were separated using 12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and visualized (Odyssey, Li-Cor).

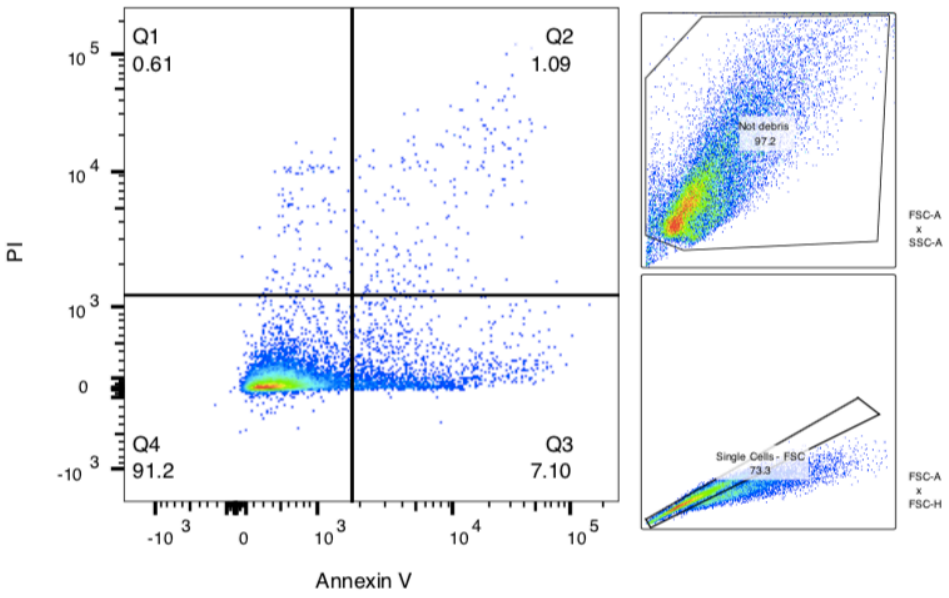
Copper Azide-Alkyne Cycloaddition (CuAAC) reaction – CuAAC on whole cell lysate was performed using 4 mM $CuSO_4$, 8 mM THPTA, 75 μM picolyl azide-conjugated fluorophore (ClickChemistryTools), and 10 mM sodium ascorbate. CuAAC reagents were added directly to lysate, incubated for 45 min at RT, then prepared for downstream analysis.



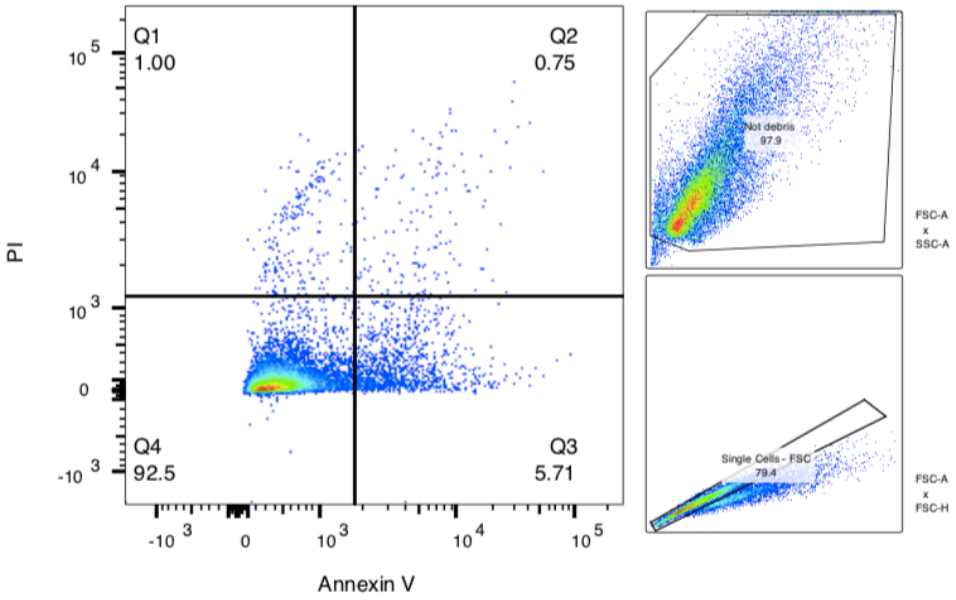
XIX. Supplementary Figure 15. Cell viability studies with 1cyne.
Annexin V/PI staining of T47D cells treated with increasing concentrations of 1c-yne.
To assay for cell death, T47D cells were seeded and treated with the indicated concentration (5 μM and 20 μM) of **1c-yne** for 24hrs, after which cells were detached using trypsin (**Biolegend**) and stained using Annexin V/PI following manufacturer's protocol (BioLegend). Cells were analyzed by flow cytometer (BDFACSymphony A3) within 1h. The reaction was done in triplicates. T47D cells treated with indicated concentrations of **1c-yne** for 24h did not show an increase in apoptosis/necrosis compared to DMSO control.



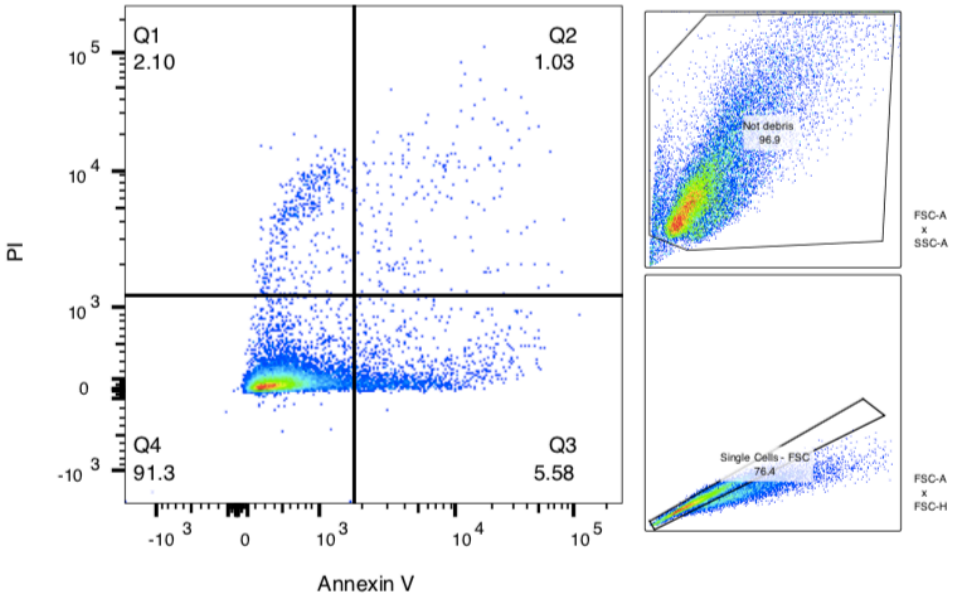
DMSO control:



TAREs-1c-yne (5 μM)



TAREs-1c-yne (20 μM)



XX. Supplementary Figure 16. Chemoproteomic analysis of residue selectivity

Proteomic sample preparation using 1c-yne. HEK293T proteome (200 μL of 2 mg/mL, prepared as described above) was labeled with **1c-yne** (10 μL of 10 mM stock solution in DMSO, final concentration = 500 μM) for 1h at ambient temperature. CuAAC was performed with biotin-azide (4 μL of 50 mM stock in DMSO, final concentration = 1 mM), TCEP (4 μL of fresh 50 mM stock in water, final concentration = 1 mM), TBTA (12 μL of 1.7 mM stock in DMSO/t-butanol 1:4, final concentration = 100 μM), and $CuSO_4$ (4 μL of 50 mM stock in water, final concentration = 1 mM). Samples were allowed to react for 1h at ambient

temperature. The samples were then subjected to SP3 sample preparation and LC-MS/MS analysis, as described below.

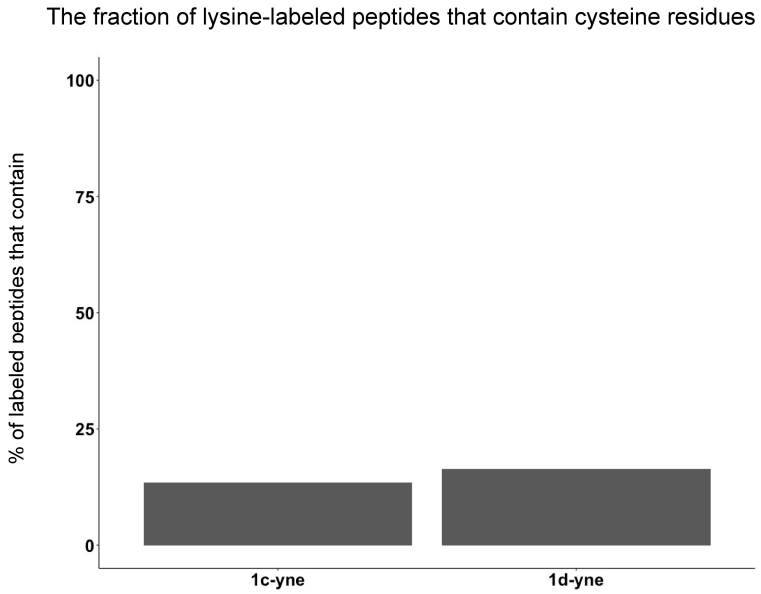
SP3 proteomic sample preparation. After CuAAC labeling, each sample was then treated with 0.5 µL benzonase (Fisher Scientific, 70-664-3) for 30 min at 37 °C. DTT (10 µL of 200 mM stock in water, final concentration = 10 mM) was added into each sample and the sample was incubated at 65 °C for 15 min. To this iodoacetamide (10 µL of 400 mM stock in water, final concentration = 20 mM) was added and the solution was incubated for 30 min at 37 °C with shaking. SP3 sample cleanup was performed at a bead/protein ratio of 10:1 (wt/wt). For each 200 µL sample (1 mg/mL protein concentration), 20 µL Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophobic (GE Healthcare, 65152105050250, 50 µg/µL, total 2 mg) and 40 µL Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophilic (GE Healthcare, 45152105050250, 50 µg/µL, total 1 mg) were aliquoted into a single microcentrifuge tube and gently mixed. Tubes were then placed on a magnetic rack until the beads settled to the tube wall, and the supernatants were removed. The beads were removed from the magnetic rack, reconstituted in 400 µL of MB water, and gently mixed. Tubes were then returned to the magnetic rack, beads allowed to settle, and the supernatants removed. Washes were repeated for two more cycles, and then the beads were reconstituted in 80 µL MB water. The bead slurries were then transferred to CuAAC samples, and incubated for 5 min at RT with shaking. Absolute ethanol (400 µL) was added to each sample, and the samples were incubated for 5 min at RT with shaking. Samples were then placed on a magnetic rack, and beads allowed to settle. Supernatants were then removed and discarded. Using the magnetic rack as described above, the beads were further washed three times with ethanol (400 µL of 80% solution in water). Beads were then resuspended in 100 µL PBS containing 2 M urea followed by addition trypsin solution (Worthington Biochemical, LS003740, 5 µL, 1 mg/mL in 666 µL of 50 mM acetic acid and 334 µL of 100 mM $CaCl_2$, final weight = 10 ng). Digest was allowed to proceed overnight at 37 ºC with shaking. The samples were then acidified to a final concentration of 3% (v/v) FA. After incubation for 5 min at RT with shaking, ~ 2 mL acetonitrile (> 95% of the final volume) was added to each sample and the mixtures were then incubated for an additional 10 min at RT with shaking. Supernatants were then removed and discarded using the magnetic rack, and the beads were washed (3 × 500 µL acetonitrile). Peptides were then eluted from SP3 beads with 100 µL of 2% DMSO in MB water for 30 min at 37 °C with shaking. The elution will be used for NeutrAvidin enrichment.

NeutrAvidin enrichment of labelled peptides. For each sample, 50 µL of NeutrAvidin® Agarose resin slurry (Pierce, 29200) was washed twice in 10 mL IAP buffer (50 mM MOPS pH 7.2, 10 mM sodium phosphate, and 50 mM NaCl buffer) and then resuspended in 500 µL IAP buffer. Peptide solutions eluted from SP3 beads were then transferred to the NeutrAvidin® Agarose resin suspension, and the samples were then rotated for 2h at RT. After incubation, the beads were pelleted by centrifugation (21,000 g, 1 min) and washed by centrifugation (6 × 700 µL water). Bound peptides were eluted with 60 µL of 80% acetonitrile in MB water containing 0.1% FA (10 min at RT). The samples were then harvested by centrifugation (21,000 g, 1 min) and residual beads separated from supernatants using Micro Bio-Spin columns (Bio-Rad). The remaining peptides were then eluted from pelleted beads with 60 µL of 80% acetonitrile in water containing 0.1% FA (10 min, 72 °C). Beads were then separated from the eluants using the same Bio-Spin column. Eluants were then collected by centrifugation (21,000 g, 1 min) and the combined eluants were dried (SpeedVac). The samples were then reconstituted in 40 µL water containing 5% acetonitrile and 1% FA and analyzed by LC-MS/MS.

Liquid-chromatography tandem mass-spectrometry (LC-MS/MS) analysis. The samples were analyzed by liquid chromatography tandem mass spectrometry using an Q Exactive™ mass spectrometer (Thermo Scientific) coupled to an Easy-nLC™ 1000 pump. Peptides were resolved on a C18 reversed phase column (3 µM, 100Å pores), packed in-house, with 100 nm internal diameter and 18 cm of packed resin. The peptides were eluted using a 140 min gradient of Buffer B in Buffer A (Buffer A: water with 3% DMSO and 0.1% FA; Buffer B: acetonitrile with 3% DMSO and 0.1% FA) and a flow rate of 220 nL/min with electrospray ionization of 2.2 kV. The regular gradient includes 0 – 5 min from 1% to 5%, 15 – 130 min from 5% to 27%, 15 – 137 min from 27% to 35%, and 137 – 138 min from 35% to 80% buffer B in

buffer A. The steep gradient for this study includes 0 – 5 min from 1% to 5%, 5 – 20 min from 5% to 15%, 20 – 130 min from 15% to 35% and from 130 – 135 min from 35 to 95% buffer B in buffer A. The detailed gradient includes 0 – 15 min from 1% to 15%, 15 – 110 min from 15% to 35% and from 130 – 135 min from 35 to 95% buffer B in buffer A. Data was collected in data-dependent acquisition mode with dynamic exclusion (15 s), and charge exclusion (1,7,8,>8) was enabled. Data acquisition consisted of cycles of one full MS scan (400 − 1800 m/z at a resolution of 70,000) followed by 12 MS2 scans of the nth most abundant ions at resolution of 17,500.

Peptide and protein identification. The MS2 spectra data were extracted from a raw file using RAW Xtractor (version 1.1.0.22; available at http://fields.scripps.edu/rawconv/). MS2 spectra data were searched using the ProLuCID algorithm (publicly available at http://fields.scripps.edu/yates/wp/?page_id=17 using a reverse concatenated, nonredundant variant of the Human UniProt database (release-2020_01). Lysine or cysteine residues were searched with a variable modification for carboxyamidomethylation (+57.02146) and an additional variable modifications at either lysine or cysteine residues, which is +526.2231 for probe 1d-yne and +483.19213 for 1c-yne. Peptides were required to have at least one tryptic terminus and to contain the biotin modification. ProLuCID data was filtered through DTASelect (version 2.0) to achieve a peptide false-positive rate below 1%. The Xcorr score was used for match confidence criteria. Mass tolerance of the peptide precursor was set to 50 ppm. The built in localization features on IP2 were used predict the PTM index.



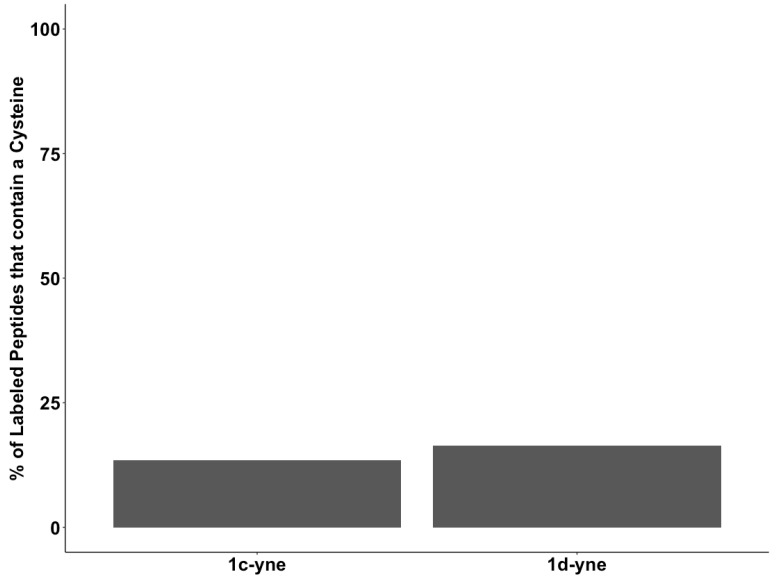
1cyne datasets were also searched using MSFragger v15 with a precursor mass window of 50 ppm and a fragment mass tolerance of 20 ppm and variable modifications of +483.19213 on K and C as well as a variable modification for carbamidomethylation on cysteine. The options --decoyprobs --ppm --accmass --nonparam –expectscore were used for PeptdieProphet and --static --em 1 --nions b --mods C:483.19213, K:483.19213 --minprob 0.5 for PTMProphet.

Distribution of labeling sites identified for 1cyne by MSFragger, using PTMProphet to score modification sites

Identified	Counts
Cysteine Protein IDs	17
Cysteine Peptide IDs	20
Lysine Protein IDs	1560
Lysine Peptide IDs	4460

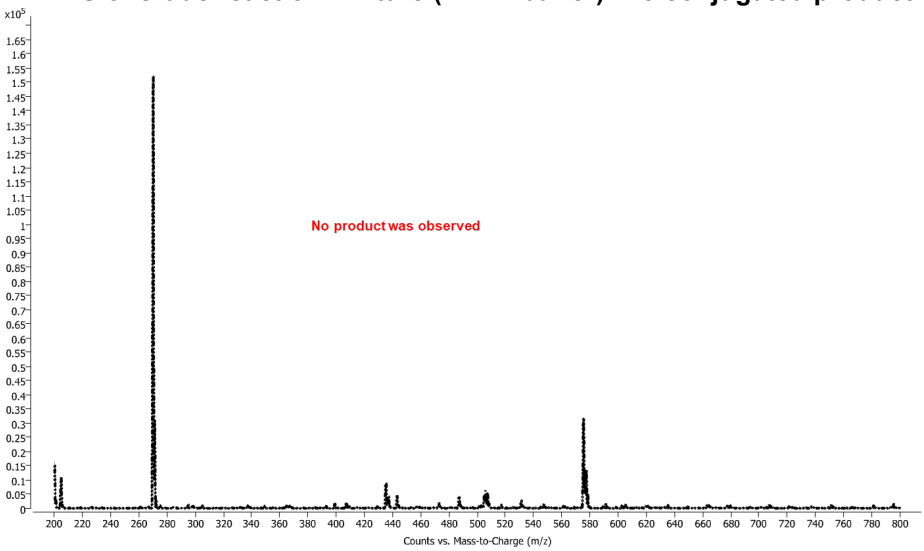
Lysine selectivity of 1c-yne and 1d-yne. To further analyze the lysine-selective labeling, we conducted an analysis of the amino acid content of all peptides identified as labeled by the TARE probes. The probability score was used for match confidence criteria. Mass tolerance of the peptide precursor was set to 50 ppm. In addition to using the build in localization features on IP2 to predict the PTM index, to further analyze the lysine-selective labeling, we conducted an analysis of the amino acid content of all peptides identified as labeled by the TARE probes. Gratifyingly <15% of the labeled peptides contained one or more cysteine residues, which again supports preferential labeling for lysine residues.

To further investigate the specificity of the modification, we additionally reprocessed the 1c-yne data using MSFragger using the built in PTMProphet tool to improve our confidence in the localization of the modifications. This analysis revealed that >95% of all labeled residues are lysines, which is consistent with our initial analysis using the ProLuCID/IP2 search.

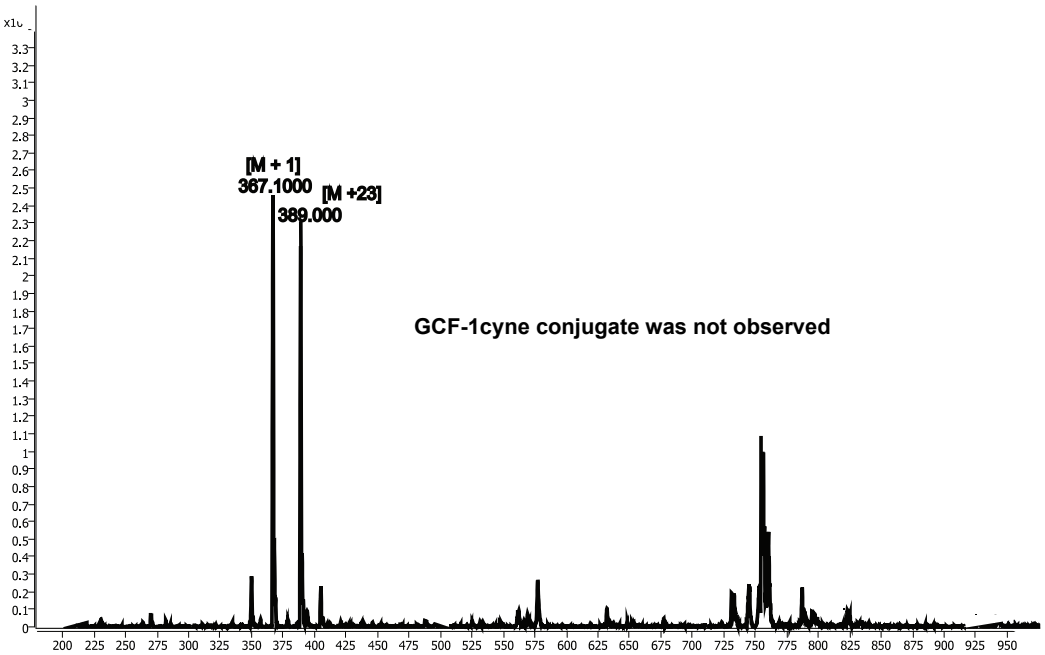


XXI. Supplementary Figure 17. Synthesis of GCF-1c-yne conjugate under click reaction conditions
Procedure for synthesis of GCF-1c-yne conjugate in presence of TCEP:
Ac-GCF-NH_2 (6.3 mM) was dissolved in 0.4 mL of 10 mM phosphate buffer (Nap, pH 7.5) with or without TCEP buffer (50 mM,pH 7.4, 200 µL), then 10 equiv. of 1c-yne was added. The mixture was stirred at room temperature for 3 hours. The reaction mixture was purified by HPLC then dried by lyophilization to afford pure modified peptide. 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.

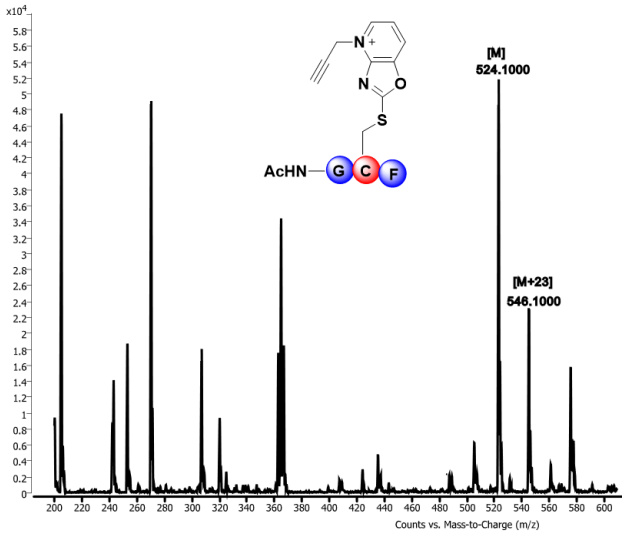
LC-MS of crude reaction mixture (TCEP buffer): No conjugated product



Mass of the unmodified GCF after the HPLC purification

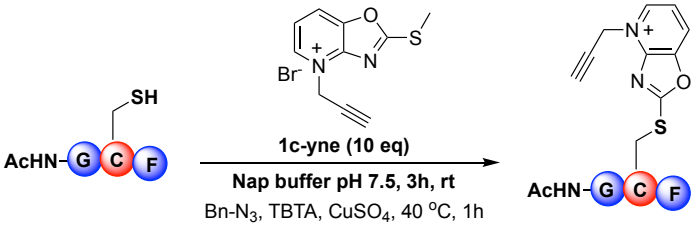


LC-MS of GCF-1c-yne conjugate reaction (without addition of TCEP buffer)

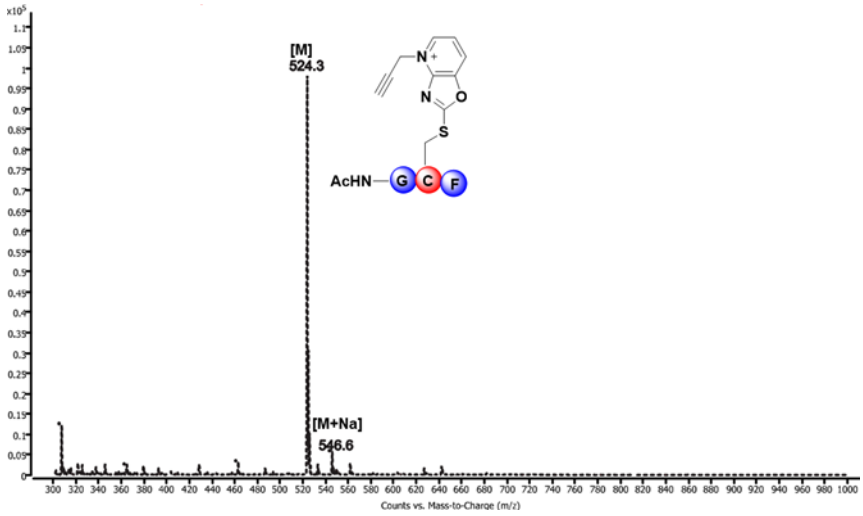


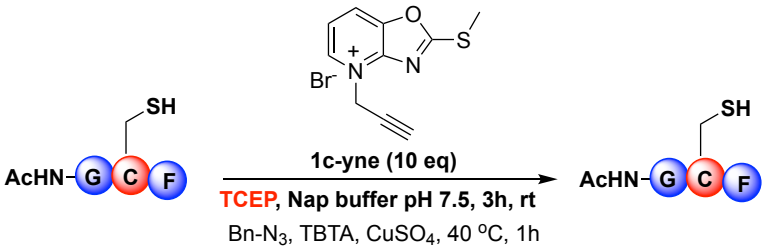
Procedure for synthesis of GCF-1c-yne conjugate under click conditions

Ac-GCF-NH_2 (6.3 mM) was dissolved in 0.4 mL of 10 mM phosphate buffer (Nap, pH 7.5) without TCEP buffer (50 mM,pH 7.4, 200 µL), then 10 equiv. of 1c-yne was added. The mixture was stirred at room temperature for 3 hours. The reaction mixture was used for click reaction directly without further modification. The click reaction was performed with benzyl-azide (1 eq), TBTA (0.1 eq) and CuSO4 (1 eq). The reaction mixture was allowed to react for 1h at 40 ^{o}C. The reaction was analyzed by HPLC and LC-MS. No degradation of the Ac-GCF-1c-yne was observed in click conditions without TCEP. Although we added benzyl azide but we did not observe any modification of the Ac-GCF-1c-yne of peptide because of the excess of 1c-yne in the reaction mixture. 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.



LC-MS of GCF-1c-yne conjugate (after click reaction without addition of TCEP buffer)

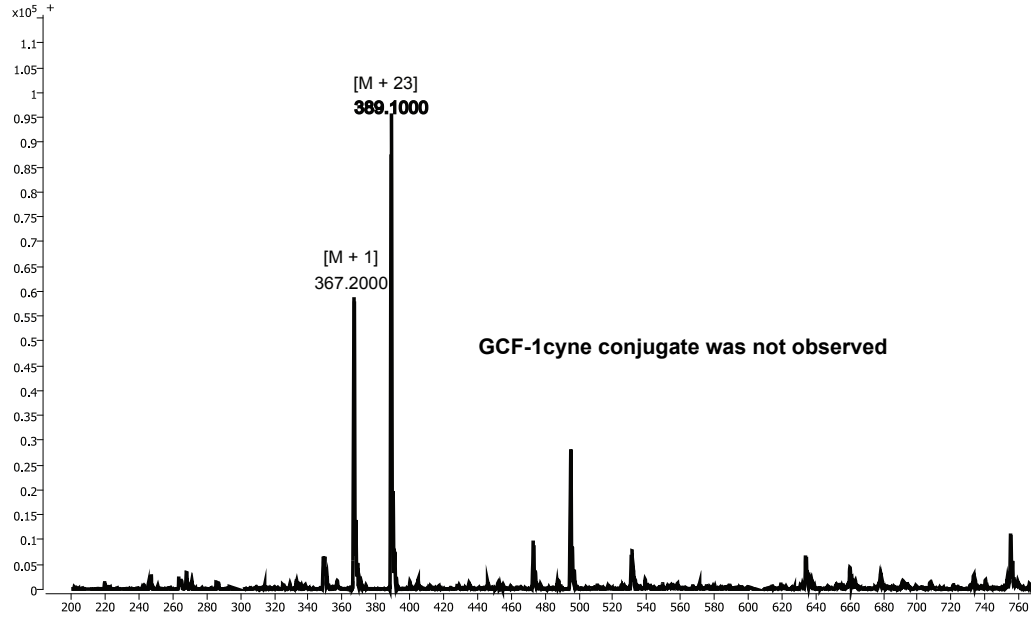




Procedure for stability study of GCF-1c-yne conjugate under click condition with TCEP

Ac-GCF-NH_2 (6.3 mM) was dissolved in 0.4 mL of 10 mM phosphate buffer (Nap, pH 7.5) with TCEP buffer (50 mM,pH 7.4, 200 µL), then 10 equiv. of 1c-yne was added. The mixture was stirred at room temperature for 3 hours. The reaction mixture was used for click reaction directly without further modification. The click reaction was performed with benzyl-azide (1 eq), TBTA (0.1 eq) and CuSO4 (1 eq). The reaction mixture was allowed to react for 1h at 40 ^{o}C. The reaction was analyzed by HPLC and LC-MS. We did not observe the formation of conjugate Ac-GCF-1c-yne under click conditions with TCEP. 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

LC-MS of GCF-1c-yne conjugate (after click reaction with addition of TCEP buffer)

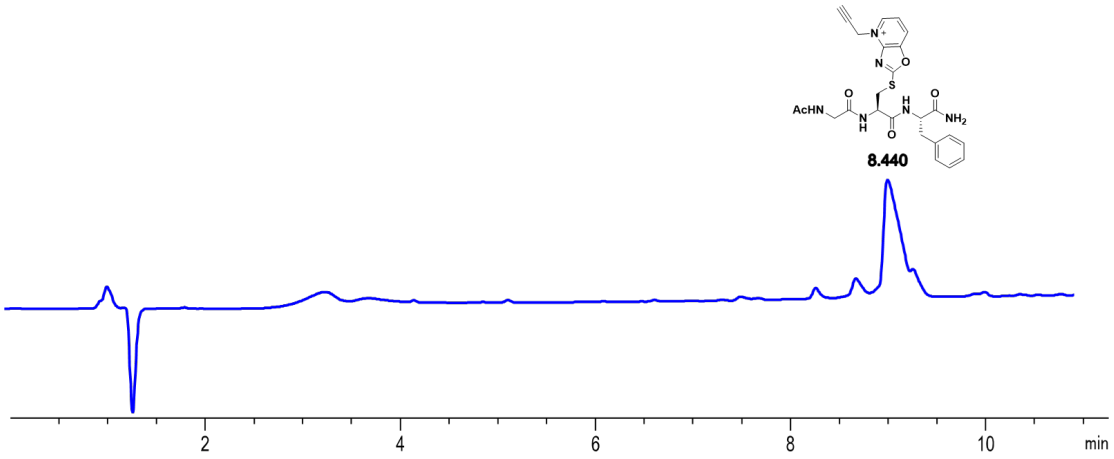


XXII. Supplementary Figure 18. Stability study of GCF-1c-yne conjugate in TCEP, and 1d-thio-conjugate under sodium phosphate buffer.

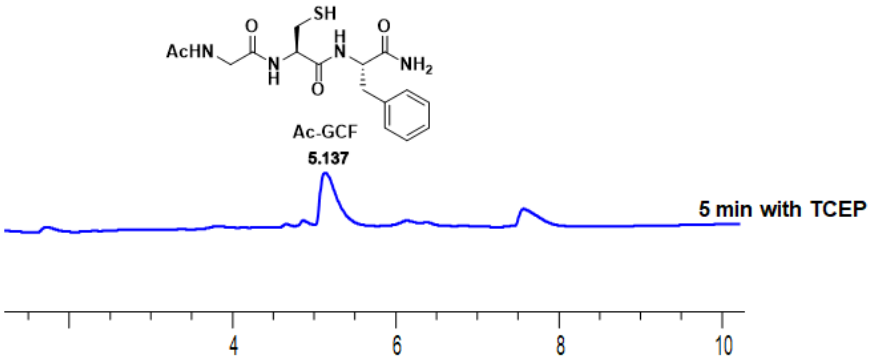
Procedure for stability study of GCF-1c-yne conjugate in TCEP buffer

Ac-GCF-NH_2 (6.3 mM) was dissolved in 0.4 mL of 10 mM phosphate buffer (Nap, pH 7.5), and then 10 equiv. of 1c-yne was added. The mixture was stirred at room temperature for 3 hours. The reaction mixture was purified by HPLC then dried by lyophilization to afford pure modified peptide, GCF-1c-yne conjugate. To a solution of pure GCF-1c-yne conjugate (6.3 mM) in Nap buffer (pH 7.5, 400 µL), TCEP buffer (pH 7.4, 200 µL) was added. The reaction was incubated at 25 ^{o}C 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 pure GCF-1c-yne conjugate



After incubation with TCEP buffer for 5 min

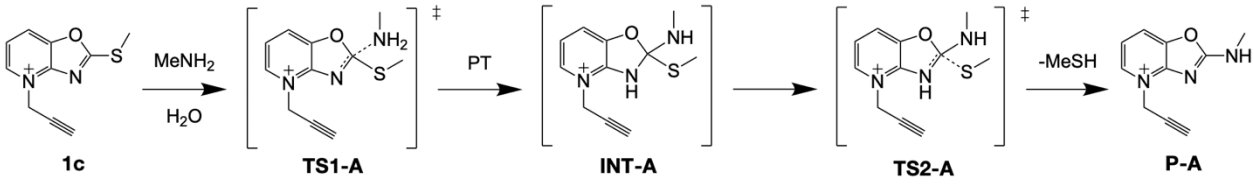


XXIII. Supplementary Figure 19. General Computation Procedure

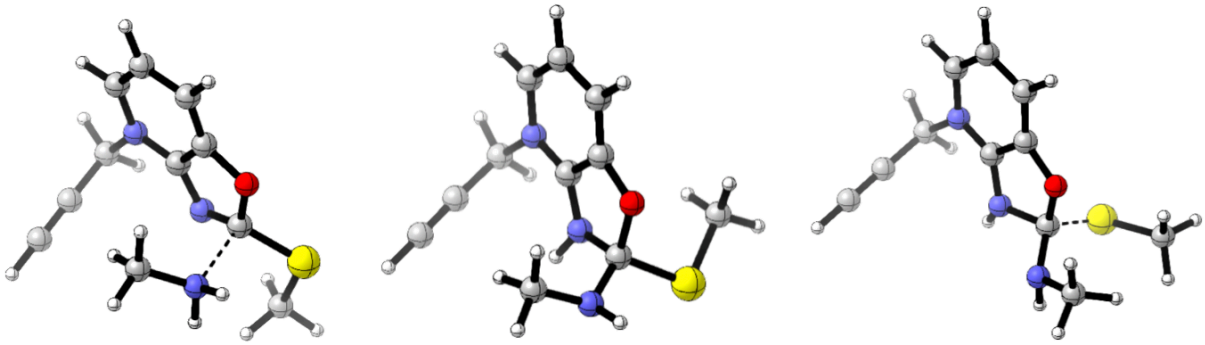
Quantum Mechanical Calculations

All conformational searches were carried out using CREST, Conformer–Rotamer Ensemble Sampling Tool version 2.7.1, of the XTB program version 6.2 RC2 (SAW190805).[4,5] The RMSD threshold for each conformational search was set to 0.5 Å. Density functional theory calculations were performed using the Gaussian 09 software package.[6] Ground state and transition state structures were optimized at the B3LYP/6-31+G(d) level with SMD solvation model for water, the D3 version of Grimme's empirical dispersion correction, and the integration grid set to ultrafine.[7] Frequency calculations were carried out at the same level of theory. The GoodVibes program was used for quasi-harmonic correction of Gibbs free energies at 298 K.[8] Single point energy calculations of the optimized geometries were performed at ωB97X-D/6-311++G(d,p) level with SMD solvation model for water and the integration grid set to ultrafine.

Proposed S_NAr mechanism of TARE probe 1c with methylamine

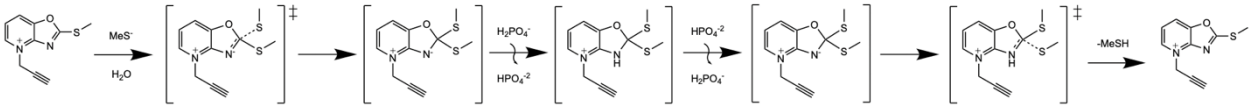


Optimized structures of methylamine calculations at ωB97X-D/6-311++G(d,p) SMD(H_2O)//B3LYP/6-31+G(d) SMD(H_2O) level



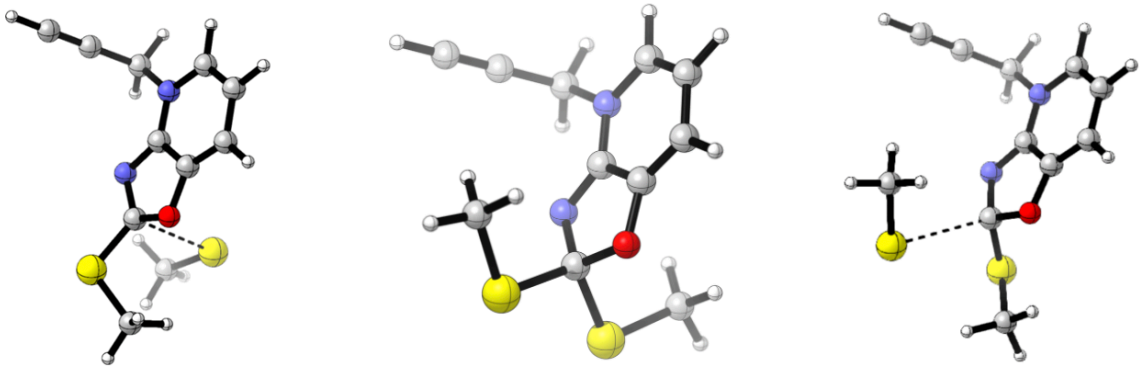
TS1-A INT-A TS2-A

Proposed S_NAr mechanism of TARE probe 1c with methyl thiolate



1c TS1-B INT-B1 INT-B2 INT-B1 TS2-B P-B

Optimized structures of methyl thiolate calculations at ωB97X-D/6-311++G(d,p) SMD(H_2O)//B3LYP/6-31+G(d) SMD(H_2O) level



TS1-B INT-B1 TS2-B

Cartesian Coordinates of Calculated Structures

MeNH2

N	-0.05479700	-0.76598500	0.00000000
C	-0.05479700	0.70827600	0.00000000

H	-0.58856500	1.07163500	0.88380800
H	-0.58856500	1.07163500	-0.88380800
H	0.94960600	1.15772000	0.00000000
H	0.46994500	-1.09437700	0.81100100
H	0.46994500	-1.09437700	-0.81100100

TS1-A

C	2.97217400	-0.83487500	-0.49303600
C	2.72513100	-2.08376000	0.02874800
C	1.40295200	-2.50588500	0.32754000
C	0.42469600	-1.59336100	0.05408800
C	0.68089700	-0.31355100	-0.48388500
N	1.95449000	0.05041200	-0.75980500
C	2.23960100	1.42570900	-1.24291900
C	2.14136200	2.40450300	-0.16104600
C	2.04472400	3.20628100	0.73877500
H	1.96146800	3.91667100	1.53662300
H	3.24184800	1.41958600	-1.67733800
H	1.53066100	1.65426400	-2.04295000
N	-0.44887800	0.39351400	-0.66899900
C	-1.40387700	-0.39410400	-0.13528000
S	-3.08928200	-0.43946300	-0.67893900
C	-3.31769700	1.31370400	-1.14750700
H	-2.64477000	1.58606500	-1.96223800
H	-3.16651900	1.97044900	-0.28752500
H	-4.35418300	1.39465700	-1.48616200
O	-0.94210100	-1.66751800	0.22068700
H	1.19314900	-3.48400300	0.74623400
H	3.56393100	-2.74468800	0.21256600
H	3.97001600	-0.48377700	-0.72337000
N	-1.63973400	0.34659000	1.79778600
H	-2.25022400	-0.32875900	2.25716700

H	-2.14672800	1.22679700	1.70368800
C	-0.38118500	0.53548000	2.53105700
H	0.08898200	-0.43629100	2.69857600
H	0.28929200	1.16545400	1.93853300
H	-0.54542300	1.02068000	3.50163200

INT-A

C	2.02374300	-2.44089600	0.87542800
C	0.67675000	-2.27144800	1.29248600
C	0.00718300	-1.19729000	0.77247300
C	0.62976100	-0.31258100	-0.13140000
N	1.90610300	-0.48850800	-0.50907900
C	2.55499500	0.48877300	-1.42331200
C	2.68133900	1.80312400	-0.79640800
C	2.73457900	2.89170400	-0.27334000
H	2.79655100	3.85608900	0.18956400
H	3.53024200	0.08144400	-1.69630000
H	1.95172700	0.54955500	-2.33580400
N	-0.25337400	0.62968900	-0.50547100
C	-1.51624000	0.47217200	0.21523800
S	-2.95502100	0.17965600	-0.89684900
C	-2.43922200	-1.40014300	-1.65396000
H	-2.41084400	-2.20039700	-0.91147300
H	-1.46542600	-1.29675400	-2.14125900
H	-3.19082500	-1.63545700	-2.41300000
O	-1.26848500	-0.77742700	0.99506200
H	0.20701400	-2.95214500	1.99374200
H	2.60947100	-3.27180600	1.25099800
H	3.63764300	-1.64448100	-0.32782800
N	-1.80484100	1.59498400	1.02125600
C	-0.77713900	1.95023700	2.01813300
H	-1.15516200	2.79338000	2.59958000

H	0.13864800	2.26703600	1.51274400
H	-0.54293300	1.12379100	2.70070200
H	-2.71047100	1.46938800	1.47004600
H	-0.02725500	1.51367600	-0.94945800

TS2-A

C	-2.78678500	1.54957400	-0.14267600
C	-2.10354700	2.58989800	0.43095400
C	-0.73383100	2.46178100	0.78428800
C	-0.15591100	1.25079400	0.52855600
C	-0.87679000	0.19168600	-0.05527200
N	-2.16564600	0.34155200	-0.39972800
C	-2.91109200	-0.77868000	-1.03719100
C	-3.18175200	-1.86711700	-0.10072600
C	-3.39687400	-2.77857400	0.66366700
H	-3.59085800	-3.58609100	1.34088200
H	-3.83722500	-0.36398700	-1.43976200
H	-2.31769400	-1.13443000	-1.88687400
N	-0.07601500	-0.88920500	-0.13765400
C	1.30225100	-0.45942100	0.17049500
S	2.10268600	-0.24382200	-1.53447800
C	3.73835700	0.48599300	-1.15300500
H	3.64151900	1.27302200	-0.40084500
H	4.08875700	0.93862000	-2.08570400
H	4.45838900	-0.26864300	-0.83151000
O	1.12042500	0.82638800	0.78638500
H	-0.18055300	3.27814800	1.23474400
H	-2.62730700	3.52203600	0.60739700
H	-3.82742900	1.60968100	-0.43225000
N	1.93243700	-1.33151500	1.10872400
C	3.06470300	-0.79793300	1.89274000
H	2.22190200	-2.17613800	0.61753900

H	-0.26192700	-1.70630200	-0.71229400
H	2.73966300	0.07543800	2.45963400
H	3.35753000	-1.57340900	2.60415000
H	3.94104100	-0.53172900	1.29508000

P-A

C	2.38324100	-0.98451400	-0.31097600
C	2.01612100	-2.24292000	0.11390600
C	0.66373700	-2.55489000	0.39308500
C	-0.21817200	-1.52718400	0.20637200
C	0.16061600	-0.24431400	-0.22854100
N	1.45967500	0.01347000	-0.49161500
C	1.88840300	1.37451000	-0.91096700
C	2.08110600	2.26185100	0.23434300
C	2.24150400	3.00320200	1.17589300
H	2.38420900	3.65899700	2.01125800
H	1.13037300	1.76839800	-1.59194100
H	2.81506700	1.25722400	-1.47724300
N	-0.90395200	0.58862500	-0.32884300
C	-1.92413200	-0.18492100	0.04157700
N	-3.19778700	0.14590400	0.11887500
H	-3.85046200	-0.56649700	0.42847000
C	-3.68776100	1.48229000	-0.22044700
H	-3.46307400	1.71891800	-1.26420500
H	-3.23245600	2.23456700	0.42958200
H	-4.76756200	1.48568400	-0.07368700
O	-1.58775900	-1.48004700	0.38154100
H	0.35666700	-3.53899200	0.72944300
H	2.78647800	-2.99580900	0.23172900
H	3.40929700	-0.71677400	-0.53012300

1c + MeS-

C	-2.11630900	0.82309400	1.43031600
C	-2.37049100	-0.50417600	1.41540100
C	-1.41694900	-1.54810700	0.89305800
C	-0.13352800	-0.85680600	0.70896800
C	0.08743300	0.48189200	0.72359600
N	-0.87470900	1.39964500	1.12403100
C	-0.84975900	2.76492600	0.57782500
C	-1.26628200	2.82382800	-0.83111600
C	-1.61182900	2.81765500	-1.99148300
H	-1.92044100	2.81451500	-3.01713200
H	0.15994800	3.16692100	0.69149700
H	-1.51215000	3.37996500	1.19476400
N	1.40577600	0.77136700	0.39366200
C	1.93255400	-0.40568100	0.18503100
S	3.56431900	-0.84711400	-0.25598500
C	4.32669700	0.81197100	-0.35339000
H	3.85352300	1.40723400	-1.13717400
H	5.37608200	0.63840900	-0.60662200
H	4.26266600	1.32370200	0.60901500
O	1.07393200	-1.45675100	0.35306300
H	-1.35277200	-2.42371300	1.54824300
H	-3.34728000	-0.84497800	1.74324500
H	-2.86911800	1.53849700	1.74567800
S	-2.06422500	-2.33424500	-0.72892000
C	-2.04885800	-0.86147200	-1.81227600
H	-2.41167200	-1.17937000	-2.79452800
H	-1.03515400	-0.46345600	-1.91958000
H	-2.71012900	-0.08182100	-1.42284800

INT-B1

C	2.75673000	-1.41929900	0.33489000
C	2.23463500	-2.07970500	1.40865800
C	0.85346200	-1.92324400	1.76865500
C	0.11949900	-1.09305800	0.98213400
C	0.65966100	-0.40015600	-0.14970900
N	-0.21513600	0.36737300	-0.76066900
C	-1.43600700	0.22568800	-0.03668000
O	-1.19047500	-0.72665900	1.07741000
S	-2.06532500	1.81624800	0.68435800
C	-0.57385100	2.30825000	1.62157200
H	-0.80262900	3.27120000	2.08776900
H	0.28586400	2.42591800	0.95672700
H	-0.35000600	1.57692800	2.40292900
S	-2.85017600	-0.42520000	-1.04865600
C	-2.12454100	-2.03246700	-1.52712700
H	-1.99200500	-2.67581000	-0.65384200
H	-1.16856600	-1.89080900	-2.03857700
H	-2.83167300	-2.50198000	-2.21730100
N	1.97529200	-0.59280600	-0.46187700
C	2.58664900	0.18297300	-1.55647700
C	2.90558800	1.55463100	-1.14897600
C	3.15770300	2.68661000	-0.80427600
H	3.38189400	3.68934300	-0.50091500
H	1.89629300	0.18658500	-2.40466800
H	3.49051400	-0.34418500	-1.87278300
H	0.42958800	-2.43620800	2.62538400
H	2.88045400	-2.72583300	1.99230000
H	3.79382700	-1.50194600	0.03430300

TS2-B

C	3.22998000	-1.04218700	0.43793600
C	2.74585900	-1.85221500	1.42320400
C	1.33165500	-2.00662100	1.62137300
C	0.52826500	-1.31337200	0.77287400
C	1.02937500	-0.46646700	-0.26773300
N	2.38144500	-0.35891300	-0.42391200
C	2.92294800	0.60026800	-1.40421800
C	2.87265000	1.97849400	-0.90722600
C	2.82048000	3.11238000	-0.48831900
H	2.77535700	4.11680600	-0.11832600
H	3.95258900	0.30803600	-1.62432900
H	2.34998800	0.50733800	-2.33115000
N	0.08582500	0.12855100	-0.96302500
C	-1.14631800	-0.29159300	-0.37470300
S	-2.16912700	-1.00048900	-1.53116400
C	-3.55708200	-1.58435600	-0.46048900
H	-4.17625600	-0.75771000	-0.10478000
H	-3.17577900	-2.16169900	0.38539700
H	-4.16534600	-2.23923300	-1.09218500
O	-0.83323900	-1.23044400	0.72695200
H	0.93538600	-2.63643600	2.41078400
H	3.44561500	-2.37820500	2.06264700
H	4.28742800	-0.89013800	0.26037800
C	-1.53726100	2.35999400	1.81127100
H	-1.35093200	1.59043500	2.56476500
H	-1.94644100	3.24934300	2.30096800
H	-0.60342800	2.62880300	1.30888600
S	-2.85440200	1.82253800	0.58654300

XXIV. References.

1. Chan, W. C. & White, P. D. *Fmoc Solid Phase Peptide Synthesis: A Practical Approach* (Oxford Univ. Press, New York, 2000).
2. a) M. N. Kamat, N. P. Rath, A. V. Demchenko, *J. Org. Chem.* **2007**, *72*, 6938-6946. b) K. Walczynski, O. P. Zuiderveld, H. Timmerman, *Eur. J Med. Chem.* **2005**, *40*, 15-23. c) L. Bethge, D. V. Jarikote, O. Seitz, *Bioorg. Med. Chem.* **2008**, *16*, 114-125.
3. Myers, M. C. *et al. J. Am. Chem. Soc*. **127**, 6152-6153 (2005).
4. a) S. Grimme, C. Bannwarth, S. Dohm, A. Hansen, J. Pisarek, P. Pracht, J. Seibert, F. Neese, *Angew. Chem. Int. Ed.* **2017**, *56*, 14763-14769. b) S. Grimme, *J. Chem. Theory Comput.* **2019**, *15*, 2847-2862.
5. a) S. Grimme, C. Bannwarth, P. Shushkov, *J. Chem. Theory Comput.* **2017**, *13*, 1989-2009. b) C. Bannwarth, S. Ehlert, S. Grimme, *J. Chem. Theory Comput.* **2019**, *15*, 1652-1671. c) Pracht, P., Caldeweyher, E., Ehlert, S. & Grimme, S. A Robust Non-Self-Consistent Tight-Binding Quantum Chemistry Method for Large Molecules. *ChemRxiv*, Preprint; **2019**. https://doi.org/10.26434/chemrxiv.8326202.v1.
6. Gaussian 09, Revision A.02, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, G. A. Petersson, H. Nakatsuji, X. Li, M. Caricato, A. Marenich, J. Bloino, B. G. Janesko, R. Gomperts, B. Mennucci, H. P. Hratchian, J. V. Ortiz, A. F. Izmaylov, J. L. Sonnenberg, D. Williams-Young, F. Ding, F. Lipparini, F. Egidi, J. Goings, B. Peng, A. Petrone, T. Henderson, D. Ranasinghe, V. G. Zakrzewski, J. Gao, N. Rega, G. Zheng, W. Liang, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, K. Throssell, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, T. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, J. M. Millam, M. Klene, C. Adamo, R. Cammi, J. W. Ochterski, R. L. Martin, K. Morokuma, O. Farkas, J. B. Foresman, and D. J. Fox, Gaussian, Inc., Wallingford CT, 2016.
7. S. Grimme, *Chem. Eur. J.* **2012**, *18*, 9955-9964.
8. Luchini, G.; Alegre-Requena, J. V.; Funes-Ardoiz, I. & Paton, R. S. (GoodVibes: Automated thermochemistry for heterogeneous computational chemistry data. *F1000Research* **9**, 291 (2020).