Peptide and Protein Cysteine Modification by Virtue of Highly Chemo-, Regio- and Stereoselective Hydrosulfuration of Ynamide

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Supplementary Information

Content

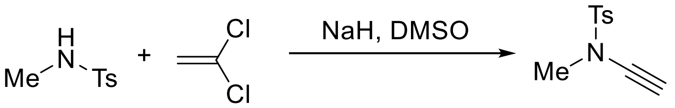
1. General Remarks

All Fmoc-amino acids were purchased from CS Bio Co. (Menlo Park, CA) and Matrix Innovation (Quebec City, Canada). Small organic molecules were purchased from Leyan, Energy Chemical. Bovine pancreatic trypsin inhibitor (WT-BPTI) was a generous gift of Bachem AG. ^{1}H- and ^{13}C spectra were recorded on a Bruker (400 MHz for ^{1}H, 101 MHz for ^{13}C respectively) instrument, and are internally referenced to residual solvent signals, $CDCl_3$ referenced at δ 7.26 and 77.00 ppm, DMSO-d_6 referenced at δ 2.50 and 39.8 ppm. Data for ^{1}H is reported as follows: chemical shift (δ ppm), integration, multiplicity (s= singlet, d = doublet, t = triplet, q = quartet, m = multiplet), broad peaks (br), coupling constant (Hz) and assignment. Data for ^{13}C NMR are reported in terms of chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (Hz) and no special nomenclature is used for equivalent carbons. HRMS (ESI) spectra were obtained by the electrospray ionization time-of-flight (ESI-TOF) mass spectrometry. Flash column chromatography purification of compound was carried out by gradient elution using ethyl acetate (EA) in light petroleum ether (PE) or gradient elution using methanol (MeOH) in dichloromethane (DCM). Unless otherwise noted, materials obtained from commercial suppliers were used without further purification. Analytical RP-HPLC was performed on an UltiMate 3000 HPLC system or Waters Alliance HPLC (220 nm UV detection) with appropriate columns and elution conditions. ESI-MS was performed on an LCQ Fleet Ion Trap mass spectrometer (Thermo Scientific) in the positive mode. The HR-MS were recorded on an Agilent 6520 QTOF analyzer (Agilent Technologies, Inc., United States) with a dual electrospray ionization source. The raw data were deconvoluted using MagTran v1.03.

2. General Procedures for Preparation of Ynamides, Peptides and Proteins

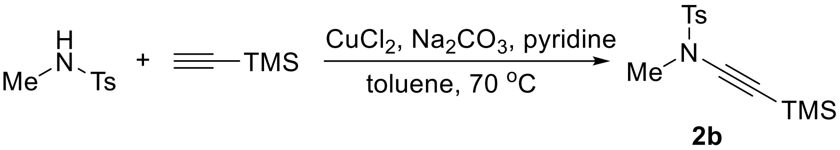
(1) General procedures for preparation of ynamides ***2a-2m***[1]

(a) *N*, 4-dimethylbenzenesulfonamide (2.0 mmol), NaH (4.0 mmol, 2.0 equiv) were combined in an oven-dried Schlenk tube equipped with a stir-bar. After the addition of all solid reagents, 20.0 mL of DMSO was added to the tube via a syringe, and the reaction mixture was allowed to stir at rt for 15 min. The Schlenk tube was heated at 70 °C. Then 1,1-dichloroethene (4.0 mmol, 2.0 equiv) was added to the Schlenk tube slowly via a syringe. The reaction mixture was stirred at 70 °C for another 48 h and then cooled to room temperature. The reaction was quenched by cold water and extracted with methylene chloride three times. The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under vacuum. The desired products were obtained in 80% yield after purification by flash chromatography on silica gel with petroleum ether/ ethyl acetate.



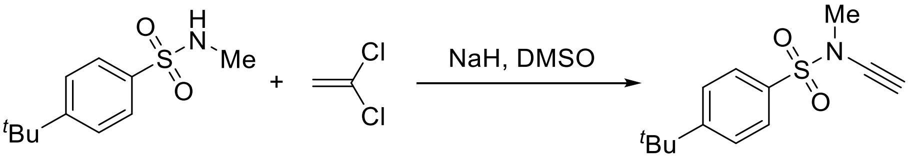
2a

(b) In a 100 mL three-neck round-bottom flask equipped with a stir-bar, $CuCl_2$ (0.4 mmol, 20%), *N*, 4-dimethylbenzenesulfonamide (10.0 mmol, 5.0 equiv) and Na_2CO_3 (4.0 mmol, 2.0 equiv) were combined. The reaction flask was purged with oxygen gas for 15 minutes. A solution of pyridine (4 mmol, 2.0 equiv) in 20.0 ml of toluene was added to the reaction flask via a syringe. Two balloons filled with oxygen gas were connected to the reaction flask via needles. The flask was placed in an oil-bath and heated to 70 °C. A solution of terminal alkyne (2.0 mmol) in 20.0 ml of toluene was added slowly to the flask over 4 h by using a syringe pump. After addition of terminal alkyne/toluene solution, the reaction mixture was allowed to stir at 70 °C for another 16 hours and then cooled to room temperature. The crude mixture was concentrated under vacuum and then purified by flash chromatography on silica gel with PE/ethyl acetate to afford the ynamide **2b**.



2b

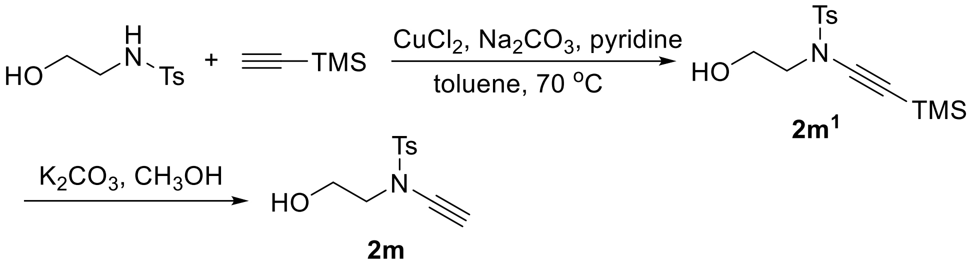
(c) N-(4-(tert-butyl)phenyl)-4-methylbenzenesulfonamide (2.0 mmol), NaH (4.0 mmol, 2.0 equiv) were combined in an oven-dried Schlenk tube equipped with a stir-bar. After the addition of all solid reagents, 20.0 mL of DMSO was added to the tube via a syringe, and the reaction mixture was allowed to stir at rt for 15 min. The Schlenk tube was heated at 70 °C. Then 1,1-dichloroethene (4.0 mmol, 2.0 equiv) was added to the Schlenk tube slowly via a syringe. The reaction mixture was stirred at 70 °C for another 48 h and then cooled to room temperature. The reaction was quenched by cold water and extracted with methylene chloride three times. The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under vacuum. The desired products were obtained in 80% yield after purification by flash chromatography on silica gel with petroleum ether/ ethyl acetate.



2l

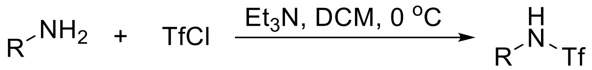
(d) Step 1: In a 100 mL three-neck round-bottom flask equipped with a stir-bar, $CuCl_2$ (0.4 mmol, 20%), N-(2-hydroxyethyl)-4-methylbenzenesulfonamide (10.0 mmol, 5.0 equiv) and Na_2CO_3 (4.0 mmol, 2.0 equiv) were combined. The reaction flask was purged with oxygen gas for 15 minutes. A solution of pyridine (4 mmol, 2.0 equiv) in 20.0 ml of toluene was added to the reaction flask via a syringe. Two balloons filled with oxygen gas were connected to the reaction flask via needles. The flask was placed in an oil-bath and heated to 70 °C. A solution of terminal alkyne (2.0 mmol) in 20.0 ml of toluene was added slowly to the flask over 4 h by using a syringe pump. After addition of terminal alkyne/toluene solution, the reaction mixture was allowed to stir at 70 °C for another 16 hours and then cooled to room temperature. The crude mixture was concentrated under vacuum and then purified by flash chromatography on silica gel with PE/ethyl acetate to afford the ynamide **2m**[1].

Step 2: In a 10 ml round-bottom flask equipped with TMS-ynamide (2 mmol), CH_3OH and K_2CO_3 (2.0 equiv) were combined. The reaction mixture was stirred at room temperature under air until TMS-ynamide was fully consumed. The reaction mixture was treated with H_2O at room temperature and aqueous layer was extracted with Et_2O for three times. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and evaporated in vacuo. Crude product was purified by flash chromatography to afford the terminal ynamide **2m** in quantitative yield.



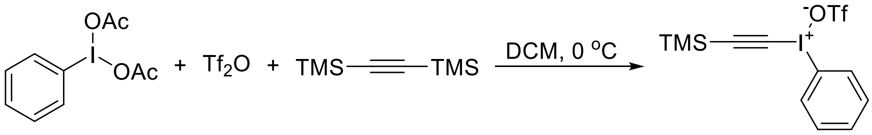
(2) General procedures for preparation of ynamides ***2n-2o, 2t***

(a) A 100 mL round-bottom flask equipped with a stir bar was charged with primary amine (10 mmol), triethylamine (2 equiv, 20 mmol) The flask was allowed to put in an ice bath, and then Trifluoromathanesulfonyl chloride (1.2 equiv, 12 mmol) was added to the flask dropwise. The reaction mixture was allowed to stir at 0 °C until primary amine was completely consumed. After the reaction had completed, the reaction mixture was treated with saturated $Na_2S_2O_3$ solution and aqueous layer was extracted with DCM (3 × 10 mL) The combined organic layer was washed with brine, dried over $MgSO_4$, filtered and evaporated under vacuum. The residue was purified by flash chromatography to afford target product RNHTf.

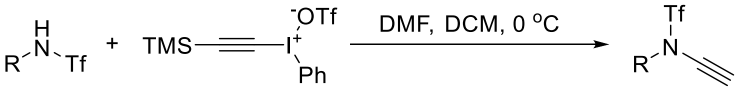


(b) In a 250 mL round-bottom flask was introduced (Diacetoxyiodo)benzene (12.88 g, 40 mmol) in DCM (200 mL), the reaction mixture was cooled down to 0 °C with ice bath. Then trifluoromethanesulfonic anhydride (5.64 g, 20 mmol) in 20 mL DCM was added dropwise to the reaction mixture for 24 min while stirring. The reaction left for 40 min for stirring at the same conditions. Later Bis(trimethylsilyl)acetylene (6.82 g, 40 mmol) were added to the reaction. Reaction was kept stirring at 0 °C for additional

5h. Upon reaction completion the volume of organic solvent was reduced under reduced pressure, then diethylether was added to help the precipitation of the product. After obtaining maximum precipitation the product hapervalent Iodine reagent was directly filtered out and dried under vacuum (12.2 g, 27 mmol, 68% yield, white solid)



(c) A 100 mL round-bottom flask was charged with RNHTf (2 mmol), K_2CO_3 (5 mmol, 2.5 equiv), 20 mL DMF. The reaction mixture left for stirring in rt for 5 min. Then reaction flask was placed in a 0 °C cold bath and stirred for 15 min. Then hapervalent Iodine reagent (2.4 mmol, 1.2 equiv) in DCM (20 mL) was added slowly to the reaction mixture, reaction was kept stirring at 0 °C for additional 40 min. Upon reaction completion, the reaction mixture was diluted with H_2O and the aqueous phase extracted with DCM (3 x 15 mL) The combined organic layers were washed with brine and dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. Purification of the product **2** was performed via flash chromatography



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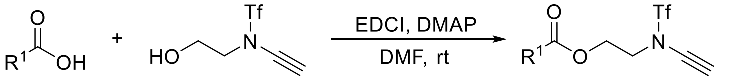
(3) General procedures for preparation of ynamides ***2r & 2s***

A 50 mL round-bottom flask was charged biotin or Indomethacin (1 mmol), **2e** (1.2 mmol, 1.2 equiv), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (1.2 mmol, 1.2 equiv), and 4-dimethylaminopyridine (DMAP) (2 mmol, 2 mmol) with 10 mL DMF. Reaction was left for stirring at room temperature for 4 h. After reaction completion the organic solvent was evaporated under reduced pressure. Later product **2** was purified by silica gel column chromatography.



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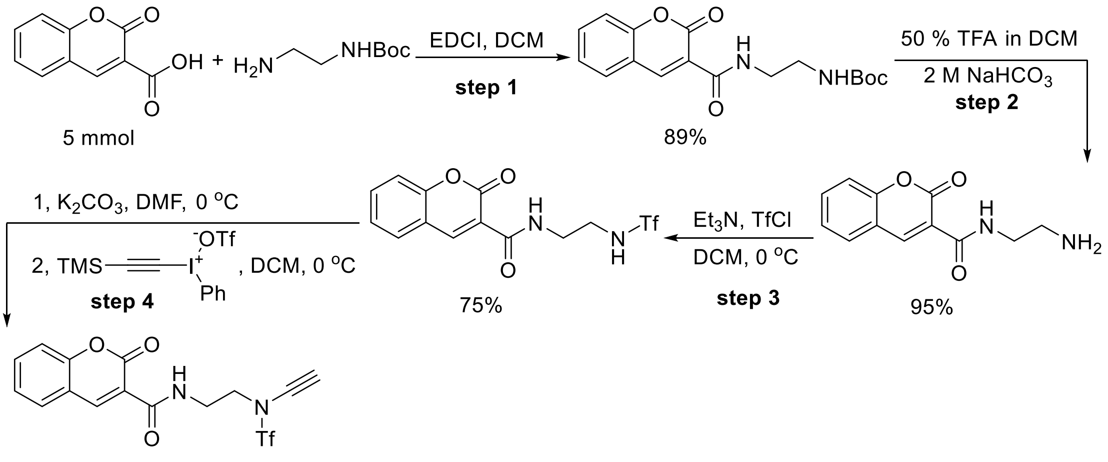
(4) General procedures for preparation of ynamide ***2q***

Step 1: A 100 mL round-bottom flask was charged 2-oxo-2H-chromene-3-carboxylic acid (5.0 mmol), *tert*-butyl (2-aminoethyl) carbamate (6.0 mmol, 1.2 equiv), EDCI (6.0 mmol, 1.2 equiv) and DCM (50.0 mL) The reaction mixture was allowed to stir for 30 min at room temperature. Later the product was purified by silica gel column chromatography.

Step 2: A 50 mL round-bottom flask was charged *tert*-butyl (2-(2-oxo-2H-chromene-3-carboxamido)ethyl)carbamate (2.0 mmol), then with the 50% TFA in DCM, after 20.0 min at room temperature, remove the all of solvent and extract the product with 2.0 M $NaHCO_3$.

Step 3: A 50.0 mL round-bottom flask was charged with N-(2-aminoethyl)-2-oxo-2H-chromene-3-carboxamide (2.0 mmol), Et_3N (2.4 mmol, 1.2 equiv), 20.0 mL DCM, the reaction flask was placed in a 0 °C cold bath and stirred for 5 min. Then TfCl (2.4 mmol, 1.2 equiv) was added slowly to the reaction mixture, after that, the reaction was kept stirring at room temperature for additional 30 min. Upon reaction completed, the product was extracted.

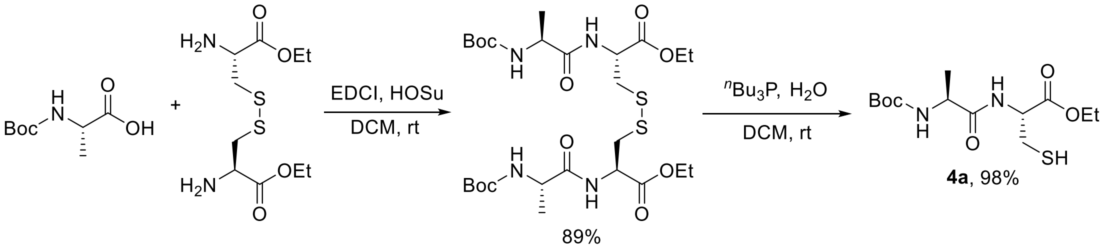
Step 4: A 25 mL round-bottom flask was charged with 2-oxo-N-(2-((trifluoromethyl) sulfonamido)ethyl)-2H-chromene-3-carboxamide (2 mmol), K_2CO_3 (5 mmol, 2.5 equiv), 10 mL DMF. The reaction mixture left for stirring at 0 °C for 5 min. Another reagent high iodine (2.4 mmol, 1.2 equiv) in DCM (10.0 mL) was added slowly to the reaction mixture, Then reaction was kept stirring at room temperature for additional 30 min. The reaction was quenched by cold water and extracted with methylene chloride three times. Later the product was purified by silica gel column chromatography.



*5) General procedures for dipeptide Boc-Ala-Cys-OEt (**4a**) synthesis*

Step 1: A 250 mL round-bottom flask was charged Boc-Ala-OH (10 mmol), EDCI (12 mmol, 1.2 equiv), 1-hydroxy-2,5-pyrrolidinedione (HOSu) (12 mmol, 1.2 equiv) and DCM (100 mL) The reaction mixture was allowed to stir for 30 min at room temperature. Then (H-Cys-OEt)$_2$ (6 mmol, 0.6 equiv) was added to solution and the reaction mixture was kept stirring for additional 30 min. After reaction completion the organic solvent was evaporated under reduced pressure. Later product (Boc-Ala-Cys-OEt)$_2$ was purified by silica gel column chromatography.

Step 2: To 100 mL round-bottom flask was charged (Boc-Ala-Cys-OEt)$_2$ (5 mmol), ^{n}Bu$_3$P (20 mmol, 4 equiv), 0.5 mL H_2O, and 30 mL DCM. The reaction mixture was allowed to stir for 1 h at room temperature. Later the product was purified by silica gel column chromatography.



6) General procedure for Fmoc solid-phase peptide synthesis

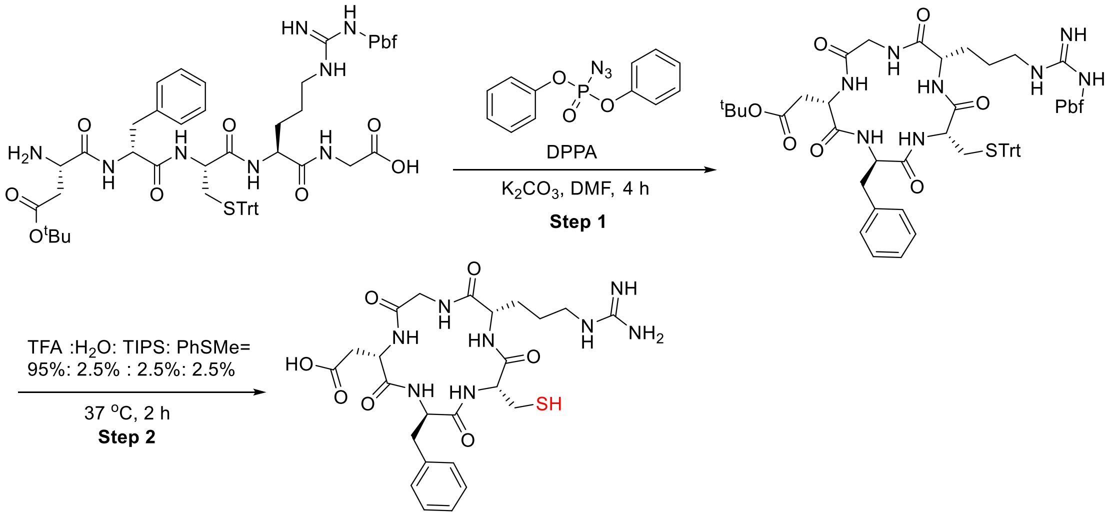
Peptides were synthesized manually on a 0.2 mmol scale by Fmoc-SPPS. Fmoc deprotection was carried out with 20% piperdine in DMF (2x10min) Fmoc-amino acids (1 mmol in 5 mL of DMF, 4 equiv) were activated with PyBOP (0.6 mmol, 3 equiv) and DIEA (1.2 mmol, 6 equiv) for 5 min and allowed to couple for 30 min with constant shaking. The resulting resins were washed with DMF (×3), DCM (×3) and dried.

The peptides were cleaved off resin using a TFA: triisopropylsilane (TIPS): PhSMe: H_2O (95: 2.5: 2.5: 2.5) cocktail for 2.5 h at room temperature. The cleavage mixture was filtered, and the resin was washed with TFA. The combined solutions were concentrated by nitrogen gas bubbling, to which 2-fold volumes of cold diethyether was added dropwise. The precipitated crude peptides were centrifuged, ether was removed, and the crude peptides were dissolved in MeCN : water (1:1) containing 0.1% TFA and lyophilized.

*7) General procedure for cyclic peptide **4o** synthesis*

Step 1: The linear peptide H-Asp(O^{t}Bu)-Phe(D)-Cys(Trt)-Arg(Pbf)-Gly-OH (1 mmol) was dissolved in DMF (400 mL, c =2.5 x10^{-3} M for very high dilution conditions), DPPA (3 equiv) and K_2CO_3 (5 equiv) were added and the mixture was stirred for 4 h at room temperature in a dark room. The DMF was evaporated under high vacuum. The protected cyclic peptide was precipitated in water and precipitates were collected by centrifugation at 5000 rpm for 5 min and washed by Et_2O 3 times.

Step 2: The protected cyclic peptide was dissolved in TFA : H_2O : TIPS : PhSMe = 95% : 2.5% : 2.5% : 2.5%. The reaction mixture was was allowed to stir for 2 h at 37 °C temperature and monitored by LC-MS. The solvent was evaporated under reduced pressure. And the residue was washed by ice-cold ether (4 × 5.0 mL) The protected cyclic peptide was obtained without further purification.



8) *The chemical synthesis of **Ubiquitin(G47C) (6)***

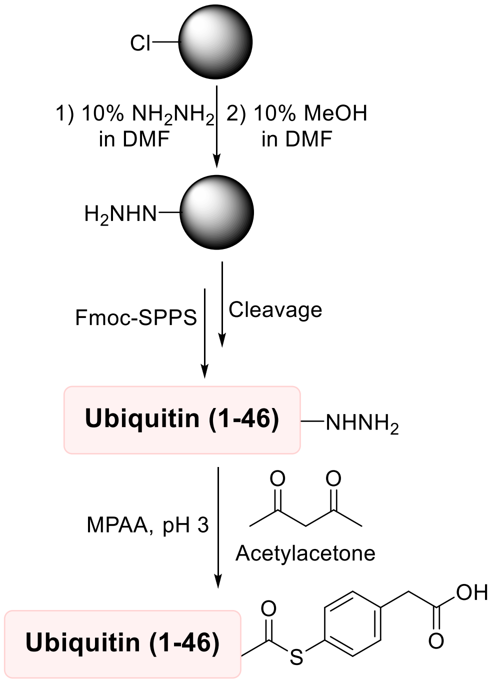
The sequence of wildtype of ubiquitin:

10 20 30 40 50
MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL

60 70 76
EDGRTLSDYN IQKESTLHLV LRLRGG

Ubiquitin consists of 76 amino acids and does not contain a Cys residue, so the solvent exposed Gly47 was substituted with Cys (shown in *red*). Ubiquitin(G47C) (**6**) was prepared from two segments: ubiquitin(1-46)-MPAA (**6a**) and ubiquitin(47-76)(G47C) (**6b**) using a single native chemical ligation (NCL) reaction[2]. The synthesis of each segment and NCL reaction are described in detail below.

9) *The synthesis of ubiquitin(1-46)-MPAA (**6a**)*



Scheme S1. Chemical synthesis of ubiquitin(1-46)-MPAA (**6a**)

Hydrazine loading on resin: 2-chlorotrityl chloride resin (0.25 mmol scale, 0.5 mmol/g) was swelled in DCM: DMF (1:1) for 2 h. The resin was treated twice with fresh 10% hydrazine in DMF for 30 min and drained. The resin was then treated twice with 10% MeOH in DMF for 30 min. After that, the resin was washed well with DCM and DMF.

Synthesis of ubiquitin(1-46)-NHNH$_2$: Ubiquitin(1-46)-NHNH$_2$ was synthesized on hydrazine-functionalized 2-chlorotrityl resin using automatic Fmoc-peptide synthesizer. After completion of synthesis the resin was washed by DCM and cleaved according to the general procedure.

Thioestrification of ubiquitin(1-46)-NHNH$_2$: Crude ubiquitin(1-46)-NHNH$_2$ was dissolved in phosphate buffer (0.2 M, 6 M Gn·HCl, pH 3), acetylacetone (25 equiv) and MPAA (50 mM) were then added to the peptide solution.[3] The reaction mixture was incubated at room temperature for 3 h. The purification of ubiquitin(1-46)-MPAA (**6a**) was conducted by RP-HPLC using C4 column with a gradient of 5-70% B (0.1% TFA in MeCN) over 20 min to obtain ubiquitin(1-46)-MPAA (**6a**) in 30% yield (mass obs. 5334.4 Da, Mass calc. 5334.8 Da) The results are shown in **Figure S1**.

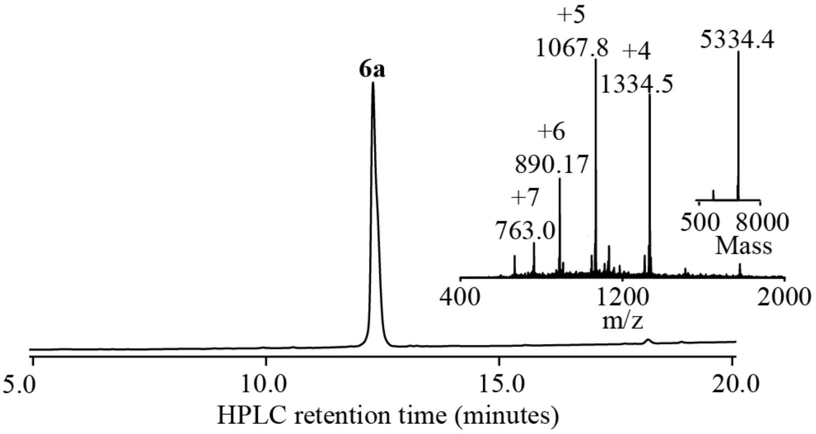
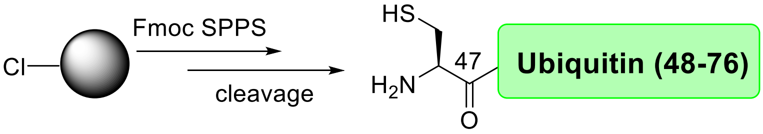


Figure S1. HPLC trace and ESI-MS of ubiquitin (1-46)- MPAA (**6a**)

10) *The synthesis of ubiquitin(47-76)(G47C)* ***(6b)***



Scheme S2. Chemical synthesis of ubiquitin(47-76)(G47C) (**6b**)

The synthesis of ubiquitin(47-76)(G47C) (**6b**) was carried out on 2-chlorotrityl chloride resin (0.25 mmol scale, 0.5 mmol/g) on automated Fmoc-peptide synthesizer. Upon synthesis completion the peptide was cleaved and treated as described previously. The crude ubiquitin(47-76)(G47C) was purified by Prep RP-HPLC (C18 column) to obtain **6b** in ~ 10% yield (mass obs. 3442.8 Da, Mass calc. 3443.8 Da) The results are shown in **Figure S2**.

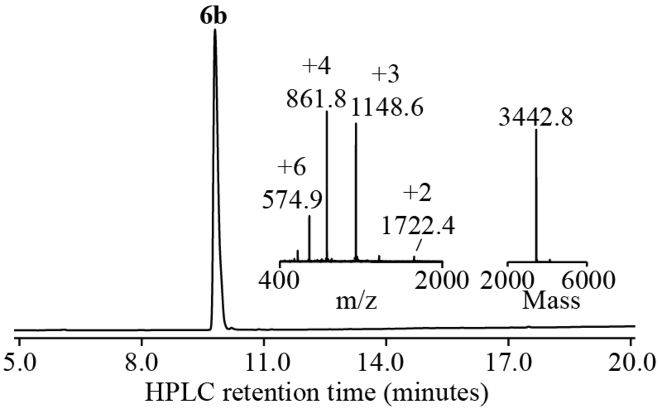
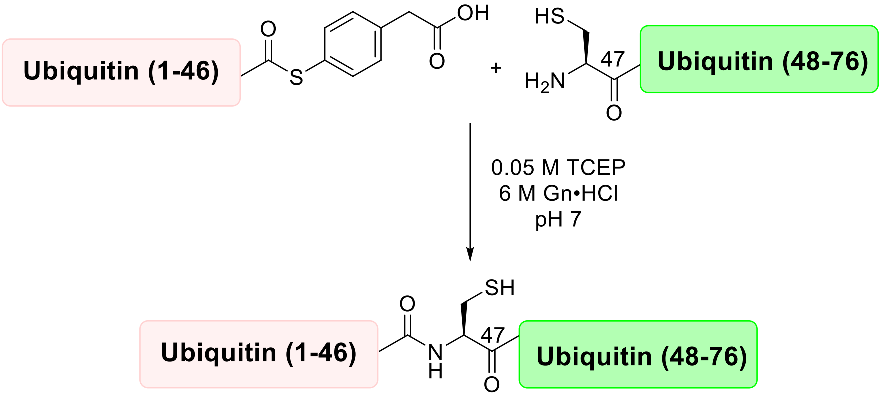


Figure S2. HPLC trace and ESI-MS ubiquitin(47-76)(G47C), **6b.**

*11) The native chemical ligation of ubiquitin(1-46)-MPAA thioester (**6a**) and ubiquitin(47-76)(G47C) (**6b**)*



Scheme S3. Chemical synthesis of ubiquitin(G47C) (**6**) by NCL

Ubiquitin(1-46)-MPAA (**6a**, 11.4 mg, 2.1 μmol, 1mM) and ubiquitin(47-76)(G47C) (**6b**, 9.8 mg, 2.8 μmol, 1.3 mM) were dissolved in 2.13 mL of degassed PB (0.1 M, 6 M Gn·HCl, 0.05 M TCEP, at pH 7) and incubated at 37 ºC for 3 h. The reaction progress was monitored by analytical RP-HPLC (C4 column) with a gradient of 5-70% B (0.1% TFA in MeCN) over 20 min at 220 nm (Figure S8) The purification was conducted by semi-prep RP-HPLC (C4 column) to afford the corresponding protein ubiquitin(G47C) (**6**) in ~ 28% yield (mass obs. 8607.1 Da, Mass calc. 8608.6 Da). The results are shown in **Figure S3.**

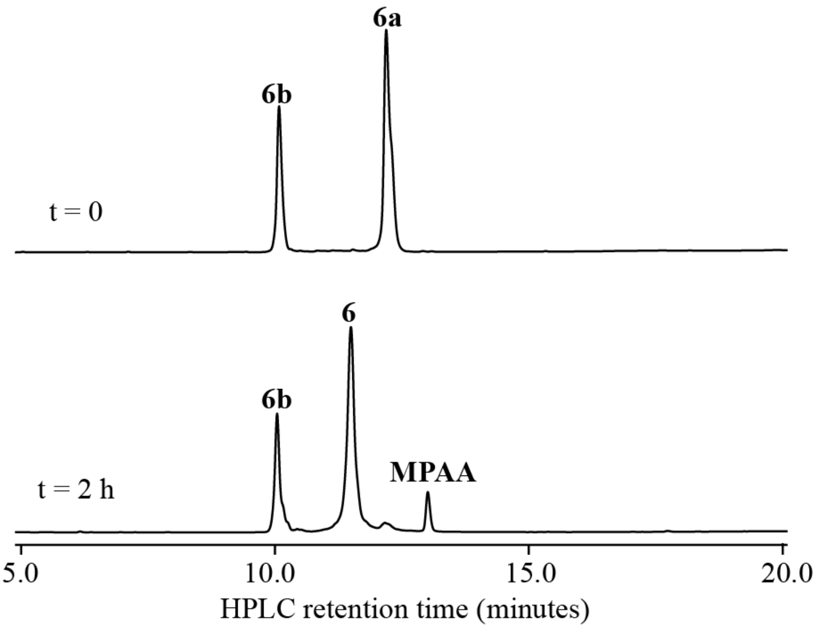
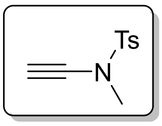


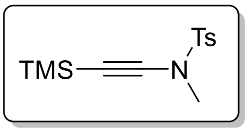
Figure S3. Chemical synthesis of ubiquitin(G47C) (**6**) by NCL.

*N-ethynyl-N,4-dimethylbenzenesulfonamide (**2a**)*[1]



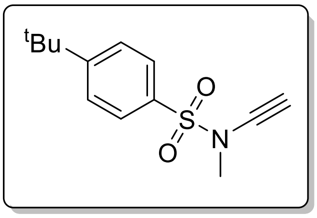
White solid, 89%, 372.0 mg, purified by flash chromatography using PA/EA (8:1) as eluent, Rf = 0.5 in PE/EA (5:1) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 8.3 Hz, 2H), 7.37 (d, *J* = 8.3 Hz, 2H), 3.06 (s, 3H), 2.69 (s, 1H), 2.46 (s, 3H) ^{3}C NMR (101 MHz, $CDCl_3$) δ 144.9, 133.3, 130.0, 127.8, 77.6, 57.5, 38.7, 21.7.

*N,4-dimethyl-N-((trimethylsilyl)ethynyl)benzenesulfonamide (**2b**)*[4]



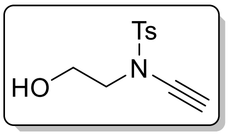
White solid, 70%, 394.0 mg, purified by flash chromatography using PA/EA (8:1) as eluent, Rf = 0.6 in PE/EA (5:1)^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (d, *J* = 8.1 Hz, 2H), 7.21 (d, *J* = 8.1 Hz, 2H), 2.89 (s, 3H), 2.31 (s, 3H), 0.00 (s, 9H) ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.7, 133.2, 129.6, 127.9, 96.6, 71.3, 39.0, 21.6, 0.0.

*4-(tert-butyl)-N-ethynyl-N-methylbenzenesulfonamide (**2l**)*[1]



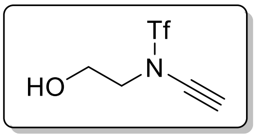
Colourless oil, 85%, 426.7 mg, purified by flash chromatography using PA/EA (6:1) as eluent, Rf = 0.4 in PE/EA (5:1)^{1}H NMR (400 MHz, CDCl$_3$) δ 7.84 (d, *J* = 8.6 Hz, 2H), 7.58 (d, *J* = 8.6 Hz, 2H), 3.08 (d, *J* = 2.7 Hz, 3H), 2.70 (s, 1H), 1.36 (s, 9H) ^{13}C NMR (101 MHz, CDCl$_3$) δ 157.8, 133.4, 127.7, 126.2, 77.7, 57.5, 38.8, 35.3, 31.0.

N-ethynyl-N-(2-hydroxyethyl)-4-methylbenzenesulfonamide ***(2m)***^{5}



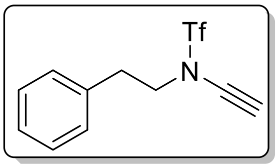
White solid, 85%, 406.3 mg, purified by flash chromatography using PA/EA (10:1) as eluent, Rf = 0.5 in PE/EA (5:1)^{1}H NMR (400 MHz, CDCl$_3$) δ 8.14 (s, 1H), 7.68 (d, *J* = 15.0 Hz, 2H), 7.37 (d, *J* = 14.9 Hz, 2H), 3.62 (t, *J* = 7.4 Hz, 2H), 3.35 (t, *J* = 7.4 Hz, 2H), 2.43 (s, 3H) ^{13}C NMR (101 MHz, CDCl$_3$) δ 145.2, 134.3, 130.0, 127.9, 76.0, 60.0, 59.5, 53.7, 21.8.

N-ethynyl-1,1,1-trifluoro-N-(2-hydroxyethyl)methanesulfonamide ***(2n)***



Yellow oil, 95%, 412.3 mg, purified by flash chromatography using PA/EA (4:1) as eluent, Rf = 0.4 in PE/EA (2:1) ^{1}H NMR (400 MHz, CDCl$_3$) δ 3.95 (d, *J* = 4.6 Hz, 2H), 3.73 (t, *J* = 5.2 Hz, 2H), 2.85 (s, 1H), 2.50 (s, 1H) ^{13}C NMR (101 MHz, CDCl$_3$) δ 119.6 (q, J = 320.0 Hz), 71.6, 59.9, 59.9, 58.8, 55.1. HRMS (ESI-TOF) m/z: $C_5H_7F_3NO_3S^+$ $[M+H]^+$ Mass calc. 218.0093; Obs.: 218.0085.

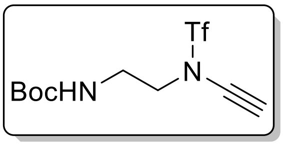
N-ethynyl-1,1,1-trifluoro-N-phenethylmethanesulfonamide ***(2o)***



White solid, 92%, 509.7 mg, purified by flash chromatography using PA/EA (10:1) as eluent, Rf = 0.5 in PE/EA (5:1) ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.33 (t, *J* = 7.2 Hz, 2H), 7.27 (d, *J* = 7.2 Hz, 1H), 7.22 (d, *J* = 8.4 Hz, 2H), 3.83 – 3.72 (m, 2H), 3.12 – 3.04 (m, 2H), 2.83 (s, 1H) ^{13}C NMR (101 MHz, CDCl$_3$) δ 136.1, 128.9, 128.85, 127.26, 119.6 (q, J = 320.0 Hz), 71.78, 60.15, 54.62, 34.37. HRMS (ESI-TOF) m/z:

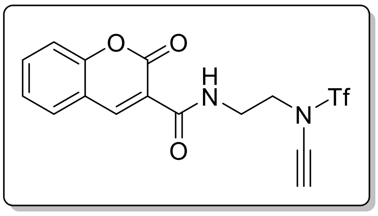
$C_{11}H_{11}F_3NO_2S^+$ $[M+H]^+$ Mass calc. 278.0457; Obs.: 278.0467.

tert-butyl (2-((N-ethynyl-1,1,1-trifluoromethyl)sulfonamido)ethyl)carbamate ***(2p)***



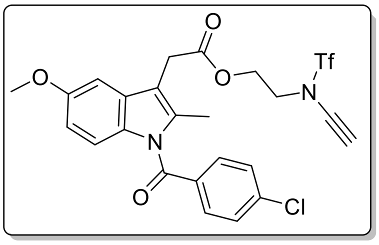
Yellow oil, 95%, 601.0 mg, purified by flash chromatography using PA/EA (5:1) as eluent, Rf = 0.4 in PE/EA (3:1) ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.93 (s, 1H), 3.72 (s, 2H), 3.47 (d, *J* = 5.0 Hz, 2H), 2.83 (s, 1H), 1.45 (s, 9H) ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.7, 119.6 (q, J = 320.0 Hz), 80.1, 71.6, 59.9, 52.7, 38.5, 28.3. HRMS (ESI-TOF) m/z: $C_{10}H_{15}F_3N_2NaO_4S^+$ $[M+Na]^+$ Mass calc. 339.0597; Obs.: 339.0599

N-(2-((N-ethynyl-1,1,1-trifluoromethyl)sulfonamido)ethyl)-2-oxo-2H-chromene-3-carboxamide ***(2q)***



Yellow solid, 92%, 714.4 mg, purified by flash chromatography using PA/EA (2:1) as eluent, Rf = 0.5 in PE/EA (1:1) ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.14 (s, 1H), 8.92 (s, 1H), 7.70 (t, *J* = 8.9 Hz, 2H), 7.48 – 7.33 (m, 2H), 3.85 (dd, *J* = 10.2, 4.6 Hz, 4H), 2.88 (d, *J* = 6.1 Hz, 1H) ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.4, 161.3, 154.6, 148.8, 134.4, 130.0, 125.4, 119.6 (q, J = 320.0 Hz), 118.5, 117.8, 116.7, 71.6, 60.2, 52.1, 37.9. HRMS (ESI-TOF) m/z: $C_{15}H_{12}F_3N_2O_5S^+$ $[M+H]^+$ Mass calc. 389.0414; Obs.: 389.0416.

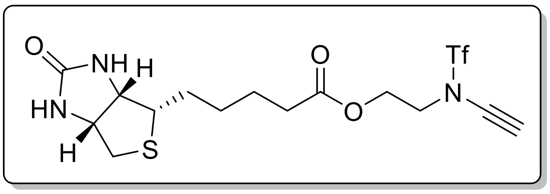
2-((N-ethynyl-1,1,1-trifluoromethyl)sulfonamido)ethyl 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetate ***(2r)***



White solid, 90%, 501.3 mg, purified by flash chromatography using PA/EA (3:1) as eluent, Rf = 0.4 in PE/EA (2:1) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (d, *J* = 8.2 Hz, 2H), 7.45 (d, *J* = 8.2 Hz, 2H), 6.95 (s, 1H), 6.87 (d, *J* = 9.0 Hz, 1H), 6.66 (d, *J* = 8.8 Hz, 1H), 4.52 – 4.27 (m, 2H), 3.82 (s, 5H), 3.72 (s, 2H), 2.81 (s, 1H), 2.37 (s, 3H) ^{13}C NMR (101

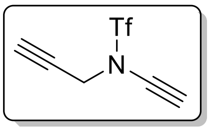
MHz, $CDCl_3$) δ 170.5, 168.3, 156.1, 139.2, 136.2, 133.9, 131.2, 130.8, 130.6, 129.1, 119.6 (q, J = 322.0 Hz), 115.0, 111.9, 111.8, 101.1, 71.2, 60.2, 55.7, 52.1, 29.9, 13.3. HRMS (ESI-TOF) m/z: $C_{24}H_{21}ClF_3N_2O_6S^+$ $[M+H]^+$ Mass calc. 557.0755; Obs.: 557.0758.

*2-((N-ethynyl-1,1,1-trifluoromethyl)sulfonamido)ethyl 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate **(2s)***



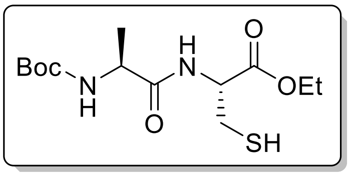
White solid, 80%, 354.4 mg, purified by flash chromatography using DCM/MeOH (200:1) as eluent, Rf = 0.6 in DCM/MeOH (10:1). ^1H NMR (400 MHz, $CDCl_3$) δ 6.20 (s, 1H), 5.79 (s, 1H), 4.55 – 4.46 (m, 1H), 4.38 (t, *J* = 4.9 Hz, 2H), 4.35 – 4.25 (m, 1H), 3.84 (t, *J* = 4.9 Hz, 2H), 3.16 (d, *J* = 4.6 Hz, 1H), 2.91 (dd, *J* = 11.4, 6.2 Hz, 2H), 2.74 (d, *J* = 12.8 Hz, 1H), 2.39 (t, *J* = 7.4 Hz, 2H), 1.80 – 1.57 (m, 4H), 1.47 (dd, *J* = 15.1, 7.5 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.2, 164.0, 119.5 (q, J = 322.0 Hz), 71.3, 62.0, 60.2, 60.2, 59.7, 55.5, 52.1, 40.6, 33.6, 28.3, 28.2, 24.5. HRMS (ESI-TOF) m/z: $C_{15}H_{21}F_3N_3O_5S_2^+$ $[M+H]^+$ Mass calc. 444.0869; obs. 444.0876.

*N-ethynyl-1,1,1-trifluoro-N-(prop-2-yn-1-yl)methanesulfonamide **(2t)***



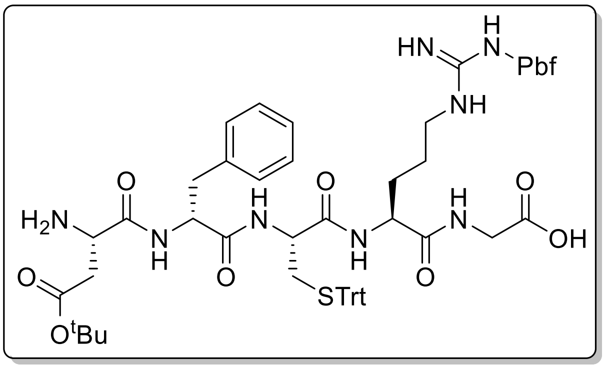
Yellow oil, 95%, 399.0 mg, purified by flash chromatography using PA/EA (10:1) as eluent, Rf = 0.5 in PE/EA (5:1). ^1H NMR (400 MHz, $CDCl_3$) δ 4.32 (d, *J* = 1.9 Hz, 2H), 2.81 (s, 1H), 2.49 (d, *J* = 2.2 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 119.4 (q, J = 322.0 Hz), 76.18, 74.19, 71.04, 60.54, 43.34. HRMS (ESI-TOF) m/z: $C_6H_5F_3NO_2S^+$ $[M+H]^+$ Mass calc. 211.9988; Obs.: 212.0003.

*ethyl (tert-butoxycarbonyl)-L-alanyl-L-cysteinate **(4a)***



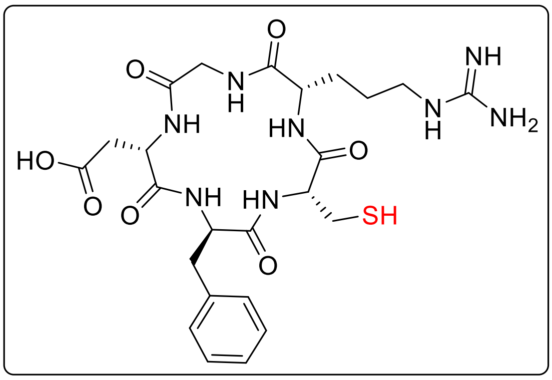
White solid, 91%, 2.9 g, purified by flash chromatography using PA/EA (2:1) as eluent, Rf = 0.5 in PE/EA (1:1). ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.45 (s, 1H), 5.62 (d, *J* = 7.1 Hz, 1H), 4.94 – 4.65 (m, 1H), 4.27 (s, 1H), 4.15 (q, *J* = 7.1 Hz, 2H), 3.28 – 2.88 (m, 2H), 1.37 (s, 9H), 1.31 (d, *J* = 7.0 Hz, 3H), 1.21 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 173.2, 170.0, 155.7, 79.9, 61.7, 52.1, 49.8, 40.7, 28.3, 18.2, 14.0. HRMS (ESI-TOF) m/z: [M+H]$^+$ Mass calc. $C_{13}H_{25}N_2O_5S^+$ 321.1479; Obs.: 321.1479.

N^2-(N-(((S)-2-amino-4-(tert-butoxy)-4-oxobutanoyl)-D-phenylalanyl)-S-trityl-L-cysteinyl)-N^w-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)-L-arginylglycine



White solid, 80%, 917.6 mg, purified by recrystallization. ^{1}H NMR (400 MHz, DMSO) δ 8.55 (s, 1H), 8.55 (s, 1H), 7.89 (d, *J* = 3.4 Hz, 2H), 7.50 – 7.28 (m, 13H), 7.22 (dd, *J* = 14.8, 8.6 Hz, 6H), 7.20 – 7.02 (m, 5H), 6.62 (s, 3H), 4.61 (s, 1H), 4.27 (dd, *J* = 17.1, 9.7 Hz, 2H), 3.68 (s, 1H), 3.61 (s, 2H), 3.02 (d, *J* = 4.4 Hz, 3H), 2.94 (s, 2H), 2.82 (d, *J* = 8.3 Hz, 1H), 2.62 – 2.44 (m, 6H), 2.40 (d, *J* = 24.6 Hz, 5H), 2.00 (s, 3H), 1.91 (s, 4H), 1.73 (d, *J* = 16.4 Hz, 2H), 1.53 (s, 3H), 1.40 (s, 6H), 1.37 (s, 9H). ^{13}C NMR (101 MHz, DMSO) δ 172.6, 172.5, 171.4, 171.2, 171.2, 169.7, 157.9, 156.7, 144.7, 137.7, 137.7, 134.7, 131.9, 129.7, 129.6, 128.5, 127.2, 126.8, 124.8, 116.8, 86.7, 81.1, 66.4, 54.5, 54.4, 52.7, 52.3, 50.9, 43.0, 38.3, 34.2, 29.8, 28.8, 28.2, 25.6, 21.6, 21.6, 19.4, 18.1, 12.7. HRMS (ESI-TOF) m/z: $C_{60}H_{75}N_8O_{11}S_2^+$ [M+H]$^+$ Mass calc. 1147.4991; Obs.: 1147.4994.

*2-((2S,5R,8R,11S)-5-benzyl-11-(3-guanidinopropyl)-8-(mercaptomethyl)-3,6,9,12,15-pentaoxo-1,4,7,10,13-pentaazacyclopentadecan-2-yl)acetic acid (**4o**)*

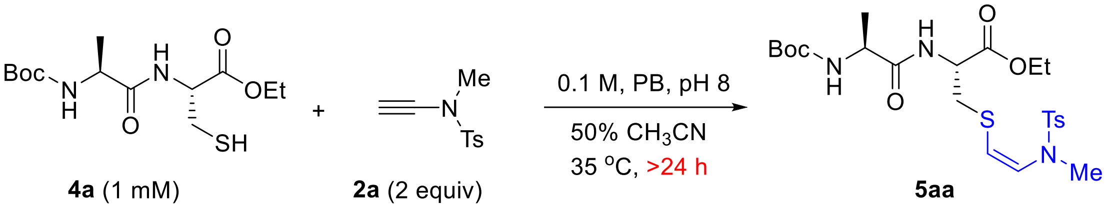


White solid, 99%, 572.2 mg, purified by recrystallization. ^{1}H NMR (400 MHz, DMSO) δ 9.01 (s, 1H), 8.25 (d, *J* = 3.8 Hz, 1H), 8.15 (t, *J* = 8.1 Hz, 2H), 7.96 (d, *J* = 8.2 Hz, 2H), 7.56 (d, *J* = 5.1 Hz, 1H), 7.22 – 6.95 (m, 5H), 4.65 (d, *J* = 6.0 Hz, 1H), 4.52 (d, *J* = 7.0 Hz, 1H), 4.36 – 4.18 (m, 1H), 4.19 – 3.93 (m, 2H), 3.32 – 3.19 (m, 1H), 3.09 (s, 2H), 3.00 (dd, *J* = 13.4, 7.6 Hz, 1H), 2.82 (dd, *J* = 13.5, 6.2 Hz, 1H), 2.70 (dd, *J* = 15.0, 9.2 Hz, 3H), 2.52 (s, 1H), 2.43 – 2.30 (m, 1H), 2.20 (t, *J* = 8.3 Hz, 1H), 1.80 (s, 1H), 1.53 (dd, *J* = 12.8, 9.0 Hz, 1H), 1.48 – 1.30 (m, 2H) ^{13}C NMR (101 MHz, DMSO) δ 172.2, 171.4, 171.3, 170.6, 170.5, 169.8, 157.1, 137.9, 129.7, 128.6, 126.7, 65.4, 57.6, 54.7, 52.7, 49.4, 43.7, 40.8, 37.4, 35.8, 28.4, 26.3. HRMS (ESI-TOF) m/z: $C_{24}H_{35}N_8O_7S^+$ $[M+H]^+$ Mass calc. 579.2344; Obs.: 579.2354.

3. Optimization of Reaction Conditions for Cys Modification with Ynamides

1.) *Preliminary study of the modification of peptide **4a** with ynamide **2a***

The peptide **4a** (0.01 mmol) and ynamide **2a** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 10 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. The peptide conjugate **5aa** was isolated in 85% after 24 h.



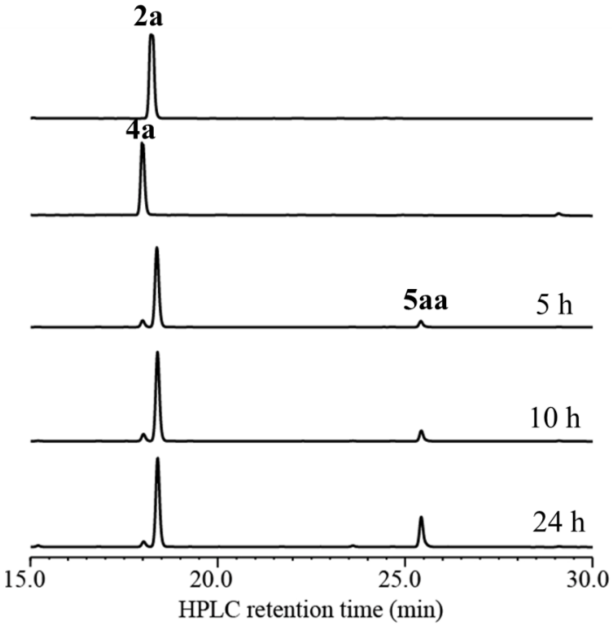


Figure S4. Preliminary study of the modification of peptide **4a** with ynamide **2a**

2) *The modification of peptide **4a** with ynamide **2l***

The peptide **4a** (0.01 mmol) and ynamide **2l** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 10 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient 30-95% solvent B in 30 min at 220 nm and LC-MS. No new products were detected within 24 h.

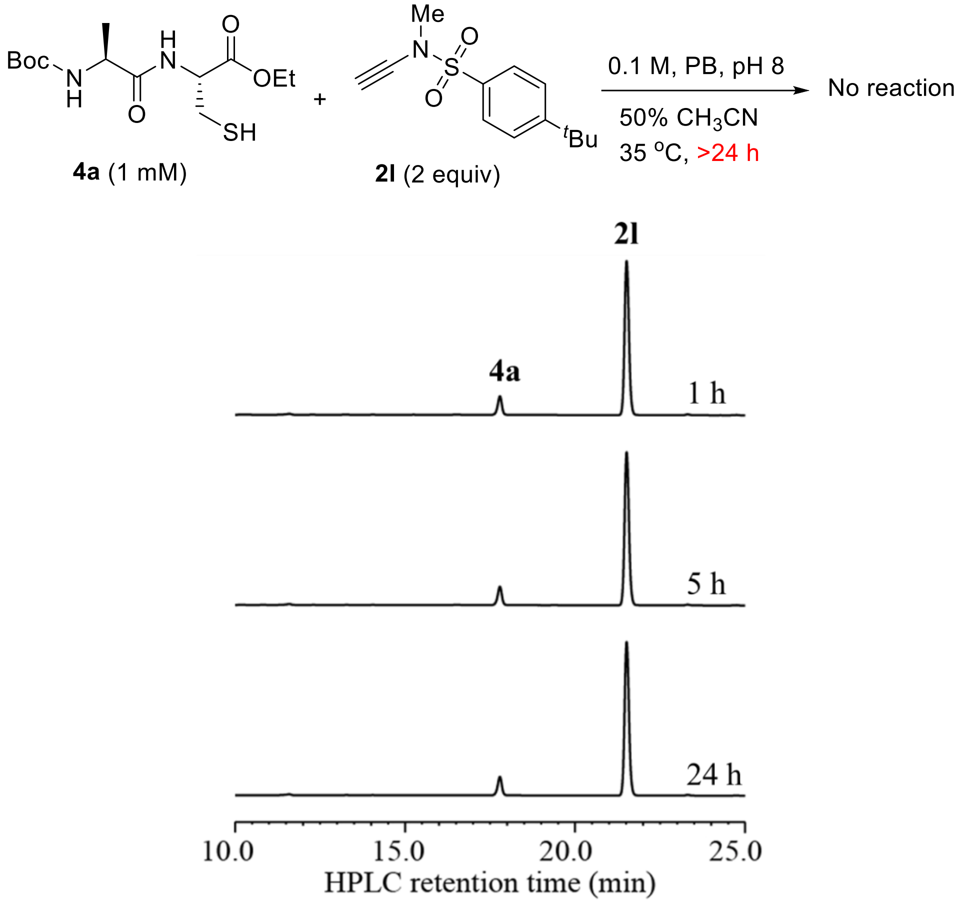
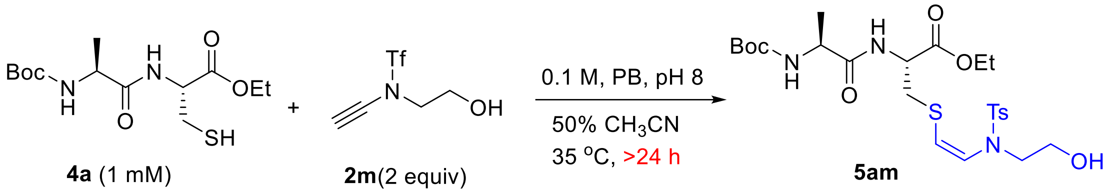


Figure S5. Preliminary study of the modification of peptide **4a** with ynamide **2l**

3) *The modification of peptide **4a** with ynamide **2m***

The peptide **4a** (0.01 mmol) and ynamide **2m** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 3 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient 30-95% solvent B in 30 min at 220 nm and LC-MS. The peptide conjugate **5am** was isolated in 89% after 24 h.



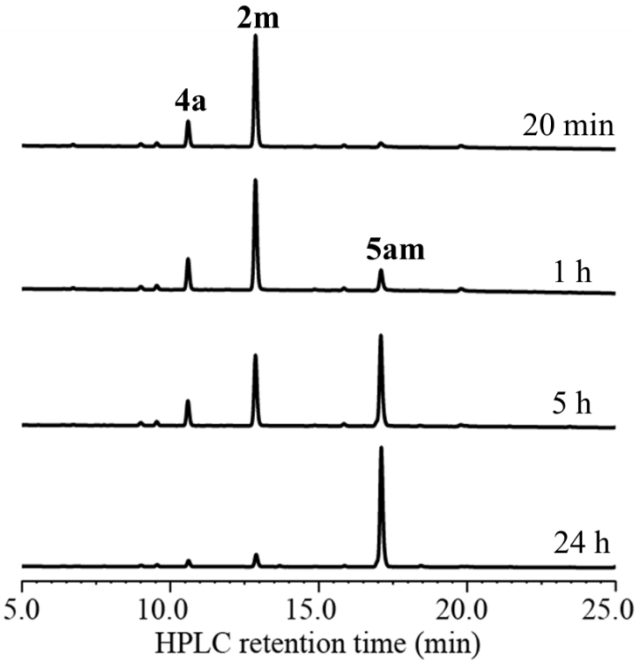
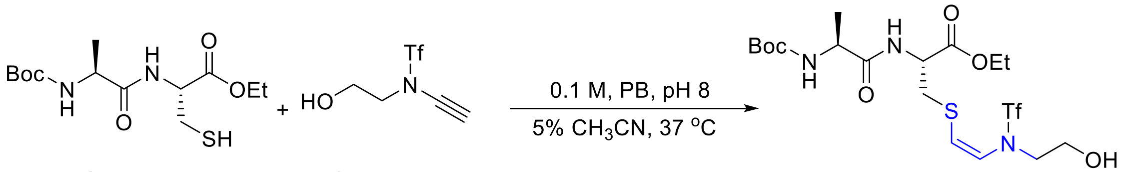


Figure S6 Preliminary study of the modification of peptide **4a** with ynamide **2m**

4) *The modification of peptide **4a** with ynamide **2n***

The peptide **4a** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. The peptide conjugate **5an** was isolated in 95% after 20 min.



4a (1 mM) **2n** (2 equiv) **5an**

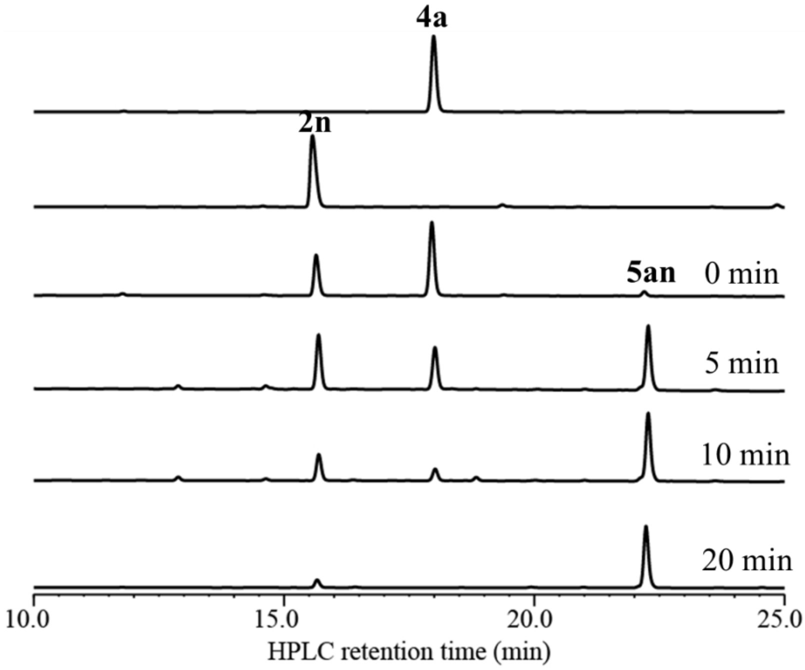


Figure S7. The modification reaction of peptide **4a** with ynamide **2n**

5) *The modification of peptide **4a** with ynamide **2o***

The peptide **4a** (0.01 mmol) and ynamide **2o** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 10 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. The peptide conjugate **5ao** was isolated in 95% after 20 min.

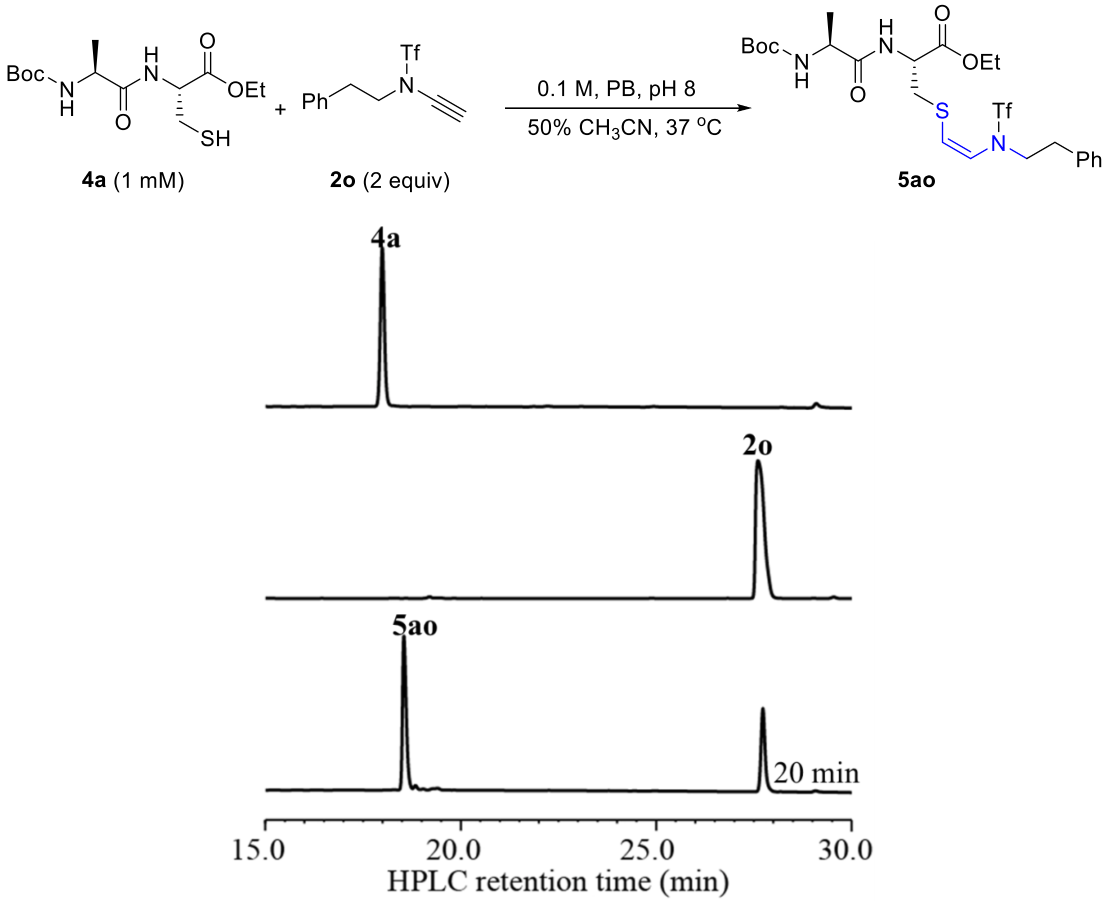


Figure S8. The modification reaction of peptide **4a** with ynamide **2o**

6) *The modification of peptide **4a** with ynamide **2p***

The peptide **4a** (0.01 mmol) and ynamide **2p** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 4 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. The peptide conjugate **5ap** was isolated in 94% after 20 min.

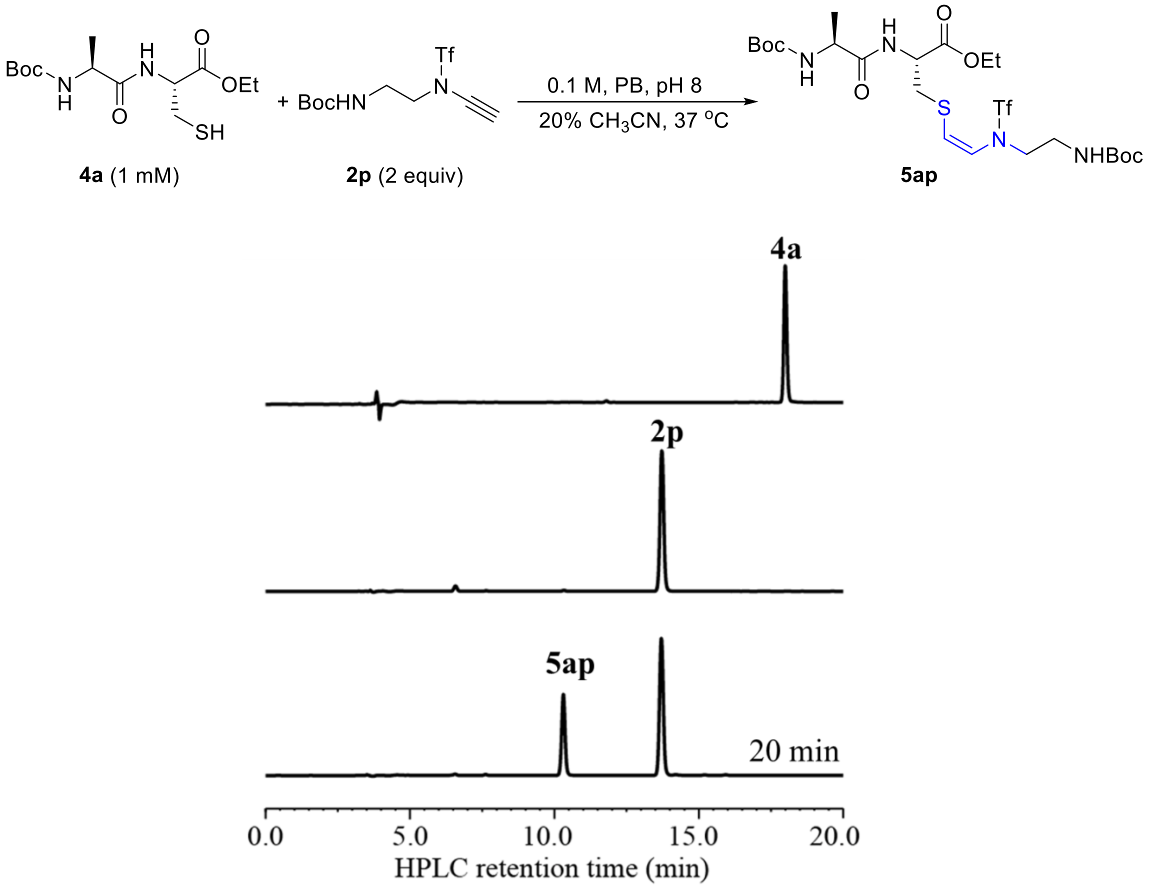
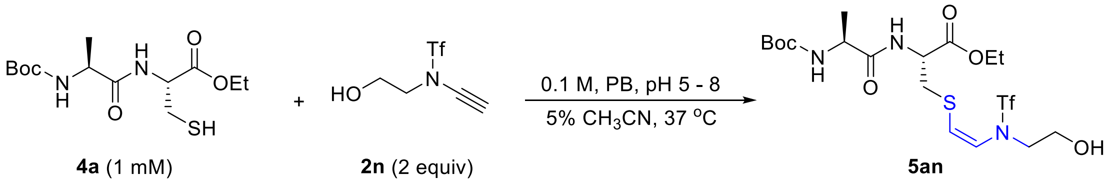


Figure S9. The modification reaction of peptide **4a** with ynamide **2p**

8) *The effect of pH on peptide modification with ynamide* ***2n***

The peptide **4a** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M), which pH were 5.0, 6.0, 7.0, and 8.0, respectively, and 0.5 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS.



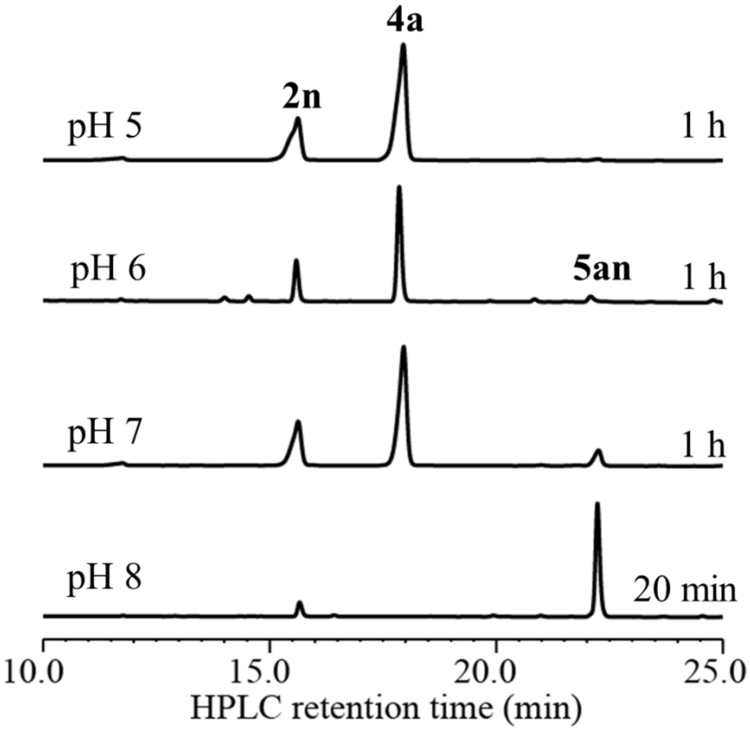
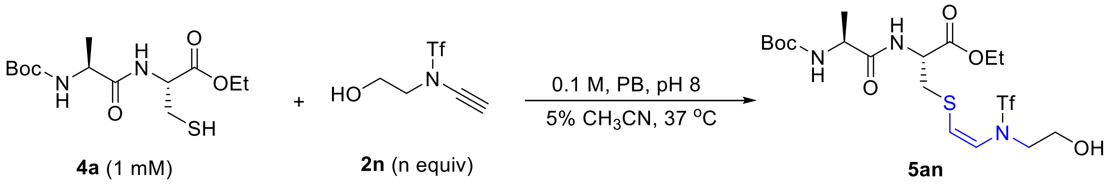


Figure S10. The effect of pH on peptide modification with ynamide **2n**

9) *The effect of the amount of ynamide **2n** on peptide modification*

The peptide **4a** (0.01 mmol) and ynamide **2n** (n equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. (n = 2.0, 3.0, 5.0, respectively)



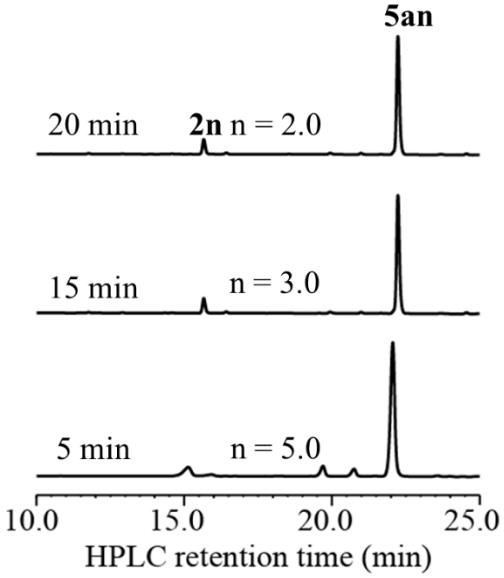


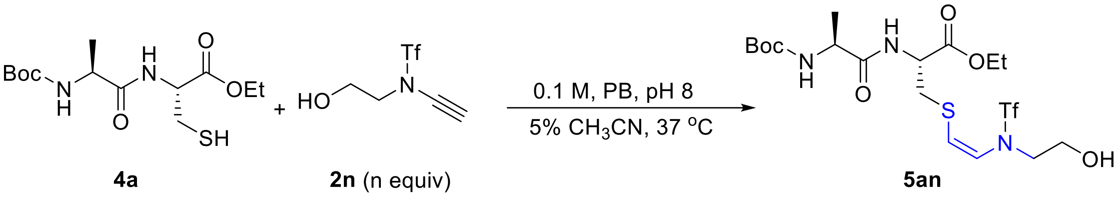
Figure S11. The effect of the amount of ynamide **2n** on peptide modification

10) *The effect of the concentration of reagents on peptide modification*

The peptide **4a** (0.01 mmol) and ynamide **2n** (n equiv) were dissolved in x mL PB (0.1 M pH 8.0) and y mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. (n = 2.0, 3.0, 5.0, respectively)

From top to bottom: 1. n = 2.0, x = 100, y = 5

2. n = 2.0, x = 200, y = 10
3. n = 2.0, x = 1000, y = 50
4. n = 5.0, x = 1000, y = 50



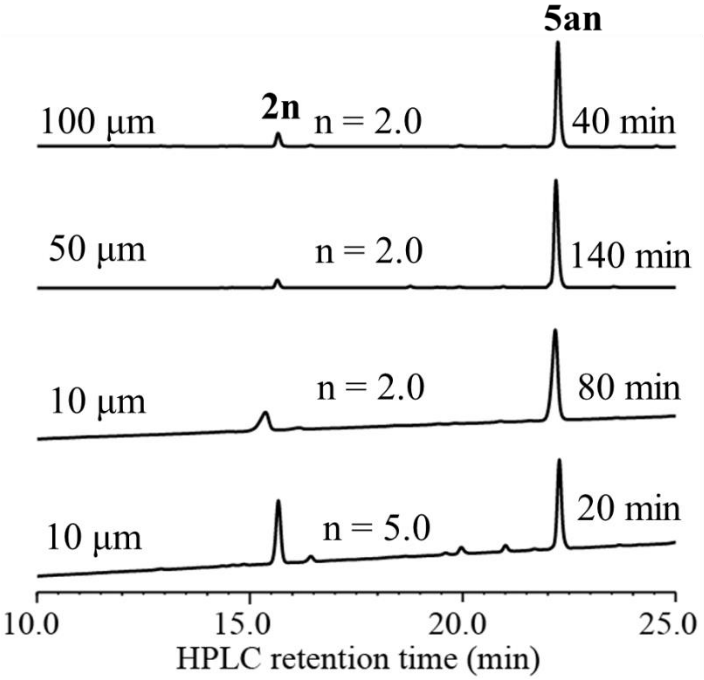
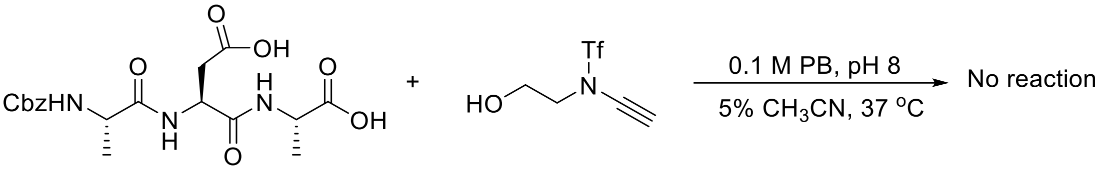


Figure S12. The effect of the concentration of reagents on peptide modification

4. Systematic Study of Cys Modification by Ynamides in the Presence of Other Amino Acid Residues

1) *Chemoselectivity study of ynamide **2n** in the presence of peptide **4b***

The peptide **4b** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. No new products were detected within 5 h.



4b (1 mM) **2n** (2 equiv)

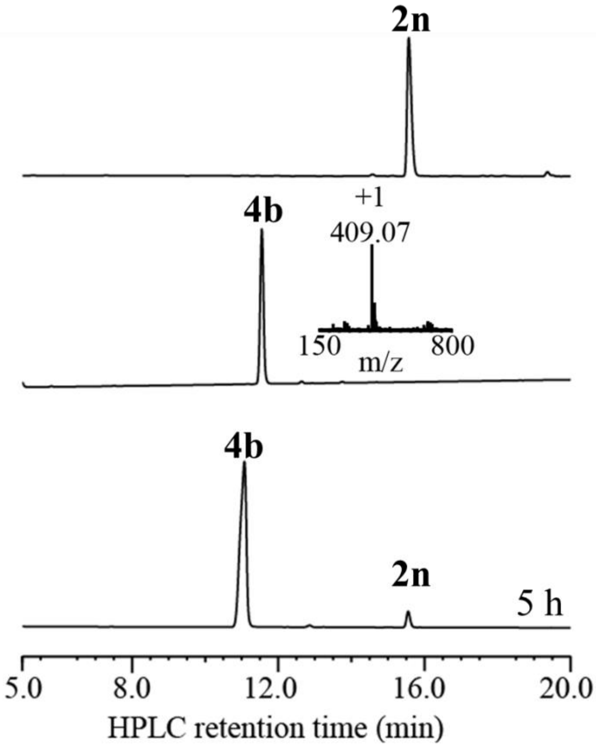
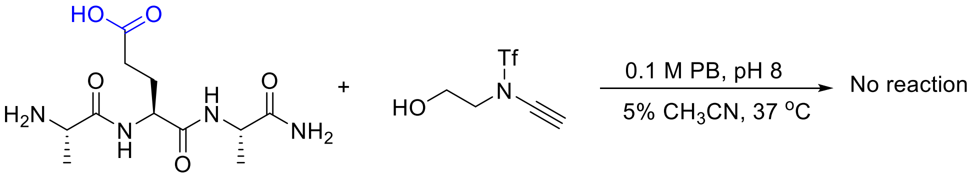


Figure S13. Chemoselectivity study of ynamide **2n** in the presence of peptide **4b**

2) *Chemoselectivity study of ynamide **2n** in the presence of peptide **4c***

The peptide **4c** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. No new products were detected within 5 h.



4c (1 mM)

2n (2 equiv)

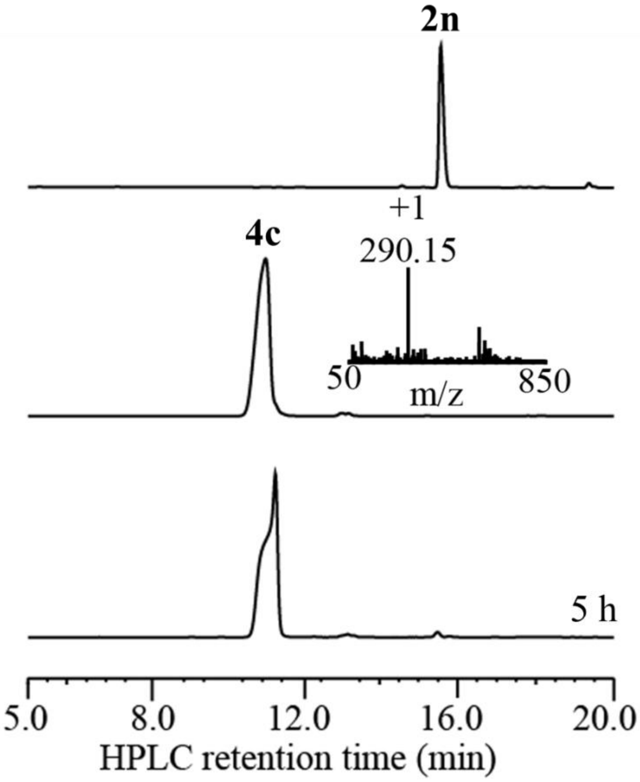
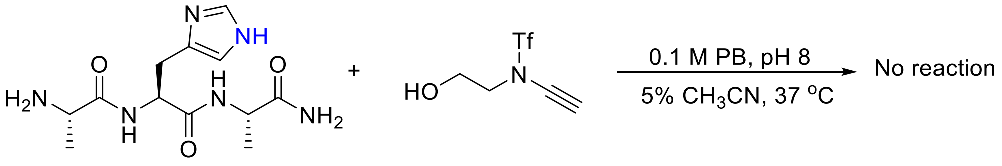


Figure S14. Chemoselectivity study of ynamide **2n** in the presence of peptide **4c**

3) *Chemoselectivity study of ynamide **2n** in the presence of peptide **4d***

The peptide **4d** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. No new products were detected within 5 h.



4d (1 mM)

2n (2 equiv)

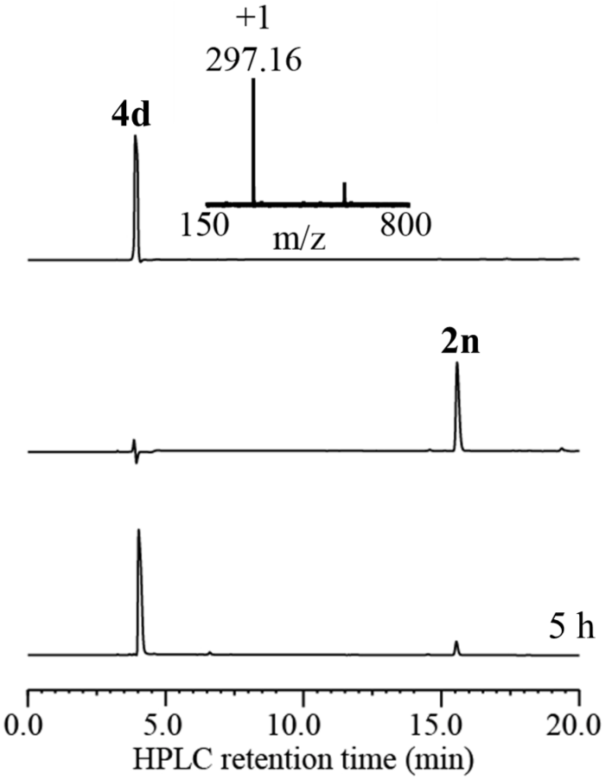
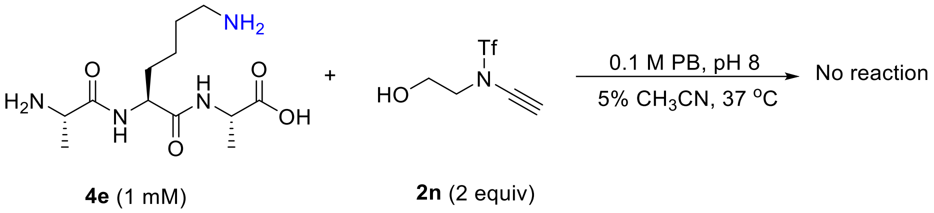


Figure S15. Chemoselectivity study of ynamide **2n** in the presence of peptide **4d**

4) *Chemoselectivity study of ynamide* ***2n*** *in the presence of peptide* ***4e***

The peptide **4e** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. No new products were detected within 5 h.



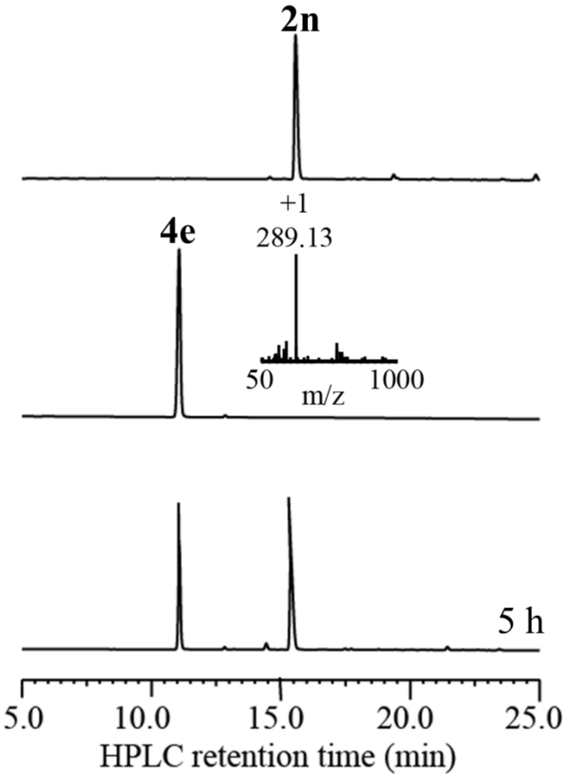
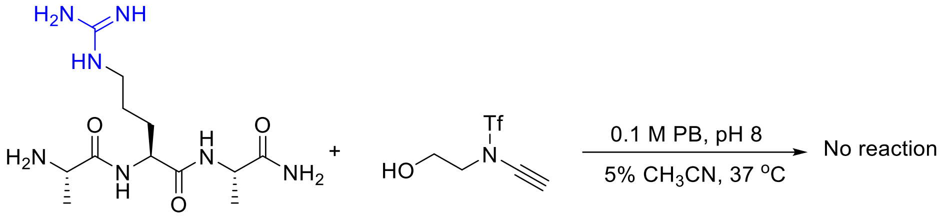


Figure S16. Chemoselectivity study of ynamide **2n** in the presence of peptide **4e**

5) *Chemoselectivity study of ynamide **2n** in the presence of peptide **4f***

The peptide **4f** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. No new products were detected within 5 h.



4f (1 mM)

2n (2 equiv)

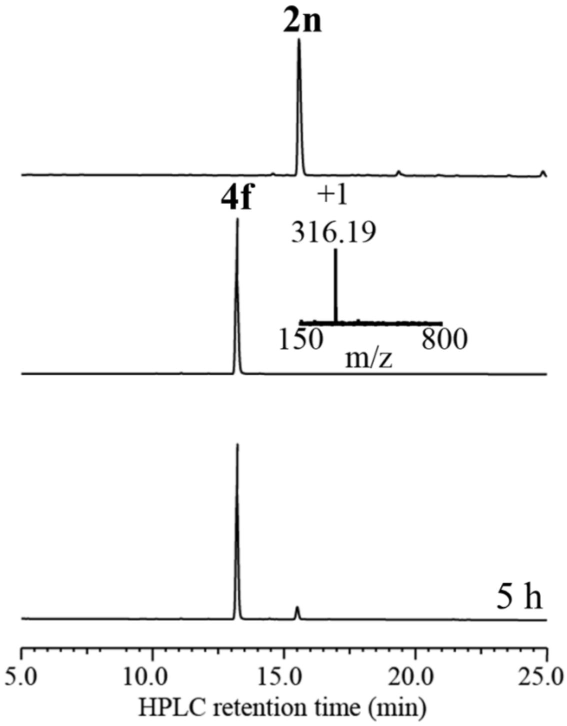
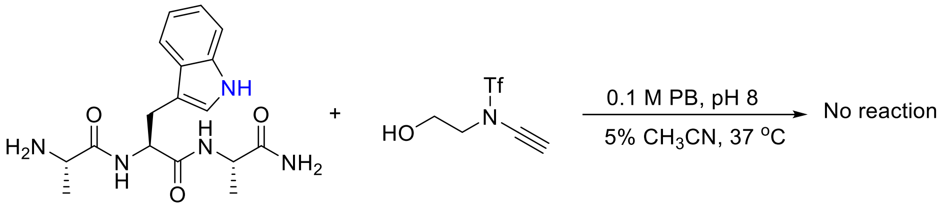


Figure S17. Chemoselectivity study of ynamide **2n** in the presence of peptide **4f**

6) *Chemoselectivity study of ynamide **2n** in the presence of peptide **4g***

The peptide **4g** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. No new products were detected within 5 h.



4g (1 mM) **2n** (2 equiv)

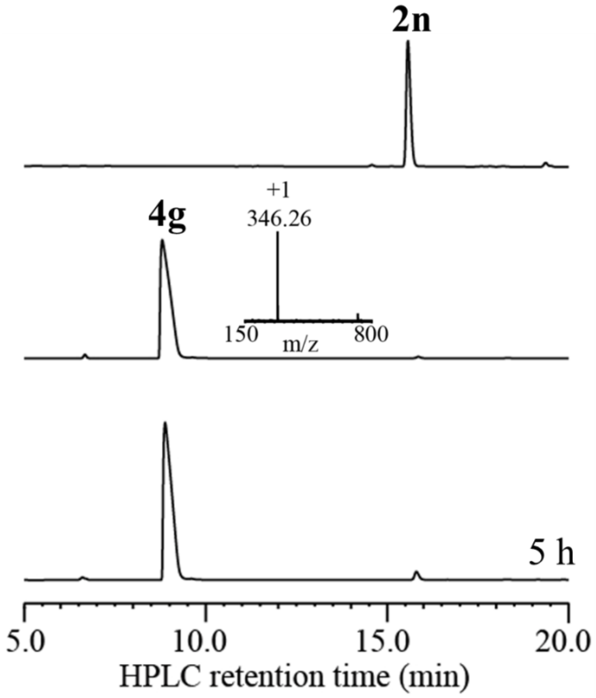
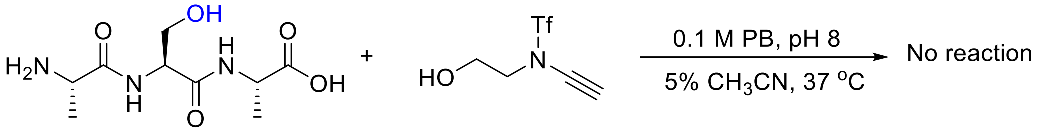


Figure S18. Chemoselectivity study of ynamide **2n** in the presence of peptide **4g**

7) *Chemoselectivity study of ynamide* ***2n*** *in the presence of peptide* ***4h***

The peptide **4h** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. No new products were detected within 5 h.



4h (1 mM)

2n (2 equiv)

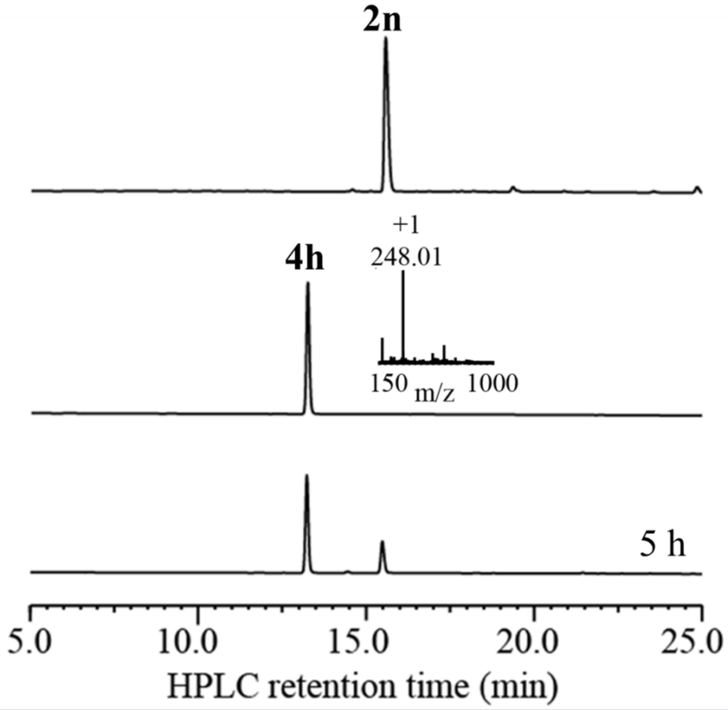
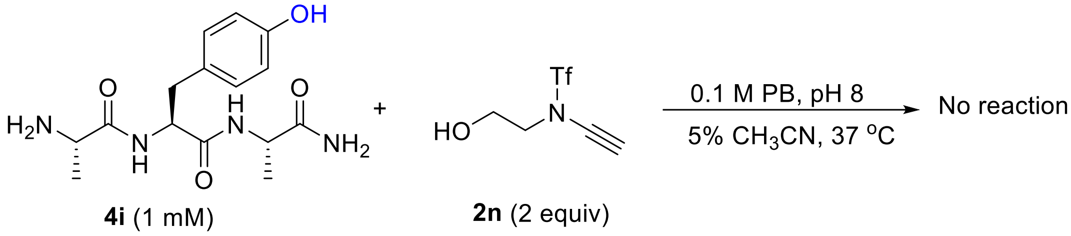


Figure S19. Chemoselectivity study of ynamide **2n** in the presence of peptide **4h**

8) *Chemoselectivity study of ynamide **2n** in the presence of peptide **4i***

The peptide **4i** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. No new products were detected within 5 h.



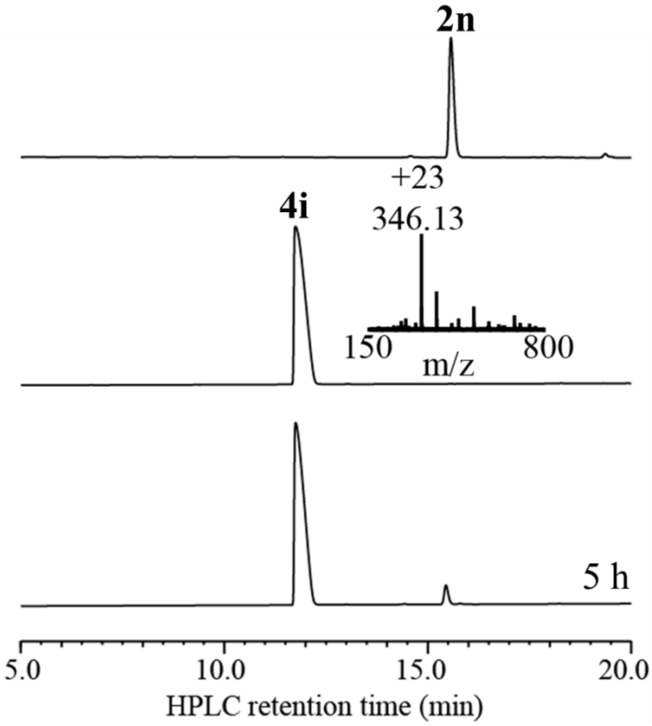
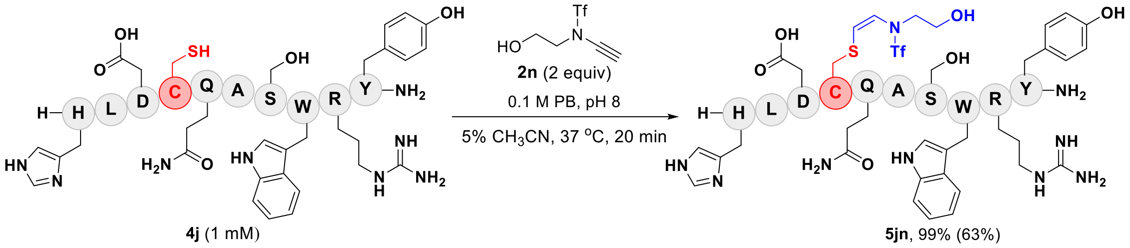


Figure S20. Chemoselectivity study of ynamide **2n** in the presence of peptide **4i**

9) *Chemoselectivity study of ynamide **2n** in the presence of peptide **4j***

The peptide **4j** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5jn** was isolated in 63% yield. The product was characterized by ESI-MS ($[M+H]^+$ Obs. 1509.99, Mass calc. 1509.60)



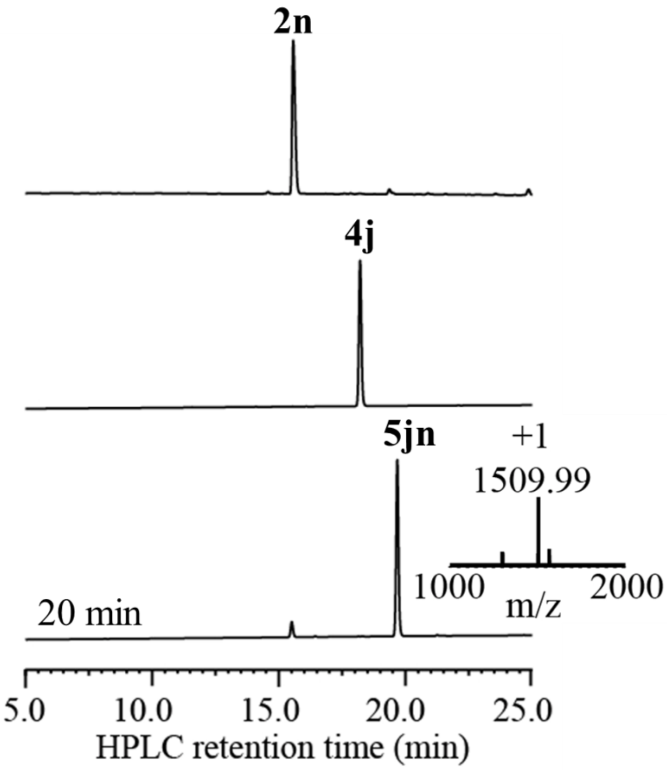
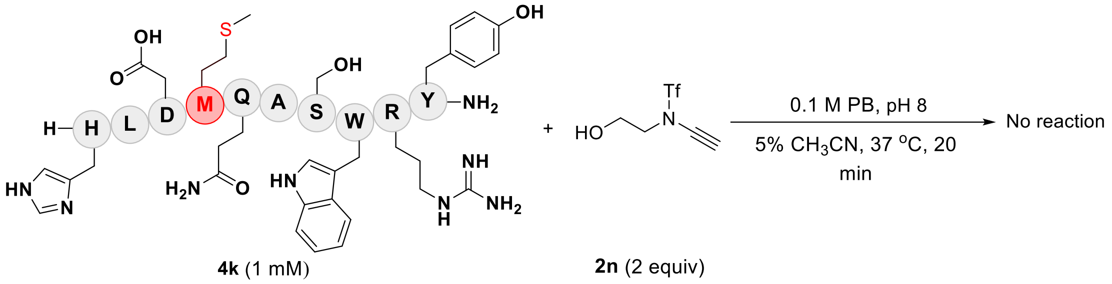


Figure S21. Chemoselectivity study of ynamide **2n** in the presence of peptide **4j**

10) *Chemoselectivity study of ynamide **2n** in the presence of peptide **4k***

The peptide **4k** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. No new products were detected within 24 h.



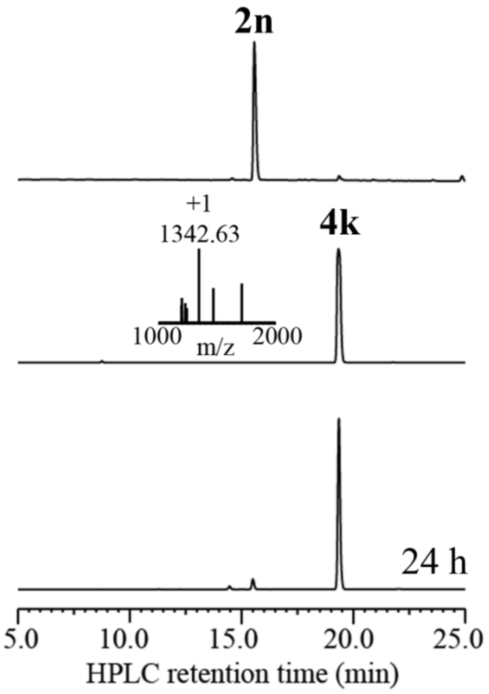


Figure S22. Chemoselectivity study of ynamide **2n** in the presence of peptide **4k**.

11) *The modification of peptide* ***4l*** *with ynamide* ***2n***

Peptide **4l** was synthesized as described in the general synthetic approach described previously. The crude peptide **4l** was purified by Prep RP-HPLC (C18 column) to obtain **5ln** in ~ 30% yield (mass Obs. 1937.5, Mass calc. 1936.8). The results are shown in **Figure S161**.

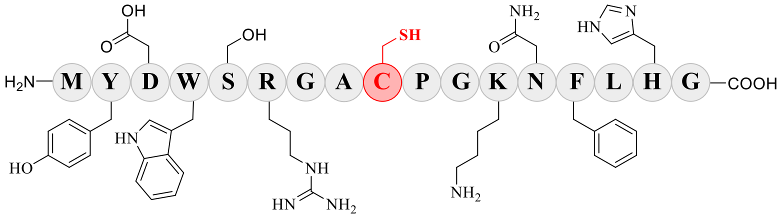
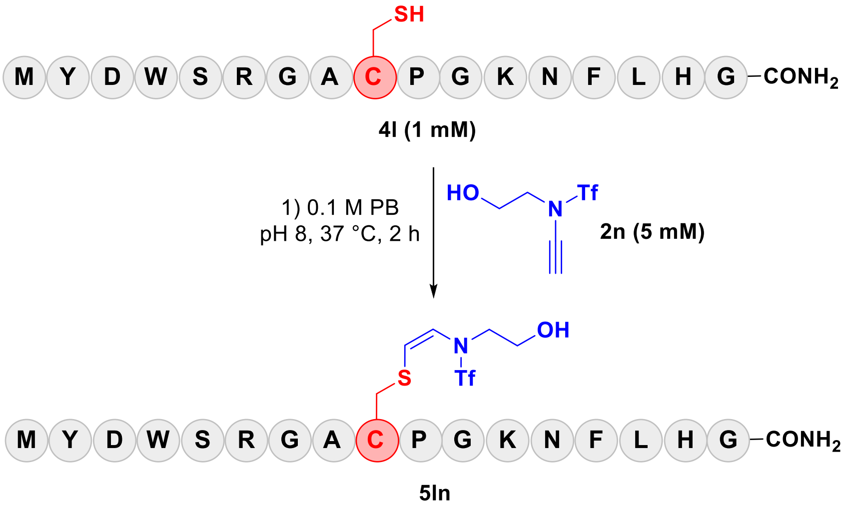


Figure S23. The sequence of peptide **4l**.

Peptide **4l** (3.8 mg, 1.96 μmol, 16.3 mM) was dissolved in 120 μL water as peptide **4l** stock solution. Ynamide **2n** (11.4 mg, 52.5 μmol, 105 mM) was dissolved in 500 μL MeCN as ynamide **2n** stock solution. 9.2 μL of peptide **4l** stock solution (0.15 μmol) and 7.1 μL of ynamide **2n** stock (5 equiv) were diluted in 133.7 μL PB (0.1 M, pH 8, final concentration of **4l** is 1mM). The reaction mixture was incubated at 37 °C, and the reaction progress was monitored by RP-HPLC (XSelect C18 column (3.5 μm, 130 Å, 4.6 × 150 mm) with a gradient of 5-70% B (0.1% TFA in MeCN) over 25 min at 220 nm (**Figure S24**). After 2 h, the reaction was completed to afford the corresponding product **5ln** with 91% (based on HPLC integration) and 62% isolated yield. (Mass Obs. 2154.5, Mass calc. 2153.9). The results are shown in **Figure S161**.



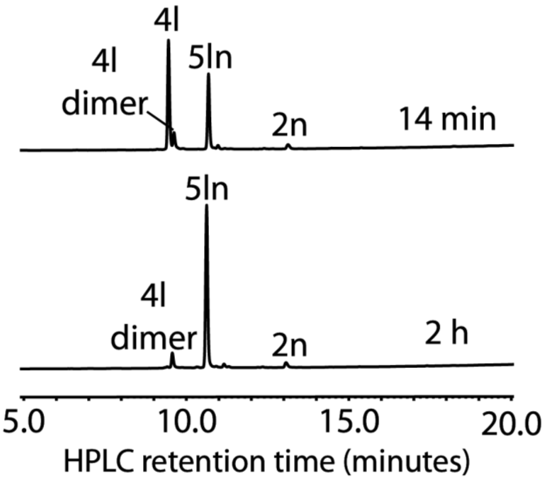


Figure S24. The modification reaction of model peptide **4l** with ynamide **2n**.

Trypsin digestion of modified peptide 5ln

The modified peptide **5ln** (1.8 mg, 0.83 μmol) was dissolved in 1.8 mL of Tris buffer (50 mM Tris-HCl, pH 8, 8 M urea) and incubated at 37 °C bath for 1 h. Then, reaction was removed from the heating bath and allowed to reach room temperature. The concentration of urea was reduced to 0.9 M by addition 14.5 mL of 50 mM NH_4HCO_3. 60 μL of trypsin stock (1 mL/mg) was then added to the reaction solution (as 1:30 ratio of trypsin:peptide), which was incubated at 37 °C for 15 min. Aliquots of the reaction were quenched by formic acid in water and checked by RP-HPLC (XSelect C18 column (3.5 μm, 130 Å, 4.6 × 150 mm) with a gradient of 5-70% B (0.1% TFA in MeCN) over 20 min at 220 nm) The results are shown in **Figures S25**.

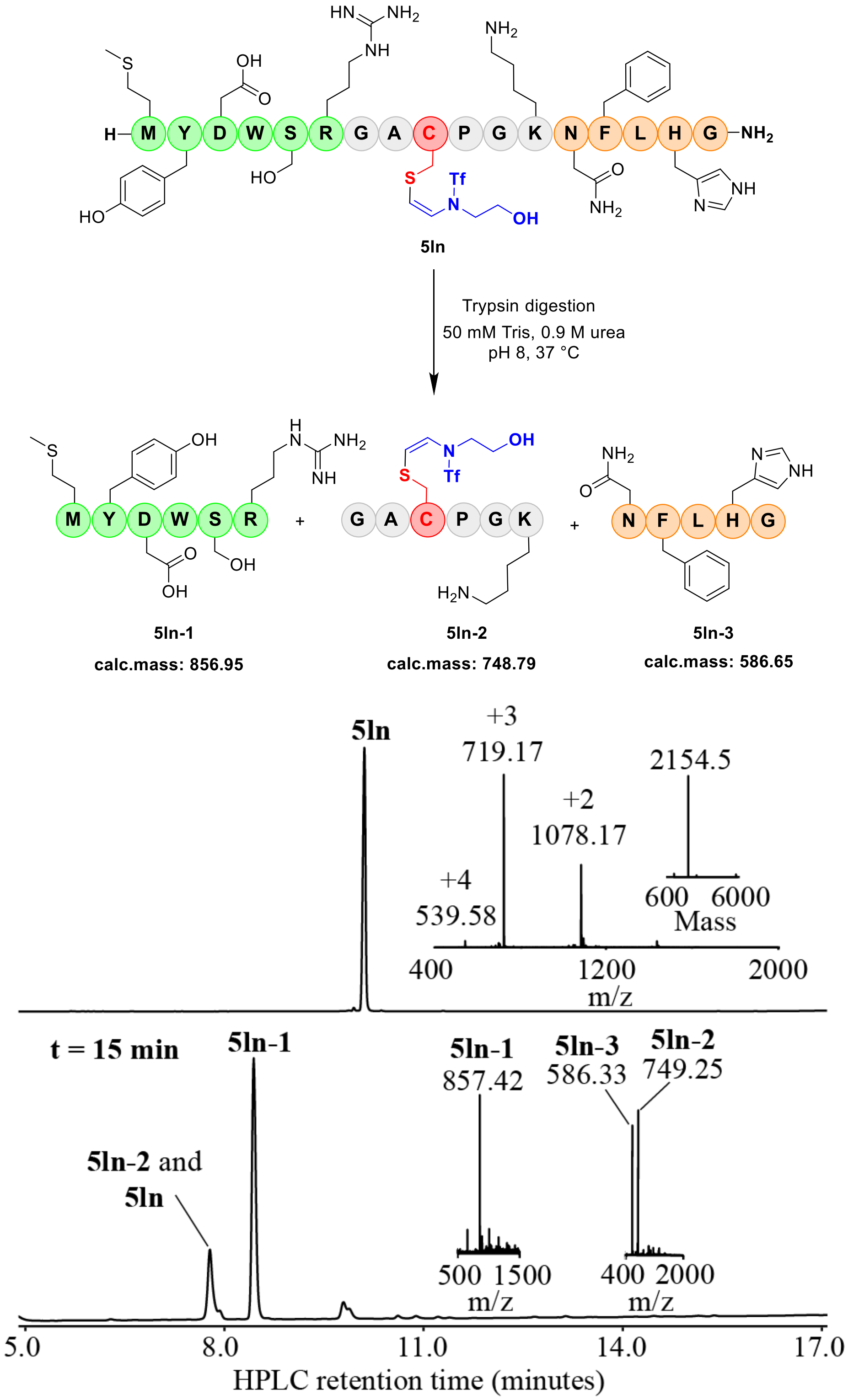
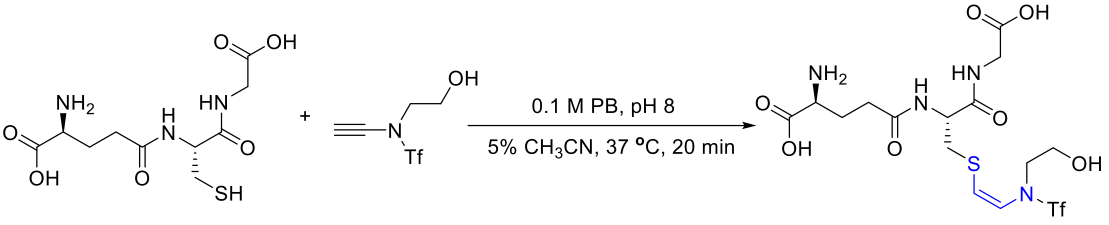


Figure S25. Trypsin digestion of the modified peptide **5ln**.

12) *The modification of peptide* ***4m*** *with ynamide* ***2n***

The peptide **4m** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5mn** was isolated in 87% yield. The product was characterized by ^1H NMR (400 MHz, D_2O) δ 6.57 (d, *J* = 6.8 Hz, 1H), 6.05 (d, *J* = 6.7 Hz, 1H), 4.57 (dd, *J* = 8.6, 5.1 Hz, 1H), 3.99 (dd, *J* = 11.0, 4.4 Hz, 1H), 3.96 (s, 2H), 3.75 – 3.52 (m, 4H), 3.29 (dd, *J* = 14.5, 5.1 Hz, 1H), 3.08 (dd, *J* = 14.5, 8.7 Hz, 1H), 2.62 – 2.44 (m, 2H), 2.26 – 2.08 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 174.3, 172.9, 172.0, 171.8, 133.4, 120.3, 119.8 (q, J = 323.0 Hz), 58.4, 53.7, 52.5, 52.3, 41.2, 34.7, 31.1, 25.6; HRMS (ESI-TOF) m/z: $C_{15}H_{24}F_3N_4O_9S_2^-$ $[M+H]^-$ Mass calc. 525.0931; Obs.: 525.0937.



4m (1 mM) **2n** (2 equiv) **5mn**

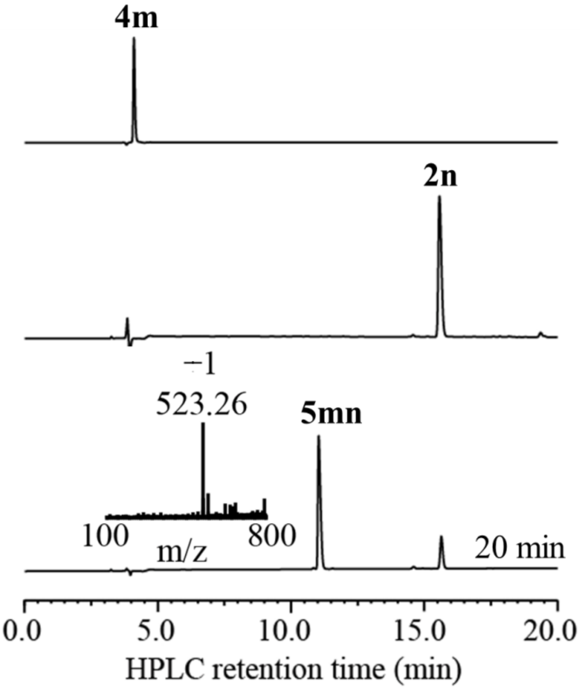
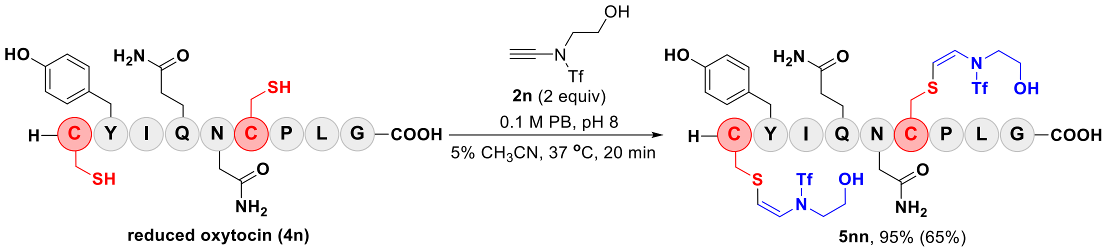


Figure S26. The modification of peptide **4m** with ynamide **2n**.

13) *The modification of reduced oxytocin* ***(4n)*** *with ynamide* ***2n***

The peptide **4n** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5nn** was isolated in 65% yield. The product was characterized by ESI-MS ($[M+H]^+$ Obs. 1443.17, Mass calc. 1443.46)



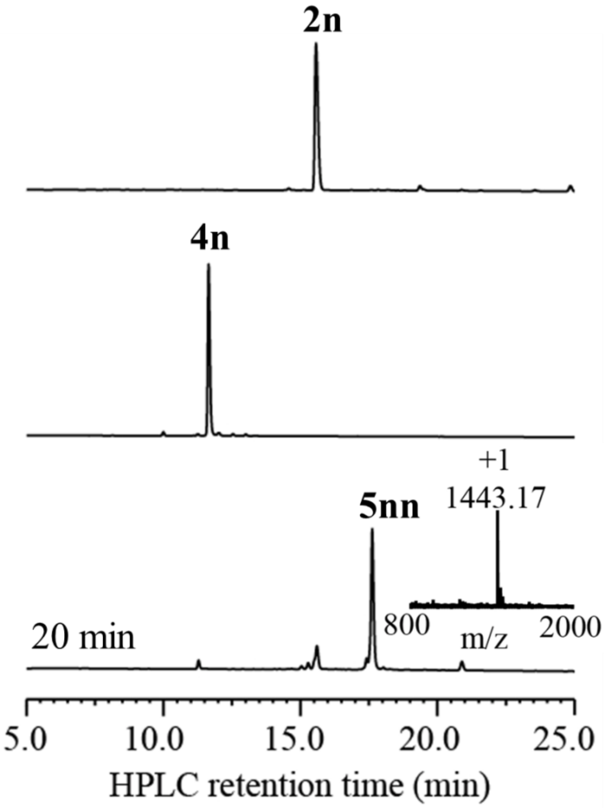
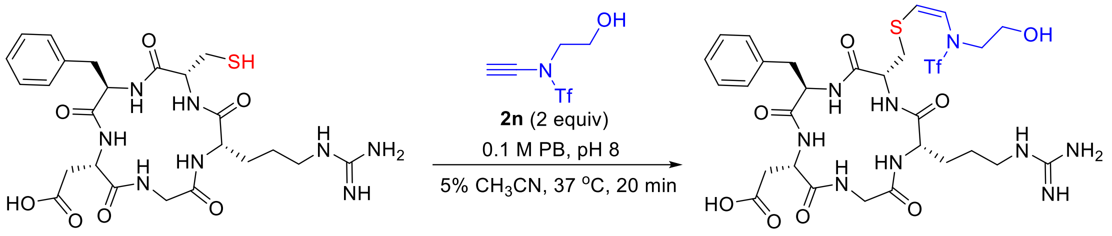


Figure S27. The modification of reduced oxytocin **4n** with ynamide **2n**.

14) *The modification of cyclic peptide **4o** with ynamide **2n***

The peptide **4o** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5on** was isolated in 76% yield. The product was characterized by ESI-MS ($[M + K]^+$ Obs. 834.17, Mass calc. 834.19)



4o (1 mM)

5on, 99% (76%)

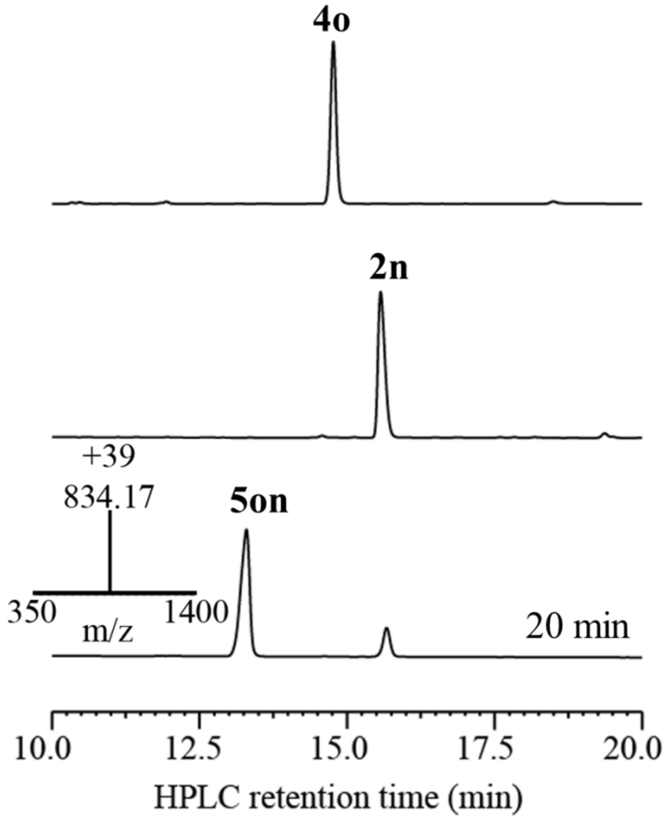
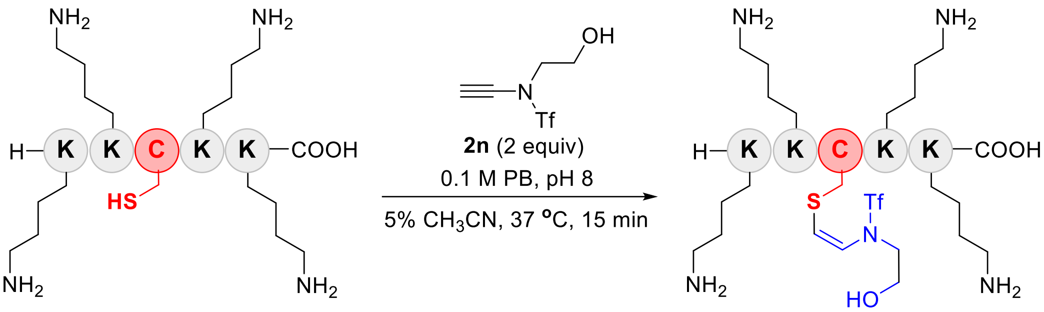


Figure S28. The modification of cyclic peptide **4o** with ynamide **2n**.

15) *The modification of peptide **4p** with ynamide **2n***

The peptide **4p** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5pn** was isolated in 76% yield. The product was characterized by ESI-MS ($[M+H]^+$ Obs. 851.44, Mass calc. 851.41)



4p (1 mM)

5pn, 99% (76%)

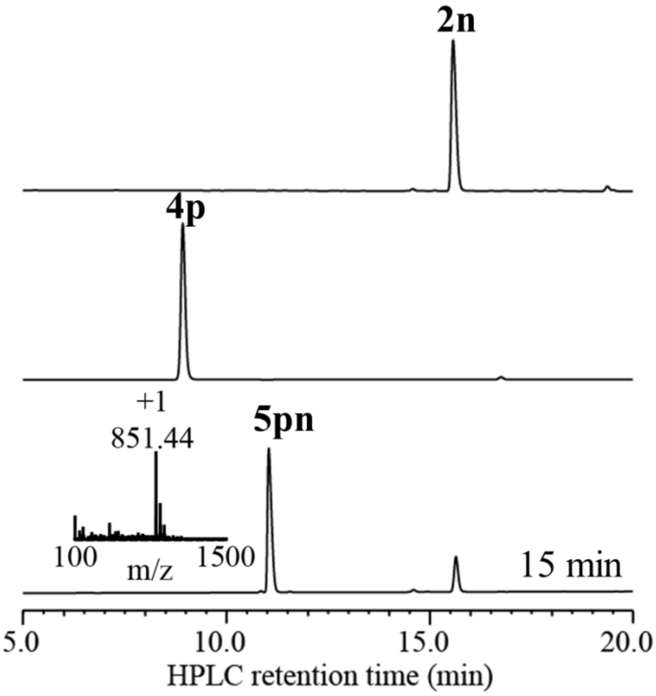
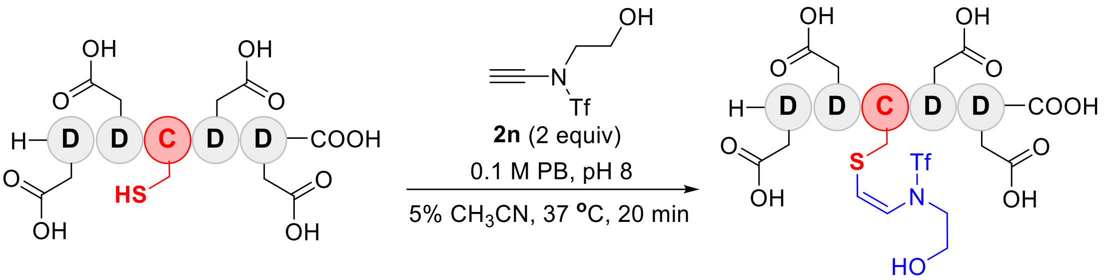


Figure S29. The modification of peptide **4p** with ynamide **2n**.

16) *The modification of peptide **4q** with ynamide **2n***

The peptide **4q** (0.01 mmol) and ynamide **2n** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5qn** was isolated in 70% yield. The product was characterized by ESI-MS ($[M-H]^-$ Obs. 797.32, Mass calc. 797.12)



4q (1 mM)

5qn, 99% (70%)

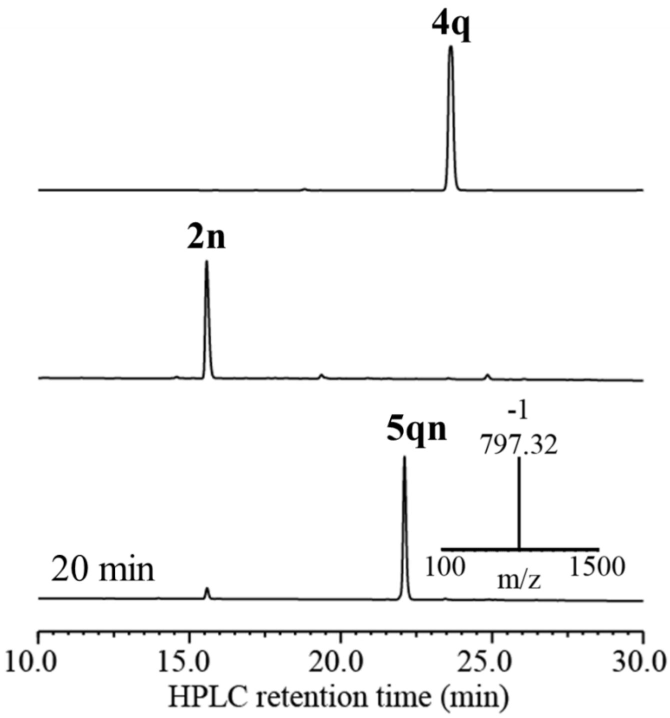


Figure S30. The modification of peptide **4q** with ynamide **2n**.

17) *The modification of peptide **4a** with ynamide **2q***

The peptide **4a** (0.1 mmol) and ynamide **2q** (0.2 mmol, 2 equiv) were dissolved in 100 mL PB (0.1 M pH 8.0) and 100 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5aq** was isolated in 92% yield. The product was characterized by ^1H NMR (400 MHz, $CDCl_3$) δ 9.18 (t, *J* = 5.7 Hz, 1H), 8.99 (s, 1H), 7.80 (d, *J* = 7.8 Hz, 1H), 7.69 (t, *J* = 7.8 Hz, 1H), 7.52 – 7.33 (m, 2H), 6.28 (d, *J* = 6.9 Hz, 1H), 5.98 (d, *J* = 6.9 Hz, 1H), 5.63 (d, *J* = 5.8 Hz, 1H), 4.90 (s, 1H), 4.49 (s, 1H), 4.21 (m, 2H), 3.82 (d, *J* = 5.0 Hz, 2H), 3.71 (s, 2H), 3.53 – 3.27 (m, 2H), 1.45 (d, *J* = 11.2 Hz, 3H), 1.40 (s, 9H), 1.29 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.4, 169.5, 162.4, 161.4, 155.3, 154.5, 149.1, 134.3, 132.4, 130.1, 125.3, 119.9 (q, J = 323.0 Hz), 119.7, 118.7, 117.9, 116.7, 79.7, 62.1, 53.1, 50.1, 48.5, 38.5, 36.6, 28.3, 18.8, 14.1; HRMS (ESI-TOF) m/z: $C_{28}H_{36}F_3N_4O_{10}S_2^+$ $[M+H]^+$ Mass calc. 709.1819; Obs.: 709.1823.

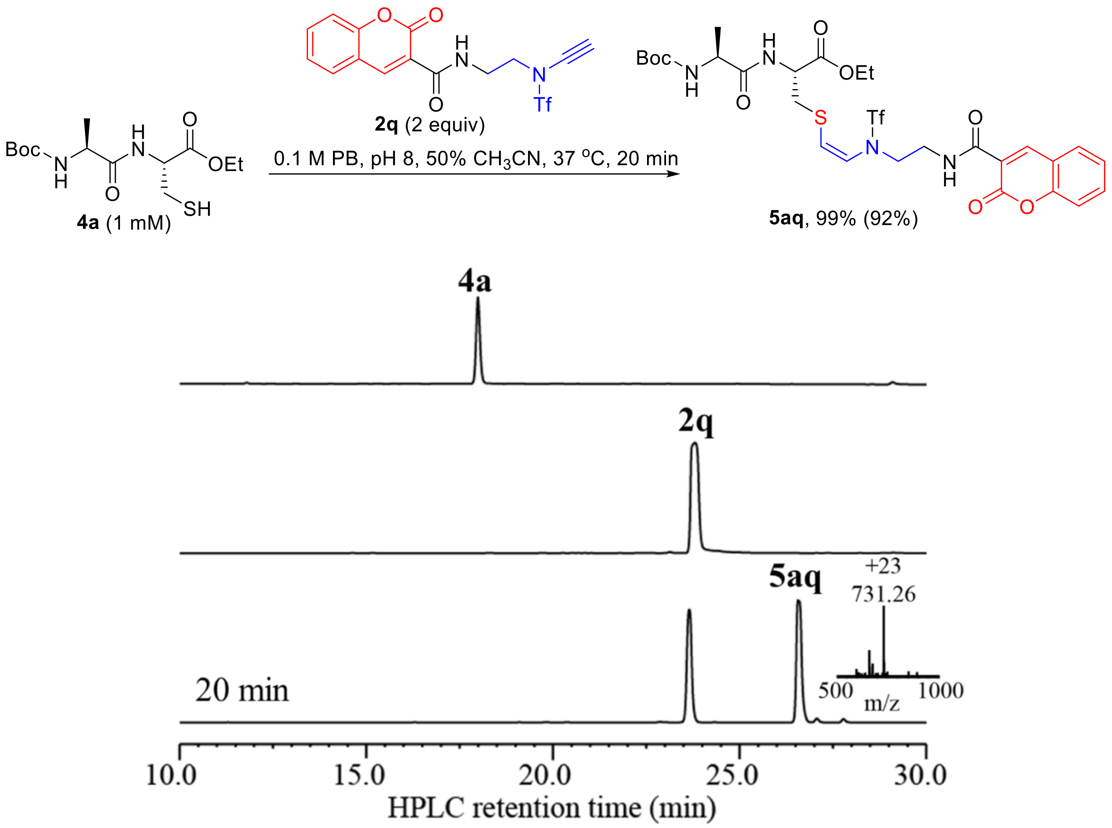


Figure S31. The modification of peptide **4a** with ynamide **2q**.

18) *The modification of peptide **4a** with ynamide **2r***

The peptide **4a** (0.1 mmol) and ynamide **2r** (0.2 mmol, 2 equiv) were dissolved in 100 mL PB (0.1 M pH 8.0) and 100 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient 30-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5ar** was isolated in 95% yield. The product was characterized by ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (d, *J* = 8.1 Hz, 2H), 7.47 (d, *J* = 8.1 Hz, 2H), 7.11 (s, 1H), 6.99 (s, 1H), 6.90 (d, *J* = 8.9 Hz, 1H), 6.67 (d, *J* = 9.0 Hz, 1H), 6.18 (d, *J* = 6.2 Hz, 1H), 5.87 (d, *J* = 6.7 Hz, 1H), 5.22 (s, 1H), 4.76 (d, *J* = 4.6 Hz, 1H), 4.21 (d, *J* = 6.8 Hz, 5H), 3.83 (s, 5H), 3.75 (s, 2H), 3.34 – 3.07 (m, 2H), 2.38 (s, 3H), 1.43 (s, 9H), 1.33 (d, *J* = 7.1 Hz, 3H), 1.27 (d, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.0, 169.5, 168.3, 156.1, 139.3, 136.2, 133.9, 132.0, 131.2, 130.9, 130.7, 129.1, 119.9 (q, J = 323.0 Hz),119.5, 114.9, 112.0, 111.6, 101.5, 80.1, 62.2, 61.1, 55.7, 52.9, 50.2, 48.1, 36.1, 30.1, 28.3, 18.1, 14.1, 13.3; HRMS (ESI-TOF) m/z: $C_{37}H_{45}ClF_3N_4O_{11}S_2^+$ $[M+H]^+$ Mass calc. 877.2161; Obs.: 877.2164.

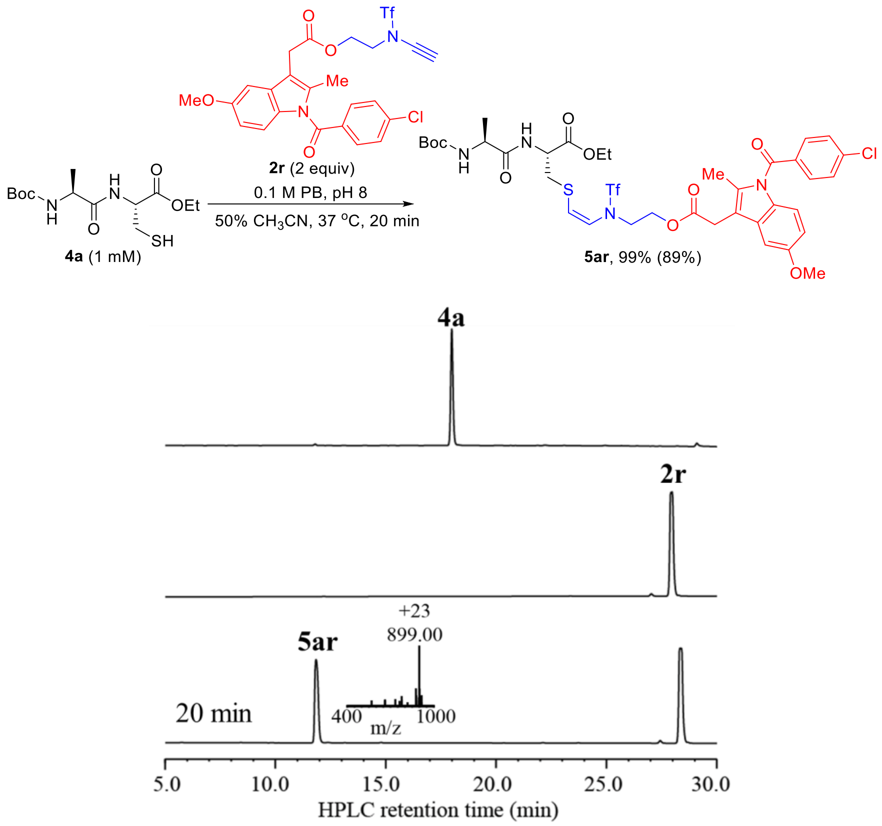


Figure S32. The modification of peptide **4a** with ynamide **2r**.

19) *The modification of peptide **4a** with ynamide **2s***

The peptide **4a** (0.1 mmol) and ynamide **2s** (0.2 mmol, 2 equiv) were dissolved in 100 mL PB (0.1 M pH 8.0) and 5 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5as** was isolated in 85% yield. The product was characterized by ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 (s, 1H), 6.33 (d, *J* = 6.2 Hz, 2H), 6.12 – 5.86 (m, 2H), 5.57 (s, 1H), 4.79 (d, *J* = 5.4 Hz, 1H), 4.51 (s, 1H), 4.32 (s, 1H), 4.23 (d, *J* = 6.4 Hz, 4H), 3.85 (s, 2H), 3.24 (m, 3H), 2.91 (dd, *J* = 12.4, 4.0 Hz, 1H), 2.75 (d, *J* = 12.7 Hz, 1H), 2.38 (t, *J* = 7.0 Hz, 2H), 1.68 (d, *J* = 6.5 Hz, 4H), 1.46 (d, *J* = 18.0 Hz, 9H), 1.36 (d, *J* = 6.7 Hz, 3H), 1.30 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.5, 173.4, 169.7, 164.1, 155.6, 132.0, 119.7 (q, J = 323.0 Hz), 119.7, 79.9, 62.2, 62.1, 60.7, 60.1, 55.6, 53.0, 50.1, 48.2, 40.5, 36.1, 33.8, 29.6, 28.4, 28.3, 28.2, 24.6, 18.2, 14.1. HRMS (ESI-TOF) m/z: $C_{27}H_{46}F_3N_6O_{10}S_3^+$ $[M+H]^+$ Mass calc. 764.2275; Obs.: 764.2296.

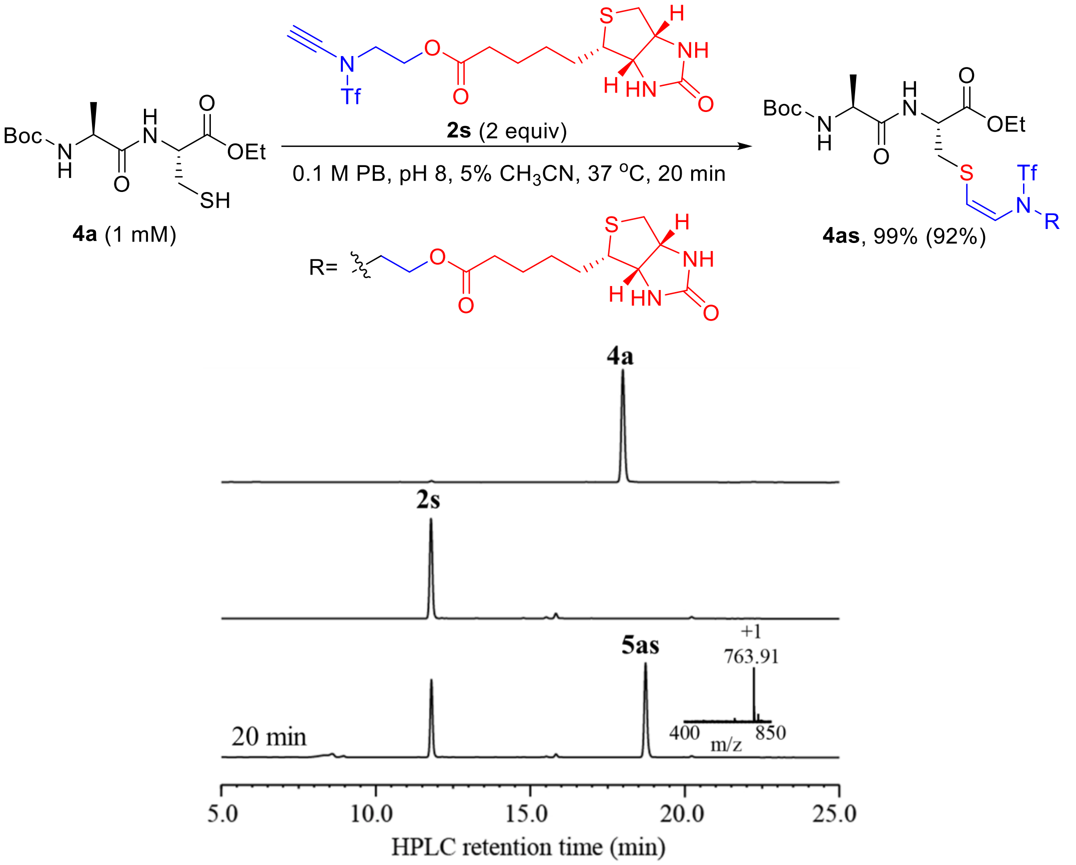


Figure S33. The modification of peptide **4a** with ynamide **2s**.

20) *The modification of peptide* ***4a*** *with ynamide* ***2t***

The peptide **4a** (0.1 mmol) and ynamide **2t** (0.2 mmol, 2 equiv) were dissolved in 100 mL PB (0.1 M pH 8.0) and 10 mL DMSO at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5at** was isolated in 92% yield. The product was characterized by ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.07 (d, *J* = 6.0 Hz, 1H), 6.37 (d, *J* = 6.6 Hz, 1H), 6.11 (d, *J* = 6.6 Hz, 1H), 5.12 (d, *J* = 7.0 Hz, 1H), 4.95 – 4.70 (m, 1H), 4.42 (q, *J* = 18.0 Hz, 2H), 4.23 (m, 3H), 3.30 (m, 2H), 2.46 (s, 1H), 1.45 (s, 9H), 1.37 (d, *J* = 6.9 Hz, 3H), 1.30 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.8, 169.5, 155.4, 133.0, 120.0, 119.7 (q, J = 323.0 Hz), 80.2, 76.1, 74.6, 62.3, 53.0, 50.3, 39.4, 36.2, 28.3, 18.1, 14.0. HRMS (ESI-TOF) m/z: $C_{19}H_{29}F_3N_3O_7S_2^+$ $[M+H]^+$ Mass calc. 532.1394; Obs.: 532.1395.

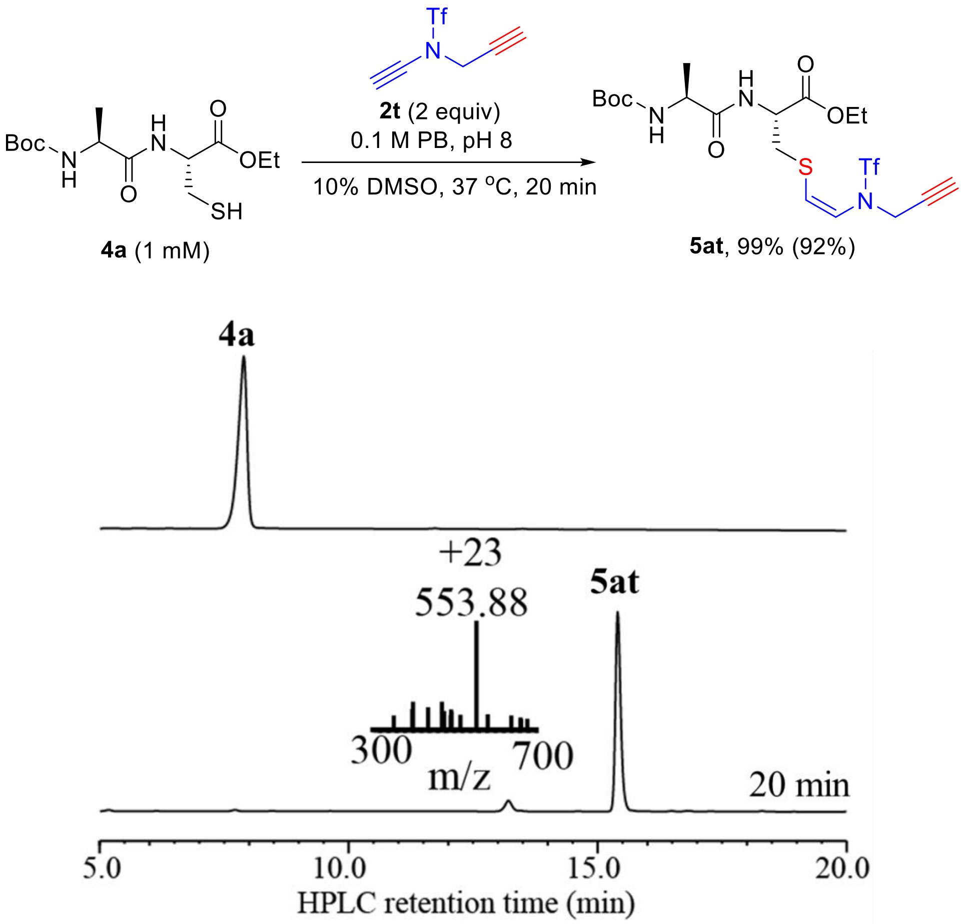
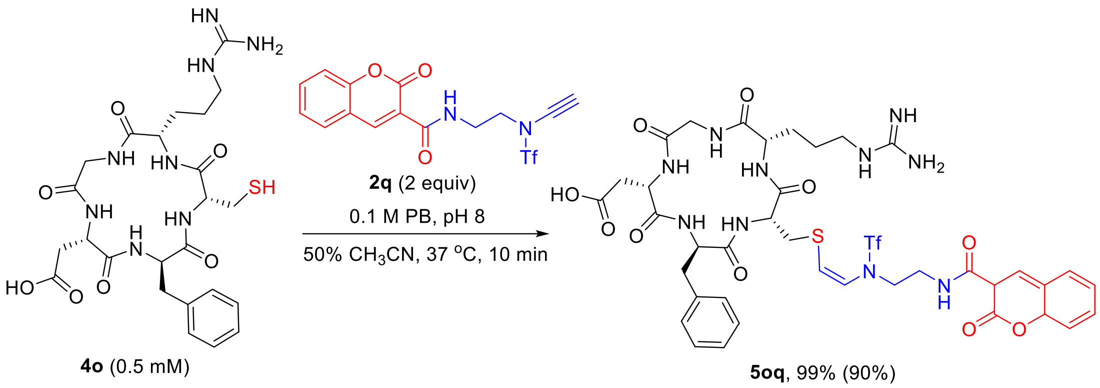


Figure S34. The modification of peptide **4a** with ynamide **2t**.

21) *The modification of cyclic peptide **4o** with ynamide **2q***

The peptide **4o** (0.1 mmol) and ynamide **2q** (0.2 mmol, 2 equiv) were dissolved in 200 mL PB (0.1 M pH 8.0) and 200 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 10 min, the product **5oq** was isolated in 90% yield. The product was characterized by ^{1}H NMR (400 MHz, DMSO) δ 8.98 (t, *J* = 5.8 Hz, 1H), 8.92 (s, 1H), 8.37 – 8.24 (m, 1H), 8.17 (t, *J* = 7.9 Hz, 2H), 8.06 – 7.96 (m, 2H), 7.78 (t, *J* = 7.8 Hz, 1H), 7.59 (s, 1H), 7.56 – 7.42 (m, 2H), 7.24 (m, 6H), 6.76 (d, *J* = 6.8 Hz, 1H), 6.13 (d, *J* = 6.7 Hz, 1H), 4.67 (d, *J* = 6.0 Hz, 1H), 4.53 (d, *J* = 7.0 Hz, 1H), 4.26 (d, *J* = 4.9 Hz, 1H), 4.21 – 4.07 (m, 1H), 3.78 (t, *J* = 5.3 Hz, 2H), 3.55 (dd, *J* = 11.1, 5.5 Hz, 2H), 3.28 (dd, *J* = 15.0, 3.1 Hz, 1H), 3.02 (dd, *J* = 13.5, 7.6 Hz, 1H), 2.82 (dd, *J* = 14.7, 7.3 Hz, 2H), 2.78 – 2.65 (m, 2H), 2.39 (dd, *J* = 16.1, 5.5 Hz, 1H), 2.22 (t, *J* = 8.4 Hz, 1H), 1.93 – 1.68 (m, 1H), 1.46 (dd, *J* = 28.1, 21.2 Hz, 3H), 1.29 – 1.12 (m, 3H). ^{13}C NMR (101 MHz, DMSO) δ 173.1, 172.4, 171.8, 171.0, 163.3, 157.6, 139.0, 129.3, 128.4, 126.4, 119.8 (q, J = 320.0 Hz), 119.2, 65.4, 61.5, 60.5, 59.7, 56.5, 55.8, 53.3, 48.4, 43.3, 36.7, 33.7, 28.4, 24.7, 19.0, 15.6. HRMS (ESI-TOF) m/z: $C_{39}H_{48}F_3N_{10}O_{12}S_2^+$ [M+H]$^+$ Mass calc. 967.2841; Obs.: 967.2844.



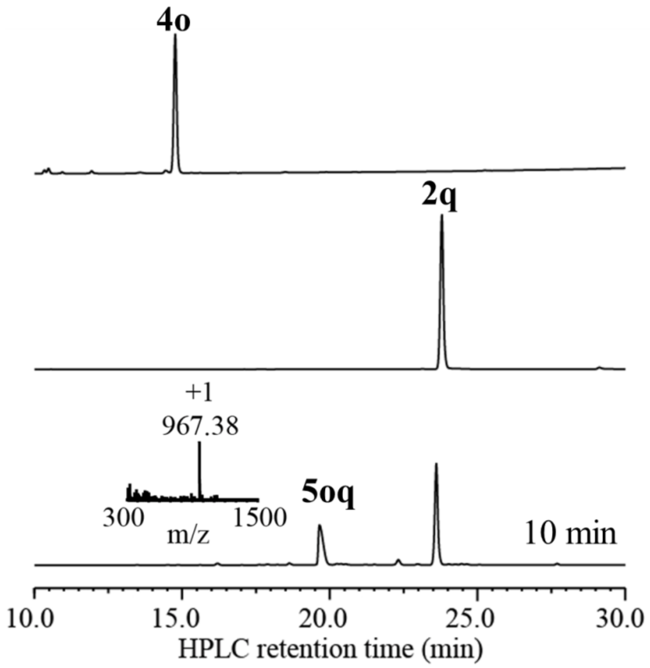


Figure S35. The modification of cyclic peptide **4o** with ynamide **2q**.

22) *The modification of cyclic peptide* ***4o*** *with ynamide* ***2r***

The peptide **4o** (0.1 mmol) and ynamide **2r** (0.2 mmol, 2 equiv) were dissolved in 200 mL PB (0.1 M pH 8.0) and 200 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 10 min, the product **5or** was isolated in 89% yield. The product was characterized by ^{1}H NMR (400 MHz, DMSO) δ 8.43 – 8.19 (m, 3H), 8.10 (d, *J* = 8.1 Hz, 1H), 7.83 (d, *J* = 8.0 Hz, 1H), 7.72 – 7.61 (m, 4H), 7.50 (d, *J* = 26.3 Hz, 1H), 7.29 – 7.08 (m, 6H), 7.02 (d, *J* = 2.1 Hz, 1H), 6.92 (d, *J* = 9.0 Hz, 1H), 6.80 – 6.59 (m, 2H), 6.04 (d, *J* = 6.8 Hz, 1H), 4.62 (d, *J* = 7.9 Hz, 1H), 4.52 (dd, *J* = 14.2, 7.0 Hz, 1H), 4.14 (m, 3H), 3.41 – 3.33 (m, 1H), 3.31 – 3.19 (m, 1H), 3.17 – 3.05 (m, 3H), 2.76 (dd, *J* = 13.9, 7.1 Hz, 1H), 2.66 (dd, *J* = 15.9, 8.4 Hz, 1H), 2.34 (dd, *J* = 16.1, 5.4 Hz, 1H), 2.23 (s, 3H), 1.83 (d, *J* = 19.3 Hz, 1H), 1.72 (d, *J* = 12.0 Hz, 2H), 1.68 – 1.59 (m, 2H), 1.54 (d, *J* = 8.7 Hz, 1H), 1.49 – 1.37 (m, 3H), 1.27 – 1.19 (m, 1H), 1.12 (m, 3H). ^{13}C NMR (101 MHz, DMSO) δ 172.1, 171.2, 171.1, 170.8, 170.5, 170.3, 169.7, 168.3, 157.2, 156.1, 138.2, 138.1, 136.1, 134.5, 133.2, 131.7, 131.0, 130.7, 129.5, 129.5, 128.5, 126.6, 119.8 (q, J = 320.0 Hz), 119.5, 117.7, 112.8, 112.0, 102.0, 78.3, 68.7, 62.3, 61.2, 55.8, 54.2, 52.9, 49.4, 48.4, 35.8, 30.7, 25.8, 24.3, 13.6. HRMS (ESI-TOF) m/z: $C_{48}H_{55}ClF_3N_{10}O_{13}S_2^+$ [M+H]$^+$ Mass calc. 1135.3027; Obs.: 1135.3031.

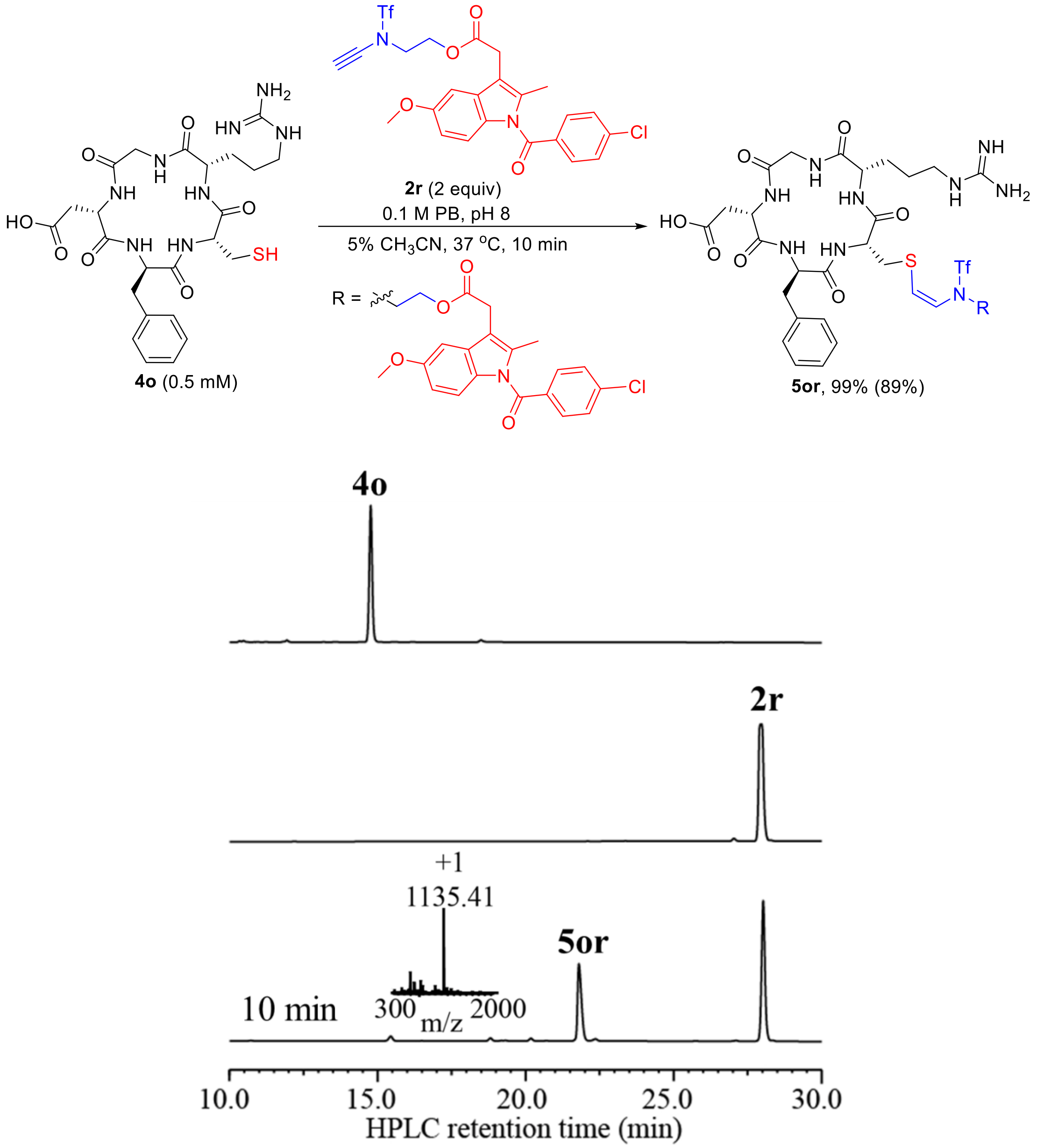


Figure S36. The modification of cyclic peptide **4o** with ynamide **2r**.

23) *The modification of cyclic peptide **4o** with ynamide **2s***

The peptide **4o** (0.1 mmol) and ynamide **2s** (0.2 mmol, 2 equiv) were dissolved in 200 mL PB (0.1 M pH 8.0) and 10 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 10 min, the product **4os** was isolated in 92% yield. The product was characterized by ^{1}H NMR (400 MHz, DMSO) δ 8.37 (d, *J* = 119.4 Hz, 2H), 7.77 (s, 2H), 7.19 (dd, *J* = 15.5, 6.5 Hz, 6H), 6.73 (d, *J* = 5.8 Hz, 1H), 6.45 (d, *J* = 32.0 Hz, 2H), 6.02 (d, *J* = 5.6 Hz, 1H), 4.57 (d, *J* = 68.1 Hz, 2H), 4.31 (s, 1H), 4.13 (t, *J* = 37.2 Hz, 4H), 3.76 (s, 1H), 3.41 (m, 6H), 3.21 (s, 2H), 3.09 (s, 3H), 2.81 (s, 1H), 2.63 – 2.53 (m, 2H), 2.30 (s, 2H), 2.05 (d, *J* = 32.0 Hz, 1H), 1.73 (d, *J* = 36.2 Hz, 1H), 1.68 – 1.20 (m, 8H), 1.12 – 0.98 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 173.1, 172.4, 171.8, 171.0, 163.3, 157.6, 139.0, 129.3, 128.4, 126.4, 119.9 (q, J = 320.0 Hz), 119.2, 116.0, 65.4, 61.5, 60.5, 59.7, 56.5, 55.8, 53.3, 48.4, 43.3, 36.7, 33.7, 28.4, 24.7, 19.0, 15.6; HRMS (ESI-TOF) m/z: $C_{39}H_{55}F_3N_{11}O_{12}S_3^+$ $[M+H]^+$ Mass calc. 1022.3140; Obs.: 1022.3117.

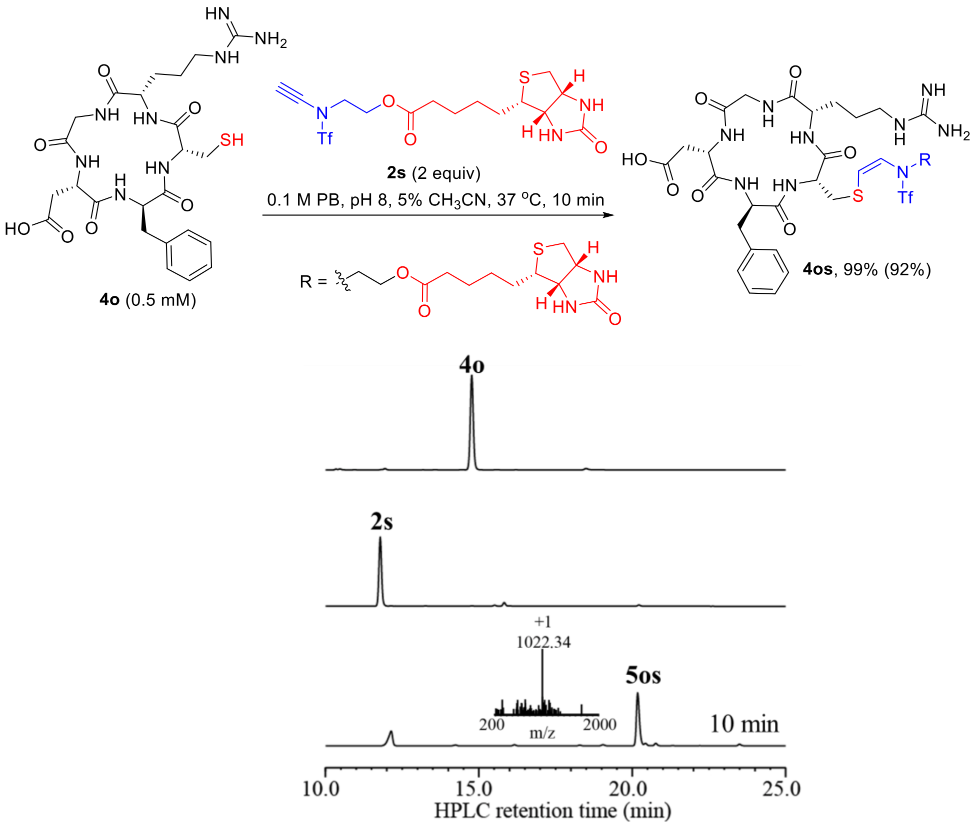


Figure S37. The modification of cyclic peptide **4o** with ynamide **2s**.

24) *The modification of cyclic peptide **4o** with ynamide **2t***

The peptide **4o** (0.1 mmol) and ynamide **2t** (0.2 mmol, 2 equiv) were dissolved in 200 mL PB (0.1 M pH 8.0) and 20 mL DMSO at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 10 min, the product **5ot** was isolated in 92% yield. The product was characterized by ^{1}H NMR (400 MHz, DMSO) δ 8.31 – 8.19 (m, 1H), 8.19 – 8.06 (m, 1H), 7.96 (dd, *J* = 8.0, 4.4 Hz, 1H), 7.57 (s, 1H), 7.35 – 7.12 (m, 5H), 6.79 (d, *J* = 6.7 Hz, 1H), 6.14 (d, *J* = 6.6 Hz, 1H), 4.64 (dd, *J* = 14.1, 8.1 Hz, 1H), 4.58 – 4.47 (m, 1H), 4.42 (d, *J* = 1.9 Hz, 2H), 4.32 – 3.95 (m, 2H), 3.52 (t, *J* = 2.1 Hz, 1H), 3.36 – 3.20 (m, 1H), 3.09 (s, 2H), 3.02 (d, *J* = 7.6 Hz, 1H), 2.82 (dd, *J* = 14.7, 7.3 Hz, 3H), 2.76 – 2.62 (m, 2H), 2.43 – 2.29 (m, 1H), 2.19 (t, *J* = 8.4 Hz, 1H), 1.78 (d, *J* = 6.9 Hz, 1H), 1.56 – 1.33 (m, 2H), 1.23 (d, *J* = 7.3 Hz, 3H) ^{13}C NMR (101 MHz, DMSO) δ 172.1, 171.4, 171.3, 170.6, 170.5, 169.8, 157.1, 137.9, 134.5, 129.7, 129.6, 128.6, 126.72, 119.8 (q, J = 320.0 Hz), 119.0, 77.4, 77.1, 57.6, 54.7, 52.7, 49.4, 43.7, 37.4, 35.8, 28.4, 27.56, 26.3, 25.1, 16.1; HRMS (ESI-TOF) m/z: $C_{30}H_{39}F_3N_9O_9S_2^+$ $[M+H]^+$ Mass calc. 790.2259; Obs.: 790.2278.

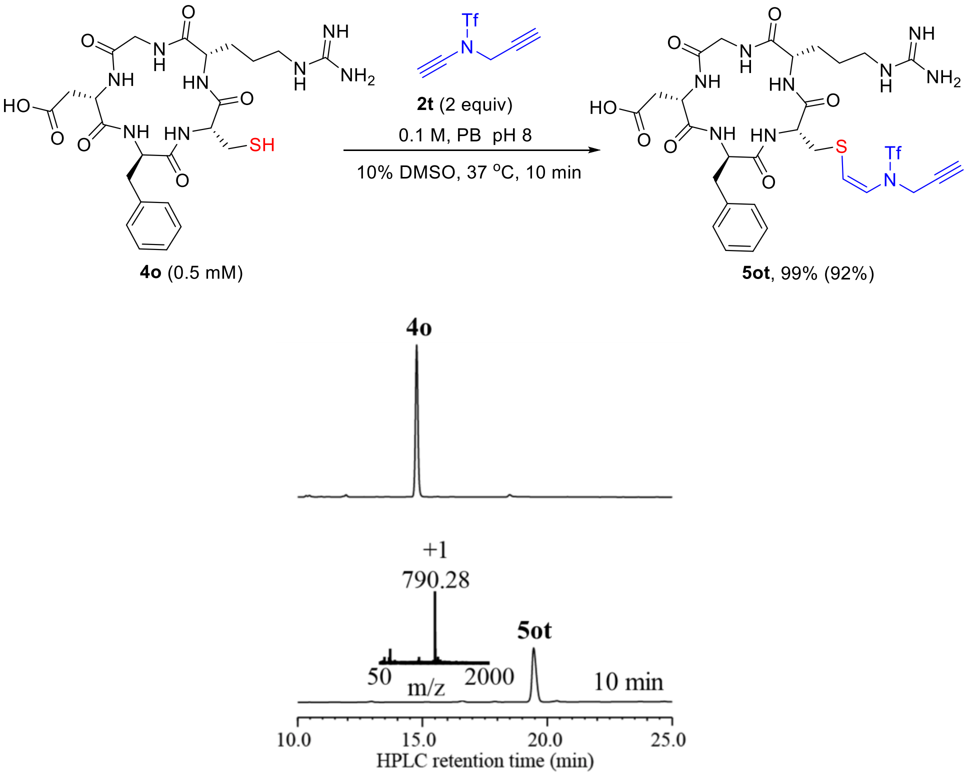
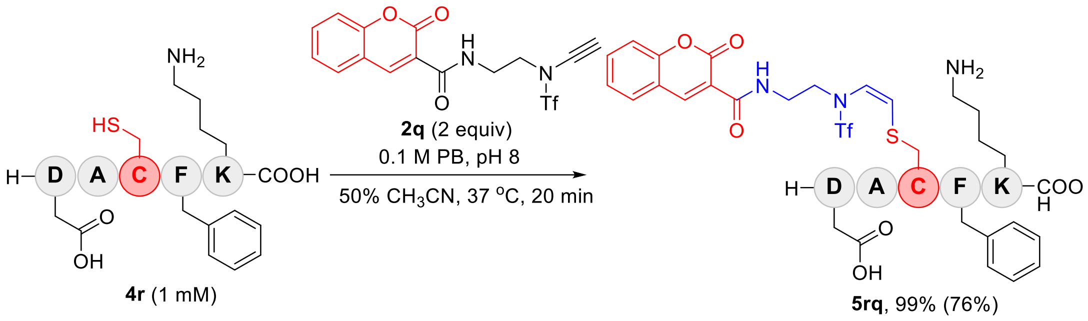


Figure S38. The modification of cyclic peptide **4o** with ynamide **2t**.

25) *The modification of peptide **4r** with ynamide **2q***

The peptide **4r** (0.01 mmol) and ynamide **2q** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 10 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5rq** was isolated in 76% yield. The product was characterized by ESI-MS ($[M+H]^+$ Obs. 971.35, Mass calc. 971.29)



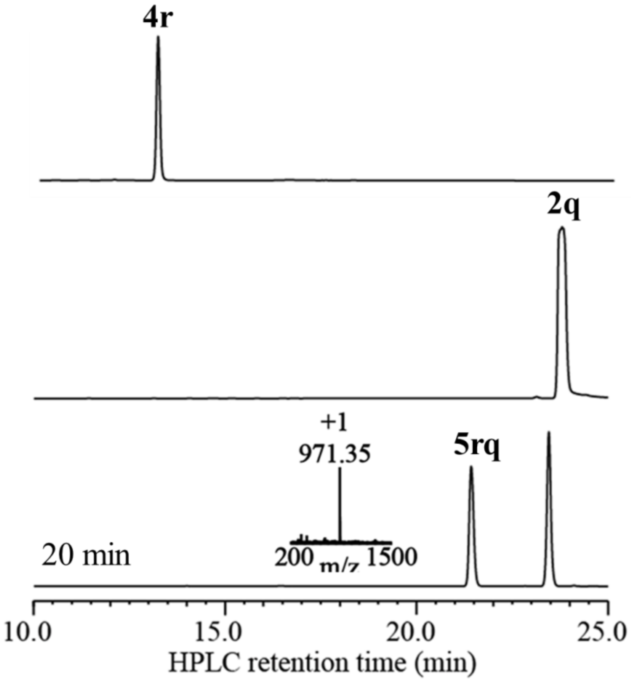


Figure S39. The modification of peptide **4r** with ynamide **2q**.

26) *The modification of peptide **4r** with ynamide **2r***

The peptide **4r** (0.01 mmol) and ynamide **2r** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 10 mL CH_3CN at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient 10-100% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5rr** was isolated in 72% yield. The product was characterized by ESI-MS ($[M+H]^+$ Obs. 1139.21, Mass calc. 1139.32)

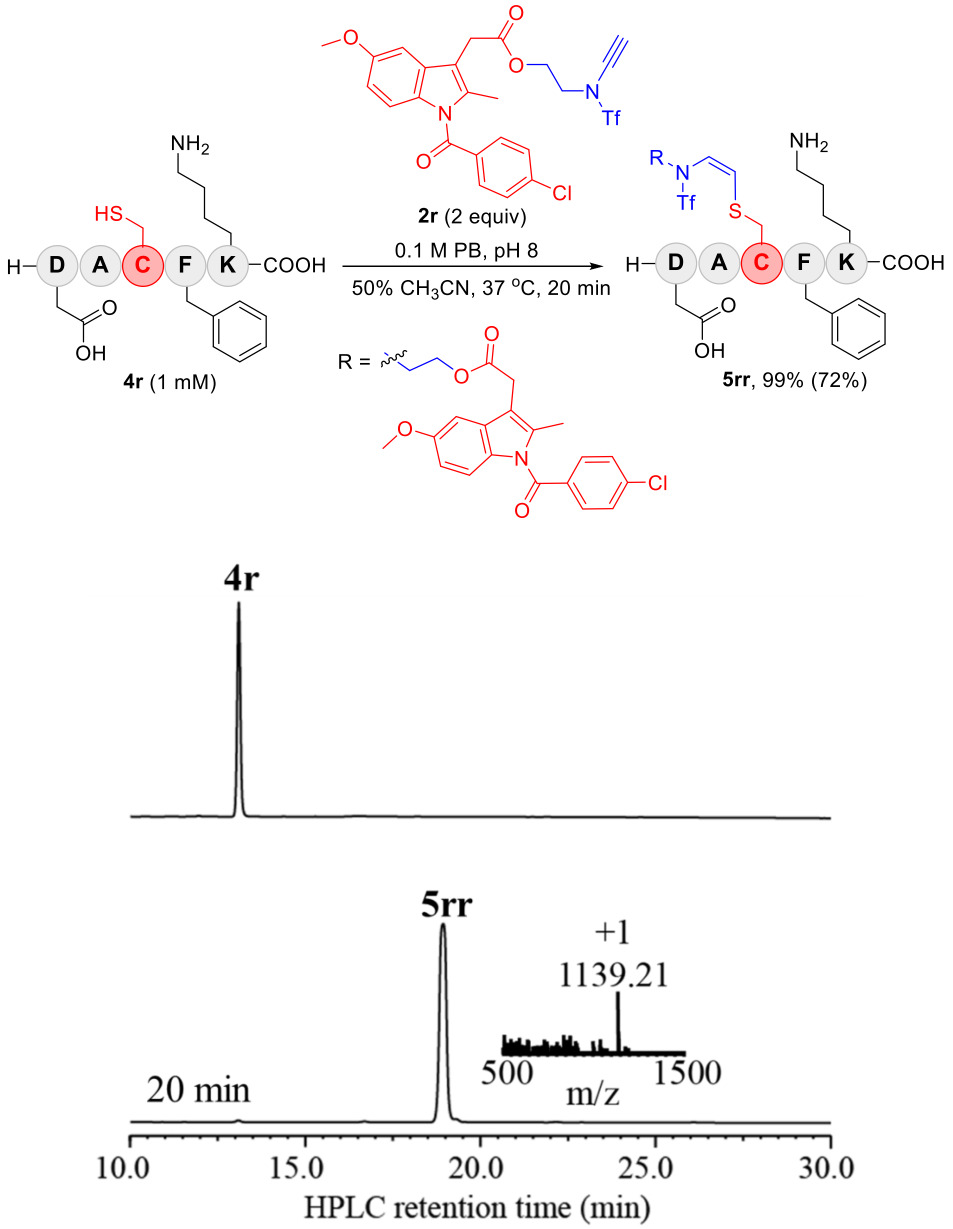
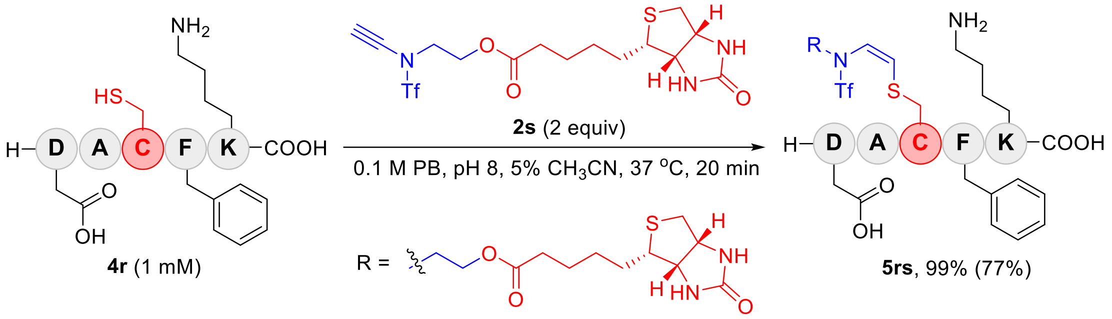


Figure S40. The modification of peptide **4r** with ynamide **2t**.

27) *The modification of peptide* ***4r*** *with ynamide* ***2s***

The peptide **4r** (0.01 mmol) and ynamide **2s** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 0.5 mL CH_3CN at 37 ºC. The reaction mixture was monitored by HPLC (using Jupiter 5μm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5rs** was isolated in 77% yield. The product was characterized by ESI-MS ($[M+H]^+$ Obs. 1026.28, Mass calc. 1026.33)



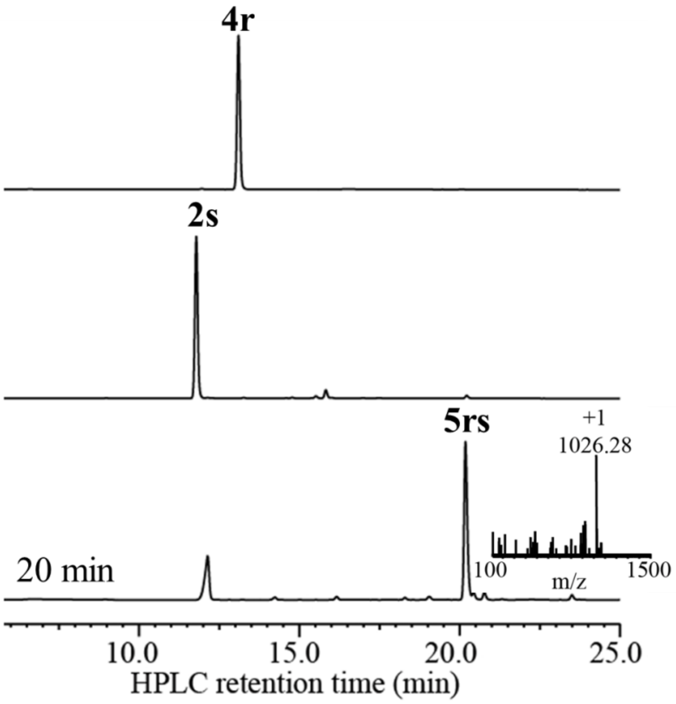
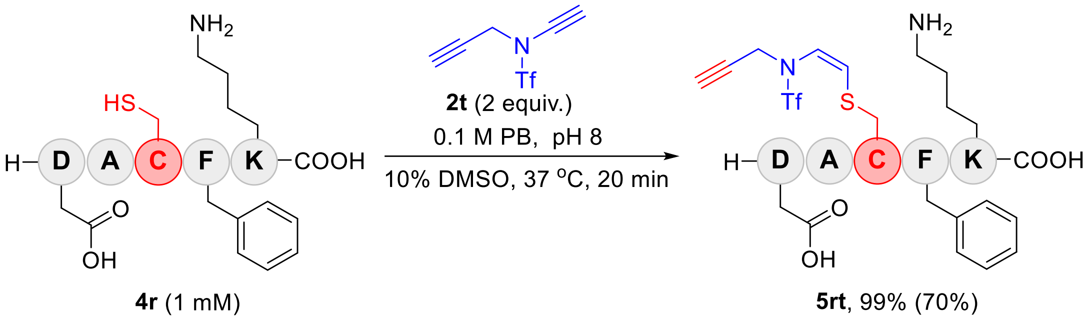


Figure S41. The modification of peptide **4r** with ynamide **2s**.

28) *The modification of peptide **4r** with ynamide **2t***

The peptide **4r** (0.01 mmol) and ynamide **2t** (0.02 mmol, 2 equiv) were dissolved in 10 mL PB (0.1 M pH 8.0) and 1 mL DMSO at 37 °C. The reaction mixture was monitored by HPLC (using Jupiter 5µm C18 4.6 × 250 mm) with a gradient of 10-95% solvent B in 30 min at 220 nm and LC-MS. After 20 min, the product **5rt** was isolated in 70% yield. The product was characterized by ESI-MS ($[M + K]^+$ Obs. 832.1, Mass calc. 832.20).



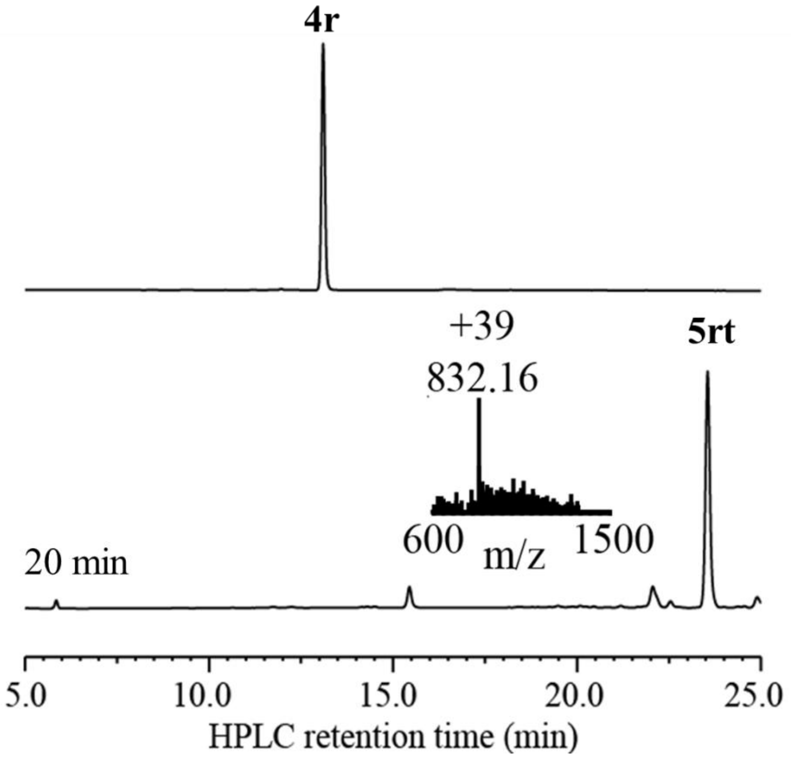
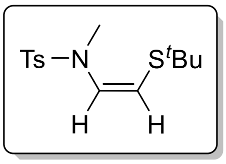


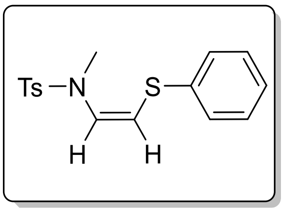
Figure S42. The modification of peptide **4r** with ynamide **2t**.

(Z)-N-(2-(tert-butylthio)vinyl)-N,4-dimethylbenzenesulfonamide ***(3aa)***



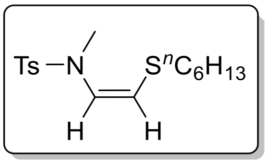
White solid, (57.4 mg, yield 96%); R_f = 0.2 (PE/EA = 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (d, *J* = 8.2 Hz, 2H), 7.31 (d, *J* = 8.0 Hz, 2H), 6.28 (d, *J* = 8.0 Hz, 1H), 5.73 (d, *J* = 8.0 Hz, 1H), 3.08 (s, 3H), 2.42 (s, 3H), 1.31 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.7, 134.9, 129.7, 127.4, 126.2, 113.8, 44.4, 36.0, 30.7, 21.6. HRMS (ESI) m/z Mass calc. for $C_{14}H_{22}NO_2S_2^+$ $[M+H]^+$: 300.1086, obs.: 300.1096.

(Z)-N,4-dimethyl-N-(2-(phenylthio)vinyl)benzenesulfonamide ***(3ba)***



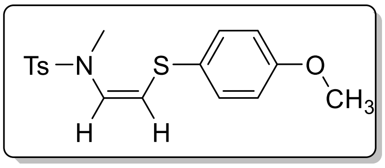
White solid, (59.9 mg, yield 94%); R*f* = 0.2 (PE/EA = 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 (d, *J* = 8.2 Hz, 2H), 7.33 (d, *J* = 8.0 Hz, 2H), 7.29 (d, *J* = 4.3 Hz, 4H), 7.22 (dq, *J* = 8.5, 4.3 Hz, 1H), 6.45 (d, *J* = 7.8 Hz, 1H), 5.79 (d, *J* = 7.8 Hz, 1H), 3.16 (s, 3H), 2.44 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.0, 135.9, 134.8, 129.8, 129.1, 128.9, 128.2, 127.4, 126.7, 113.6, 35.8, 21.6. HRMS (ESI) m/z Mass calc. for $C_{16}H_{18}NO_2S_2^+$ $[M+H]^+$: 320.0773, obs.: 320.0783

(Z)-N-(2-(hexylthio)vinyl)-N,4-dimethylbenzenesulfonamide ***(3ca)***



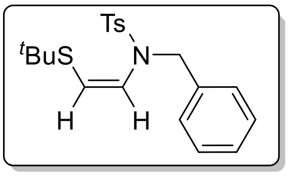
White solid, (61.5 mg, yield 94%); R*f* = 0.2 (PE/EA = 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (d, *J* = 8.2 Hz, 2H), 7.30 (d, *J* = 8.0 Hz, 2H), 6.13 (d, *J* = 7.7 Hz, 1H), 5.58 (d, *J* = 7.7 Hz, 1H), 3.06 (s, 3H), 2.60 (t, *J* = 7.4 Hz, 2H), 2.42 (s, 3H), 1.56 (dt, *J* = 14.9, 7.3 Hz, 2H), 1.31 (ddd, *J* = 18.9, 11.0, 5.7 Hz, 8H), 0.87 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.7, 134.9, 129.7, 127.4, 124.8, 119.0, 35.6, 34.8, 31.3, 30.0, 28.1, 22.5, 21.5, 14.0. HRMS (ESI) m/z Mass calc. for $C_{16}H_{25}NNaO_2S_2^+$ $[M+Na]^+$: 350.1219, obs.: 350.1229.

(Z)-N-(2-((4-methoxyphenyl)thio)vinyl)-N,4-dimethylbenzenesulfonamide ***(3da)***



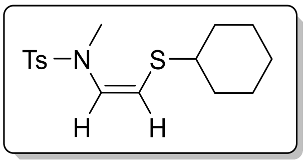
White solid, (66.3 mg, yield 95%); R*f* = 0.2 (PE/EA = 20:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 (d, *J* = 8.3 Hz, 2H), 7.35 – 7.30 (m, 2H), 7.29 – 7.23 (m, 2H), 6.83 (d, *J* = 8.8 Hz, 2H), 6.21 (d, *J* = 7.7 Hz, 1H), 5.77 (d, *J* = 7.6 Hz, 1H), 3.78 (s, 3H), 3.12 (s, 3H), 2.43 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.2, 143.8, 134.6, 132.1, 129.7, 127.3, 125.9, 125.8, 118.3, 114.8, 55.3, 35.9, 21.5. HRMS (ESI) m/z Mass calc. for $C_{17}H_{19}NNaO_3S_2^+$ $[M+Na]^+$: 372.0699, obs.: 372.0710

(Z)-N-benzyl-N-(2-(tert-butylthio)vinyl)-4-methylbenzenesulfonamide ***(3ea)***



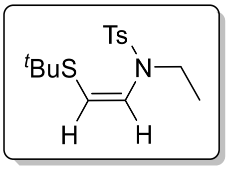
White solid, (57.9 mg, yield 87%); R*f* = 0.2 (PE/EA =10:1); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.72 (d, *J* = 8.2 Hz, 2H), 7.33 – 7.22 (m, 7H), 5.96 (d, *J* = 7.4 Hz, 1H), 5.90 (d, *J* = 7.4 Hz, 1H), 4.60 (s, 2H), 2.42 (s, 3H), 1.20 (s, 9H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 143.4, 136.4, 136.4, 129.5, 128.2, 128.2, 127.4, 127.4, 123.3, 121.9, 51.9, 44.2, 30.7, 21.5. HRMS (ESI) m/z Mass calc. for $C_{17}H_{19}NNaO_2S_2^+$ [M+Na]$^+$: 356.0749, obs.: 356.0757.

(Z)-N-(2-(cyclohexylthio)vinyl)-N,4-dimethylbenzenesulfonamide ***(3fa)***



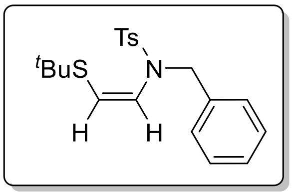
White solid, (60.5 mg, yield 93%); R$_f$ = 0.2 (PE/EA = 10:1); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.68 (d, *J* = 8.3 Hz, 2H), 7.30 (d, *J* = 8.0 Hz, 2H), 6.16 (d, *J* = 7.8 Hz, 1H), 5.65 (d, *J* = 7.8 Hz, 1H), 3.06 (s, 3H), 2.78 – 2.67 (m, 1H), 2.42 (s, 3H), 1.99 – 1.89 (m, 2H), 1.72 (dt, *J* = 6.8, 3.3 Hz, 2H), 1.40 – 1.16 (m, 6H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 143.6, 134.9, 129.6, 127.4, 125.1, 116.8, 46.7, 35.7, 33.5, 25.9, 25.6, 21.6. HRMS (ESI) m/z Mass calc. for $C_{16}H_{23}NNaO_2S_2^+$ [M+Na]$^+$: 348.1062, obs.: 348.1073.

(Z)-N-(2-(tert-butylthio)vinyl)-N-ethyl-4-methylbenzenesulfonamide ***(3ab)***



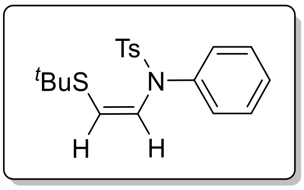
White solid, (57.0 mg, yield 91%); R*f* = 0.2 (PE/EA = 20:1); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.70 (d, *J* = 8.2 Hz, 2H), 7.27 (d, *J* = 8.1 Hz, 2H), 6.04 (d, *J* = 7.8 Hz, 1H), 5.94 (d, *J* = 7.7 Hz, 1H), 3.48 (q, *J* = 7.1 Hz, 2H), 2.41 (s, 3H), 1.32 (s, 9H), 1.12 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 143.3, 136.5, 129.5, 127.2, 123.1, 118.4, 44.1, 43.2, 30.7, 21.5, 14.0. HRMS (ESI) m/z Mass calc. for $C_{15}H_{23}NNaO_2S_2^+$ [M+Na]$^+$: 336.1062, obs.: 336.1064

(Z)-N-benzyl-N-(2-(tert-butylthio)vinyl)-4-methylbenzenesulfonamide ***(3ac)***



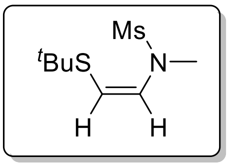
White solid, (69.0 mg, yield 92%); R*f* = 0.2 (PE/EA = 20:1); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.72 (d, *J* = 8.2 Hz, 2H), 7.33 – 7.22 (m, 7H), 5.96 (d, *J* = 7.4 Hz, 1H), 5.90 (d, *J* = 7.4 Hz, 1H), 4.60 (s, 2H), 2.42 (s, 3H), 1.20 (s, 9H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 143.4, 136.4, 136.4, 129.5, 128.2, 128.2, 127.4, 127.4, 123.3, 121.9, 51.9, 44.2, 30.7, 21.5. HRMS (ESI) m/z Mass calc. for $C_{20}H_{25}NNaO_2S_2^+$ [M+Na]$^+$: 398.1229, obs.: 398.1227

(Z)-N-(2-(tert-butylthio)vinyl)-4-methyl-N-phenylbenzenesulfonamide ***(3ad)***



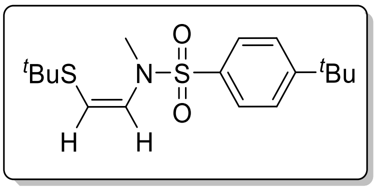
White solid, (57.8 mg, yield 80%); R*f* = 0.2 (PE/EA = 20:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 – 7.43 (m, 2H), 7.30 – 7.18 (m, 5H), 7.16 – 7.00 (m, 2H), 6.61 (d, *J* = 7.9 Hz, 1H), 5.73 (d, *J* =7.9 Hz, 1H), 2.40 (s, 3H), 1.18 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.8, 139.0, 135.2, 129.4, 128.7, 128.6, 127.7, 127.4, 124.4, 114.6, 43.9, 30.7, 21.6. HRMS (ESI) m/z Mass calc. for $C_{19}H_{23}NNaO_2S_2^+$ $[M+Na]^+$: 384.1062, obs.: 384.1044

(Z)-N-(2-(tert-butylthio)vinyl)-N-methylmethanesulfonamide ***(3ae)***



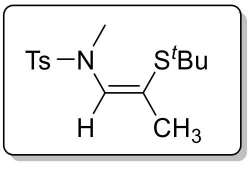
Yellow oil, (41.5 mg, yield 93%); R*f* = 0.2 (PE/EA = 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.27 (d, *J* = 7.8 Hz, 1H), 5.84 (d, *J* = 7.8 Hz, 1H), 3.17 (s, 3H), 2.87 (s, 3H), 1.36 (s, 9H); ^{13}C NMR (100MHz, $CDCl_3$) δ 125.5, 115.6, 44.5, 37.7, 35.6, 30.7. HRMS (ESI) m/z Mass calc. for $C_8H_{18}NO_2S_2^+$ $[M+H]^+$: 224.0773, obs.: 224.0788

(Z)-4-(tert-butyl)-N-(2-(tert-butylthio)vinyl)-N-methylbenzenesulfonamide ***(3af)***



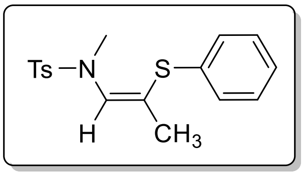
White solid, (66.8 mg, yield 98%); R*f* = 0.2 (PE/EA = 20:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (d, *J* = 8.5 Hz, 2H), 7.51 (d, *J* = 8.6 Hz, 2H), 6.32 (d, *J* = 8.0 Hz, 1H), 5.72 (d, *J* = 7.9 Hz, 1H), 3.11 (s, 3H), 1.34 (s, 9H), 1.29 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.6, 134.9, 127.2, 126.3, 126.0, 113.5, 44.2, 36.0, 35.1, 31.1, 30.6. HRMS (ESI) m/z Mass calc. for $C_{17}H_{27}NNaO_2S_2^+$ $[M+Na]^+$: 364.1375, obs.: 364.1371.

(Z)-N-(2-(tert-butylthio)prop-1-en-1-yl)-N,4-dimethylbenzenesulfonamide ***(3ag)***



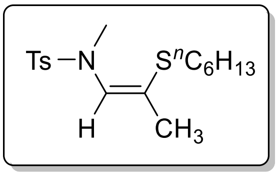
White solid, (57.0 mg, yield 91%); R*f* = 0.2 (PE/EA =10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (d, *J* = 8.3 Hz, 2H), 7.30 (d, *J* = 8.0 Hz, 2H), 6.36 (d, *J* = 1.4 Hz, 1H), 3.09 (s, 3H), 2.41 (s, 3H), 2.04 (d, *J* = 1.3 Hz, 3H), 1.32 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.6, 135.1, 131.2, 129.7, 127.5, 125.1, 47.1, 37.2, 32.1, 26.3, 21.6. HRMS (ESI) m/z Mass calc. for $C_{15}H_{23}NNaO_2S_2^+$ $[M+Na]^+$: 336.1056, obs.: 336.1062.

(Z)-N,4-dimethyl-N-(2-(phenylthio)prop-1-en-1-yl)benzenesulfonamide (3bg)



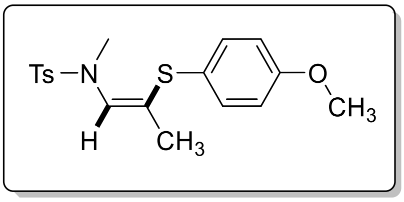
White solid, (55.9 mg, yield 84%); R*f* = 0.2 (PE/EA =20:1); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.73 (d, *J* = 8.3 Hz, 2H), 7.34 – 7.25 (m, 7H), 5.97 (d, *J* = 1.5 Hz, 1H), 3.02 (s, 3H), 2.43 (s, 3H), 1.77 (d, *J* = 1.4 Hz, 3H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 143.7, 134.6, 132.7, 132.4, 131.6, 129.6, 129.2, 128.9, 127.6, 125.7, 37.1, 21.6, 21.1. HRMS (ESI) m/z Mass calc. for $C_{17}H_{19}NNaO_2S_2^+$ [M+Na]$^+$: 356.0749, obs.: 356.0745.

(Z)-N-(2-(hexylthio)prop-1-en-1-yl)-N,4-dimethylbenzenesulfonamide ***(3cg)***



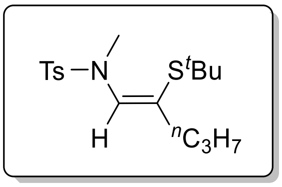
White solid, (62.0 mg, yield 91%); R*f* = 0.2 (PE/EA =10:1); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.69 (d, *J* = 8.3 Hz, 2H), 7.29 (d, *J* = 8.1 Hz, 2H), 5.70 (d, *J* = 1.7 Hz, 1H), 2.91 (s, 3H), 2.70 – 2.63 (m, 2H), 2.41 (s, 3H), 1.95 (d, *J* = 1.4 Hz, 3H), 1.56 – 1.44 (m, 2H), 1.39 – 1.24 (m, 6H), 0.87 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 143.4, 134.6, 134.6, 129.5, 127.6, 123.0, 36.8, 31.4, 30.2, 30.0, 28.5, 22.5, 21.5, 20.3, 14.0. HRMS (ESI) m/z Mass calc. for $C_{17}H_{27}NNaO_2S_2^+$ [M+Na]$^+$: 364.1375, obs.: 364.1371

(Z)-N-(2-((4-methoxyphenyl)thio)prop-1-en-1-yl)-N,4-dimethylbenzenesulfonamide ***(3dg)***



White solid, (60.3 mg, yield 83%); R*f* = 0.2 (PE/EA =10:1); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.73 (d, *J* = 8.3 Hz, 2H), 7.35 – 7.27 (m, 4H), 6.82 (d, *J* = 8.7 Hz, 2H), 5.71 (q, *J* = 1.4 Hz, 1H), 3.79 (s, 3H), 2.98 (s, 3H), 2.43 (s, 3H), 1.66 (d, *J* = 1.4 Hz, 3H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 159.9, 143.6, 136.0, 136.0, 134.5, 129.6, 127.7, 122.8, 122.1, 114.5, 55.3, 37.2, 21.5, 20.6. HRMS (ESI) m/z Mass calc. for $C_{18}H_{21}NNaO_3S_2^+$ [M+Na]$^+$: 386.0855, obs.: 386.0851.

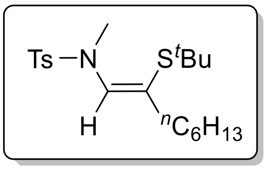
(Z)-N-(2-(tert-butylthio)hex-1-en-1-yl)-N,4-dimethylbenzenesulfonamide ***(3ah)***



White solid, (67.5 mg, yield 99%); R*f* = 0.2 (PE/EA =10:1); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.70 (d, *J* = 7.9 Hz, 2H), 7.32 (d, *J* = 7.9 Hz, 2H), 6.51 (d, *J* = 1.2 Hz, 1H), 3.15 (d, *J* = 1.1 Hz, 3H), 2.43 (s, 3H), 2.24 (t, *J* = 7.4 Hz, 2H), 1.53 (q, *J* = 7.6 Hz, 2H), 1.30 (s, 9H), 0.84 (td, *J* = 7.4, 1.1 Hz, 3H). ^{13}C NMR (100 MHz, CDCl$_3$) δ

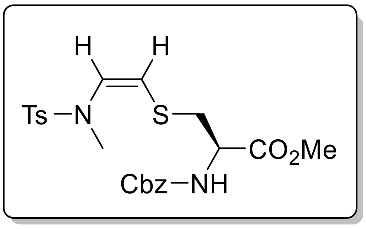
143.6, 134.9, 132.5, 129.7, 127.9, 127.4, 47.5, 41.3, 37.4, 31.9, 21.9, 21.5, 13.3. HRMS (ESI) m/z Mass calc. for $C_{17}H_{27}NNaO_2S_2^+$ $[M+Na]^+$: 364.1375, obs.: 364.1369.

(Z)-N-(2-(tert-butylthio)oct-1-en-1-yl)-N,4-dimethylbenzenesulfonamide ***(3ai)***



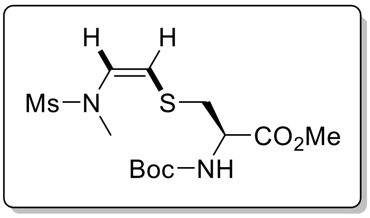
White solid, (75.8 mg, yield 99%); R*f* = 0.2 (PE/EA =10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (d, *J* = 8.0 Hz, 2H), 7.30 (d, *J* = 7.9 Hz, 2H), 6.50 (s, 1H), 3.13 (s, 3H), 2.41 (s, 3H), 2.24 (t, *J* = 7.5 Hz, 2H), 1.48 (p, *J* = 7.2 Hz, 2H), 1.29 (s, 9H), 1.23 (dq, *J* = 8.9, 4.8, 4.1 Hz, 6H), 0.85 (t, *J* = 6.7 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.6, 135.0, 132.4, 129.7, 128.1, 127.4, 47.5, 39.4, 37.4, 31.9, 31.6, 28.8, 28.5, 22.6, 21.5, 14.1. HRMS (ESI) m/z Mass calc. for $C_{20}H_{33}NNaO_2S_2^+$ $[M+Na]^+$: 406.1845, obs.: 406.1840.

Methyl (Z)-N-((benzyloxy)carbonyl)-S-(2-((N,4-dimethylphenyl) sulfonamido) vinyl)-L-cysteinate ***(3ga)***



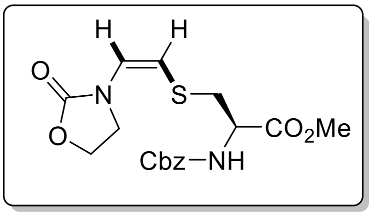
Colorless Oil, (79.3 mg, yield 83%); R*f* = 0.2 (PE/EA =4:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (d, *J* = 8.1 Hz, 2H), 7.38 – 7.26 (m, 7H), 6.06 (d, *J* = 7.6 Hz, 1H), 5.63 (d, *J* = 7.9 Hz, 1H), 5.49 (d, *J* = 7.7 Hz, 1H), 5.14 – 5.06 (m, 2H), 4.60 (dt, *J* = 8.7, 4.8 Hz, 1H), 3.72 (s, 3H), 3.11 (t, *J* = 3.9 Hz, 2H), 2.99 (s, 3H), 2.40 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.4, 155.5, 143.8, 136.1, 134.5, 129.7, 128.5, 128.2, 128.1, 127.3, 126.2, 117.4, 67.1, 54.1, 52.7, 37.1, 35.6, 21.5. HRMS (ESI) m/z Mass calc. for $C_{22}H_{26}N_2NaO_6S_2^+$ $[M+Na]^+$: 501.1124, obs.: 501.1125.

Methyl-(Z)-N-(tert-butoxycarbonyl)-S-(2-(N-methylmethylsulfonamido)vinyl)-L-cysteinate ***(3ge)***



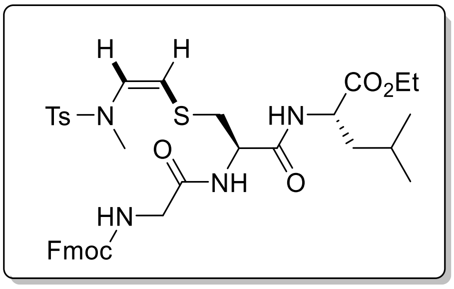
Colorless Oil, (50.0 mg, yield 68%); R*f* = 0.2 (PE/EA =3:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.17 (d, *J* = 7.6 Hz, 1H), 5.55 (d, *J* = 7.6 Hz, 1H), 5.37 (d, *J* = 7.9 Hz, 1H), 4.51 (dt, *J* = 8.7, 4.9 Hz, 1H), 3.70 (s, 3H), 3.12 (s, 3H), 3.04 (dd, *J* = 14.0, 5.0 Hz, 2H), 2.85 (s, 3H), 1.38 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.7, 154.9, 125.8, 117.5, 80.2, 53.6, 52.6, 37.5, 37.1, 35.4, 28.2. HRMS (ESI) m/z Mass calc. for $C_{13}H_{25}N_2O_6S_2^+$ $[M+H]^+$: 369.1149, obs.: 369.1153.

Methyl-(Z)-N-((benzyloxy)carbonyl)-S-(2-(2-oxooxazolidin-3-yl)vinyl)-L-cysteinate ***(3ij)***



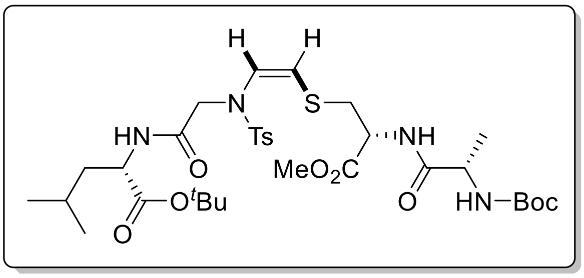
Colorless Oil, (60.0 mg, yield 79%); R*f* = 0.2 (PE/EA =2:1); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.33 (d, *J* = 4.0 Hz, 5H), 6.56 (d, *J* = 8.6 Hz, 1H), 5.69 (d, *J* = 7.8 Hz, 1H), 5.16 – 5.12 (m, 1H), 5.09 (d, *J* = 5.9 Hz, 2H), 4.61 (dd, *J* = 8.2, 4.5 Hz, 1H), 4.30 (td, *J* = 7.8, 2.4 Hz, 2H), 4.11 (t, *J* = 8.0 Hz, 2H), 3.72 (s, 3H), 3.09 (ddd, *J* = 48.5, 14.2, 4.9 Hz, 2H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 170.6, 156.3, 155.5, 136.0, 128.5, 128.3, 128.0, 125.0, 103.7, 67.1, 62.6, 53.9, 52.6, 44.6, 37.9. HRMS (ESI) m/z Mass calc. for $C_{14}H_{22}N_2NaO_6S^+$ [M+Na]$^+$: 369.1091, obs.: 369.1096.

Ethyl-N-((((9H-fluoren-9-yl)methoxy)carbonyl)glycyl)-S-((Z)-2-((N,4-dimethylphenyl)sulfonamido)vinyl)-L-cysteinyl-L-leucinate ***(3ja)***



White solid, (148.5 mg, yield 99%); R*f* = 0.2 (PE/EA =1:1); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.66 (d, *J* = 8.0 Hz, 2H), 7.59 (d, *J* = 7.5 Hz, 2H), 7.38 (t, *J* = 7.5 Hz, 2H), 7.30 (d, *J* = 7.8 Hz, 4H), 7.27 (d, *J* = 2.7 Hz, 1H), 7.24 (d, *J* = 7.6 Hz, 1H), 7.14 (d, *J* = 8.0 Hz, 1H), 5.99 (d, *J* = 7.1 Hz, 1H), 5.86 (d, *J* = 7.1 Hz, 1H), 5.80 (d, *J* = 5.8 Hz, 1H), 4.67 (q, *J* = 6.7 Hz, 1H), 4.51 (q, *J* = 7.4 Hz, 1H), 4.39 (d, *J* = 7.1 Hz, 2H), 4.26 – 4.17 (m, 2H), 4.14 (ddd, *J* = 10.8, 6.9, 3.3 Hz, 2H), 3.95 (d, *J* = 5.6 Hz, 2H), 3.21 (dd, *J* = 14.2, 4.8 Hz, 1H), 2.97 (dd, *J* = 14.2, 7.1 Hz, 1H), 2.89 (s, 3H), 2.41 (s, 3H), 1.68 (q, *J* = 6.4 Hz, 1H), 1.64 (d, *J* = 6.9 Hz, 2H), 1.26 (d, *J* = 7.2 Hz, 3H), 0.89 (dd, *J* = 6.4, 2.9 Hz, 6H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 172.3, 169.4, 169.2, 156.7, 144.1, 143.8, 141.3, 133.6, 129.8, 127.8, 127.7, 127.6, 127.1, 126.5, 125.1, 123.3, 120.0, 67.3, 61.4, 53.2, 51.5, 47.1, 44.5, 40.7, 36.5, 36.0, 24.8, 22.7, 21.8, 21.6, 14.2. HRMS (ESI) m/z Mass calc. for $C_{38}H_{47}N_4O_8S_2^+$ [M+H]$^+$: 751.2830, obs.: 751.2834.

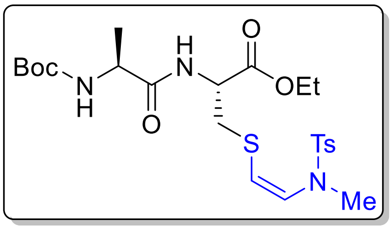
Tert-butyl-N-((Z)-2-(((R)-2-((S)-2-((tert-butoxycarbonyl)amino)propanamido)-3-methoxy-3-oxopropyl)thio)vinyl)-N-tosylglycyl-L-leucinate ***(3kk)***



Yellow Oil, (111.6 mg, yield 75%); R*f* = 0.2 (CH$_3$OH/CH$_2$Cl$_2$ =50:1); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.70 (d, *J* = 8.3 Hz, 2H), 7.52 (d, *J* = 7.1 Hz, 1H), 7.32 (d, *J* = 8.5 Hz, 2H), 7.22 (d, *J* = 8.5 Hz, 1H), 6.11 (d, *J* = 7.5 Hz, 1H), 5.80 (d, *J* = 7.5 Hz, 1H), 5.47 (d, *J* =

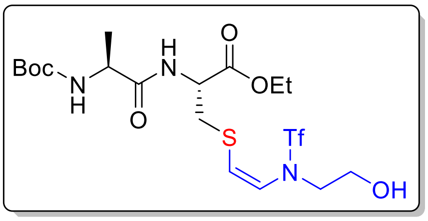
8.2 Hz, 1H), 4.72 – 4.63 (m, 1H), 4.51 (td, *J* = 8.6, 5.5 Hz, 1H), 4.34 – 4.24 (m, 1H), 4.08 (d, *J* = 7.8 Hz, 2H), 3.72 (s, 3H), 3.21 (dd, *J* = 14.2, 4.5 Hz, 1H), 3.10 (dd, *J* = 14.2, 6.1 Hz, 1H), 2.43 (s, 3H), 1.76 – 1.69 (m, 1H), 1.63 – 1.57 (m, 2H), 1.45 (d, *J* = 9.9 Hz, 18H), 0.95 (t, *J* = 6.4 Hz, 6H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 173.2, 172.2, 170.1, 167.5, 155.5, 144.4, 134.6, 129.8, 127.5, 125.5, 121.3, 81.9, 79.8, 52.6, 51.5, 51.2, 49.9, 41.2, 36.0, 28.3, 27.9, 24.8, 22.8, 21.9, 21.5, 18.3. HRMS (ESI) m/z Mass calc. for $C_{35}H_{61}N_4O_{10}S_2^+$ [M+H]$^+$: 761.3824, obs.: 761.3825.

*Ethyl-N-((tert-butoxycarbonyl)-L-alanyl)-S-((Z)-2-((N,4-dimethylphenyl)sulfonamido)vinyl)-L-cysteinate (**5aa**)*



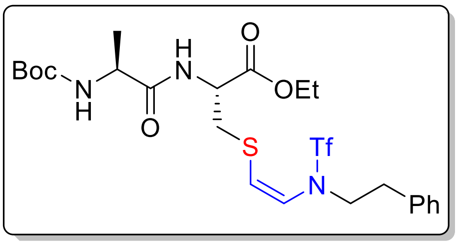
White solid, 89%, 41.7 mg, purified by flash chromatography using PA/EA (3:1) as eluent, Rf = 0.5 in PE/EA (2:1)^{1}H NMR (400 MHz, CDCl$_3$) δ 7.62 (d, *J* = 8.2 Hz, 2H), 7.26 (d, *J* = 8.1 Hz, 2H), 6.94 (d, *J* = 5.4 Hz, 1H), 5.98 (d, *J* = 7.5 Hz, 1H), 5.57 (d, *J* = 7.4 Hz, 1H), 5.02 (s, 1H), 4.73 (dt, *J* = 7.6, 4.6 Hz, 1H), 4.14 (qd, *J* = 7.1, 3.3 Hz, 3H), 3.17 – 3.01 (m, 2H), 2.92 (s, 3H), 2.37 (s, 3H), 1.38 (s, 9H), 1.30 (d, *J* = 7.1 Hz, 3H), 1.21 (t, *J* = 7.1 Hz, 3H) ^{13}C NMR (101 MHz, CDCl$_3$) δ 171.7, 168.8, 154.4, 142.9, 133.2, 128.8, 126.4, 125.0, 118.5, 79.0, 61.0, 51.7, 49.1, 35.6, 34.8, 27.3, 20.5, 17.2, 13.1. HRMS (ESI-TOF) m/z: $C_{23}H_{35}N_3NaO_7S_2^+$ [M+Na]$^+$ Mass calc. 552.1809; Obs.: 552.1814.

*Ethyl-N-((tert-butoxycarbonyl)-L-alanyl)-S-((Z)-2-((1,1,1-trifluoro-N-(2-hydroxyethyl)methyl)sulfonamido)vinyl)-L-cysteinate (**5an**)*



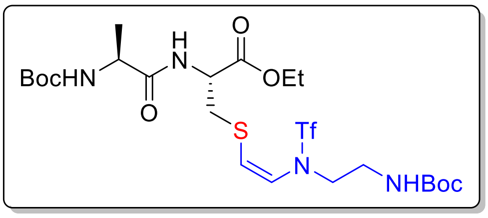
White solid, 95%, 51.0 mg, purified by flash chromatography using PA/EA (2:1) as eluent, Rf = 0.5 in PE/EA (1:1) ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.47 (t, *J* = 17.0 Hz, 1H), 6.22 (d, *J* = 6.8 Hz, 1H), 6.04 (d, *J* = 6.8 Hz, 1H), 5.46 (t, *J* = 10.5 Hz, 1H), 4.84 (dt, *J* = 7.7, 4.6 Hz, 1H), 4.22 (tt, *J* = 31.5, 15.9 Hz, 3H), 3.82 (d, *J* = 6.9 Hz, 3H), 3.67 (t, *J* = 8.6 Hz, 1H), 3.31 (dd, *J* = 14.6, 4.2 Hz, 1H), 3.27 – 3.15 (m, 1H), 1.45 (s, 9H), 1.37 – 1.32 (m, 3H), 1.28 (dd, *J* = 14.3, 5.9 Hz, 3H) ^{13}C NMR (101 MHz, CDCl$_3$) δ 173.2, 169.8, 155.6, 131.2, 121.6, 120.0 (q, J = 323.0 Hz), 80.3, 62.3, 60.9, 53.1, 52.0, 49.9, 36.3, 28.3, 18.2, 14.0. HRMS (ESI-TOF) m/z: $C_{18}H_{30}F_3N_3NaO_8S_2^+$ $[M+Na]^+$ Mass calc. 560.1319; Obs.: 560.3034.

*Ethyl-N-((tert-butoxycarbonyl)-L-alanyl)-S-((Z)-2-((1,1,1-trifluoro-N-phenethylmethyl)sulfonamido)vinyl)-L-cysteinate **(5ao)***



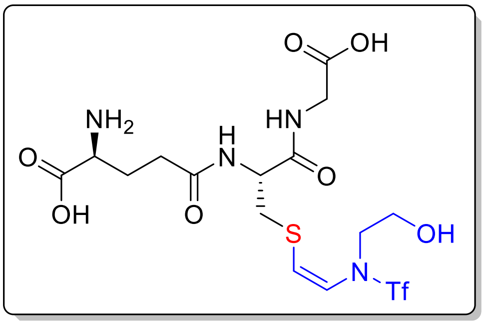
White solid, 95%, 56.8 mg, purified by flash chromatography using PA/EA (3:1) as eluent, Rf = 0.6 in PE/EA (2:1) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 (dd, *J* = 11.1, 4.2 Hz, 2H), 7.16 (dd, *J* = 15.1, 4.4 Hz, 3H), 7.00 (s, 1H), 6.15 (d, *J* = 6.9 Hz, 1H), 5.92 (d, *J* = 7.0 Hz, 1H), 4.98 (d, *J* = 9.4 Hz, 1H), 4.82 – 4.56 (m, 1H), 4.14 (m, 2H), 4.10 (s, 1H), 3.71 (dd, *J* = 9.0, 5.2 Hz, 2H), 3.36 – 3.04 (m, 2H), 2.85 (t, *J* = 7.8 Hz, 2H), 1.36 (s, 9H), 1.27 (d, *J* = 7.1 Hz, 3H), 1.20 (t, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.8, 169.5, 155.4, 137.1, 130.4, 128.9, 128.7, 126.9, 120.3, 119.9 (q, J = 320.0 Hz), 80.2, 62.3, 53.0, 51.5, 50.2, 36.3, 35.5, 28.3, 18.0, 14.1. HRMS (ESI-TOF) m/z: for $C_{24}H_{35}F_3N_3O_7S_2^+$ $[M+H]^+$ Mass calc. 598.1863; Obs.: 598.1855.

*Ethyl-(R,Z)-13-(2-((tert-butoxycarbonyl)amino)acetamido)-2,2-dimethyl-4-oxo-8-((trifluoromethyl)sulfonyl)-3-oxa-11-thia-5,8-diazatetradec-9-en-14-oate **(5ap)***



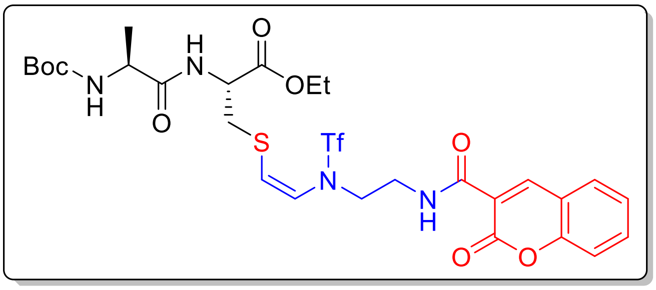
White solid, 94%, 59.8 mg, purified by flash chromatography using PA/EA (3:1) as eluent, Rf = 0.6 in PE/EA (2:1) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 (d, *J* = 24.1 Hz, 1H), 6.13 (s, 1H), 5.51 (s, 1H), 5.10 (s, 1H), 4.73 (s, 1H), 4.17 (dd, *J* = 14.1, 7.0 Hz, 3H), 3.69 (s, 1H), 3.61 – 3.45 (m, 1H), 3.24 (dt, *J* = 40.6, 14.2 Hz, 4H), 1.37 (s, 18H), 1.31 (d, *J* = 7.0 Hz, 3H), 1.23 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.3, 169.5, 156.0, 155.6, 131.2, 120.2, 119.9 (q, J = 323.0 Hz), 80.0, 62.2, 53.5, 50.1, 48.8, 38.9, 36.2, 28.3, 28.3, 18.0, 14.1. HRMS (ESI-TOF) m/z: $C_{23}H_{39}F_3N_4NaO_9S_2$ $[M+Na]^+$ Mass calc. 659.2003; Obs.: 659.2006.

N^5-((R)-1-((carboxymethyl)amino)-1-oxo-3-(((Z)-2-((1,1,1-trifluoro-N-(2-hydroxyethyl)methyl)sulfonamido)vinyl)thio)propan-2-yl)-L-glutamine ***(5mn)***



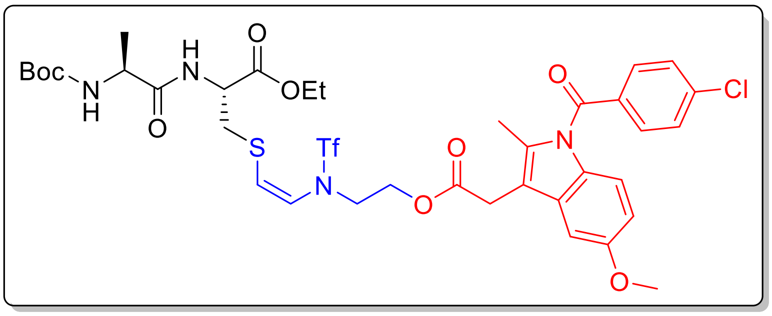
White solid, 95%, 56.8 mg, purified by recrystallization. ^{1}H NMR (400 MHz, D_2O) δ 6.57 (d, *J* = 6.8 Hz, 1H), 6.05 (d, *J* = 6.7 Hz, 1H), 4.57 (dd, *J* = 8.6, 5.1 Hz, 1H), 3.99 (dd, *J* = 11.0, 4.4 Hz, 1H), 3.96 (s, 2H), 3.75 – 3.52 (m, 4H), 3.29 (dd, *J* = 14.5, 5.1 Hz, 1H), 3.08 (dd, *J* = 14.5, 8.7 Hz, 1H), 2.62 – 2.44 (m, 2H), 2.26 – 2.08 (m, 2H) ^{13}C NMR (101 MHz, D_2O) δ 174.3, 172.9, 172.0, 171.8, 133.4, 120.3, 119.8 (q, J = 323.0 Hz), 58.4, 53.7, 52.5, 52.3, 41.2, 34.7, 31.1, 25.6. HRMS (ESI-TOF) m/z: $C_{15}H_{24}F_3N_4O_9S_2^+$ $[M+H]^+$ Mass calc. 525.0931; Obs.: 525.0937.

Ethyl-N-((tert-butoxycarbonyl)-L-alanyl)-S-((Z)-2-((1,1,1-trifluoro-N-(2-(2-oxo-2H-chromene-3-carboxamido)ethyl)methyl)sulfonamido)vinyl)-L-cysteinate ***(5aq)***



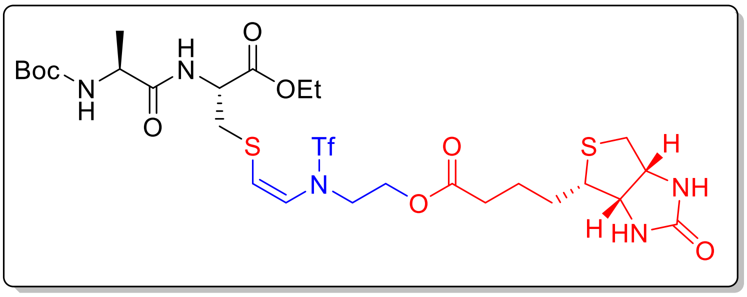
Yellow solid, 92%, 65.2 mg, purified by flash chromatography using PA/EA (2:1) as eluent, Rf = 0.6 in PE/EA (1:1) ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.18 (t, *J* = 5.7 Hz, 1H), 8.99 (s, 1H), 7.80 (d, *J* = 7.8 Hz, 1H), 7.69 (t, *J* = 7.8 Hz, 1H), 7.52 – 7.33 (m, 2H), 6.28 (d, *J* = 6.9 Hz, 1H), 5.98 (d, *J* = 6.9 Hz, 1H), 5.63 (d, *J* = 5.8 Hz, 1H), 4.90 (s, 1H), 4.49 (s, 1H), 4.21 (m, 2H), 3.82 (d, *J* = 5.0 Hz, 2H), 3.71 (s, 2H), 3.53 – 3.27 (m, 2H), 1.45 (d, *J* = 11.2 Hz, 3H), 1.40 (s, 9H), 1.29 (s, 3H) ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.4, 169.5, 162.4, 161.4, 155.3, 154.5, 149.1, 134.3, 132.4, 130.1, 125.3, 119.9 (q, J = 323.0 Hz), 119.7, 118.7, 117.9, 116.7, 79.7, 62.1, 53.1, 50.1, 48.5, 38.5, 36.6, 28.3, 18.8, 14.1. HRMS (ESI-TOF) m/z: $[M+H]^+$ Mass calc. $C_{28}H_{36}F_3N_4O_{10}S_2^+$ 709.1819; Obs.: 709.1823.

*Ethyl-N-((tert-butoxycarbonyl)-L-alanyl)-S-((Z)-2-((N-(2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetoxy)ethyl)-1,1,1-trifluoromethyl)sulfonamido)vinyl)-L-cysteinate (**5ar**)*



White solid, 95%, 83.3 mg, purified by flash chromatography using PA/EA (3:1) as eluent, Rf = 0.6 in PE/EA (2:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (d, J = 8.1 Hz, 2H), 7.47 (d, J = 8.1 Hz, 2H), 7.11 (s, 1H), 6.99 (s, 1H), 6.90 (d, J = 8.9 Hz, 1H), 6.67 (d, J = 9.0 Hz, 1H), 6.18 (d, J = 6.2 Hz, 1H), 5.87 (d, J = 6.7 Hz, 1H), 5.22 (s, 1H), 4.76 (d, J = 4.6 Hz, 1H), 4.21 (d, J = 6.8 Hz, 5H), 3.83 (s, 5H), 3.75 (s, 2H), 3.34 – 3.07 (m, 2H), 2.38 (s, 3H), 1.43 (s, 9H), 1.33 (d, J = 7.1 Hz, 3H), 1.27 (d, J = 7.4 Hz, 3H) ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.0, 169.5, 168.3, 156.1, 139.3, 136.2, 133.9, 132.0, 131.2, 130.9, 130.7, 129.1, 119.9 (q, J = 323.0 Hz),119.5, 114.9, 112.0, 111.6, 101.5, 80.1, 62.2, 61.1, 55.7, 52.9, 50.2, 48.1, 36.1, 30.1, 28.3, 18.1, 14.1, 13.3. HRMS (ESI-TOF) m/z: $C_{37}H_{45}ClF_3N_4O_{11}S_2^+$ $[M+H]^+$ Mass calc. 877.2161; Obs.: 877.2164.

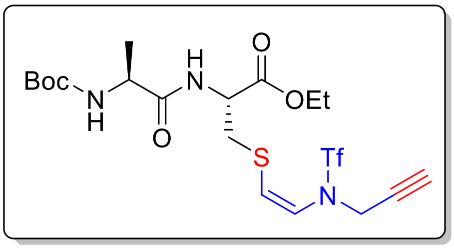
*(6S,9R,Z)-9-(ethoxycarbonyl)-2,2,6-trimethyl-4,7-dioxo-14-((trifluoromethyl)sulfonyl)-3-oxa-11-thia-5,8,14-triazahexadec-12-en-16-yl-4-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)butanoate (**5as**)*



White solid, 85%, 63.8 mg, purified by flash chromatography using DCM/MeOH (20:1) as eluent, Rf = 0.7 in DCM/MeOH (10:1). ^{1}H

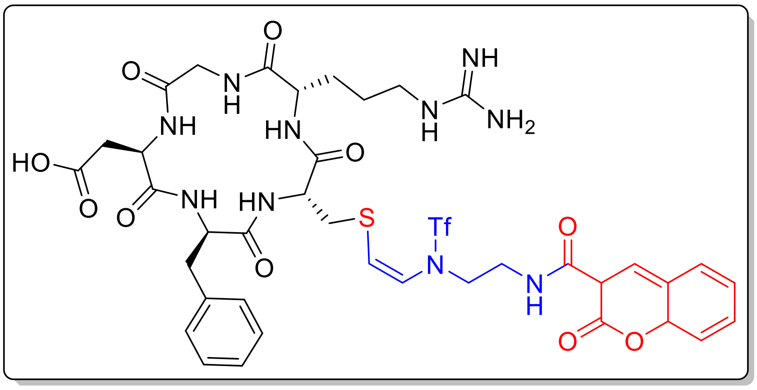
NMR (400 MHz, $CDCl_3$) δ 7.55 (s, 1H), 6.33 (d, *J* = 6.2 Hz, 2H), 6.12 – 5.86 (m, 2H), 5.57 (s, 1H), 4.79 (d, *J* = 5.4 Hz, 1H), 4.51 (s, 1H), 4.32 (s, 1H), 4.23 (d, *J* = 6.4 Hz, 4H), 3.85 (s, 2H), 3.24 (m, 3H), 2.91 (dd, *J* = 12.4, 4.0 Hz, 1H), 2.75 (d, *J* = 12.7 Hz, 1H), 2.38 (t, *J* = 7.0 Hz, 2H), 1.68 (d, *J* = 6.5 Hz, 4H), 1.46 (d, *J* = 18.0 Hz, 9H), 1.36 (d, *J* = 6.7 Hz, 3H), 1.30 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.5, 173.4, 169.7, 164.1, 155.6, 132.0, 119.7 (q, J = 323.0 Hz), 119.7, 79.9, 62.2, 62.1, 60.7, 60.1, 55.6, 53.0, 50.1, 48.2, 40.5, 36.1, 33.8, 29.6, 28.4, 28.3, 28.2, 24.6, 18.2, 14.1. HRMS (ESI-TOF) m/z: $C_{27}H_{46}F_3N_6O_{10}S_3^+$ $[M + NH_4]^+$ Mass calc. 764.2384; Obs.: 764.2296.

Ethyl-N-((tert-butoxycarbonyl)-L-alanyl)-S-((Z)-2-((1,1,1-trifluoro-N-(prop-2-yn-1-yl)methyl)sulfonamido)vinyl)-L-cysteinate ***(5at)***



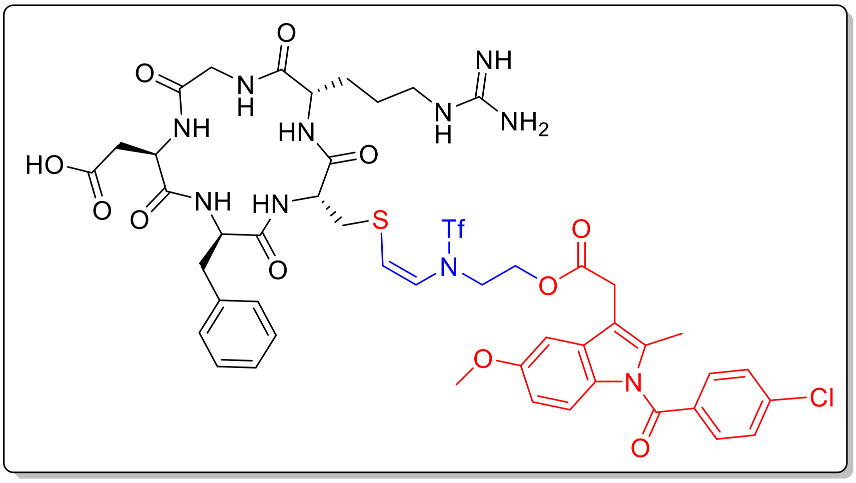
White solid, 92%, 48.9 mg, purified by flash chromatography using PA/EA (5:1) as eluent, Rf = 0.5 in PE/EA (3:1). ^1H NMR (400 MHz, $CDCl_3$) δ 7.07 (d, *J* = 6.0 Hz, 1H), 6.37 (d, *J* = 6.6 Hz, 1H), 6.11 (d, *J* = 6.6 Hz, 1H), 5.12 (d, *J* = 7.0 Hz, 1H), 4.95 – 4.70 (m, 1H), 4.42 (q, *J* = 18.0 Hz, 2H), 4.23 (m, 3H), 3.30 (m, 2H), 2.46 (s, 1H), 1.45 (s, 9H), 1.37 (d, *J* = 6.9 Hz, 3H), 1.30 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.8, 169.5, 155.4, 133.0, 120.0, 119.7 (q, J = 323.0 Hz), 80.2, 76.1, 74.6, 62.3, 53.0, 50.3, 39.4, 36.2, 28.3, 18.1, 14.0. HRMS (ESI-TOF) m/z: $C_{19}H_{29}F_3N_3O_7S_2^+$ $[M+H]^+$ Mass calc. 532.1394; Obs.: 532.1395.

2-((2R,5R,8R,11S)-5-benzyl-11-(3-guanidinopropyl)-3,6,9,12,15-pentaoxo-8-((((Z)-2-((1,1,1-trifluoro-N-(2-(2-oxo-3,8a-dihydro-2H-chromene-3-carboxamido)ethyl)methyl)sulfonamido)vinyl)thio)methyl)-1,4,7,10,13-pentaazacyclopentadecan-2-yl)acetic acid ***(5oq)***



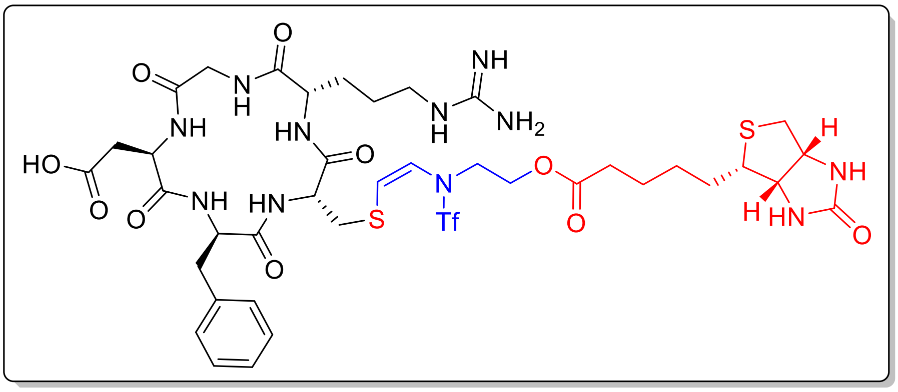
Yelow solid, 90%, 87.2 mg, purified by recrystallization. ^{1}H NMR (400 MHz, DMSO) δ 8.98 (t, *J* = 5.8 Hz, 1H), 8.92 (s, 1H), 8.37 – 8.24 (m, 1H), 8.17 (t, *J* = 7.9 Hz, 2H), 8.06 – 7.96 (m, 2H), 7.78 (t, *J* = 7.8 Hz, 1H), 7.59 (s, 1H), 7.56 – 7.42 (m, 2H), 7.24 (m, 6H), 6.76 (d, *J* = 6.8 Hz, 1H), 6.13 (d, *J* = 6.7 Hz, 1H), 4.67 (d, *J* = 6.0 Hz, 1H), 4.53 (d, *J* = 7.0 Hz, 1H), 4.26 (d, *J* = 4.9 Hz, 1H), 4.21 – 4.07 (m, 1H), 3.78 (t, *J* = 5.3 Hz, 2H), 3.55 (dd, *J* = 11.1, 5.5 Hz, 2H), 3.28 (dd, *J* = 15.0, 3.1 Hz, 1H), 3.02 (dd, *J* = 13.5, 7.6 Hz, 1H), 2.82 (dd, *J* = 14.7, 7.3 Hz, 2H), 2.78 – 2.65 (m, 2H), 2.39 (dd, *J* = 16.1, 5.5 Hz, 1H), 2.22 (t, *J* = 8.4 Hz, 1H), 1.93 – 1.68 (m, 1H), 1.46 (dd, *J* = 28.1, 21.2 Hz, 3H), 1.29 – 1.12 (m, 3H) ^{13}C NMR (101 MHz, DMSO) δ 173.1, 172.4, 171.8, 171.0, 163.3, 157.6, 139.0, 129.3, 128.4, 126.4, 119.8 (q, J = 320.0 Hz), 119.2, 65.4, 61.5, 60.5, 59.7, 56.5, 55.8, 53.3, 48.4, 43.3, 36.7, 33.7, 28.4, 24.7, 19.0, 15.6. HRMS (ESI-TOF) m/z: $C_{39}H_{48}F_3N_{10}O_{12}S_2^+$ $[M+H]^+$ Mass calc. 967.2841; Obs.: 967.2844.

*2-((2R,5R,8R,11S)-5-benzyl-8-((((Z)-2-((N-(2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetoxy)ethyl)-1,1,1-trifluoromethyl)sulfonamido)vinyl)thio)methyl)-11-(3-guanidinopropyl)-3,6,9,12,15-pentaoxo-1,4,7,10,13-pentaazacyclopentadecan-2-yl)acetic acid (**5or**)*



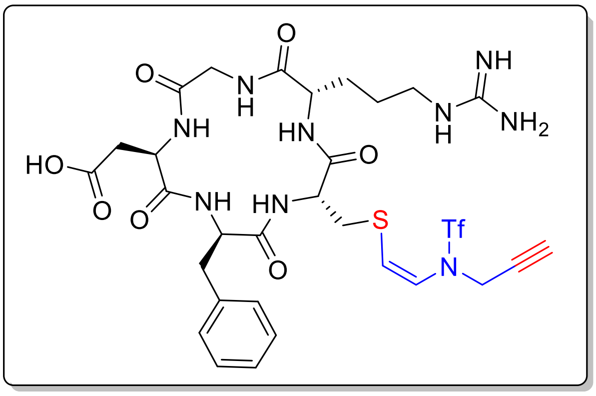
White solid, 89%, 101.0 mg, purified by recrystallization. ^{1}H NMR (400 MHz, DMSO) δ 8.43 – 8.19 (m, 3H), 8.10 (d, *J* = 8.1 Hz, 1H), 7.83 (d, *J* = 8.0 Hz, 1H), 7.72 – 7.61 (m, 4H), 7.50 (d, *J* = 26.3 Hz, 1H), 7.29 – 7.08 (m, 6H), 7.02 (d, *J* = 2.1 Hz, 1H), 6.92 (d, *J* = 9.0 Hz, 1H), 6.80 – 6.59 (m, 2H), 6.04 (d, *J* = 6.8 Hz, 1H), 4.62 (d, *J* = 7.9 Hz, 1H), 4.52 (dd, *J* = 14.2, 7.0 Hz, 1H), 4.14 (m, 3H), 3.41 – 3.33 (m, 1H), 3.31 – 3.19 (m, 1H), 3.17 – 3.05 (m, 3H), 2.76 (dd, *J* = 13.9, 7.1 Hz, 1H), 2.66 (dd, *J* = 15.9, 8.4 Hz, 1H), 2.34 (dd, *J* = 16.1, 5.4 Hz, 1H), 2.23 (s, 3H), 1.83 (d, *J* = 19.3 Hz, 1H), 1.72 (d, *J* = 12.0 Hz, 2H), 1.68 – 1.59 (m, 2H), 1.54 (d, *J* = 8.7 Hz, 1H), 1.49 – 1.37 (m, 3H), 1.27 – 1.19 (m, 1H), 1.12 (m, 3H). ^{13}C NMR (101 MHz, DMSO) δ 172.1, 171.2, 171.1, 170.8, 170.5, 170.3, 169.7, 168.3, 157.2, 156.1, 138.2, 138.1, 136.1, 134.5, 133.2, 131.7, 131.0, 130.7, 129.5, 129.5, 128.5, 126.6, 119.8 (q, J = 320.0 Hz), 119.5, 117.7, 112.8, 112.0, 102.0, 78.3, 68.7, 62.3, 61.2, 55.8, 54.2, 52.9, 49.4, 48.4, 35.8, 30.7, 25.8, 24.3, 13.6. HRMS (ESI-TOF) m/z: $C_{48}H_{55}ClF_3N_{10}O_{13}S_2^+$ [M+H]$^+$ Mass calc. 1135.3027; Obs.: 1135.3031.

*2-((2R,5R,8R,11S)-5-benzyl-11-(3-guanidinopropyl)-3,6,9,12,15-pentaoxo-8-((((Z)-2-((1,1,1-trifluoro-N-(2-((5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoyl)oxy)ethyl)methyl)sulfonamido)vinyl)thio)methyl)-1,4,7,10,13-pentaazacyclopentadecan-2-yl)acetic acid (**5os**)*



White solid, 92%, 94.4 mg, purified by recrystallization. ^{1}H NMR (400 MHz, DMSO) δ 8.37 (d, *J* = 119.4 Hz, 2H), 7.77 (s, 2H), 7.19 (dd, *J* = 15.5, 6.5 Hz, 6H), 6.73 (d, *J* = 5.8 Hz, 1H), 6.45 (d, *J* = 32.0 Hz, 2H), 6.02 (d, *J* = 5.6 Hz, 1H), 4.57 (d, *J* = 68.1 Hz, 2H), 4.31 (s, 1H), 4.13 (t, *J* = 37.2 Hz, 4H), 3.76 (s, 1H), 3.41 (m, 6H), 3.21 (s, 2H), 3.09 (s, 3H), 2.81 (s, 1H), 2.63 – 2.53 (m, 2H), 2.30 (s, 2H), 2.05 (d, *J* = 32.0 Hz, 1H), 1.73 (d, *J* = 36.2 Hz, 1H), 1.68 – 1.20 (m, 8H), 1.12 – 0.98 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 173.1, 172.4, 171.8, 171.0, 163.3, 157.6, 139.0, 129.3, 128.4, 126.4, 119.9 (q, J = 320.0 Hz), 119.2, 116.0, 65.4, 61.5, 60.5, 59.7, 56.5, 55.8, 53.3, 48.4, 43.3, 36.7, 33.7, 28.4, 24.7, 19.0, 15.6. HRMS (ESI-TOF) m/z: $C_{39}H_{55}F_3N_{11}O_{12}S_3^+$ [M+H]$^+$ Mass calc. 1022.3140; Obs.: 1022.3117.

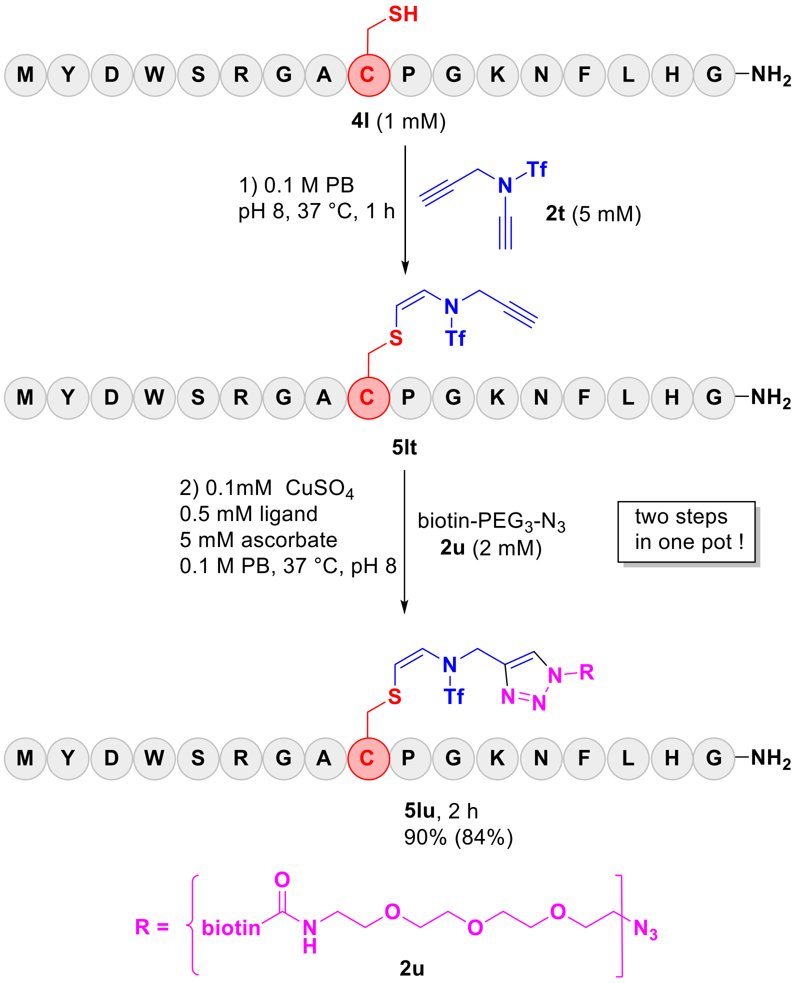
2-((2R,5R,8R,11S)-5-benzyl-11-(3-guanidinopropyl)-3,6,9,12,15-pentaoxo-8-((((Z)-2-((1,1,1-trifluoro-N-(prop-2-yn-1-yl)methyl)sulfonamido)vinyl)thio)methyl)-1,4,7,10,13-pentaazacyclopentadecan-2-yl)acetic acid ***(5ot)***



White solid, 92%, 72.7 mg, purified by recrystallization. ^{1}H NMR (400 MHz, DMSO) δ 8.31 – 8.19 (m, 1H), 8.19 – 8.06 (m, 1H), 7.96 (dd, *J* = 8.0, 4.4 Hz, 1H), 7.57 (s, 1H), 7.35 – 7.12 (m, 5H), 6.79 (d, *J* = 6.7 Hz, 1H), 6.14 (d, *J* = 6.6 Hz, 1H), 4.64 (dd, *J* = 14.1, 8.1 Hz, 1H), 4.58 – 4.47 (m, 1H), 4.42 (d, *J* = 1.9 Hz, 2H), 4.32 – 3.95 (m, 2H), 3.52 (t, *J* = 2.1 Hz, 1H), 3.36 – 3.20 (m, 1H), 3.09 (s, 2H), 3.02 (d, *J* = 7.6 Hz, 1H), 2.82 (dd, *J* = 14.7, 7.3 Hz, 3H), 2.76 – 2.62 (m, 2H), 2.43 – 2.29 (m, 1H), 2.19 (t, *J* = 8.4 Hz, 1H), 1.78 (d, *J* = 6.9 Hz, 1H), 1.56 – 1.33 (m, 2H), 1.23 (d, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 172.1, 171.4, 171.3, 170.6, 170.5, 169.8, 157.1, 137.9, 134.5, 129.7, 129.6, 128.6, 126.72, 119.8 (q, J = 320.0 Hz), 119.0, 77.4, 77.1, 57.6, 54.7, 52.7, 49.4, 43.7, 37.4, 35.8, 28.4, 27.56, 26.3, 25.1, 16.1. HRMS (ESI-TOF) m/z: $C_{30}H_{39}F_3N_9O_9S_2^+$ $[M+H]^+$ Mass calc. 790.2259; Obs.: 790.2278.

5. One-Pot Dual Functionalization of Cys with Click Chemistry

Ynamide **2t** (1.9 mg, 9 µmol, 0.45 M) was dissolved in 200 µL MeCN as **2t** stock solution. $CuSO_4\cdot 5H_2O$ (5.1 mg, 20.4 µmol, 20 mM) was dissolved in water as $CuSO_4$ stock solution. Sodium ascorbate (41 mg, 207 µmol, 100 mM) was dissolved in 2.07 ml water as ascorbate stock solution. Bathophenanthroline disulfonic acid disodium salt (BPS ligand, 1.6 mg, 2.98 µmol, 50 mM) was dissolved in 59.6 µl water as BPS ligand stock solution.



Scheme S4. One-pot dual modification of peptide **4l** with **2t** and **2u**

Peptide **4l** (3.6 mg, 1.86 µmol, 1 mM) was dissolved in 1836.4 µL PB (0.1 M, pH 8) and 20.6 µL of **2t** stock solution was then added to the peptide solution. The reaction mixture was incubated at 37 °C and monitored by using RP-HPLC (XSelect C18 column (3.5 µm, 130 Å, 4.6 × 150 mm) with a gradient of 5-70% B (0.1% TFA in

MeCN) over 20 min at 220 nm), in which 10 µL aliquots of the reaction mixture were taken and quenched by 50 µL 0.1% TFA in water. The reaction was completed after 1 h and the conjugated product **5lt** was obs. (mass obs. 2148.4 Da, Mass calc. 2147.8 Da) The results are shown in Figure S5. Next, copper catalyzed azide-alkyne cyclization (CuAAC)[6] was performed in one-pot by adding the following: 12.3 µL of $CuSO_4$ stock solution (final concentration 0.1 mM), 123 µL of ascorbate stock solution (final concentration 5 mM), 24.6 µL of BPS ligand stock solution (final concentration 0.5 mM) and biotin-PEG_3-N_3 (1 mg, 1.2 equiv, 2.232 µmol) The reaction mixture was incubated at 37 °C. The click reaction took 30 min to give a single desired product in 84% isolated yield (mass obs. 2593.6 Da, Mass calc. 2593.9 Da) The results are shown in Figure S43.

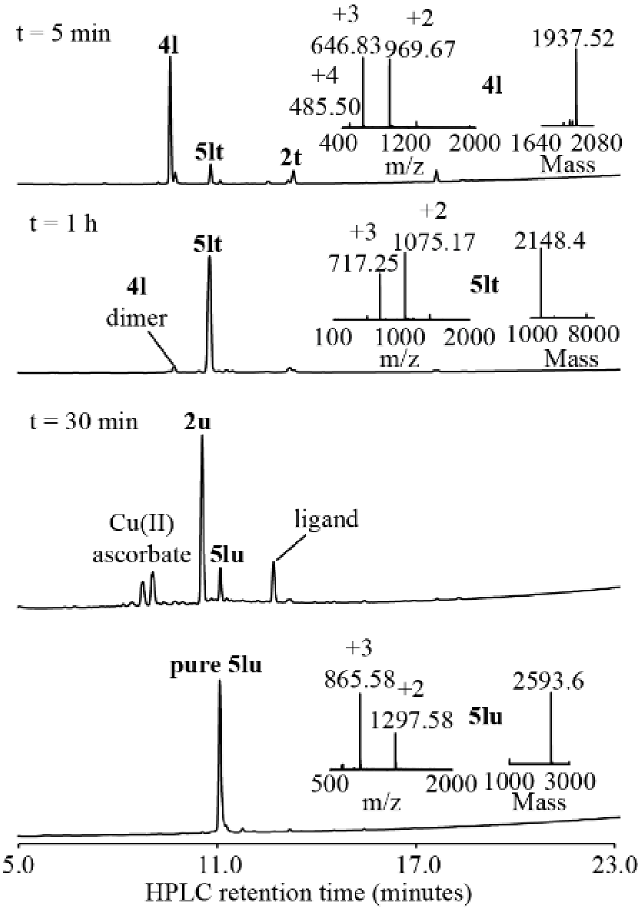


Figure S43. One pot dual modification of peptide **4l** with click chemistry.

6. Modification of Cys-Containing Proteins.

1) *Modification of ubiquitin(G47C) (**6**) by ynamide **2s***

Ynamide **2s** (11.7 mg, 26.4 μmol, 53 mM) was dissolved in 500 μL MeCN as **2s** stock solution.

Ubiquitin(G47C) (**6**, 0.12 mg, 0.014 μmol) was dissolved in 8.4 μL degassed phosphate buffer (PB, 0.1 M, 6 M Gn·HCl, pH 8), which was then diluted in 46.3 mL PB (0.1 M, pH 8) to afford ubiquitin(G47C) (**6**) solution (250 μM, 0.9 M Gn·HCl, pH 8). The protein solution was incubated for 1 h at room temperature to allow folding. Next, 1.3 μL of **2s** stock solution (5 equiv) was added to the above protein solution. The reaction mixture was incubated at 37 °C and the reaction progress was monitored by RP-HPLC (X-Bridge C4 column, 3.5 μm, 4.6 × 150 mm) with a gradient of 5-70% of B (1% TFA in MeCN) over 25 min at 220 nm (Figure S9). After 12 h, the modified ubiquitin(G47C*) (**7**) was isolated in 43% yield (mass obs. 9054.1 Da, calc. 9054.4 Da). The results are shown in Figure S44.

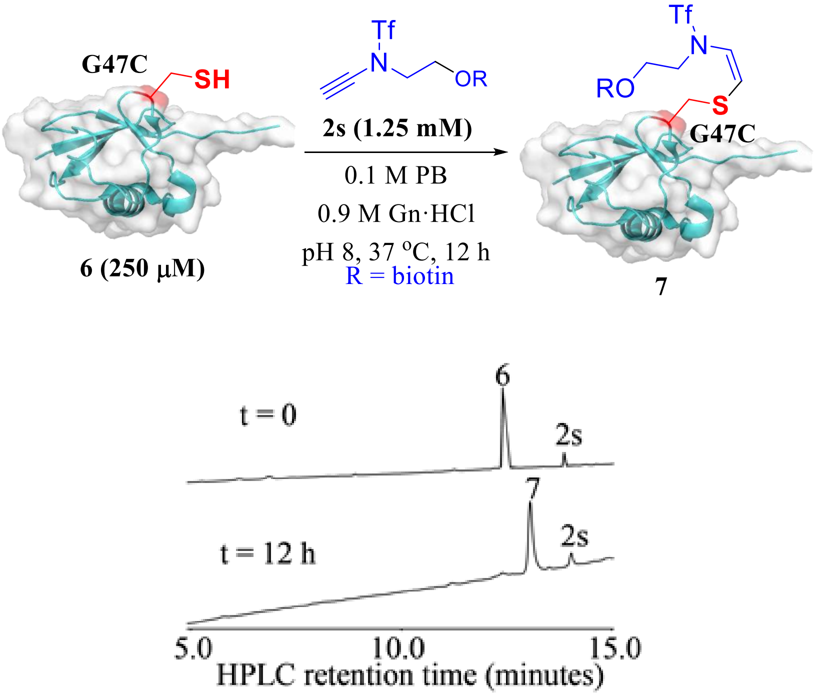


Figure S44. Modification of ubiquitin(G47C) (**6**) with biotinylated ynamide **2s**.

2) *The reaction of ubiquitin(G47A) by ynamide **2s***

The preparation of ubiquitin(G47A) (**6'**) was reported previously by the deselenization[7] of selenocysteine (Sec, U)-containing ubiquitin(G47U) variant[8]. The modification of ubiquitin(G47A) was performed under the same conditions as with ubiquitin(G47C) (**6**), however, no modification of ubiquitin(G47A) was obs. even after 18 h incubation (Figure S45)

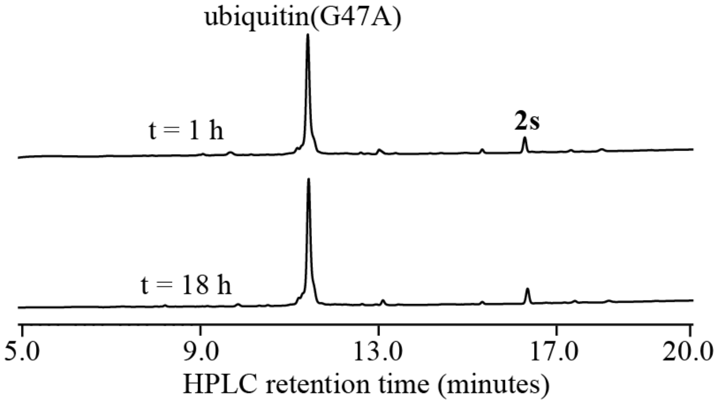


Figure S45. The modification reaction of ubiquitin(G47A) with ynamide **2s**.

3) *The modification of BPTI by ynamide* ***2n***

Ynamide **2n** (15.7 mg, 72.4 µmol, 241 mM) was dissolved in 300 µL MeCN as **2n** stock solution. Folded BPTI (0.3 mg, 0.046 µmol, 250 µM) was dissolved in 182 µL PB (0.1 M, pH 8) and 1.5 equiv of TCEP was added to the reaction at room temperature, as it was reported[9]. After 3 h, the solvent exposed disulfide bond (Cys14-Cys38) was reduced (other two disulfide bonds, Cys5-Cys55 and Cys30-Cys51, remained intact). Next, 1.87 µL of **2n** stock solution (10 equiv) was added to the above reaction. The reaction mixture was incubated at 37 °C and the reaction progress was monitored by RP-HPLC (X-Bridge C4 column, 3.5 µm, 4.6 × 150 mm) using a gradient of 5-70% B (0.1% TFA in MeCN) over 25 min at 220 nm (Figure S46). After the completion of the reaction, the doubly modified BPTI was isolated in 21.5% yield by semi-prep HPLC.

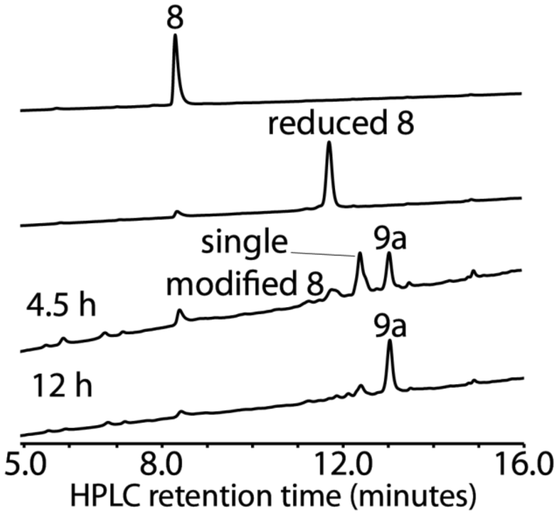


Figure S46. Selective modification of the Cys14 and Cys38 residues in BPTI with **2n**.

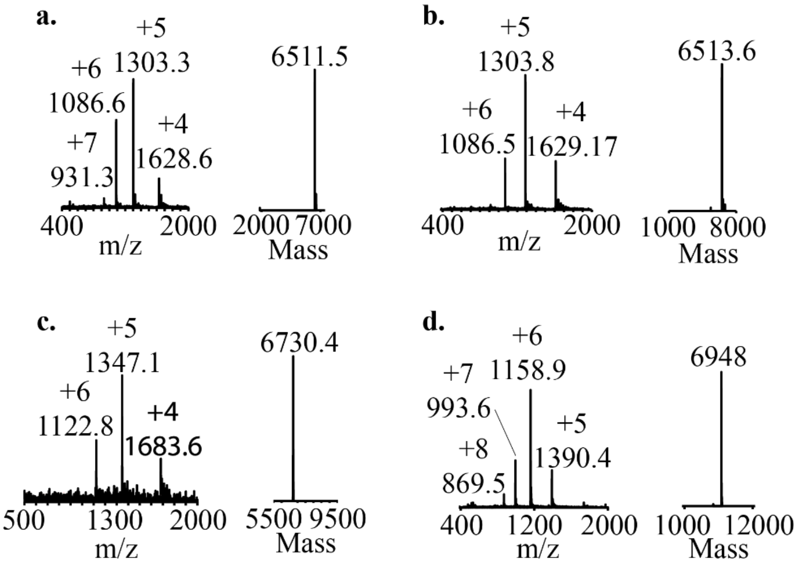


Figure S47. ESI-MS of WT-BPTI (**8**), partly reduced **8**, single modified BPTI and doubly modified BPTI (**9a**). **a**. WT-BPTI (**8**, obs. 6511.5 Da, Mass calc. 6511.5 Da), **b**. partly reduced **8** (obs. 6513.6 Da, Mass calc. 6513.5 Da), **c**. Singly modified BPTI with **2e** (obs. 6730.4 Da, Mass calc. 6730.7 Da), **d**. doubly modified BPTI (at Cys14 & Cys38) with **2n** (obs. 6948.0 Da, Mass calc. 6947.8 Da)

4) *The dual biotinylation of BPTI with biotinylated ynamide* ***2s***

The dual biotinylation of the BPTI was performed by using **2s** under the same conditions as with **2n**. After 12 h, the reaction was completed and purified to afford the dual biotinylated BPTI (**9b**) in 29% isolated yield (mass obs. 7400.6 Da, Mass calc.

7400.4 Da). The results are shown in Figure S48.

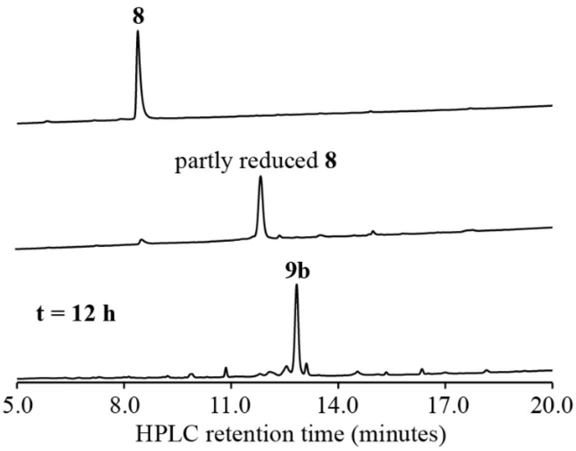


Figure S48. Dual biotinylation of BPTI by ynamide **2s**.

5) The biotinylation of Bovine Serum Albumin (BSA) with biotinylated ynamide ***2s***

BSA, **10**, (500 µM) was incubated with 2.5 mM ynamide 2s at 37 °C in PB (0.1 M, pH 8), the reaction was followed by MALDI-TOF MS (Bruker autoflex® max). After 12 h, the reaction was completed.

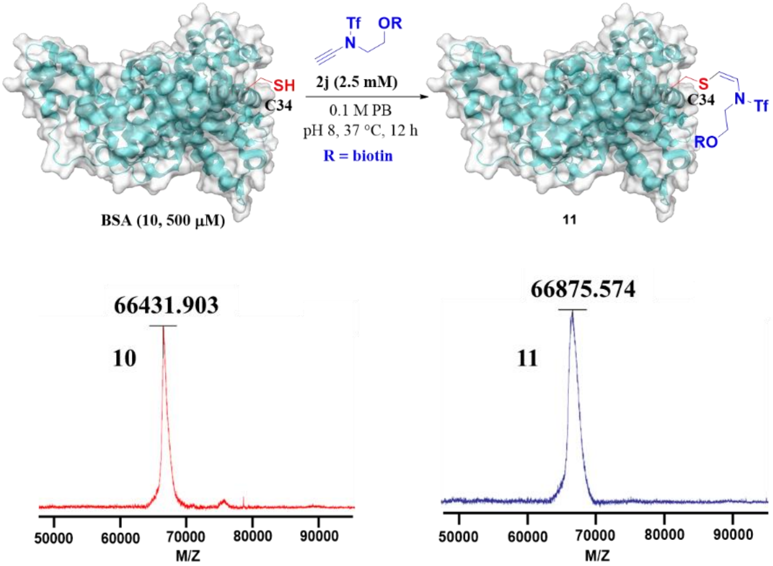


Figure S49. The modification of BSA by ynamide **2s**.

7. Stability Study of the Peptide Conjugates

1) Stability of **5an** (1 mM) in buffers at pH 2.0, pH 8.0, pH 10.0, pH 13.0 at 37 °C for 10 h

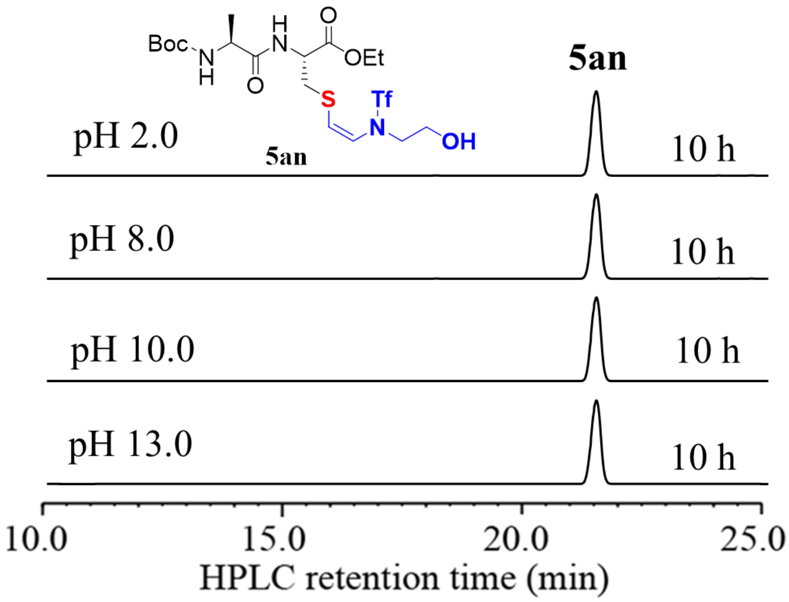


Figure S50. Stability of **5an** (1 mM) in buffers at pH 2.0, pH 8.0, pH 10.0, pH 13.0.

2) Stability of **5an** (1 mM) in the presence of external thiol (2-mercaptoethanol)

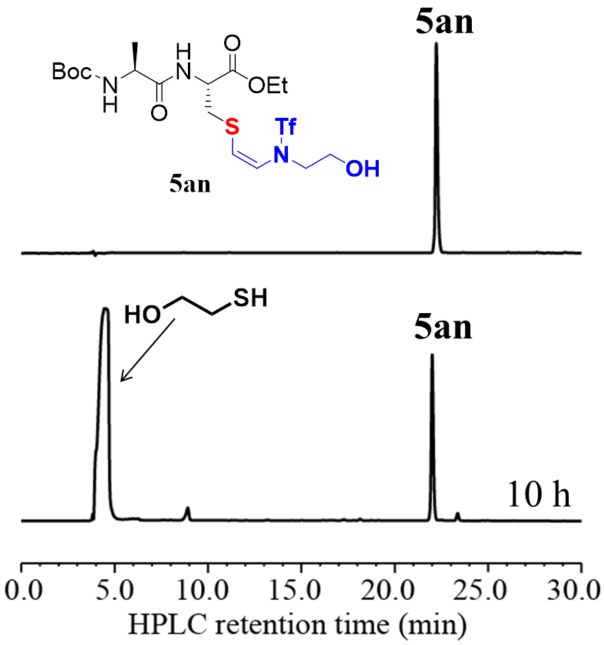


Figure S51. Stability of **5an** (1 mM) in the presence of external thiol.

3) Stability of **5an** (10 µM) when treated with large excess of H_2O_2 (at pH 4, 8 and 10 at 37 ºC) for 24 h

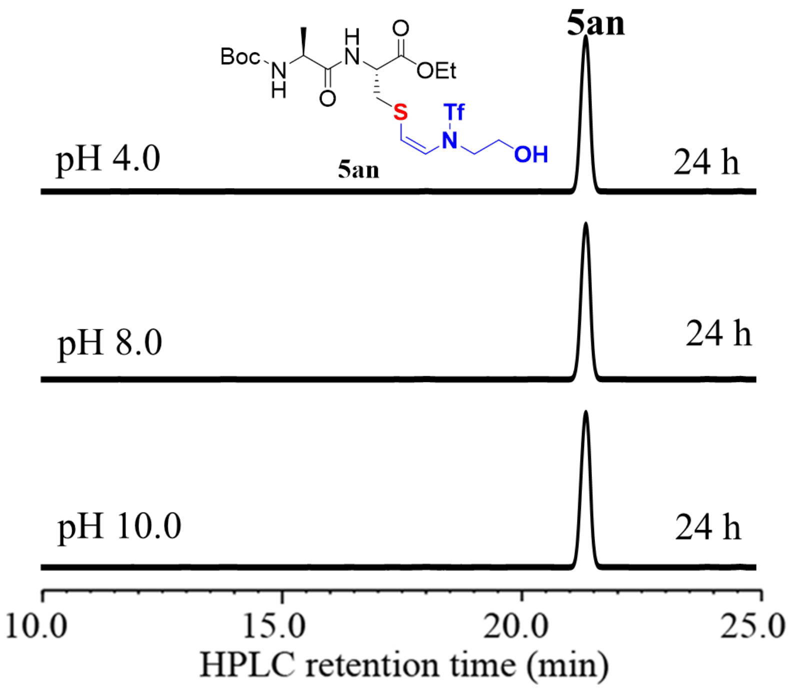


Figure S52. Stability of **5an** (10 µM) when treated with large excess of H_2O_2 (at pH 4, 8 and 10 at 37 ºC).

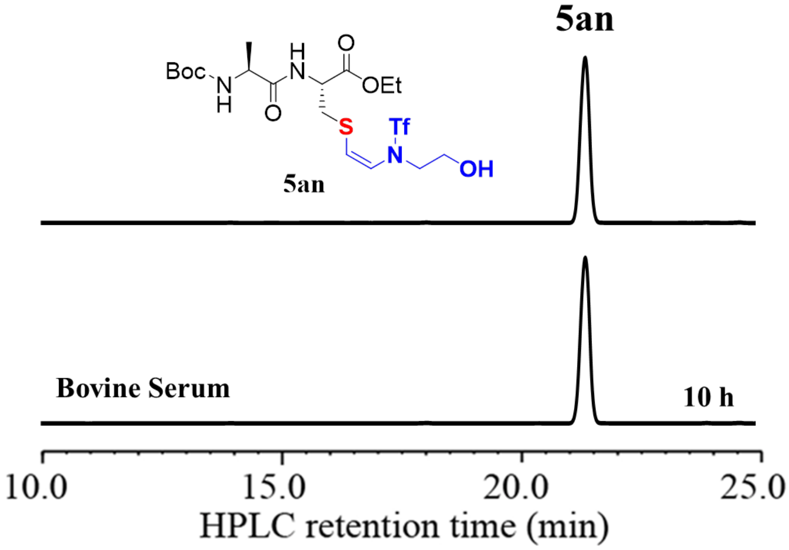


Figure S53. Stability of **5an**(10 µM) when treated with bovine serum.

8. Trastuzumab reduction

TCEP-HCl (0.8 μL out of a 1 mg/mL stock solution in H_2O, 3 nmol, 10 equiv) was added to 88 μL of Trastuzumab (0.044 mg, 0.3 nmol, c = 0.5 mg/ mL in 0.1 M PB at pH 8.0) and incubated at 37 °C for 40 min. For control reaction, this reduction step was skipped.

Trastuzumab conjugation to ynamide 2n

To the above reduced Trastuzumab was added the biotinylated ynamide (2.7 μL from 5 mg/mL solution in 30 % MeCN/H_2O, 60 nmol, 200 equiv), and the reaction was incubated at 37 °C overnight. After conjugation, the Trastuzumab was separated from excessive ynamide and buffer exchanged to 0.1 M PB, pH 8.0 by Amicon Ultra-0.5 mL centrifugal filter (3K MWCO, Merck). To do so, we first fill the sample with PB buffer to make the final volume 500 μL, and transfer the 500 μL of sample to the centrifugal filter device and cap it. Spin the device at 14,000 x g for 10 min. The remaining volume is approximately 114 μL (antibody concentration is around 0.4 mg/ mL). Discard the filtrate and refill the volume to 500 μL with pH 8.0 PB. Repeat the centrifugation using the above-described procedure. To recover the concentrated sample, place the device upside down in a clean microcentrifuge tube. Centrifuge at 1,500 x g for 2 min to transfer the sample from the device to the tube.

MS analysis of sample

To simplify the analysis, deglycosylation was first performed to remove the N-glycan heterogeneity of antibody. To do so, 30 μL of antibody was mixed with 0.5 μL of PNGase F solution (500,000 units/mL, New England Biolabs). The enzymatic reaction was incubated at 37 °C for 2-4 hours after which samples were diluted with water to a final volume of 100 μL. Prior to LC-MS analysis, 2 μL of TCEP (1 mg/mL) was added and let the reaction stand for 30 mins to maintain the reaction in a reducing environment (except for analyses of intact mass and negative control). 5 μL of the above sample was subjected for LC-MS analysis.

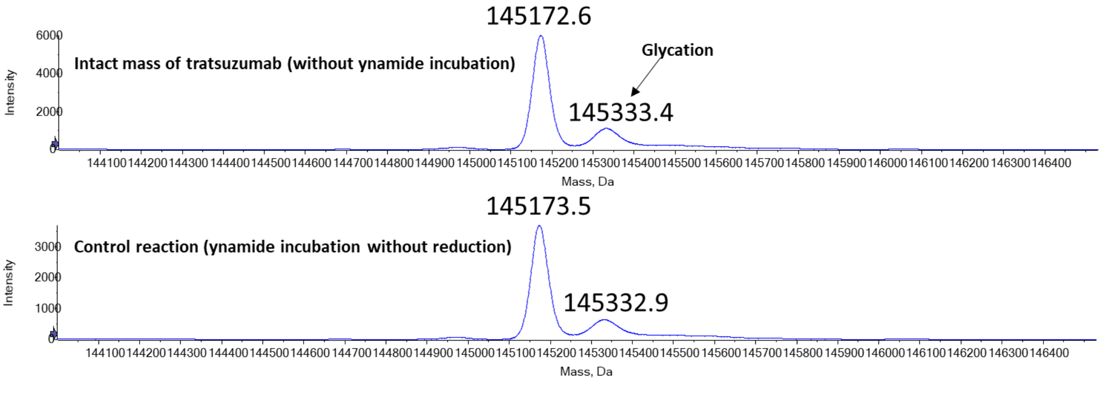


Figure S54. Deconvoluted deglycosylated intact MS spectra of tratsuzumab starting material and negative control without TCEP reduction.

9. The Chemoselectivity Evaluation of Cys Modification by Different Reagents

We investigated the chemoselectivity of three classical reactive reagents toward Cys residue. The reactions were performed with two model peptides (**14** and **22**) at different pH, which were monitored by analytical RP-HPLC (C18 column) with a gradient of 5-70% B (0.1% TFA in MeCN) over 20 min at 220 nm.

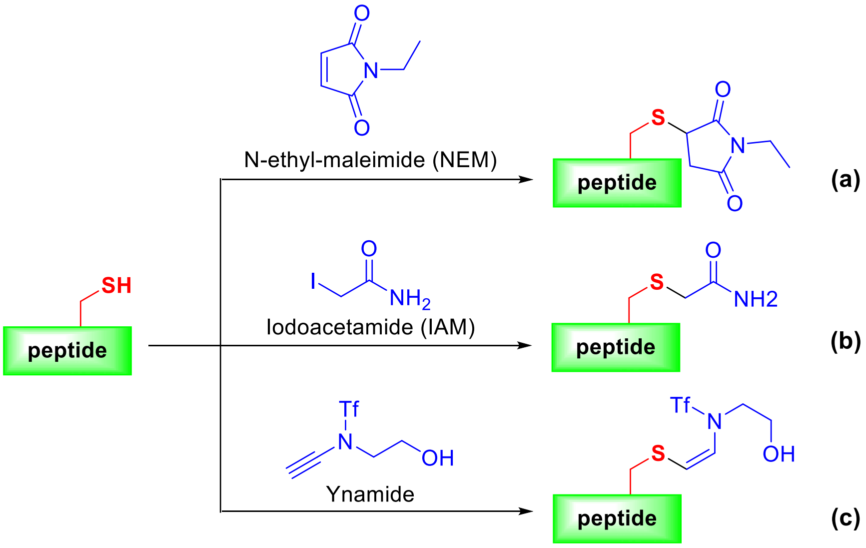


Figure S55. Modification reactions of Cys via different reagents: (**a**) *N*-ethylmaleimide (NEM), (**b**) iodoacetamide (IAM), and (**c**) ynamide **2n**.

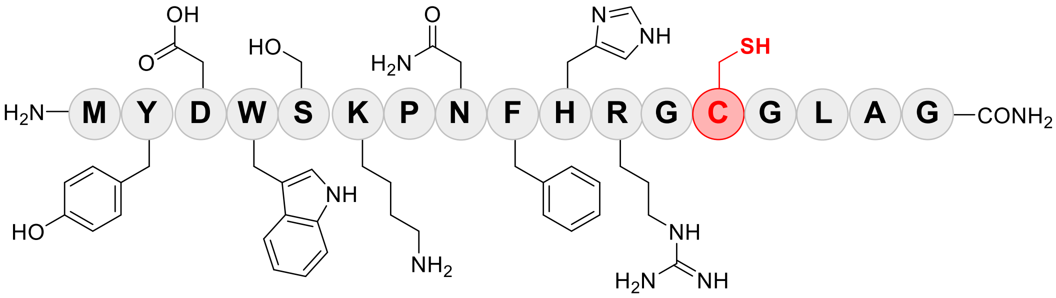


Figure S56. The sequence of model peptide **14**.

Model peptide **14**, containing all reactive amino acid side chains, was prepared according to the general procedure described previously. The crude peptide **14** was purified by Prep RP-HPLC (C18 column) to obtain **14** in ~ 38% yield (mass obs. 1937.5 Da, Mass calc. 1936.8 Da) The results are shown in Figure S156. Peptide **14** (3.5 mg, 1.8 μmol, 16.3 mM) was dissolved in 110.4 μL water as peptide **14** stock solution. Ynamide (**2n,** 8.9 mg, 41 μmol, 105 mM) was dissolved in 390.6 μL MeCN as ynamide

2n stock solution. NEM (22.8 mg, 182.2 μmol, 105 mM) was dissolved in 1735.7 μL MeCN as NEM stock solution. IAM (56.6 mg, 306 μmol, 105 mM) was dissolved in 2914.4 μL MeCN as IAM stock solution. Three aliquots of 9.2 μL of peptide **14** stock solution (0.15 μmol) were added to three aliquots of 133.7 μL PB (0.1 M, peptide final conc. 1 mM) at pH 4, 6 and 8, respectively. To these solutions, three aliquots of 7.1 μL of reactive reagents (ynamide **2n**, NEM and IAM, 5 equiv**)** were added, respectively. The reactions were incubated at 37 °C and were monitored by analytical RP-HPLC (XSelect C18 column (3.5 μm, 130 Å, 4.6 × 150 mm)) with a gradient of 5-70% B (0.1% TFA in MeCN) over 25 min at 220 nm) The yields were calculated according to integrated areas of HPLC peaks.

1) *The reaction of peptide **14** with ynamide **2n***

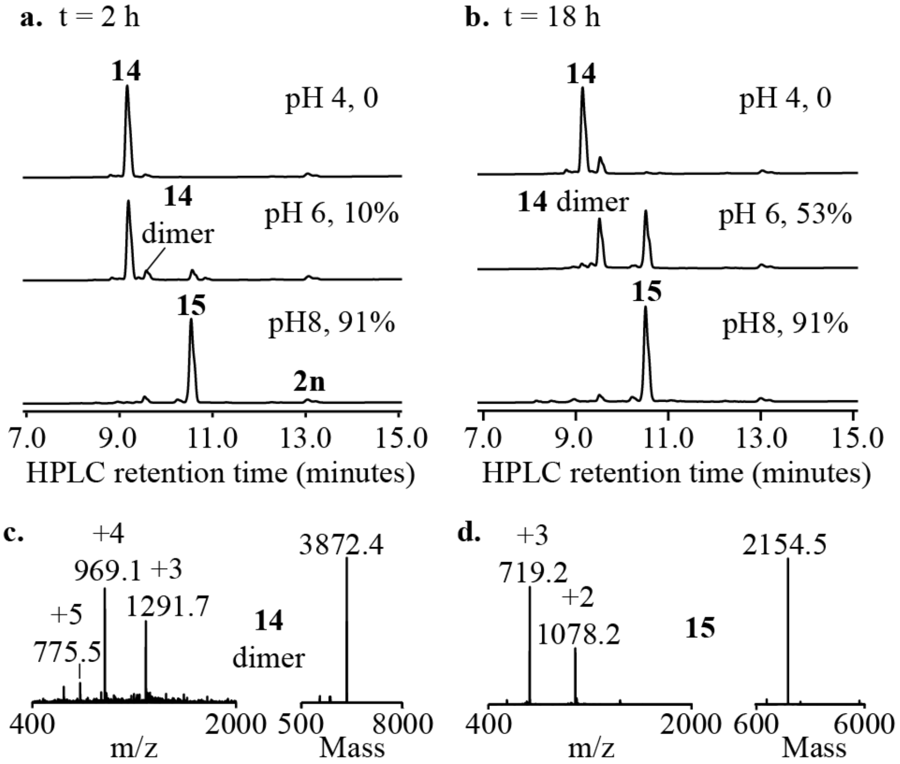


Figure S57. The modification reactions of model peptide **14** (1 mM) with ynamide (**2n**, 5 equiv) at pH 4, 6 and 8, respectively. **a**. The progress of the reactions after 2 h; **b**. The progress of reactions after 18 h. **c**. ESI-MS of peptide **14** dimer side-product (Obs. 3872.4 Da, Mass calc. 3872.8 Da) **d**. ESI-MS of modified product **15** (Obs. 2154.5 Da, Mass calc. 2154.9 Da)

2) *The reaction of peptide **14** with N-ethylmaleimide (NEM)*

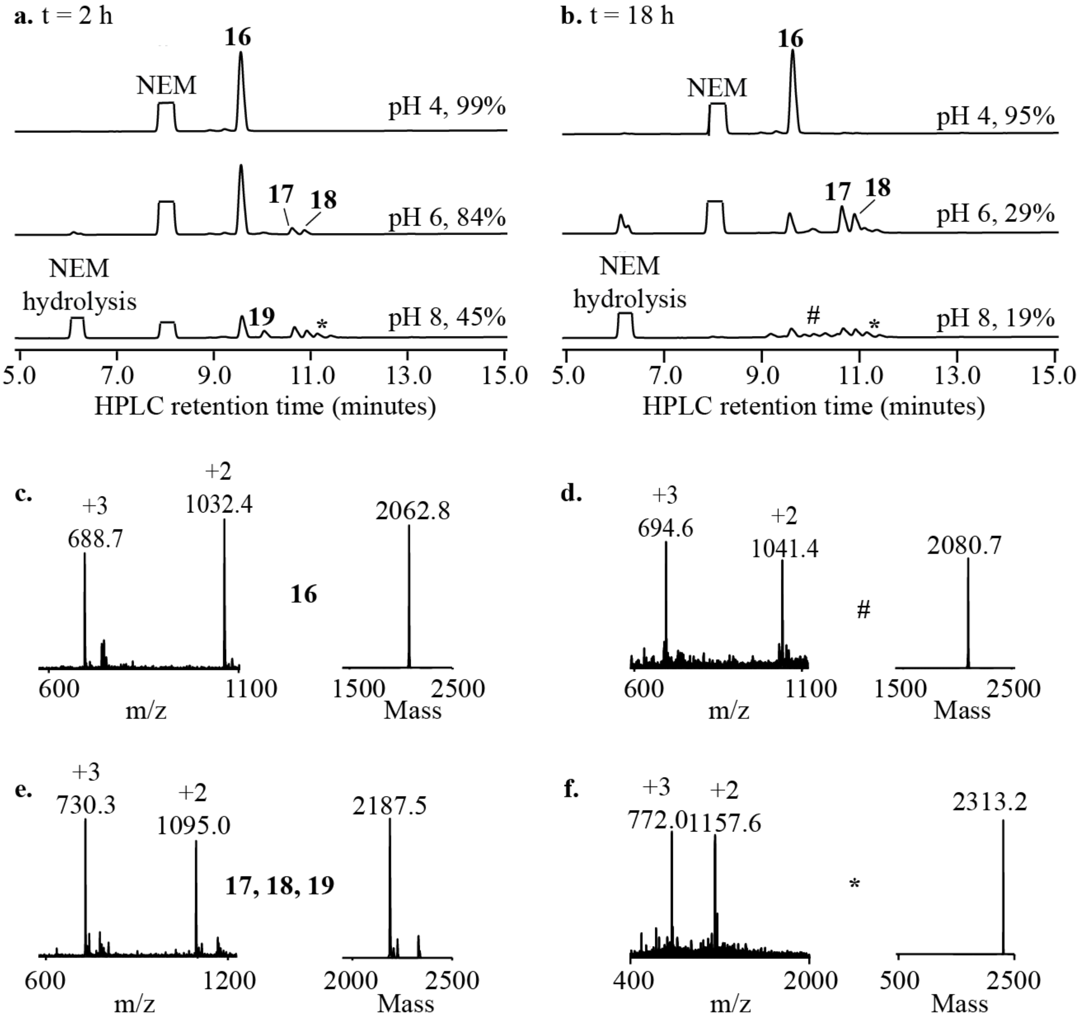


Figure S58. The modification reactions of model peptide **14** (1 mM) with NEM (5 equiv) at pH 4, 6 and 8, respectively. **a**. The progress of the reactions after 2 h; **b**. The progress of the reactions after 18 h. **126** is single modified product, **17**, **18** and **19** are doubly modified products. The product of peptide **10** with hydrolyzed NEM was observed from peak # and triple modified product was observed from peak *. **c.** ESI-MS of single NEM modified product, **16** (Obs. 2062.8 Da, Mass calc. 2062.9 Da); d. ESI-MS of the modified product of peptide **14** with hydrolyzed NEM (Obs. 2080.7 Da, Mass calc. 2080.9 Da); **e**. ESI-MS of doubly NEM modified products **17**, **18** and **19** (Obs. 2187.5 Da, Mass calc. 2188.0 Da); f. ESI-MS of triple NEM modified product

(Obs. 2313.2 Da, Mass calc. 2313.0 Da)

3) *The reaction of peptide **14** with iodoacetamide (IAM)*

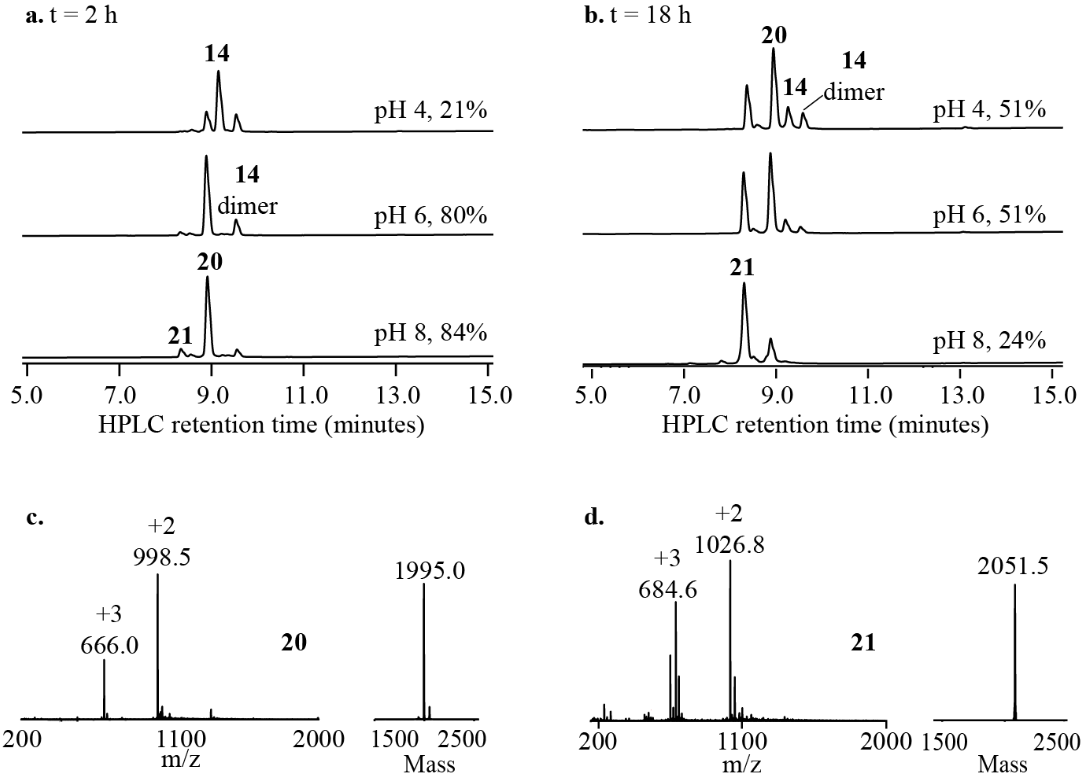


Figure S59. The modification of model peptide **14** (1 mM) with IAM (5 equiv), at pH 4, 6 and 8, respectively. **a**. The progress of the reactions after 2 h; **b**. The progress of the reactions after 18 h. **20** is singly IAM modified product, **21** is doubly IAM modified product. **c**. ESI-MS of singly IAM modified product, **20** (Obs. 1995.0 Da, Mass calc. 1995.3 Da); d. ESI-MS of doubly IAM modified product, **21** (Obs. 2051.5 Da, Mass calc. 2050.9 Da)

These results show the excellent chemoselectivity of peptide **14** modification with ynamide **2n** under the above reaction conditions, even after 18 h (Figure S60); whereas the other two classical and extensively used reagents, NEM and IAM, presented worse chemoselectivity toward Cys. Considerable amount of double and triple modification of peptide **14** with NEM and IAM were found under the same conditions (Figure S60), and only few singly modified products with NEM or IAM were obtained after 18 h.

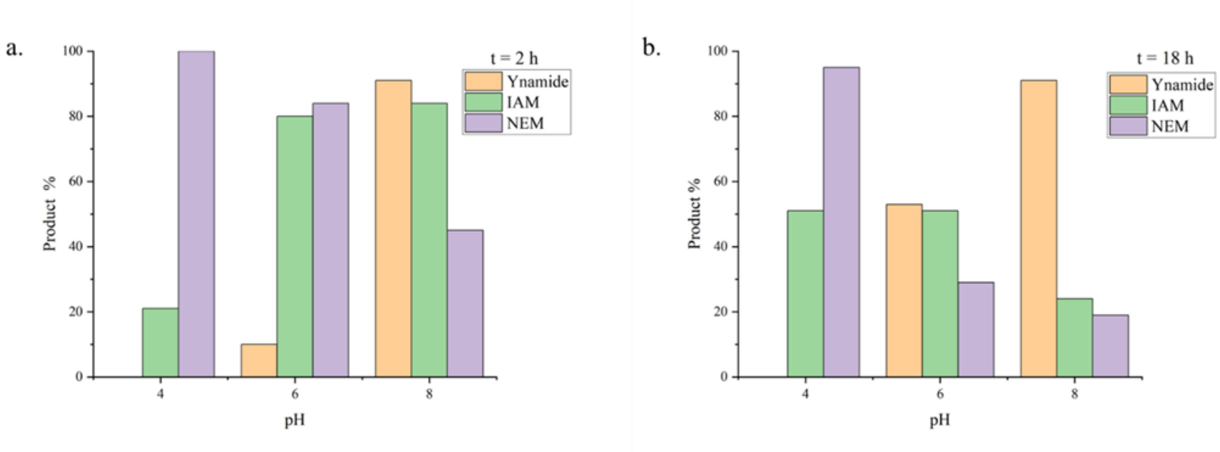


Figure S60. Modification of peptide **14** with ynamide **2n**, NEM and IAM. The graphs show only the percentage of singly modified peptide **14** with the different reagents; ynamide, NEM and IAM at different pH and after 2 h **(a)** or 18 h (**b**).

4) *Chemoselectivity evaluation of different reactive reagents with Cys-free peptide* ***22***

To further confirm the above conclusion (reactions with model peptide **14**), we designed a negative control experiments. A cysteine-free model peptide **22** with the same sequence as peptide **14** except that the Cys in peptide **14** was replace with Ala, was utilized to examine the rectivity of the three reagnets (ynamide, NEM, and IAM) toward additional amino acids. The same reaction conditions were tested for peptide **22** as with **14**.

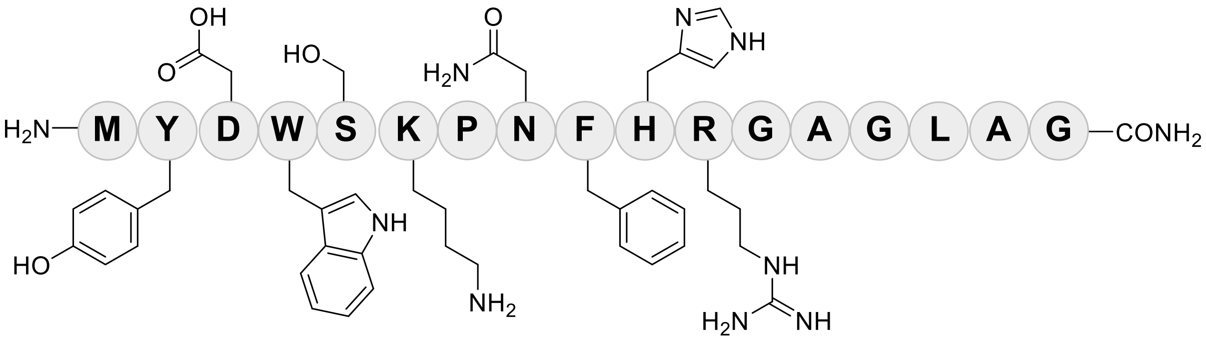


Figure S61. The sequence of peptide **22**.

Peptide **22**, containing all amino acid side chains except Cys residue, was prepared by desulfurization reaction of peptide **14**. Peptide **14** (10 mg, 5.16 µmol, 1 mM) were dissolved in 5.16 mL PB (0.1 M, pH 5) TCEP (200 equiv), V-044 initiator (10 equiv) and 10 µL of tert-butyl mercaptane (17 equiv) were then added to the peptide solution.

The reaction mixture was incubated at 37 °C for 2 h. Upon reaction completion (confirmed by HPLC and MS), peptide **22** was purified by semi-prep RP-HPLC (C18 column) to obtain **22** in ~ 84% yield (8.3 mg, Figure S190) Peptide **22** (3.3 mg, 1.73 μmol, 16.3 mM) was dissolved in 106.3 μL water as peptide **22** stock solution. The stock solutions (105 mM in MeCN) of ynamide **2n**, NEM and IAM were prepared as described previously. Three aliquots of 9.2 μL of peptide **22** stock solution (0.15 μmol) were added to three aliquots of 133.7 μL PB (0.1 M, peptide final conc. 1 mM) at pH 4, 6 and 8, respectively. To these solutions, three aliquots of 7.1 μL of reactive reagents (ynamide **2n**, NEM and IAM, 5 equiv) were added, respectively. The reactions were incubated at 37 °C and were monitored by analytical RP-HPLC (XSelect C18 column (3.5 μm, 130 Å, 4.6 × 150 mm)) with a gradient of 5-70% B (0.1% TFA in ACN) over 25 min at 220 nm). The yields were calculated according to integrated areas of HPLC peaks.

5) *The reaction of peptide **22** with ynamide **2n***

No modification of peptide **22** with ynamide **2n** was observed under the above conditions, even after 18 h.

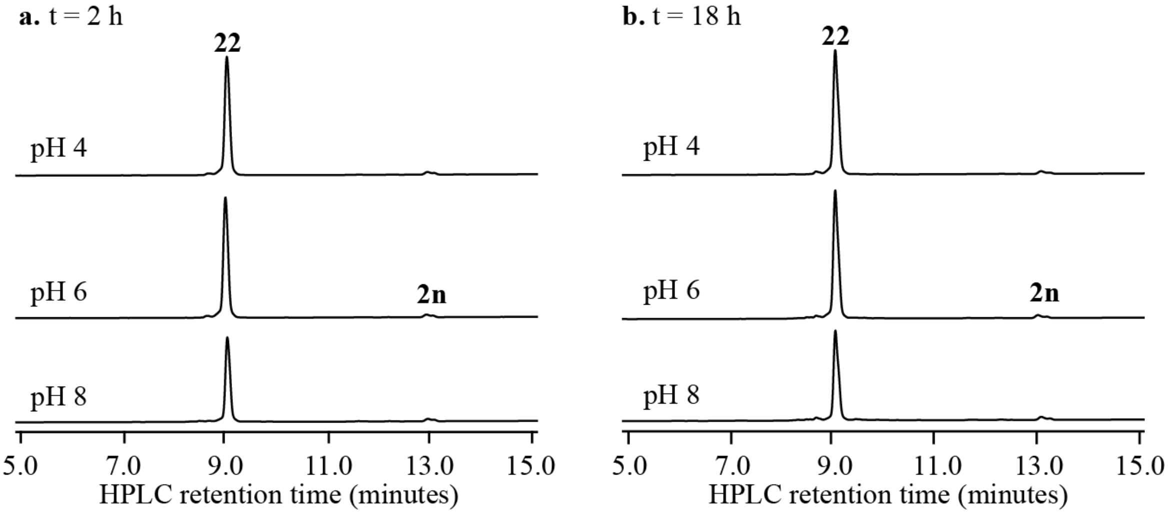


Figure S62. The reaction of peptide **22** and ynamide **2n**.

6) *The reaction of peptide **22** with N-ethylmaleimide (NEM)*

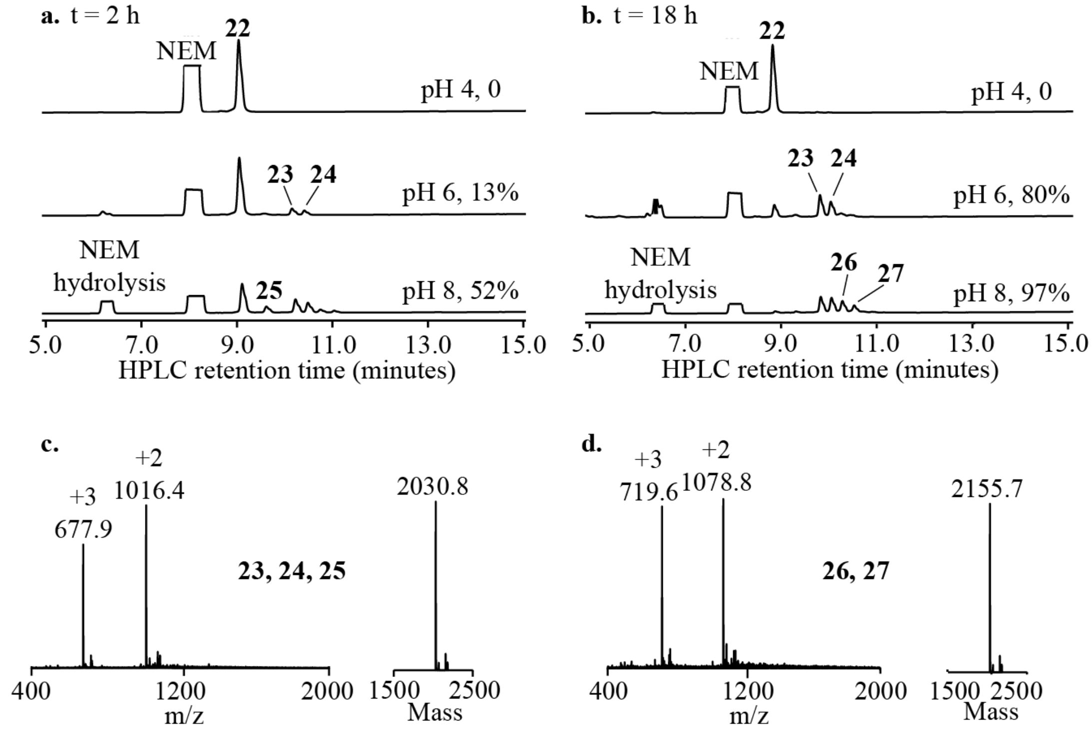


Figure S63. The modification reaction of model peptide **22** (1 mM) with NEM (5 equiv) at pH 4, 6 and 8, respectively. **a**. The progress of the reactions after 2 h; **b**. The progress of the reactions after 18 h. **23**, **24** and **25** are singly NEM modified products, **26** and **27** are doubly NEM modified products. **c**. ESI-MS of the singly NEM modified products **23**, **24** and **25** (Obs. 2030.8 Da, Mass calc. 2031.0 Da); **d**. ESI-MS of doubly NEM modified products **26** and **27** (Obs. 2155.7 Da, Mass calc. 2156.0 Da)

7) *The reaction of peptide **22** with iodoacetamide (IAM)*

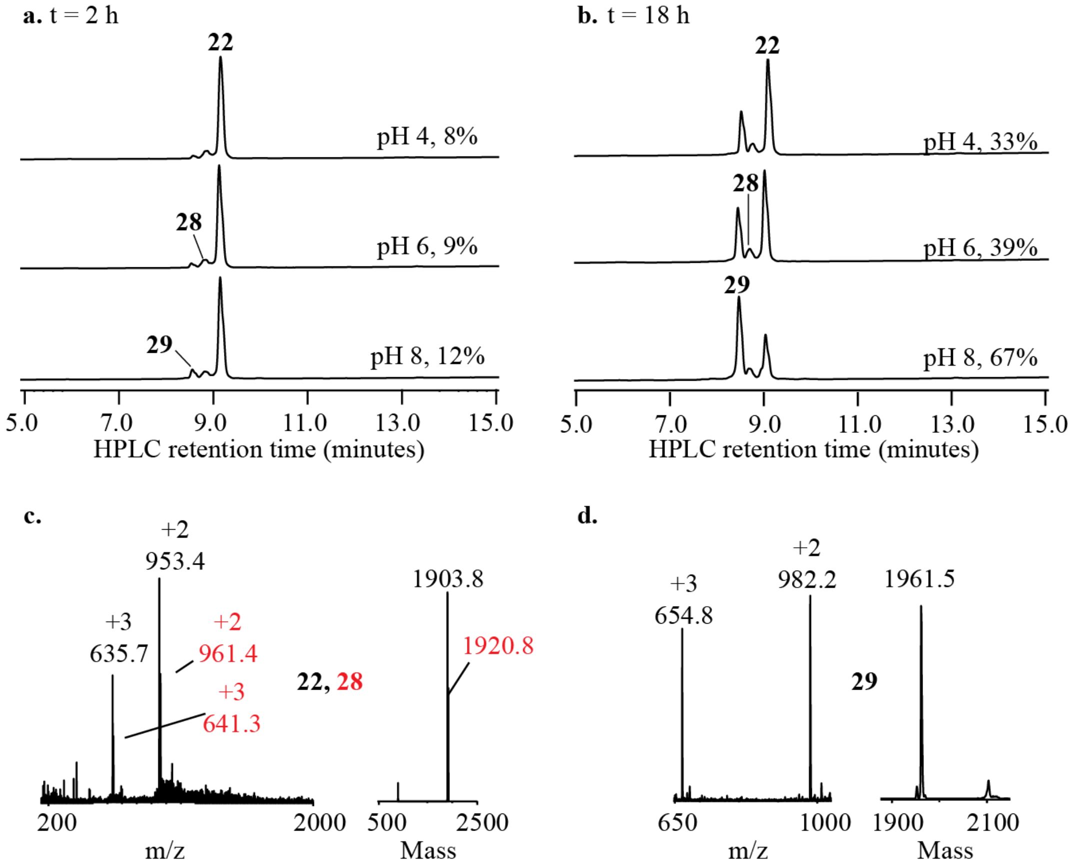


Figure S64. The modification reaction of model peptide **22** (1 mM) with IAM (5 equiv) at pH 4, 6 and 8, respectively. **a**. The progress of the reactions after 2 h; **b**. The progress of the reactions after 18 h. **28** is the oxidized peptide **22**, **29** is single NEM modified product. **c**. ESI-MS of peptide **22** with oxidation of methionine residue **28** (Obs. 1920.8 Da, Mass calc. 1920.9 Da); **d**. ESI-MS of the single IAM modified product **29** (Obs. 1961.5 Da, Mass calc. 1961.9 Da)

Based on the above results, peptide **22** did not react with ynamide **2n** (Figure S73), which supports our conclusions for the high chemoselectivity of ynamide toward Cys. However, large amount of modified products, including single and double modification, were observed from the reaction of peptide **22** with NEM and IAM (Figure S73), which showed NEM and IAM could also be reactive toward other amino acids except Cys.

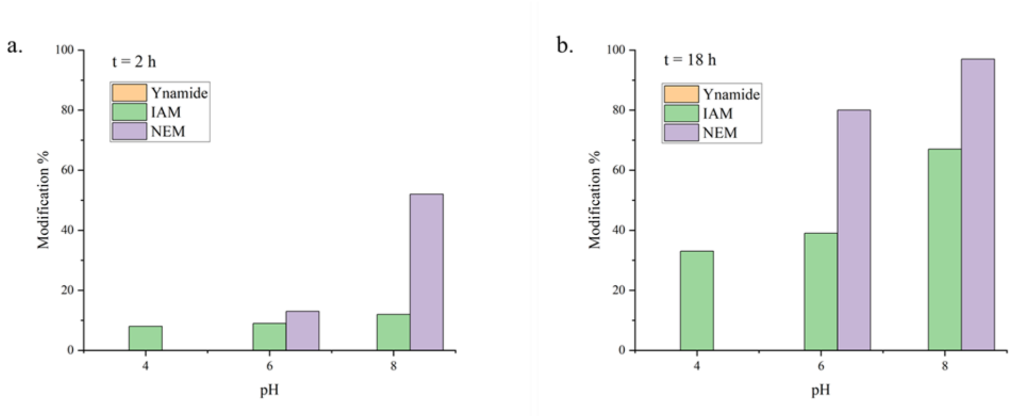
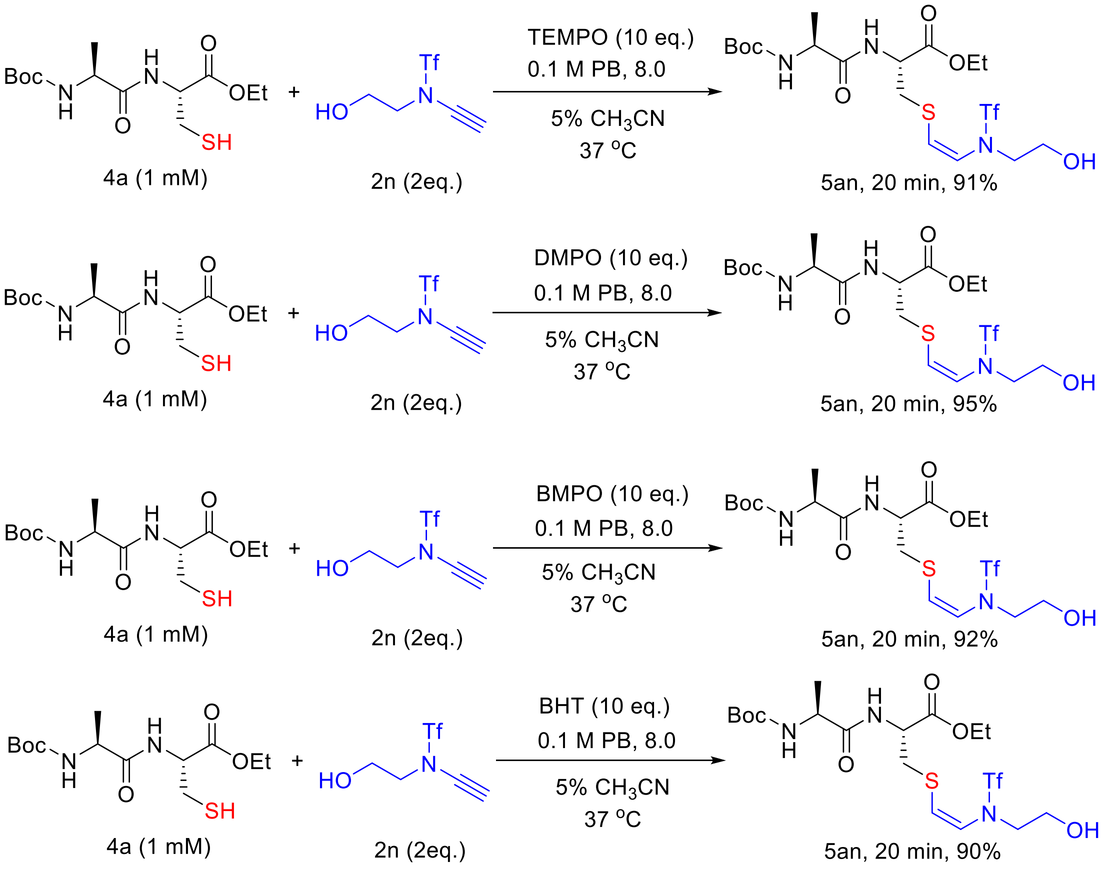
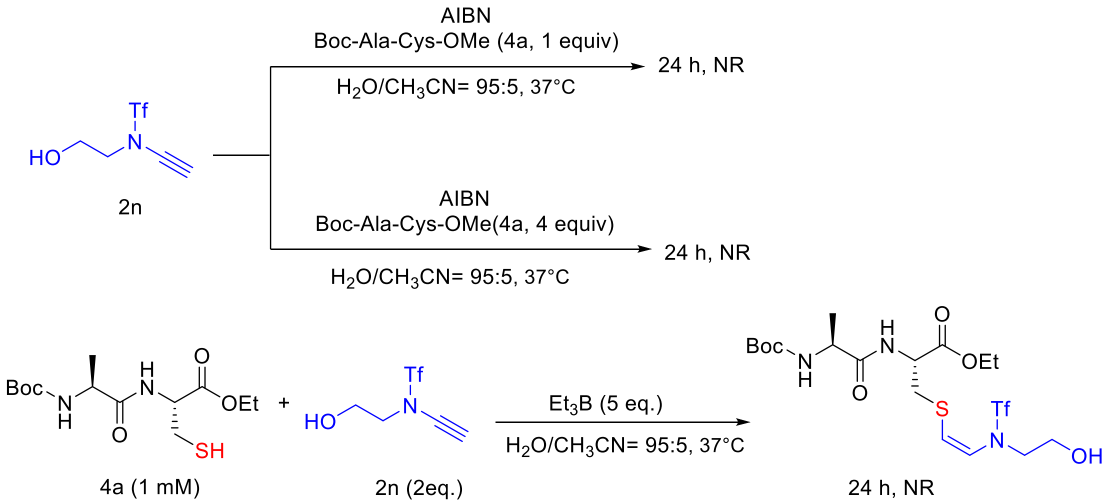


Figure S65. Modification of Cys-free model peptide **22** with ynamide **2n**, NEM and IAM. The graphs show the percentage of modified peptide **22** (including single and double) with the different reagents; ynamide, NEM and IAM at different pH after 2 h (**a**) and 18 h **(b)**

10. Radical Experiment



Scheme S5. The control experiment with different radical inhibitor between **4a** and **2n**.



Scheme S6. The control experiment with different radical initiator between **4a** and **2n**.

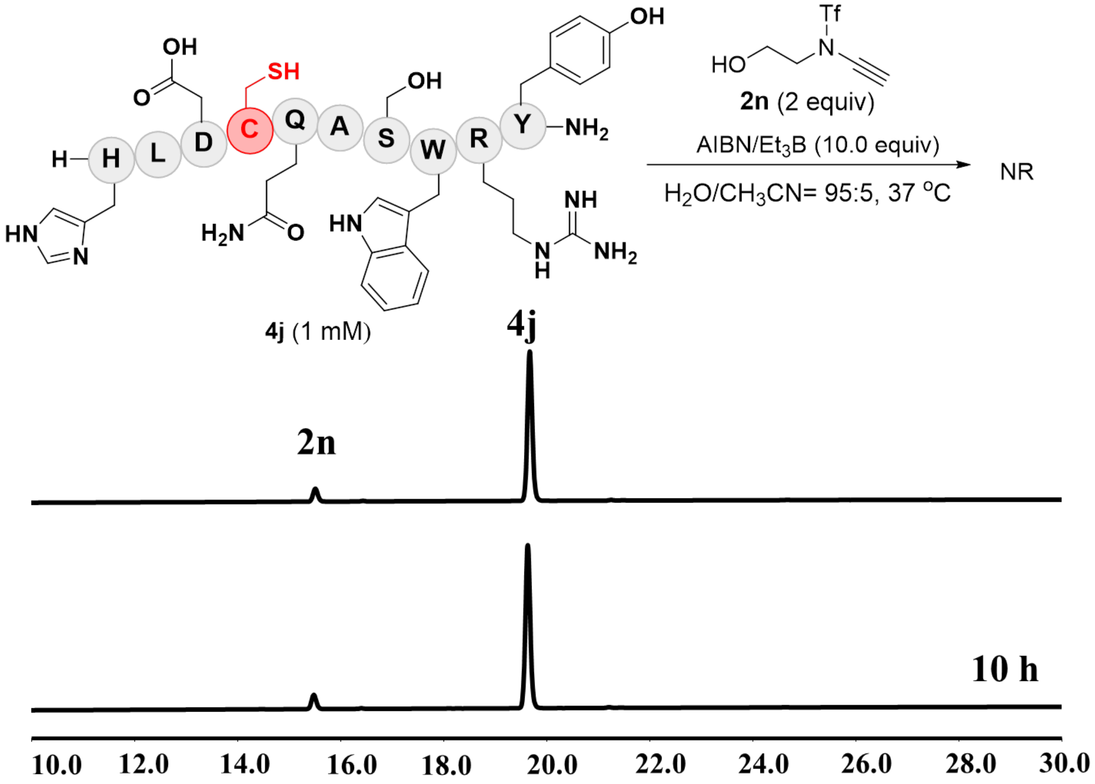


Figure S66. The control experiment with different radical initiator between **4j** and **2n**.

11. NMR Spectrum

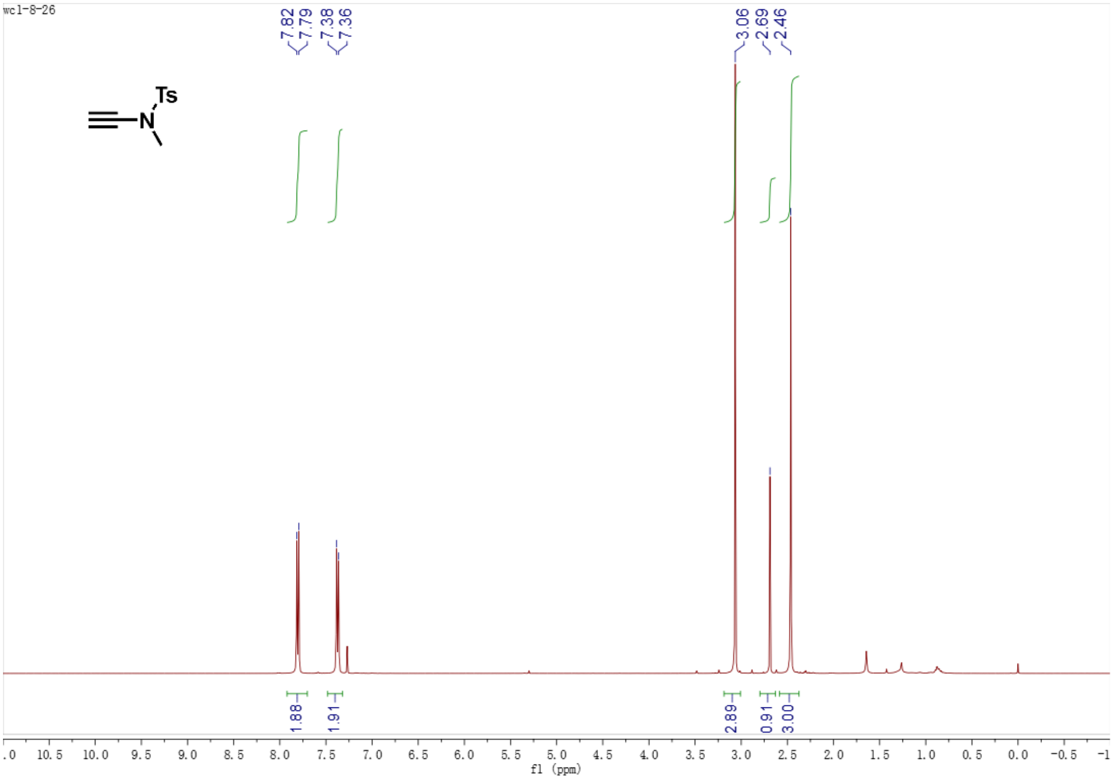


Figure S67. ^{1}H-NMR (400 MHz) spectrum of compound **2a** in CDCl$_3$

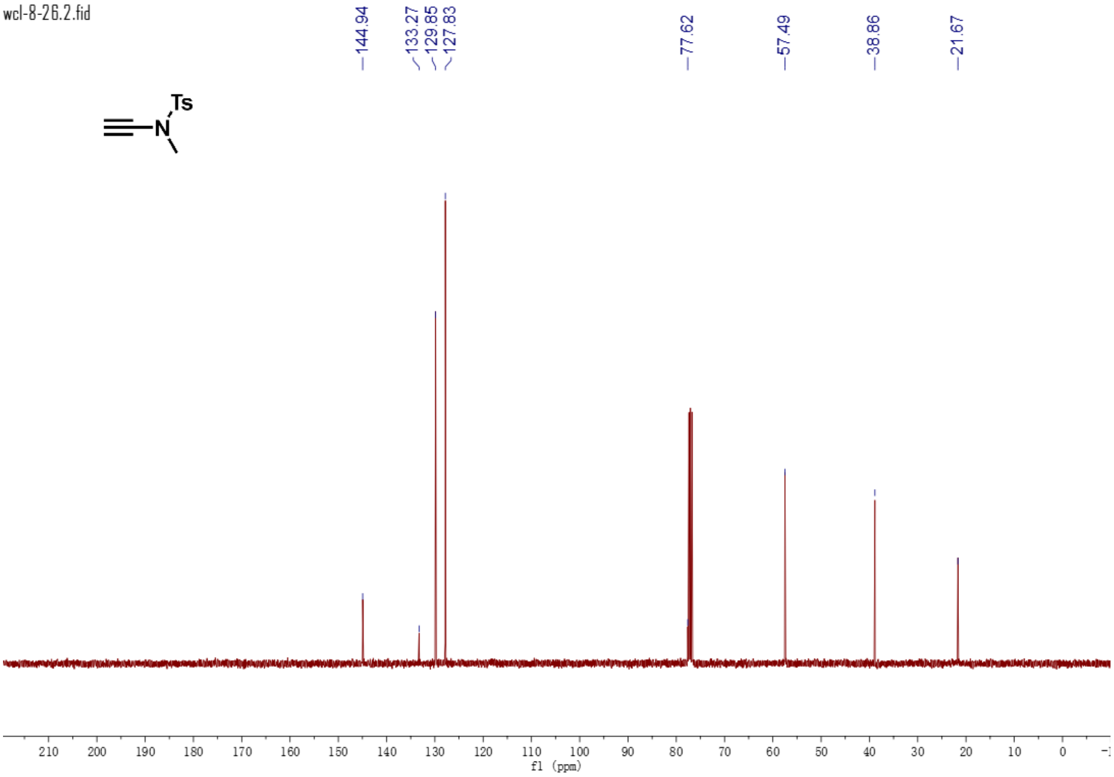


Figure S68. ^{13}C-NMR (100 MHz) spectrum of compound **2a** in CDCl$_3$

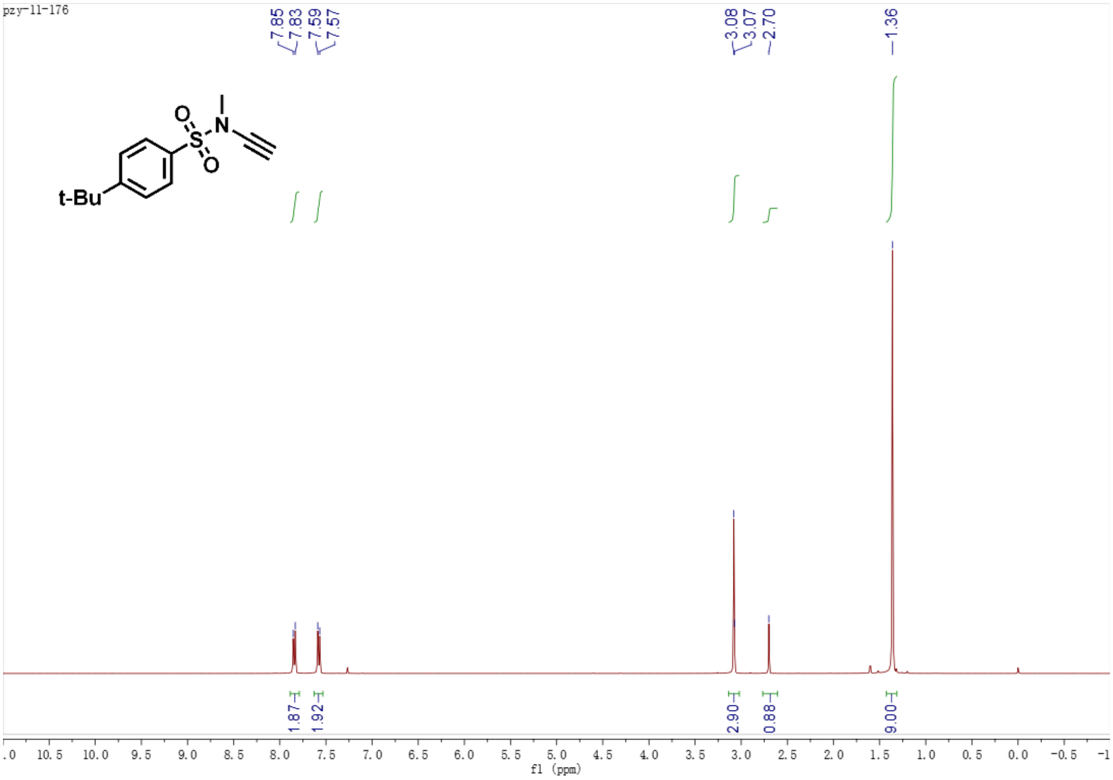


Figure S69. ^{1}H-NMR (400 MHz) spectrum of compound **2l** in $CDCl_3$

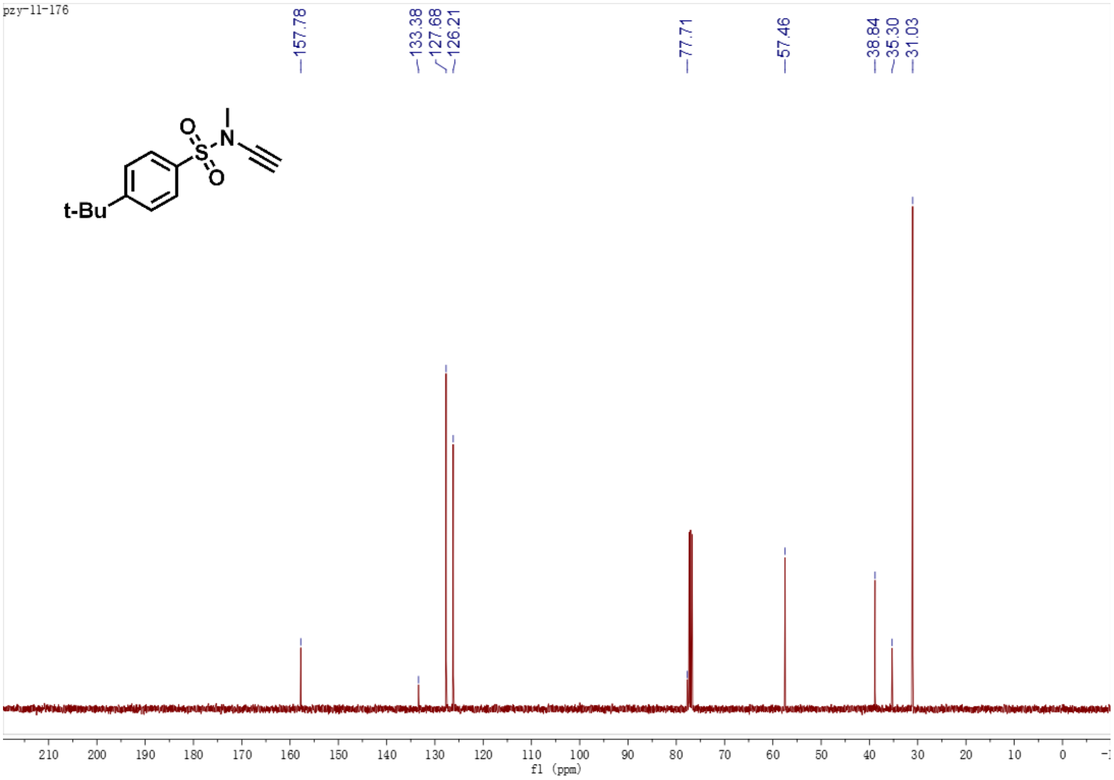


Figure S70. ^{13}C-NMR (100 MHz) spectrum of compound **2l** in $CDCl_3$

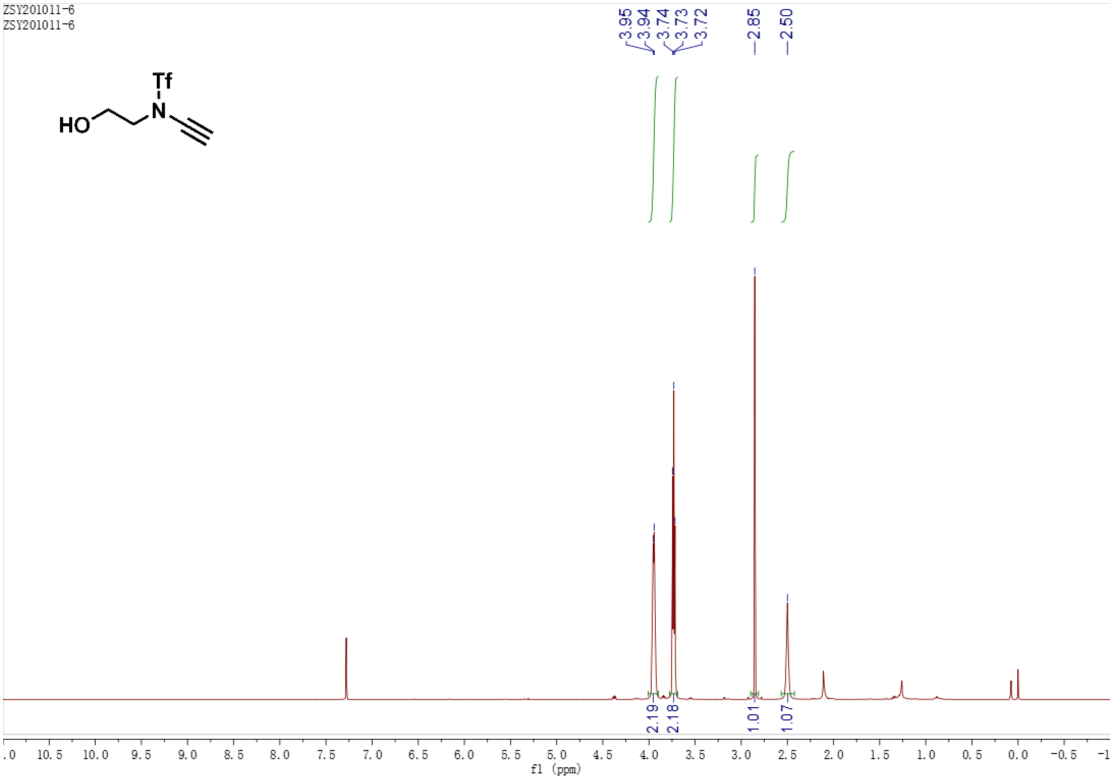


Figure S71. ^{1}H-NMR (400 MHz) spectrum of compound **2n** in $CDCl_3$

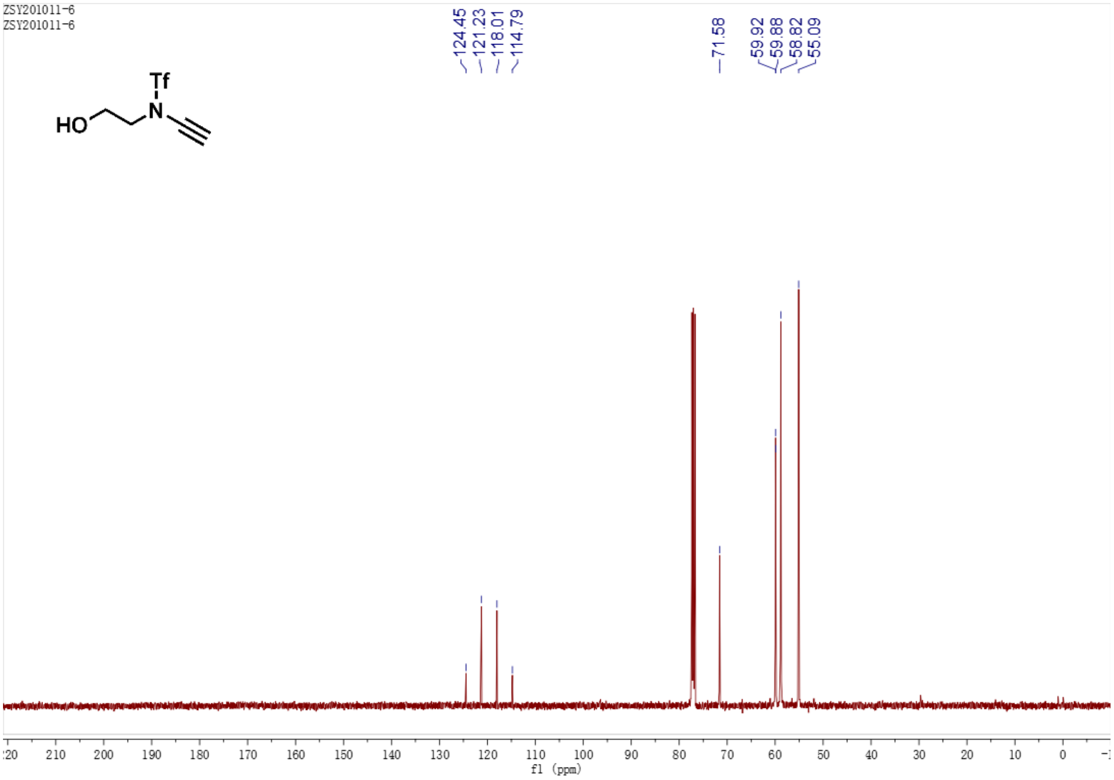


Figure S72. ^{13}C-NMR (100 MHz) spectrum of compound **2n** in $CDCl_3$

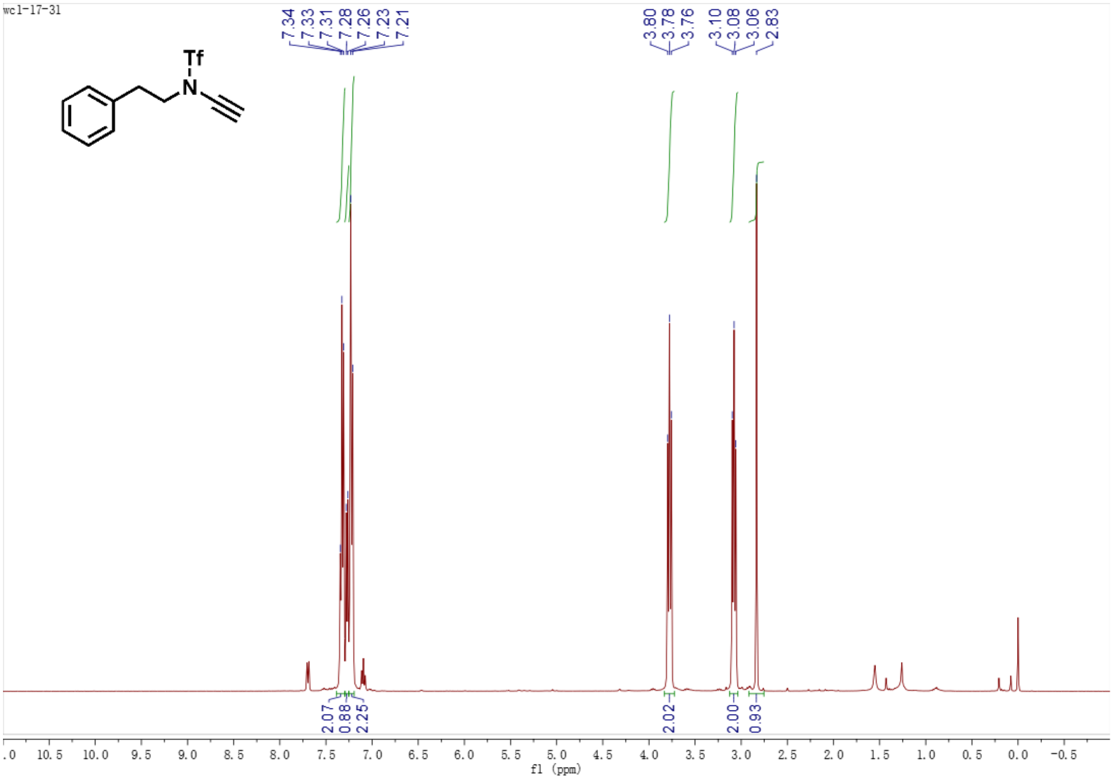


Figure S73. ^{1}H-NMR (400 MHz) spectrum of compound **2o** in $CDCl_3$

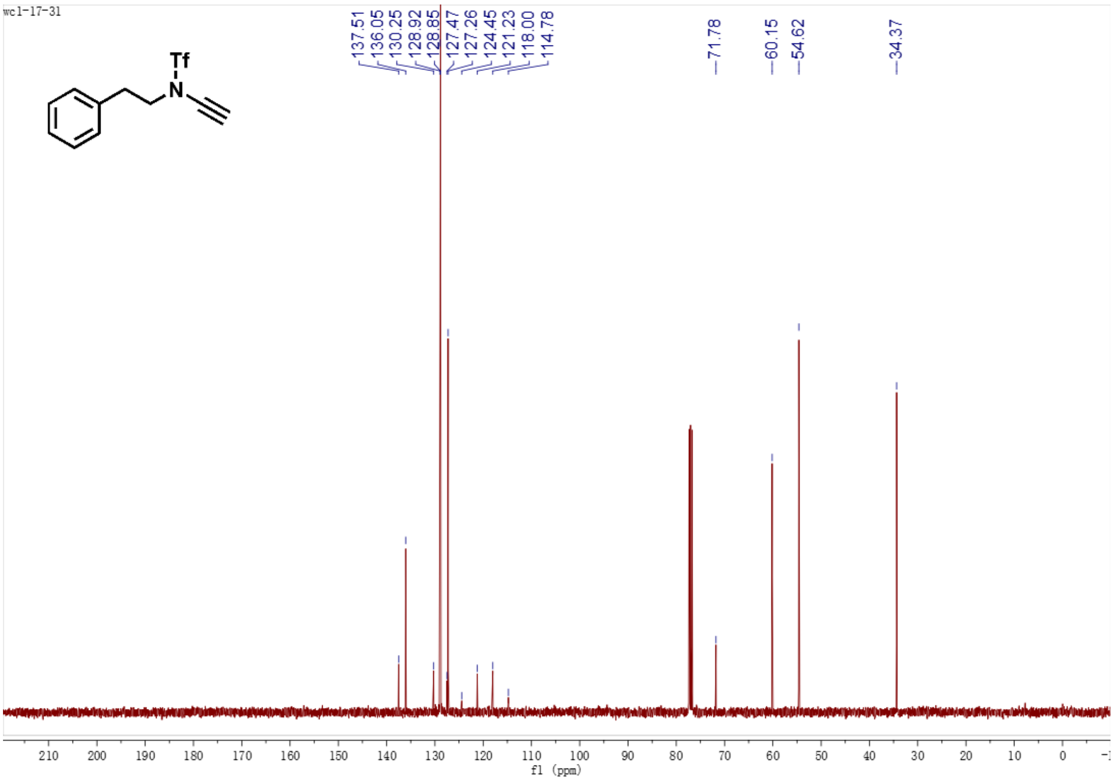


Figure S74. ^{13}C-NMR (100 MHz) spectrum of compound **2o** in $CDCl_3$

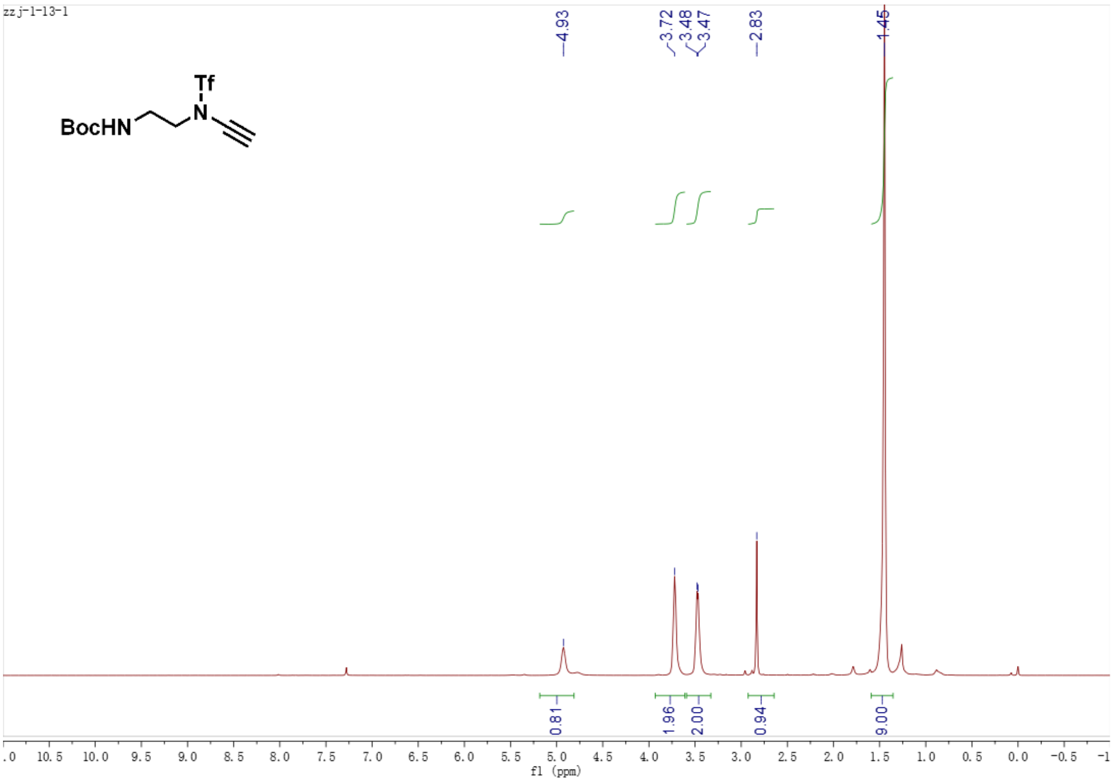


Figure S75. ^{1}H-NMR (400 MHz) spectrum of compound **2p** in $CDCl_3$

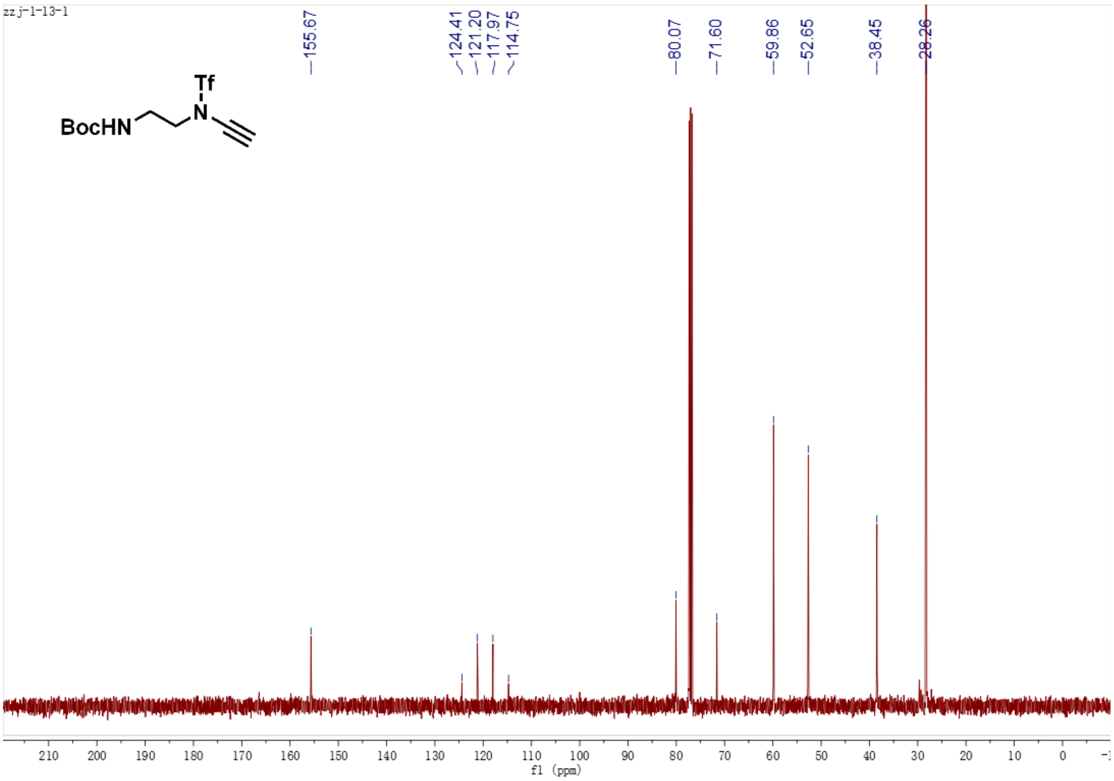


Figure S76. ^{13}C-NMR (100 MHz) spectrum of compound **2p** in $CDCl_3$

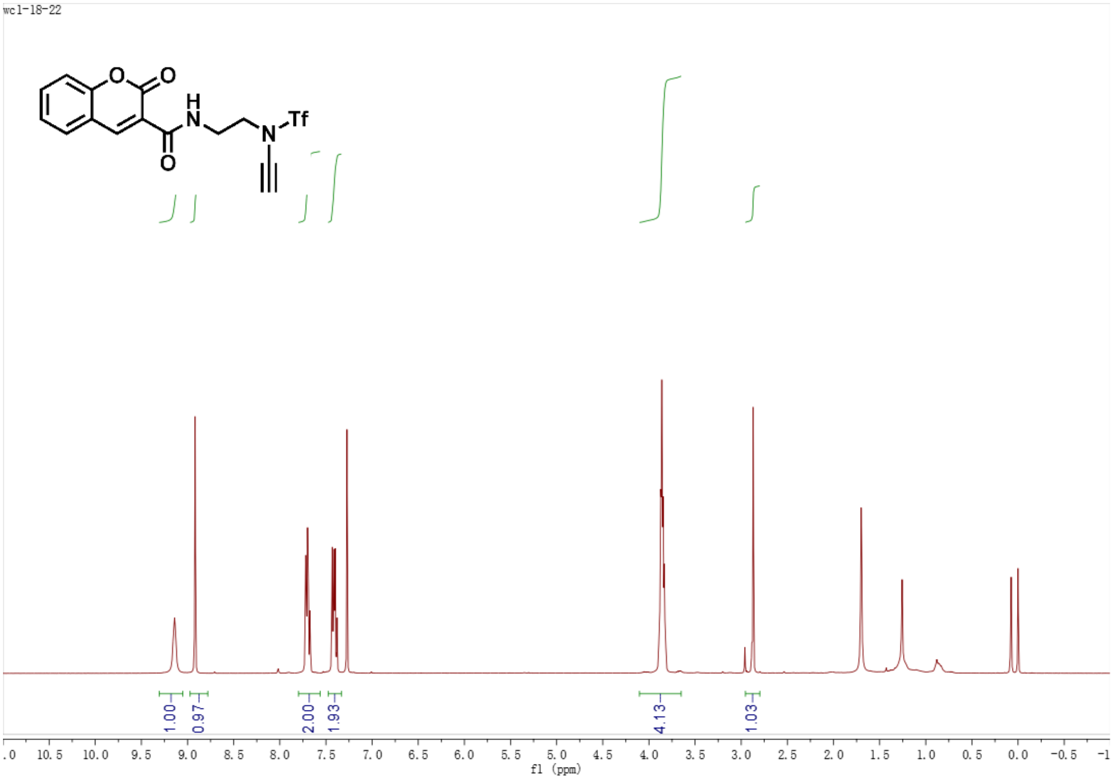


Figure S77. ^{1}H-NMR (400 MHz) spectrum of compound **2q** in CDCl$_3$

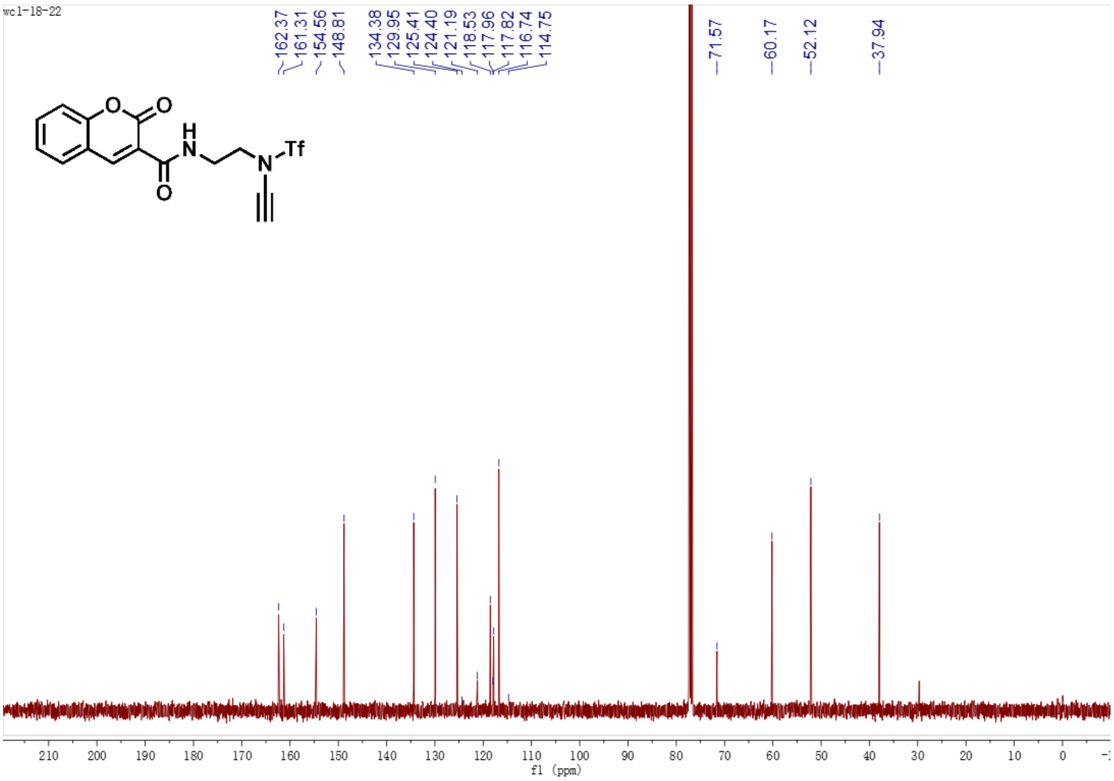


Figure S78. ^{13}C-NMR (100 MHz) spectrum of compound **2q** in CDCl$_3$

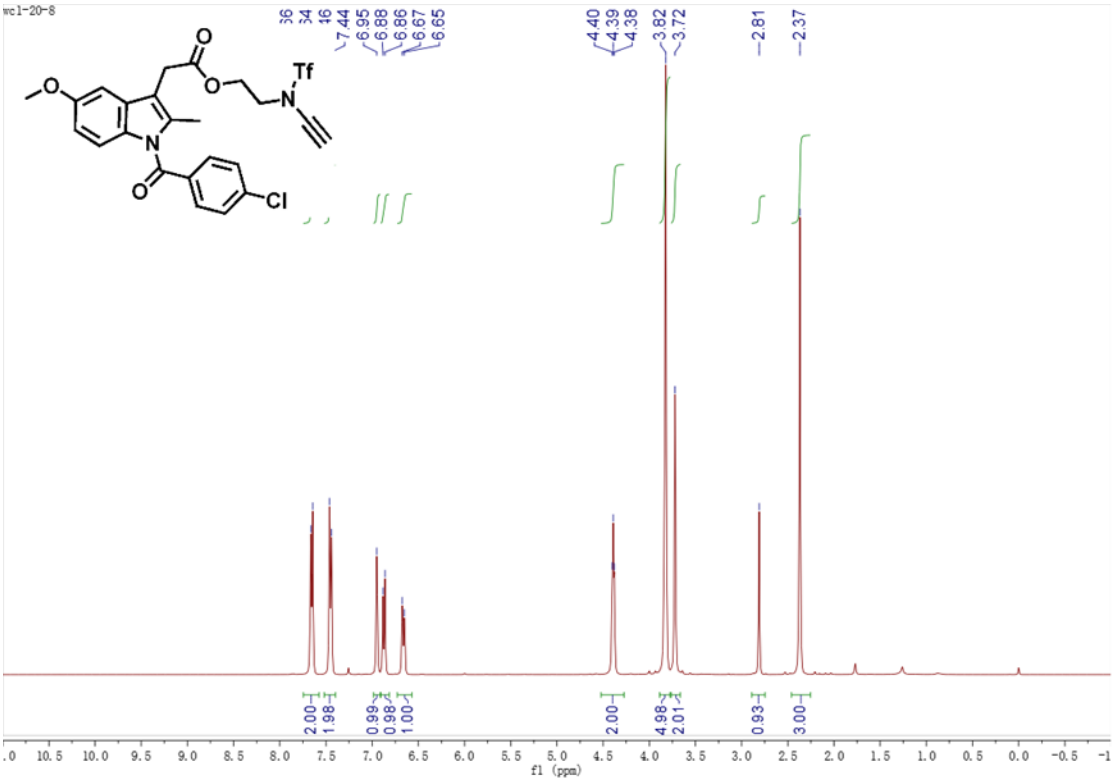


Figure S79. ^{1}H-NMR (400 MHz) spectrum of compound **2r** in CDCl$_3$

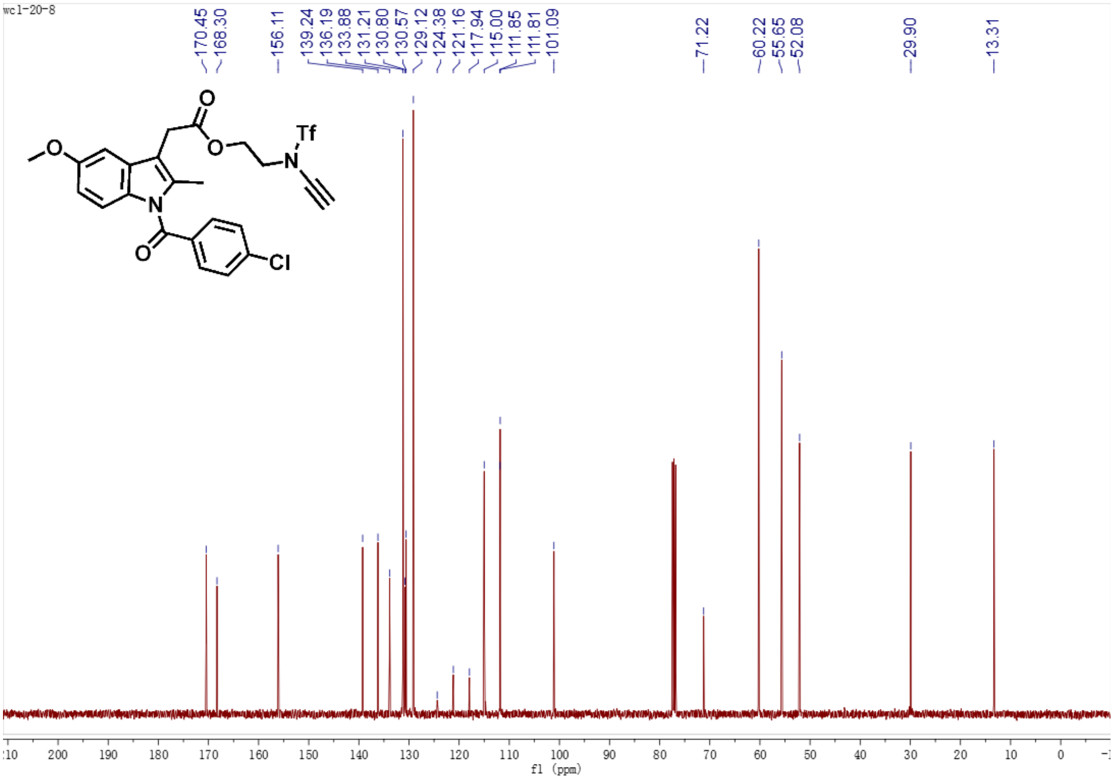


Figure S80. ^{13}C-NMR (100 MHz) spectrum of compound **2r** in CDCl$_3$

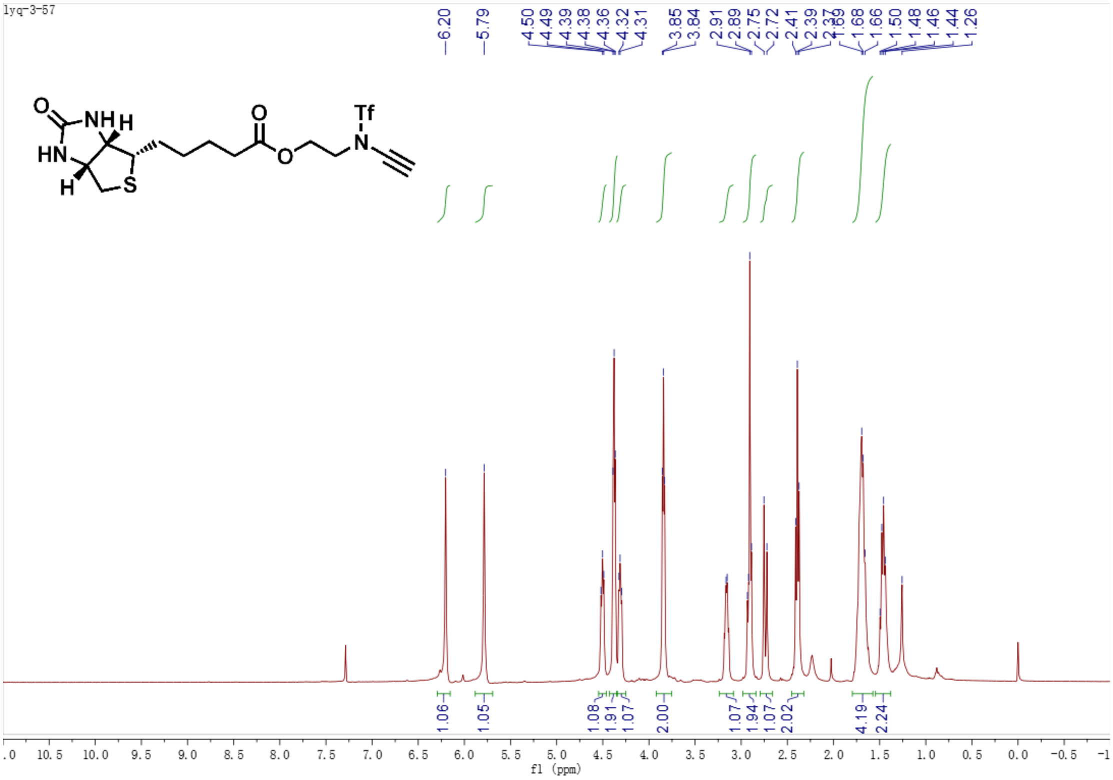


Figure S81. ^{1}H-NMR (400 MHz) spectrum of compound **2s** in $CDCl_3$

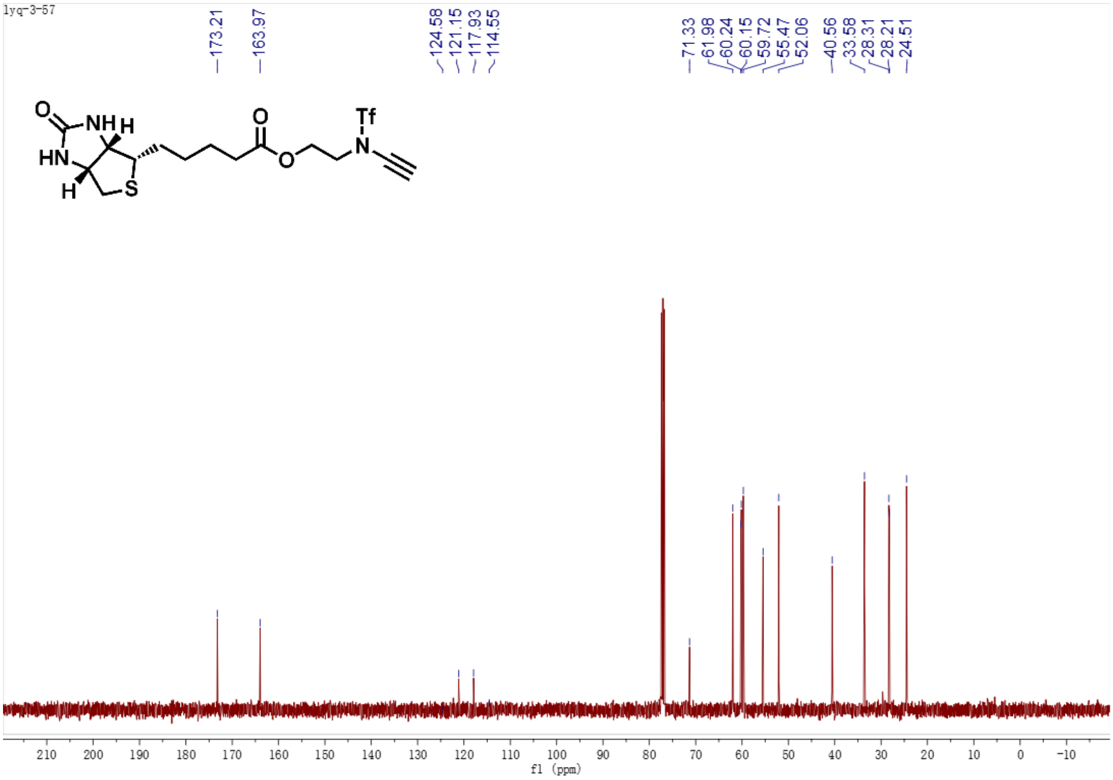


Figure S82. ^{13}C-NMR (100 MHz) spectrum of compound **2s** in $CDCl_3$

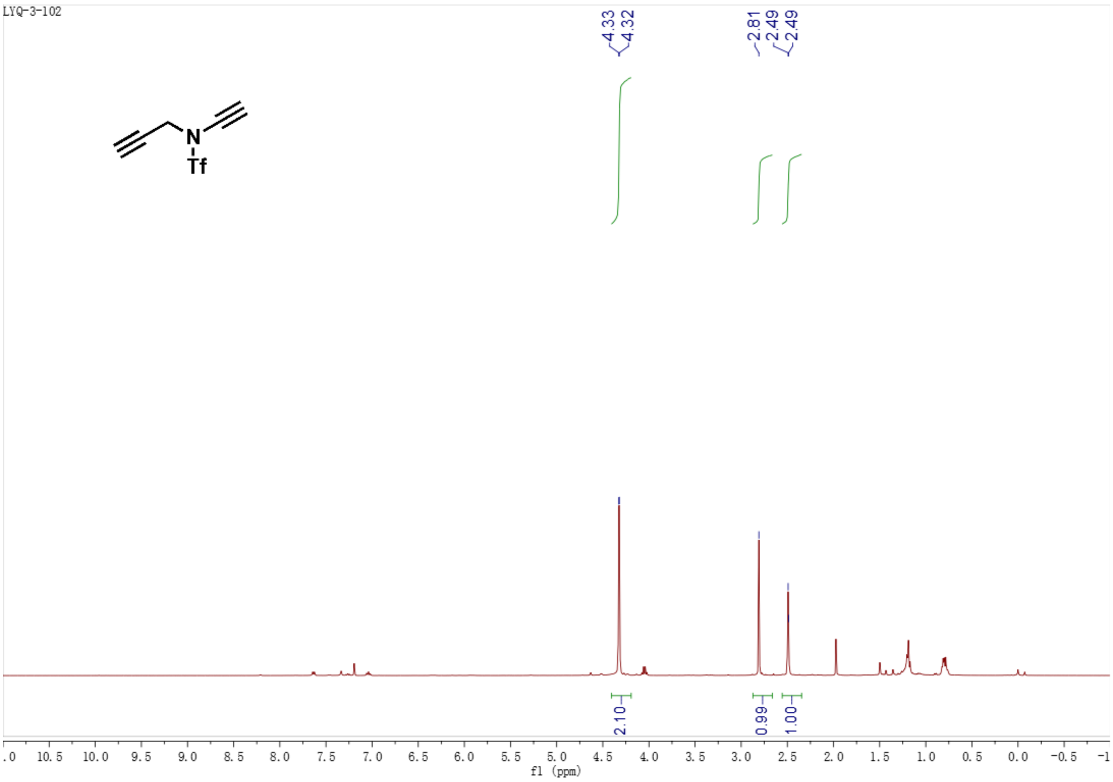


Figure S83. ^{1}H-NMR (400 MHz) spectrum of compound **2t** in CDCl$_3$

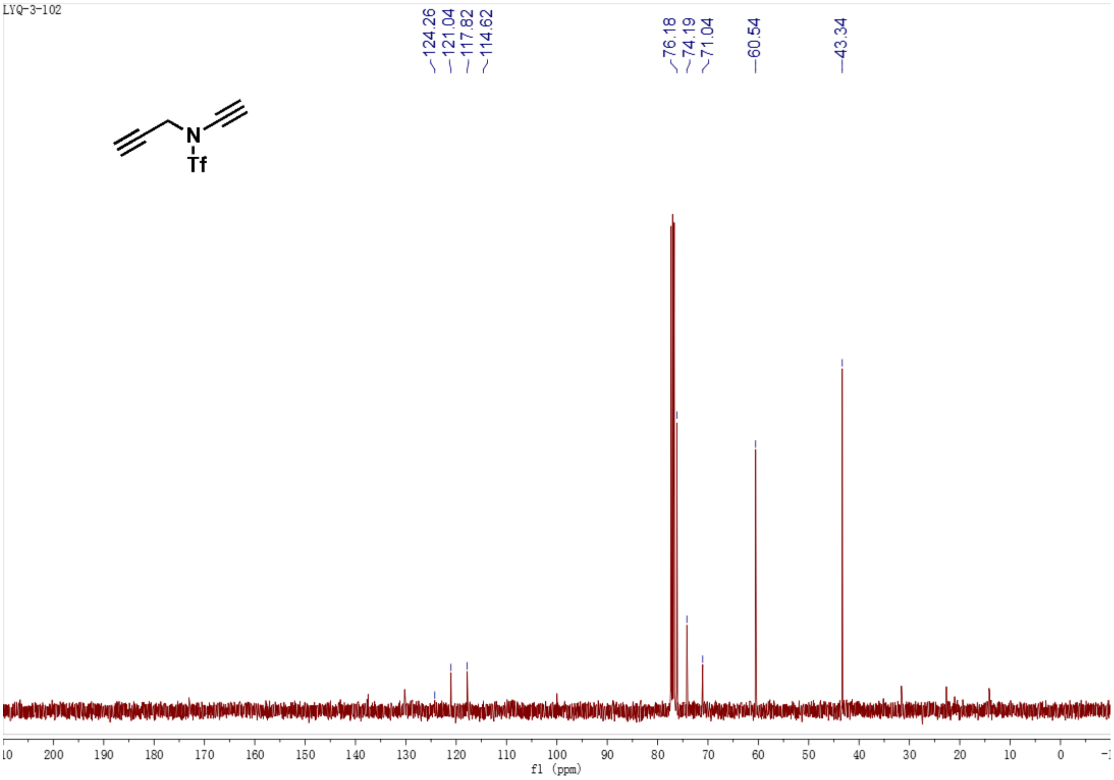


Figure S84. ^{13}C-NMR (100 MHz) spectrum of compound **2t** in CDCl$_3$

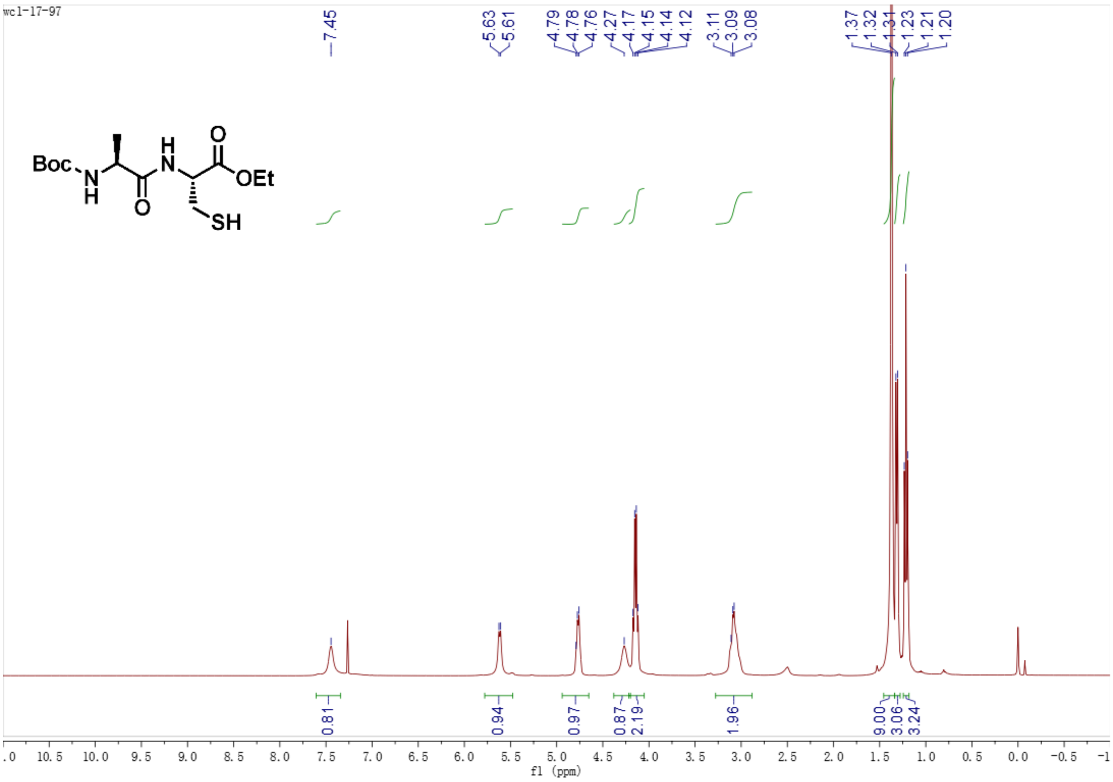


Figure S85. ^{1}H-NMR (400 MHz) spectrum of compound **4a** in CDCl$_3$

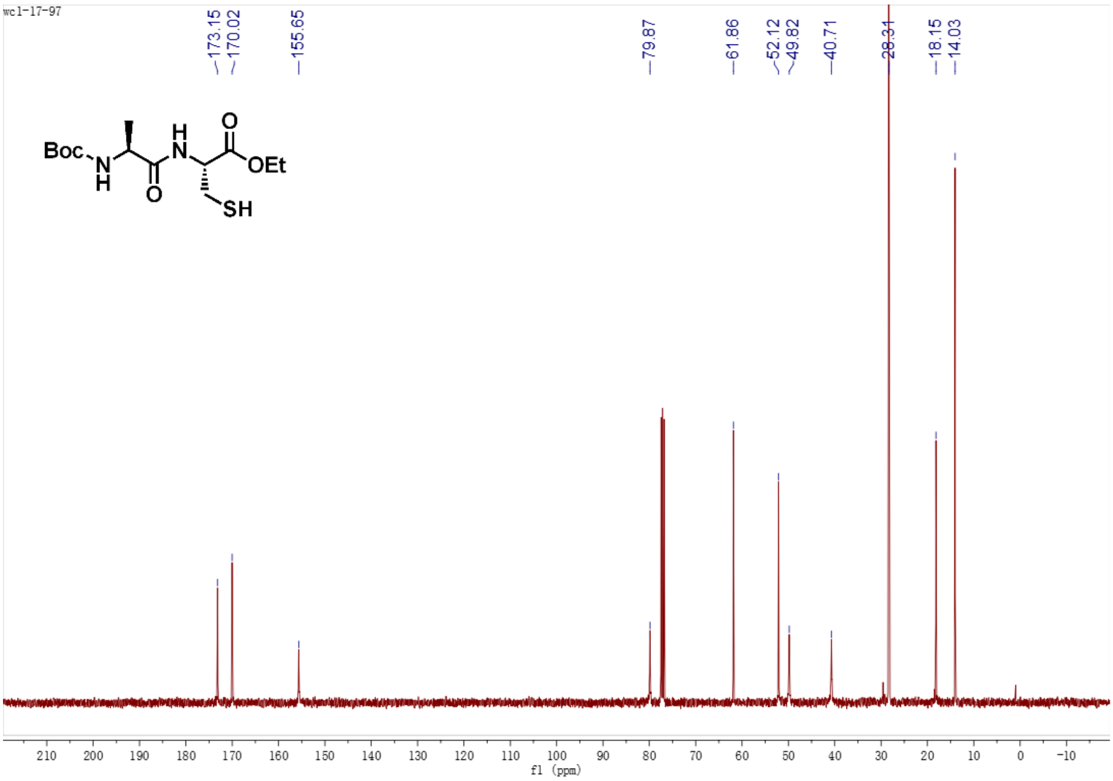


Figure S86. ^{13}C-NMR (100 MHz) spectrum of compound **4a** in CDCl$_3$

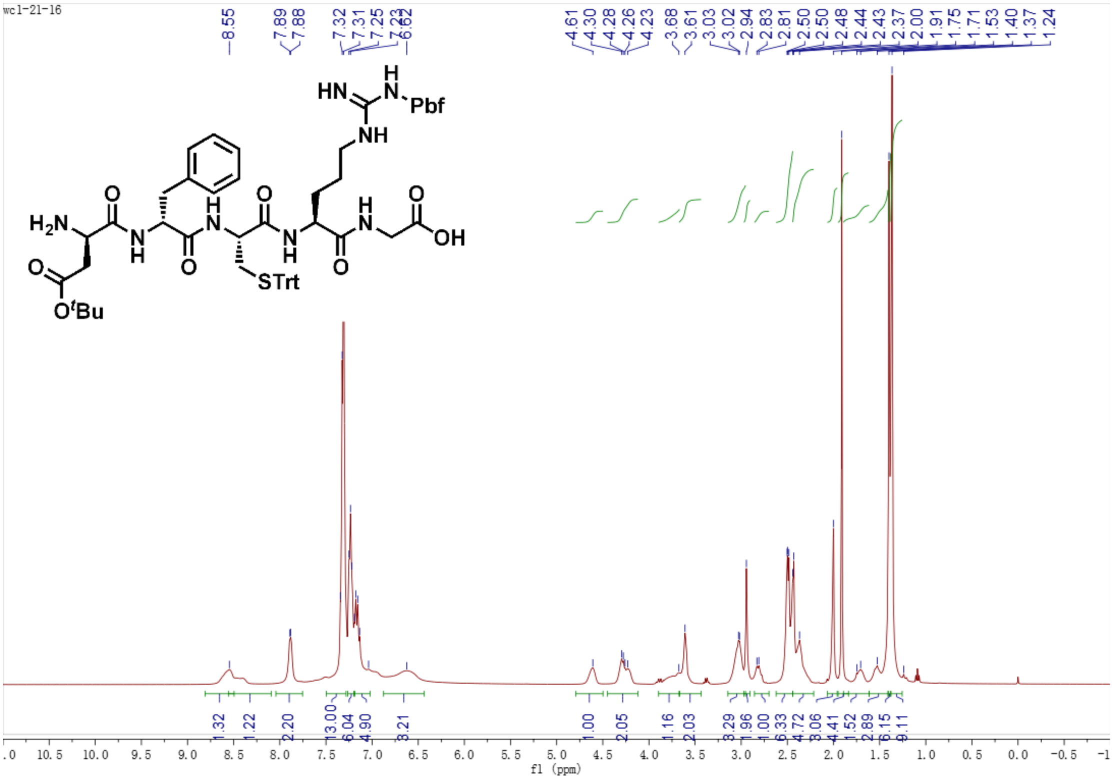


Figure S87. ^{1}H-NMR (400 MHz) spectrum of compound **4o^{1}** in DMSO-d_6

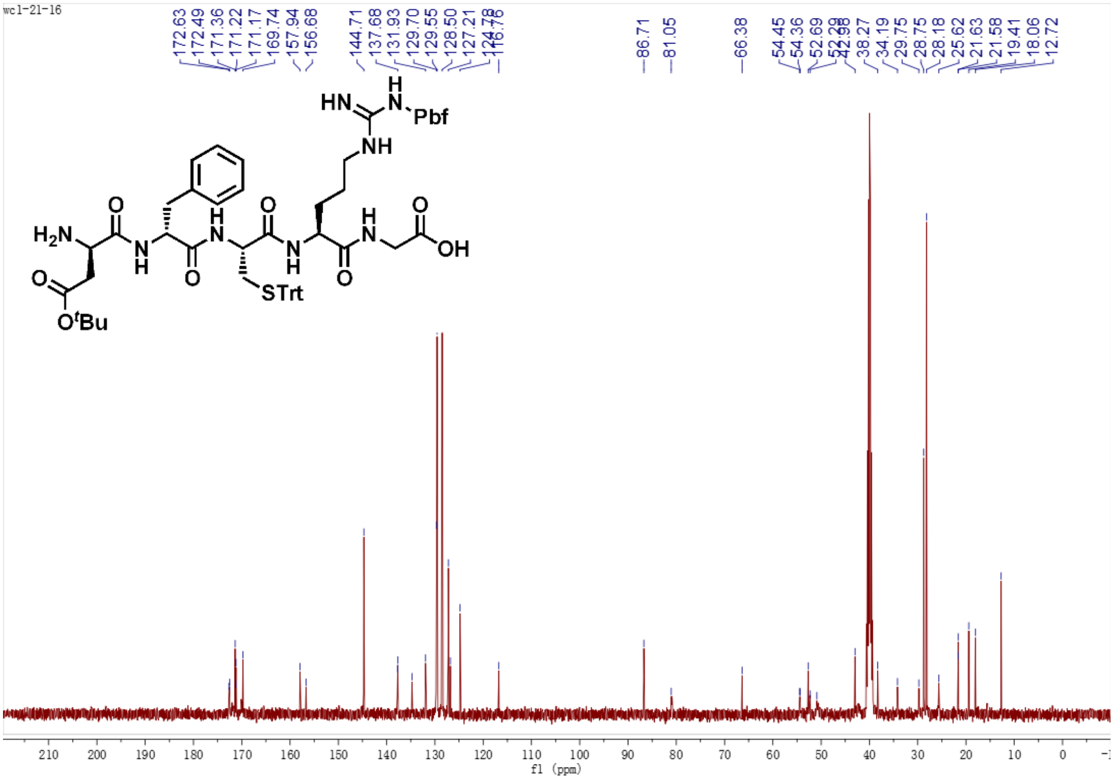


Figure S88. ^{13}C-NMR (100 MHz) spectrum of compound **4o^{1}** in DMSO-d_6

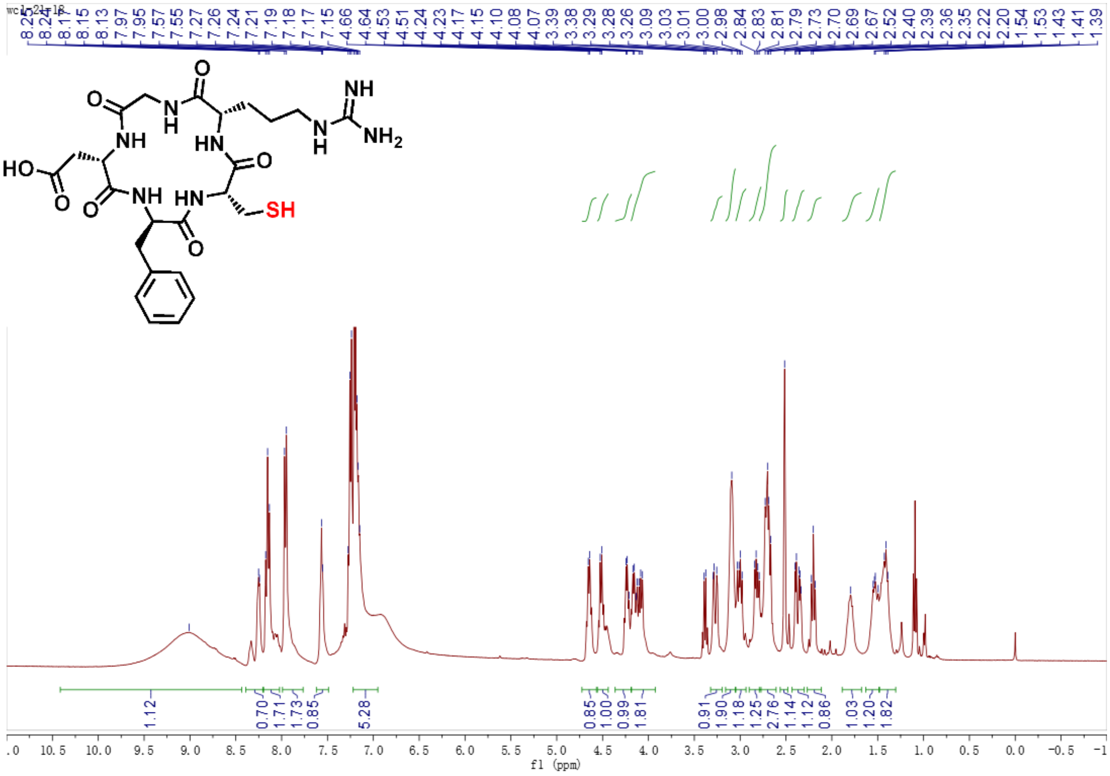


Figure S89. ^{1}H-NMR (400 MHz) spectrum of compound **4o** in DMSO-d_6

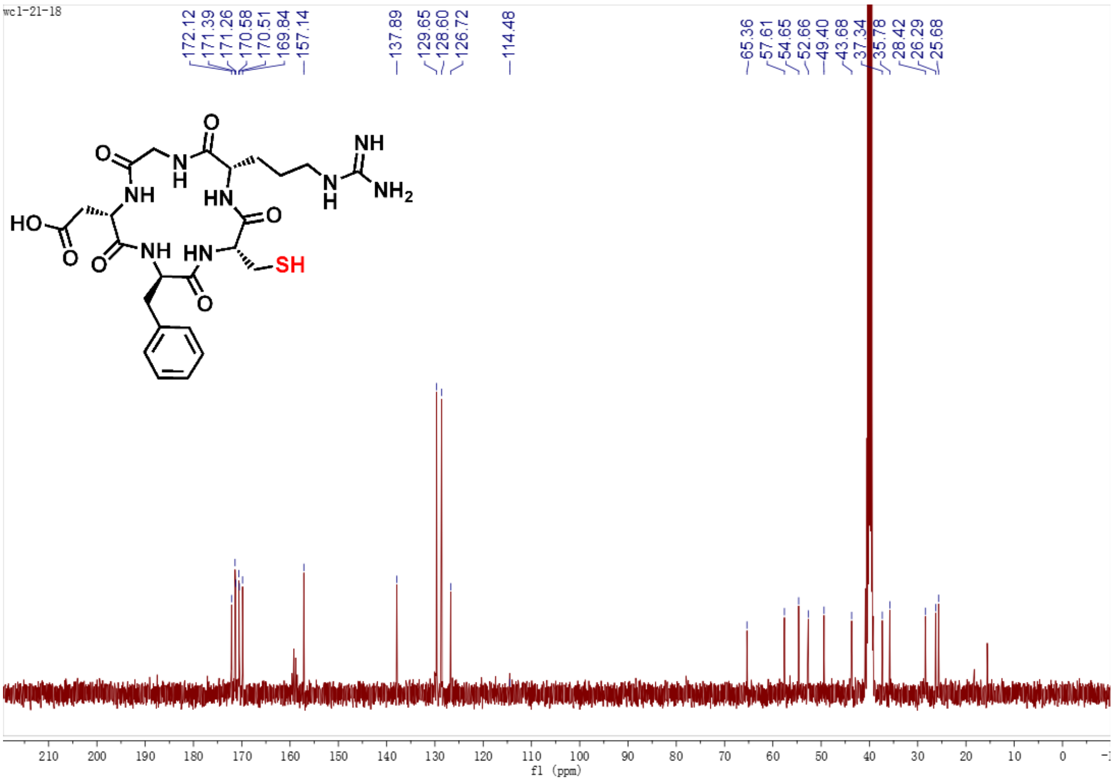


Figure S90. ^{13}C-NMR (100 MHz) spectrum of compound **4o** in DMSO-d_6

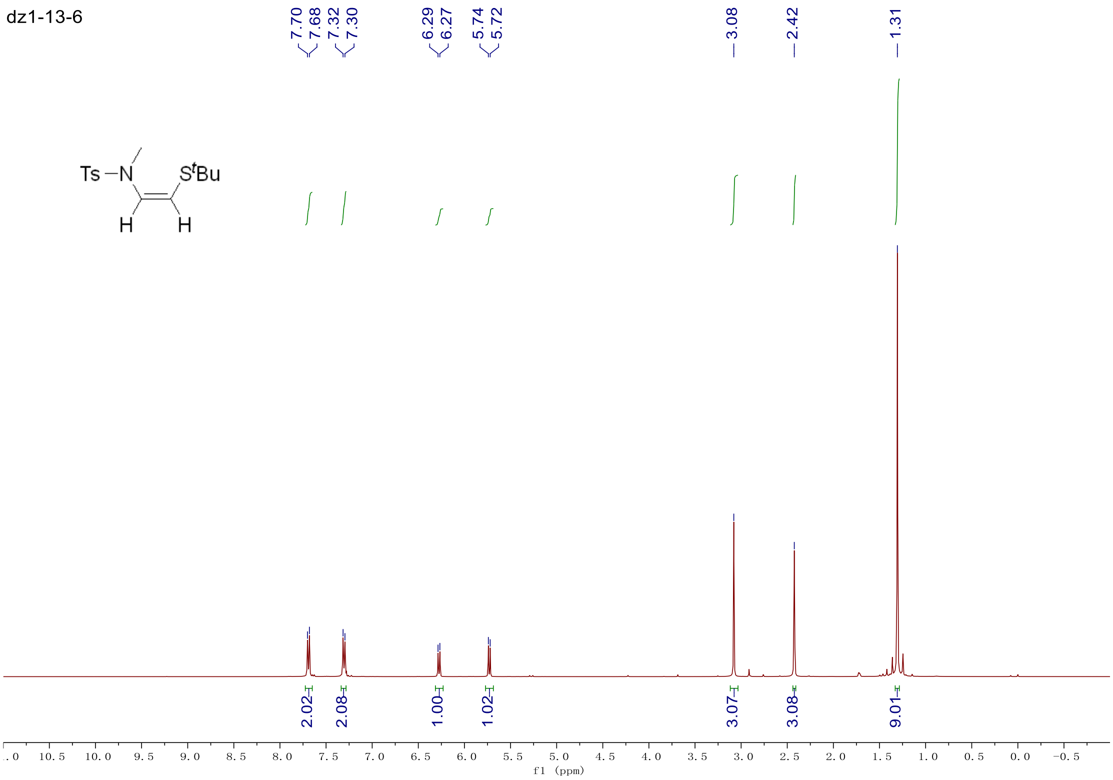


Figure S91. ^{1}H-NMR (400 MHz) spectrum of compound **3aa** in $CDCl_3$

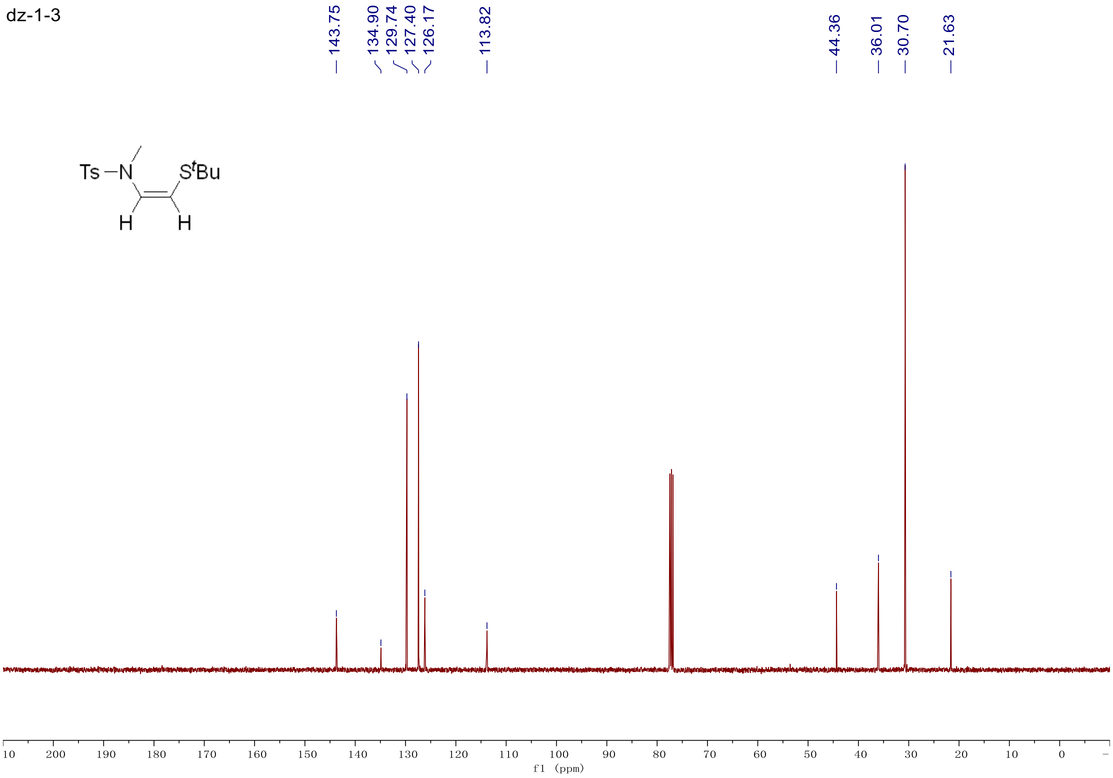


Figure S92. ^{13}C-NMR (100 MHz) spectrum of compound **3aa** in $CDCl_3$

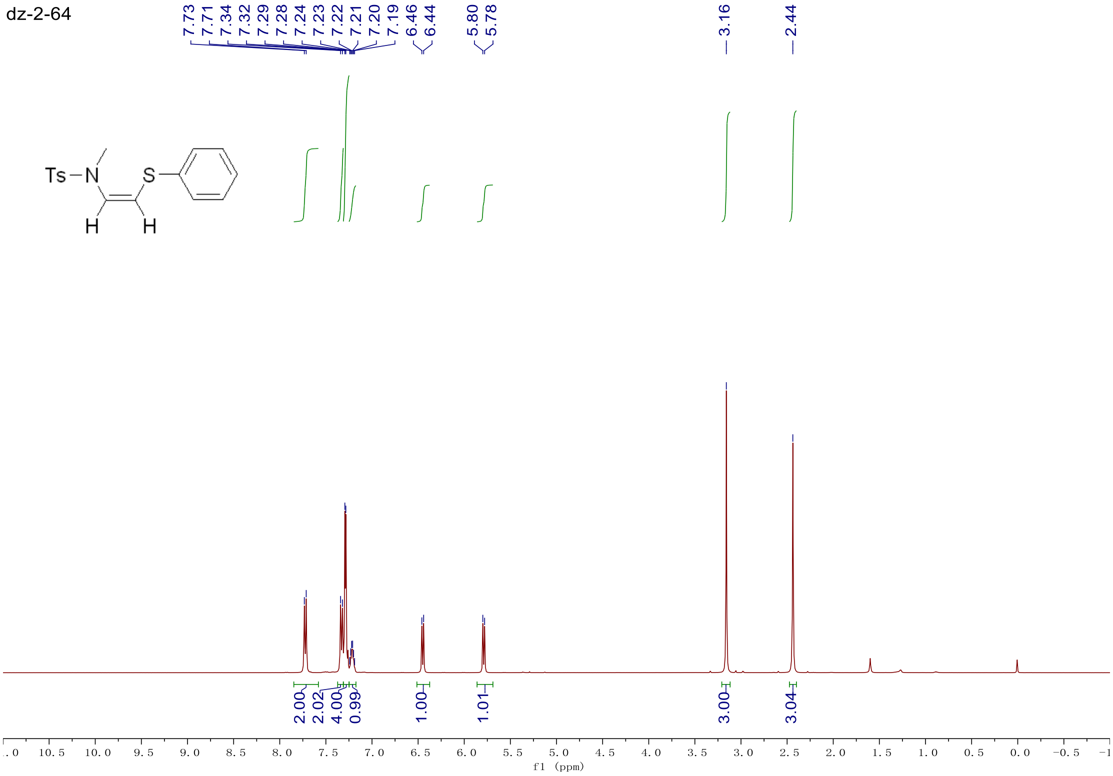


Figure S93. ^{1}H-NMR (400 MHz) spectrum of compound **3ba** in $CDCl_3$

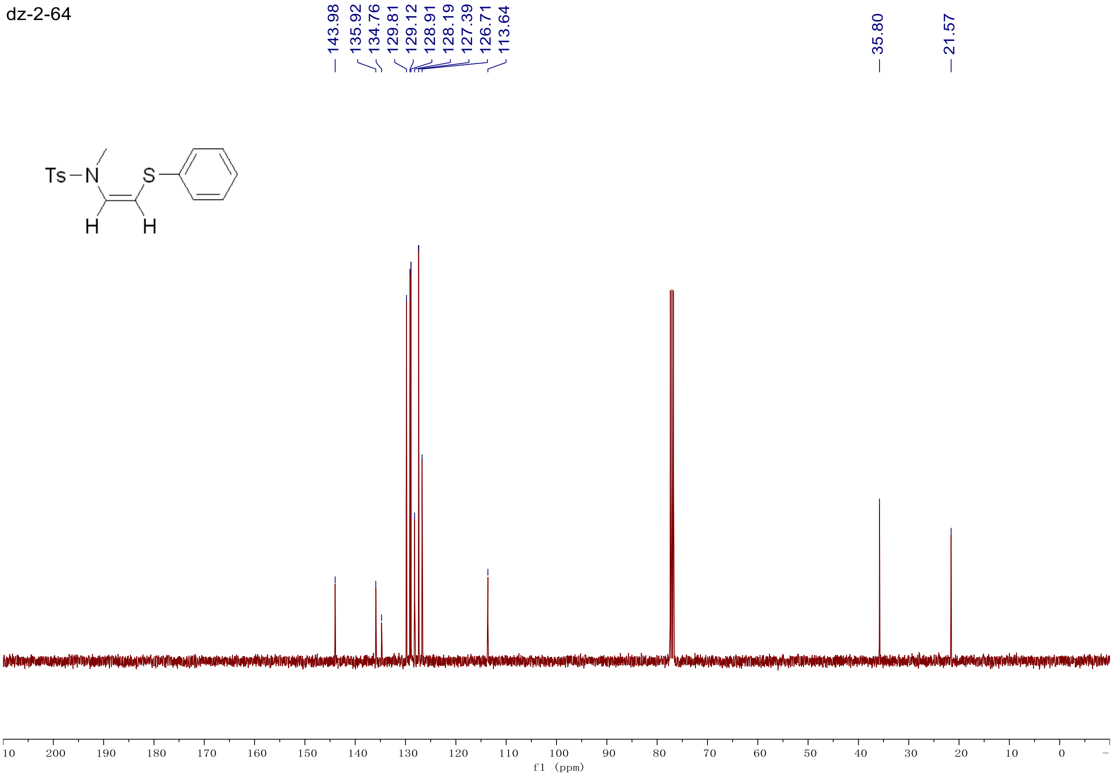


Figure S94. ^{13}C-NMR (100 MHz) spectrum of compound **3ba** in $CDCl_3$

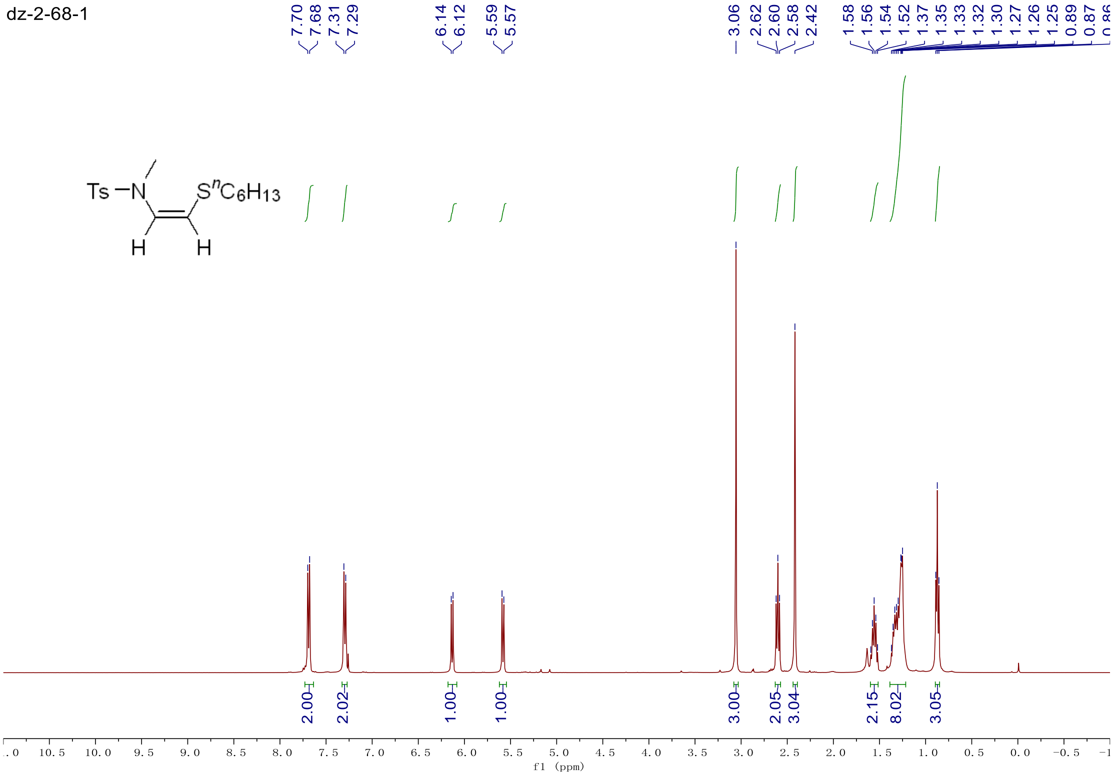


Figure S95. ^{1}H-NMR (400 MHz) spectrum of compound **3ca** in CDCl$_3$

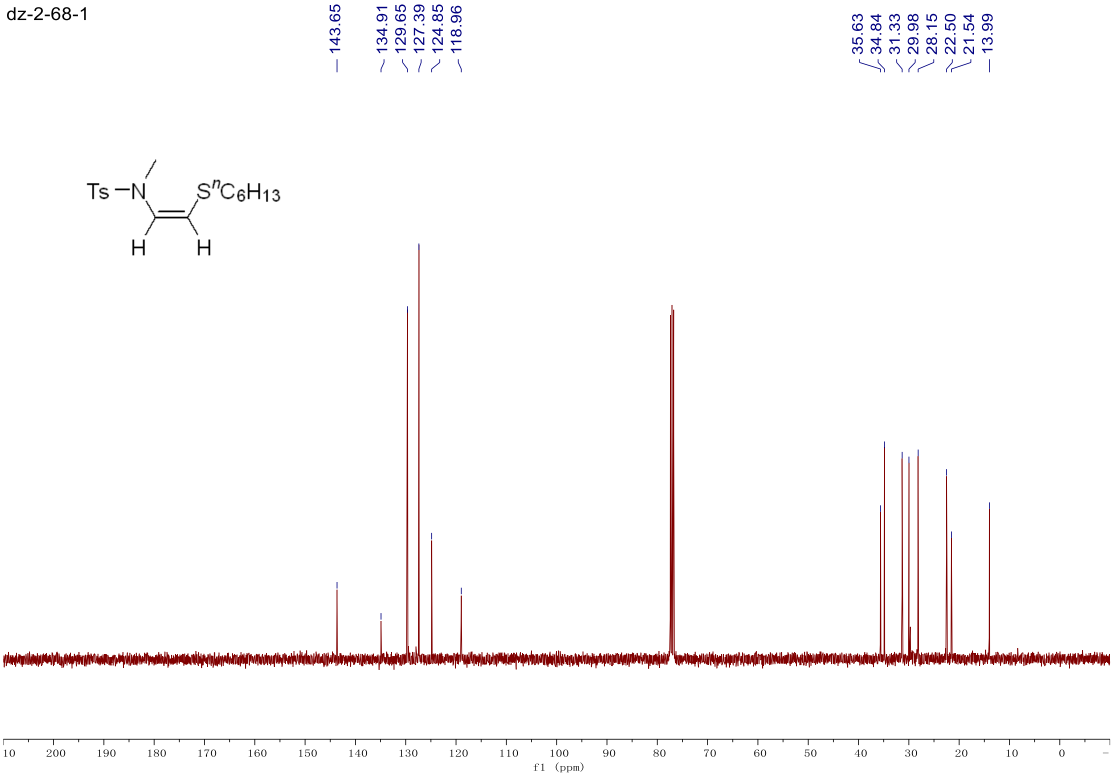


Figure S96. ^{13}C-NMR (100 MHz) spectrum of compound **3ca** in CDCl$_3$

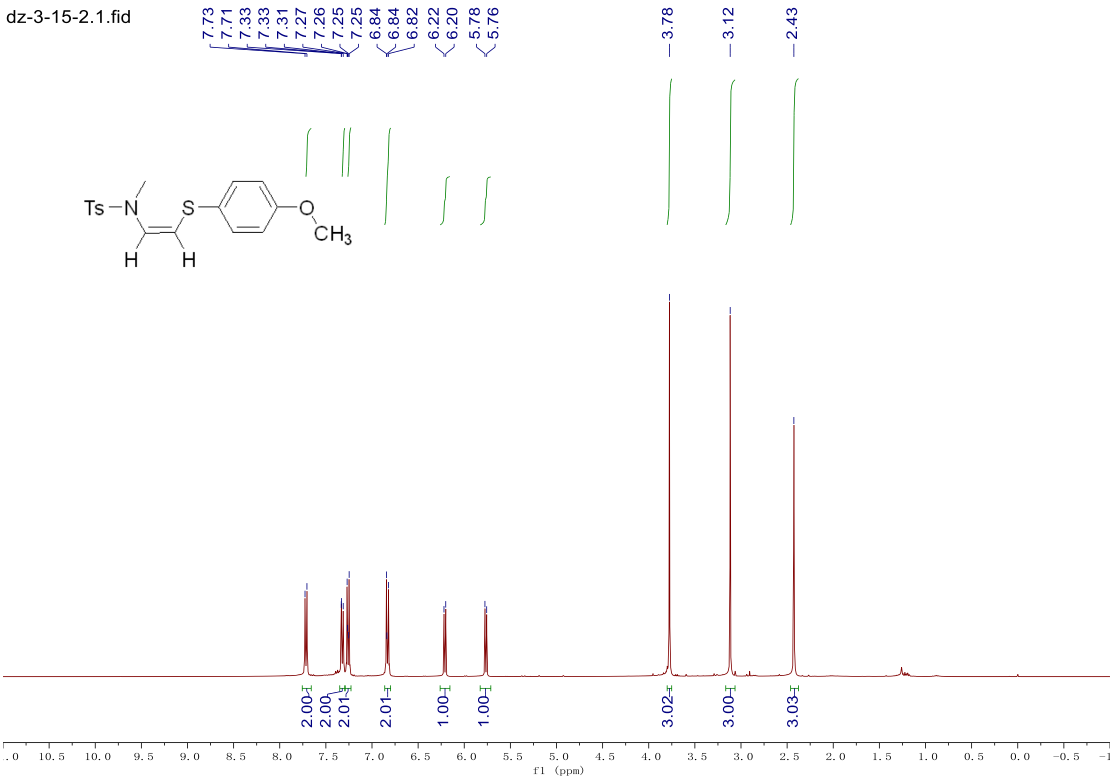


Figure S97. ^{1}H-NMR (400 MHz) spectrum of compound **3da** in CDCl$_3$

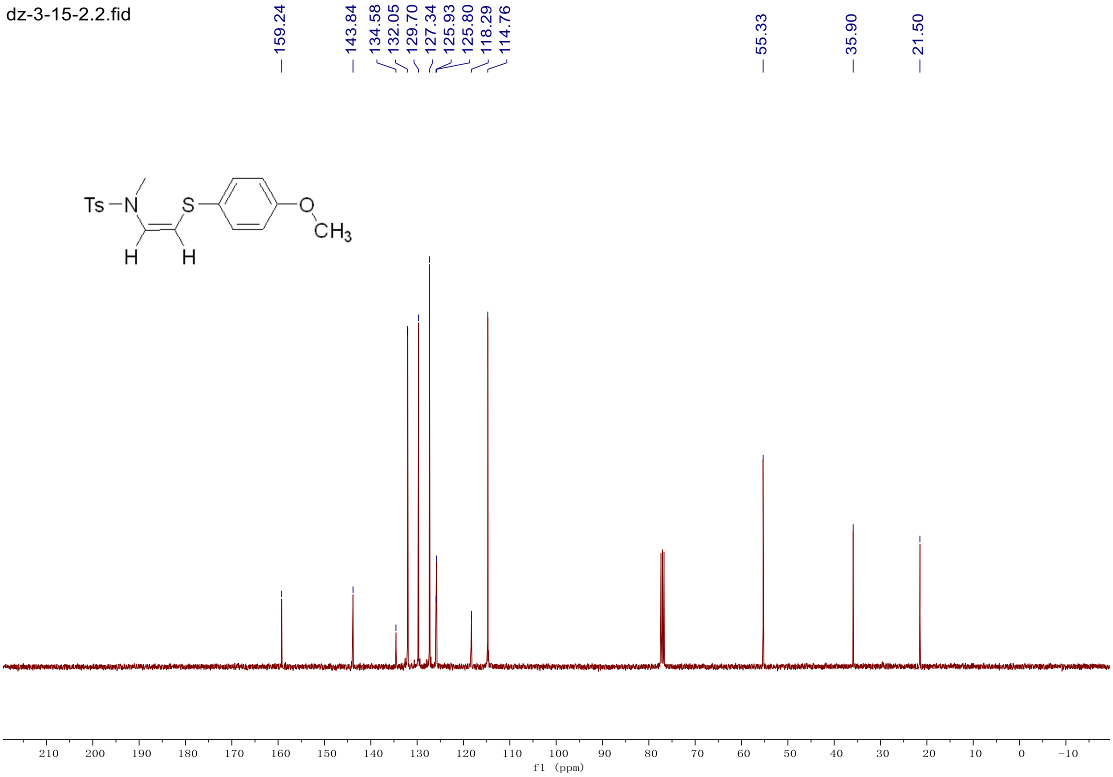


Figure S98. ^{13}C-NMR (100 MHz) spectrum of compound **3da** in CDCl$_3$

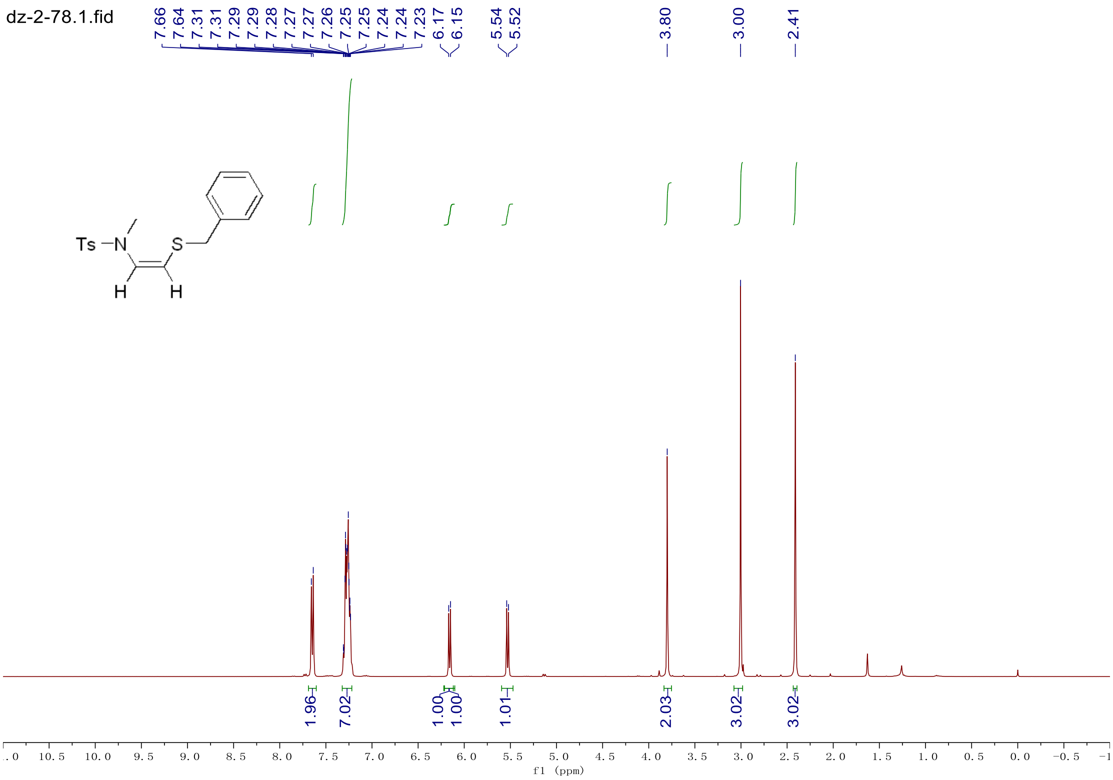


Figure S99. ^{1}H-NMR (400 MHz) spectrum of compound **3ea** in CDCl$_3$

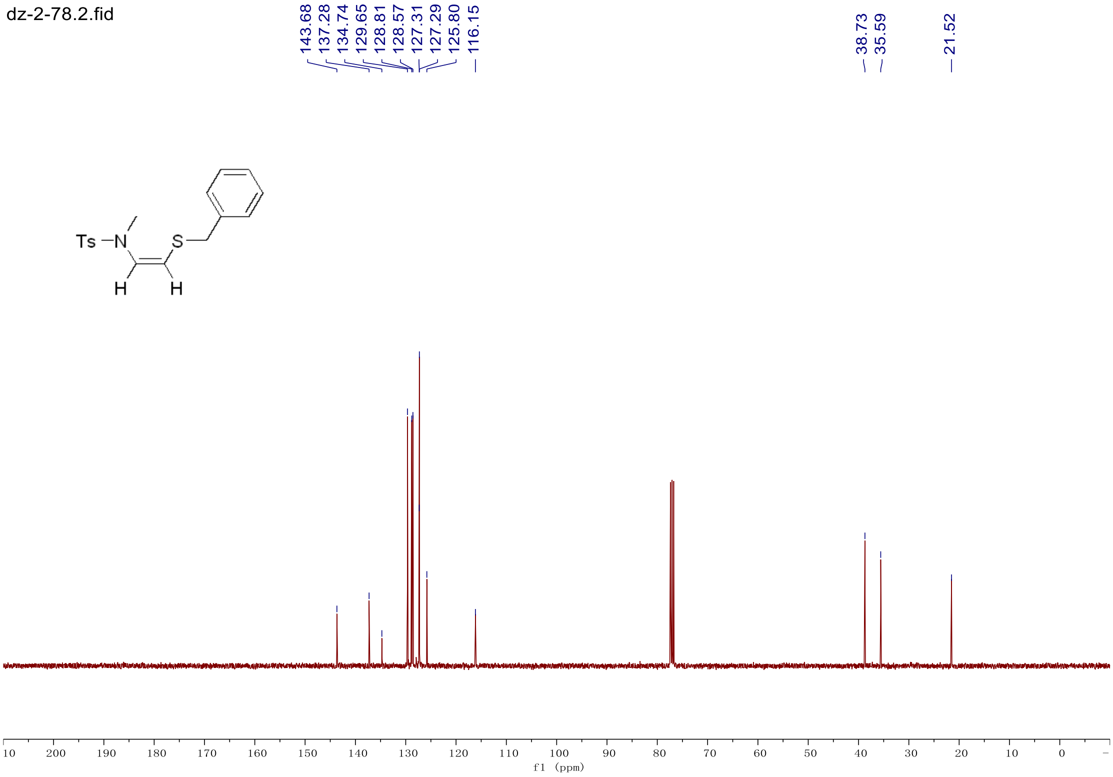


Figure S100. ^{13}C-NMR (100 MHz) spectrum of compound **3ea** in CDCl$_3$

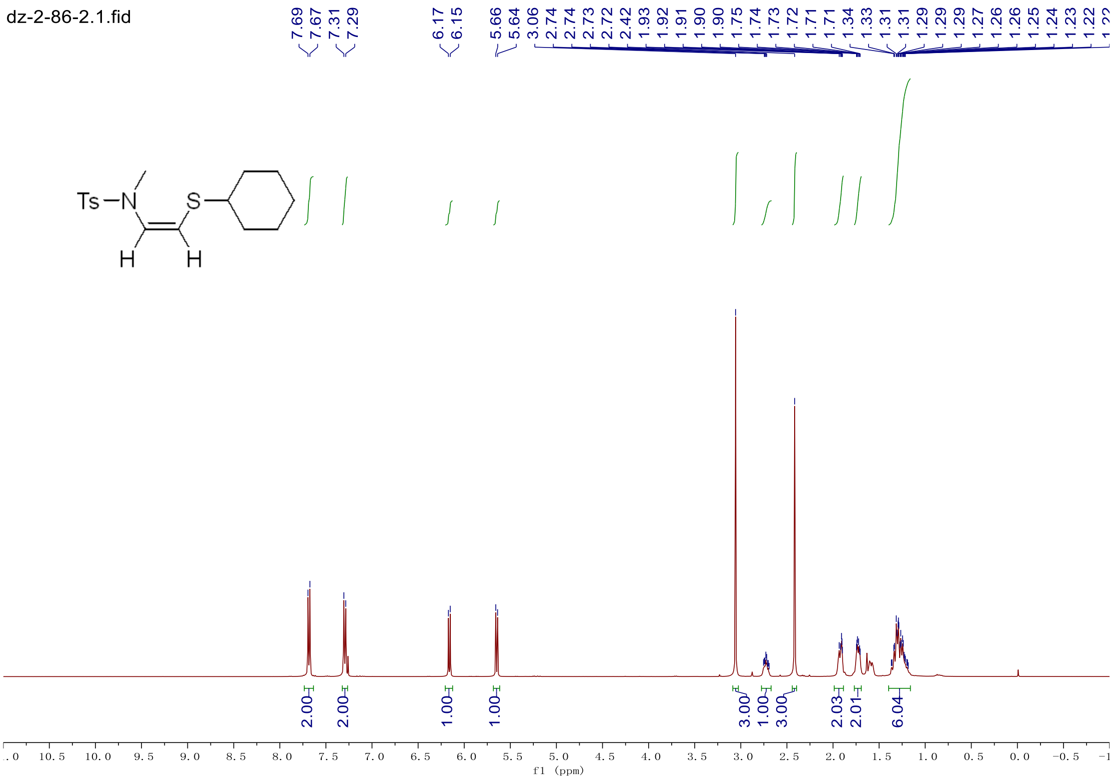


Figure S101. ^{1}H-NMR (400 MHz) spectrum of compound **3fa** in $CDCl_3$

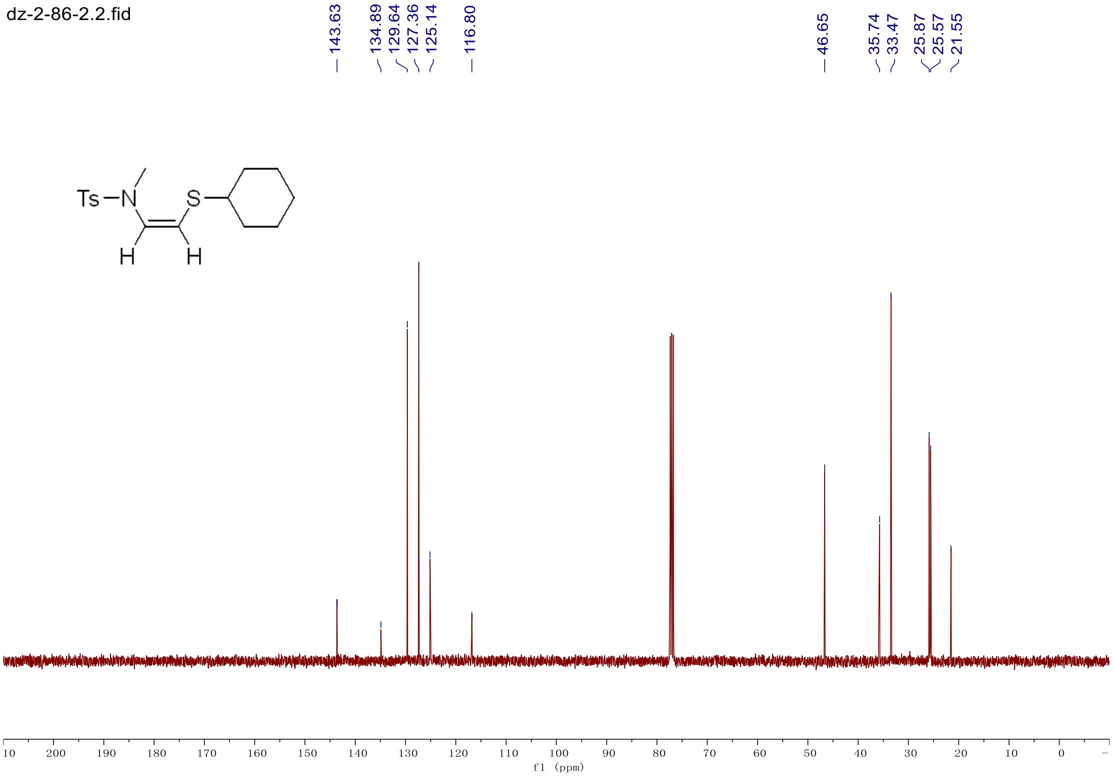


Figure S102. ^{13}C-NMR (100 MHz) spectrum of compound **3fa** in $CDCl_3$

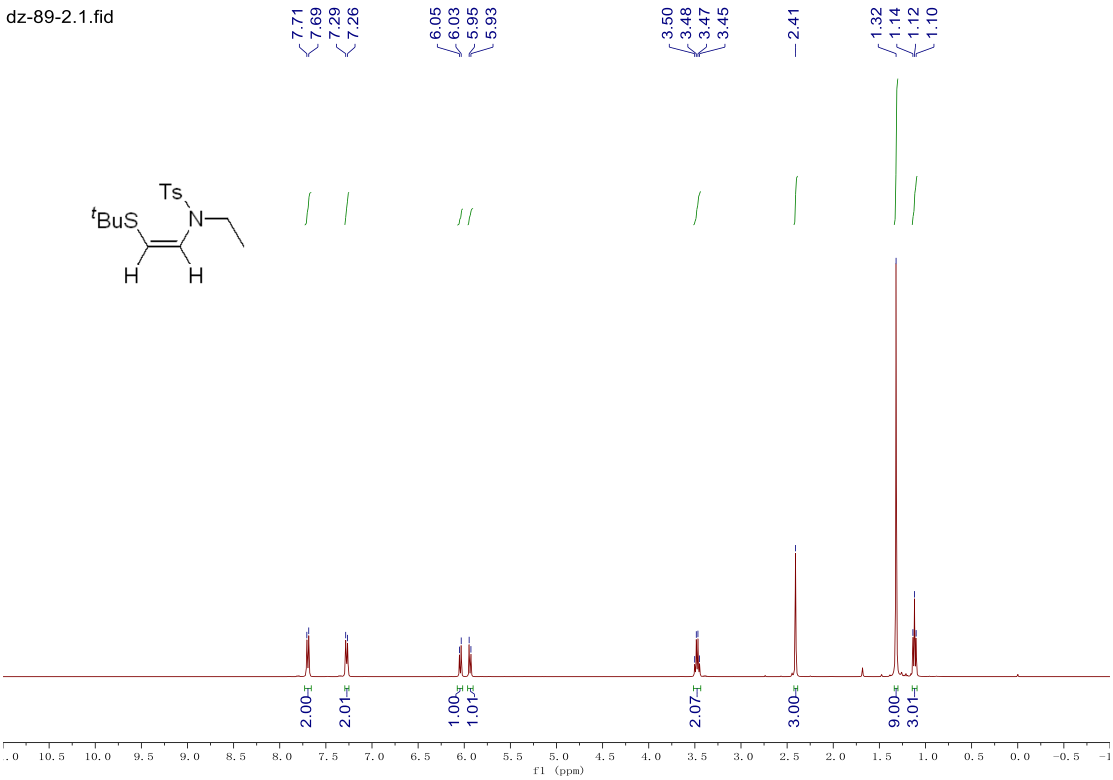


Figure S103. ^{1}H-NMR (400 MHz) spectrum of compound **3ab** in CDCl$_3$

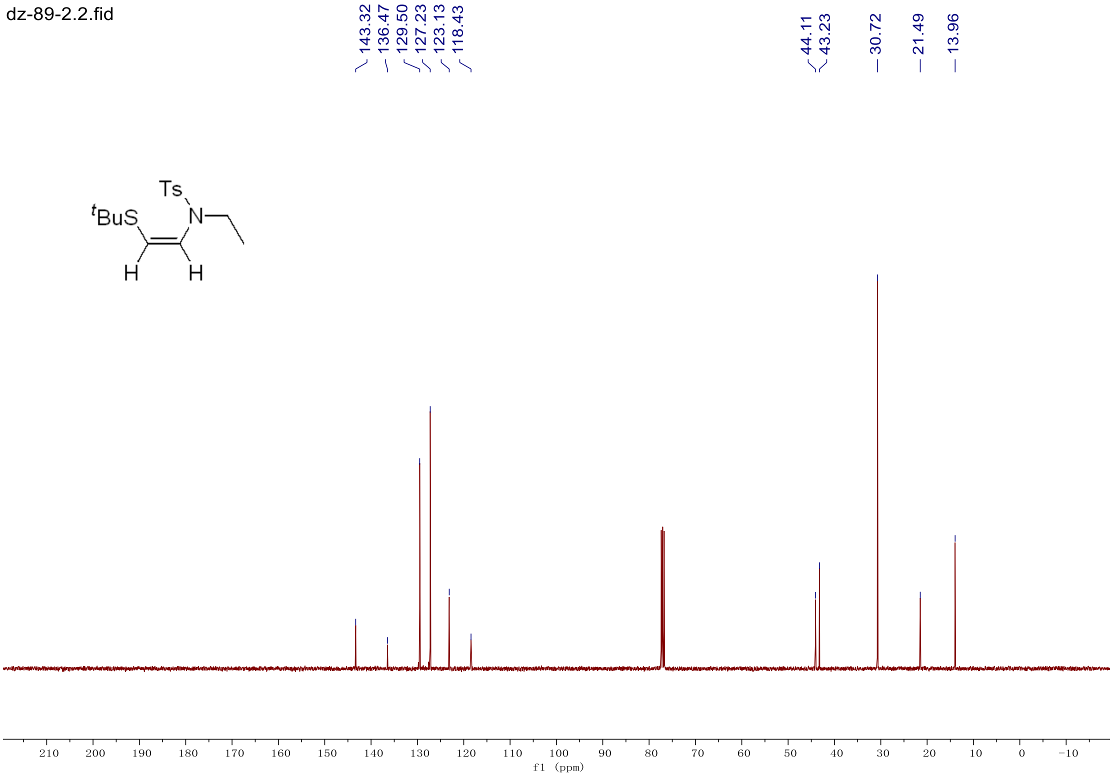


Figure S104. ^{13}C-NMR (100 MHz) spectrum of compound **3ab** in CDCl$_3$

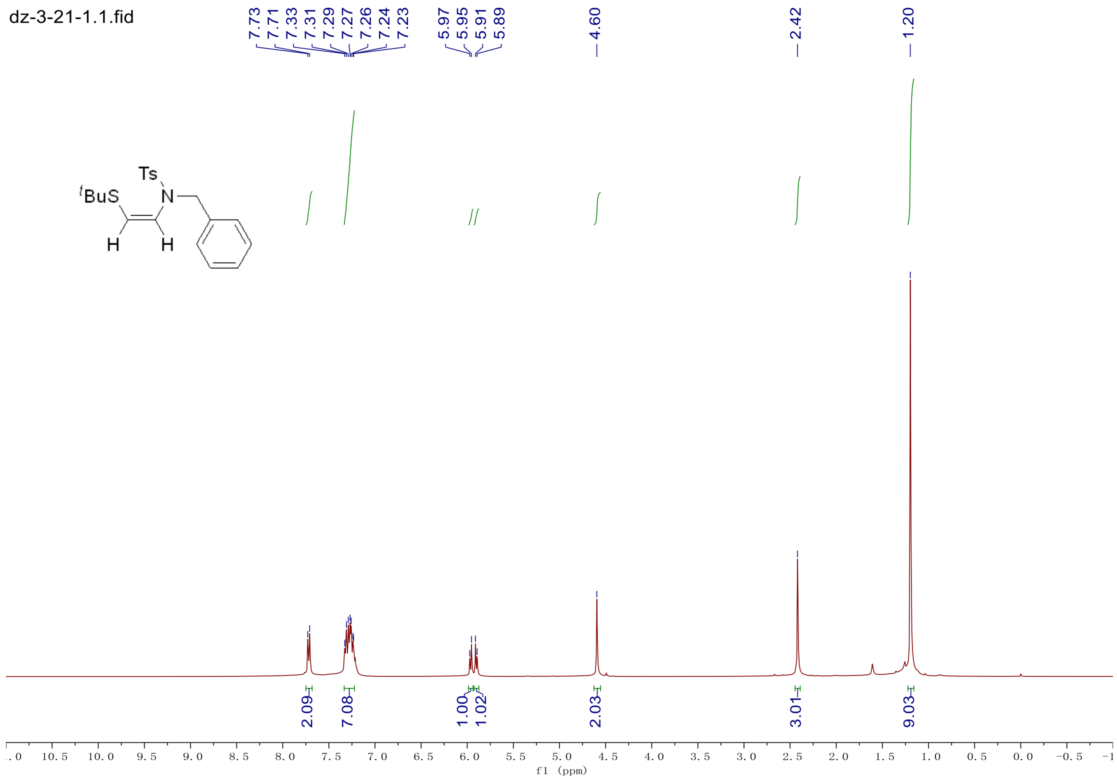


Figure S105. ^{1}H-NMR (400 MHz) spectrum of compound **3ac** in $CDCl_3$

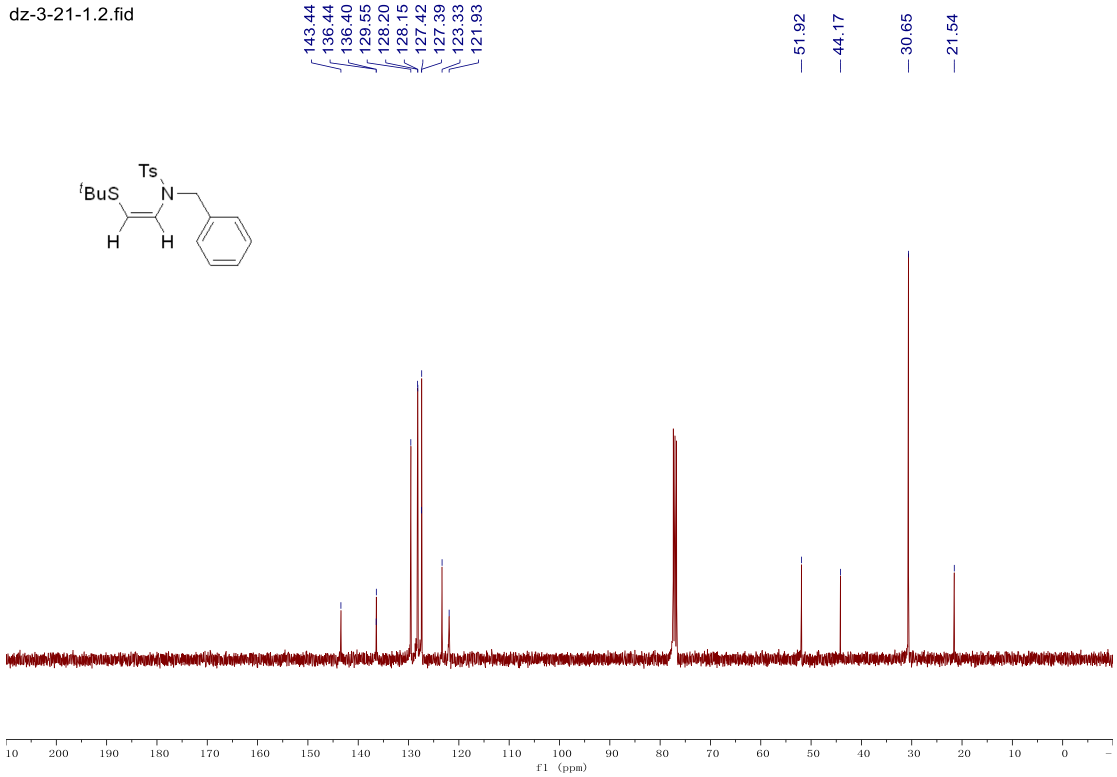


Figure S106. ^{13}C-NMR (100 MHz) spectrum of compound **3ac** in $CDCl_3$

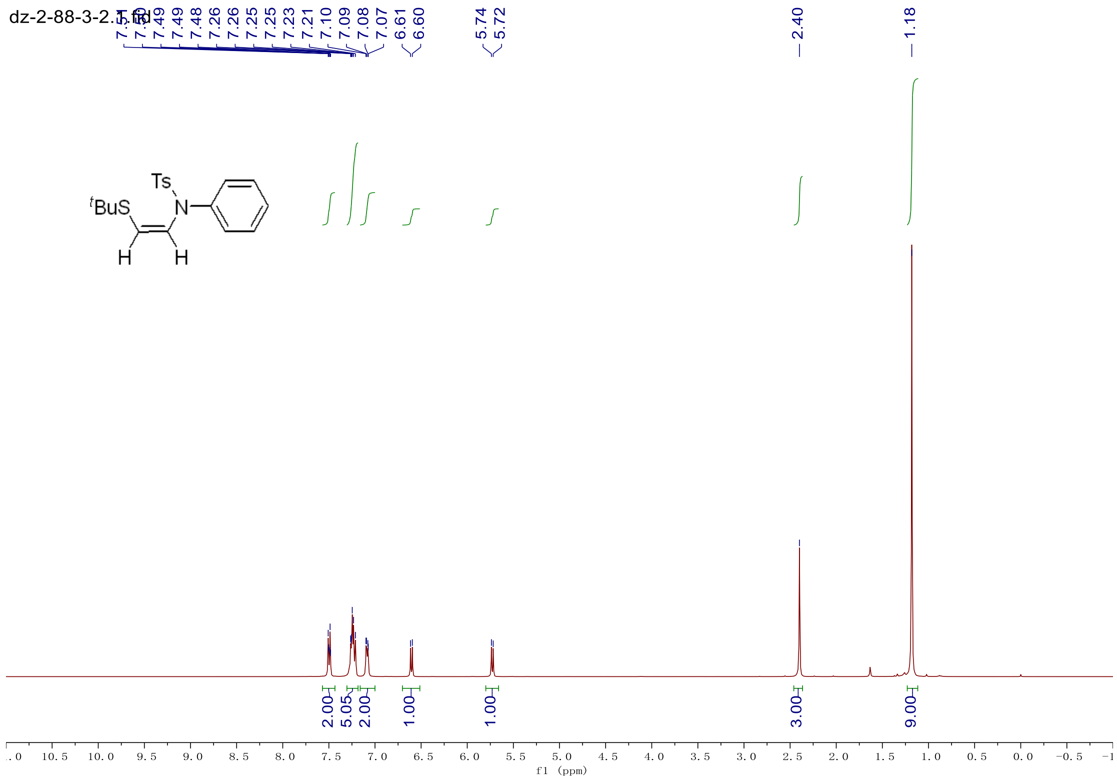


Figure S107. ^{1}H-NMR (400 MHz) spectrum of compound **3ad** in $CDCl_3$

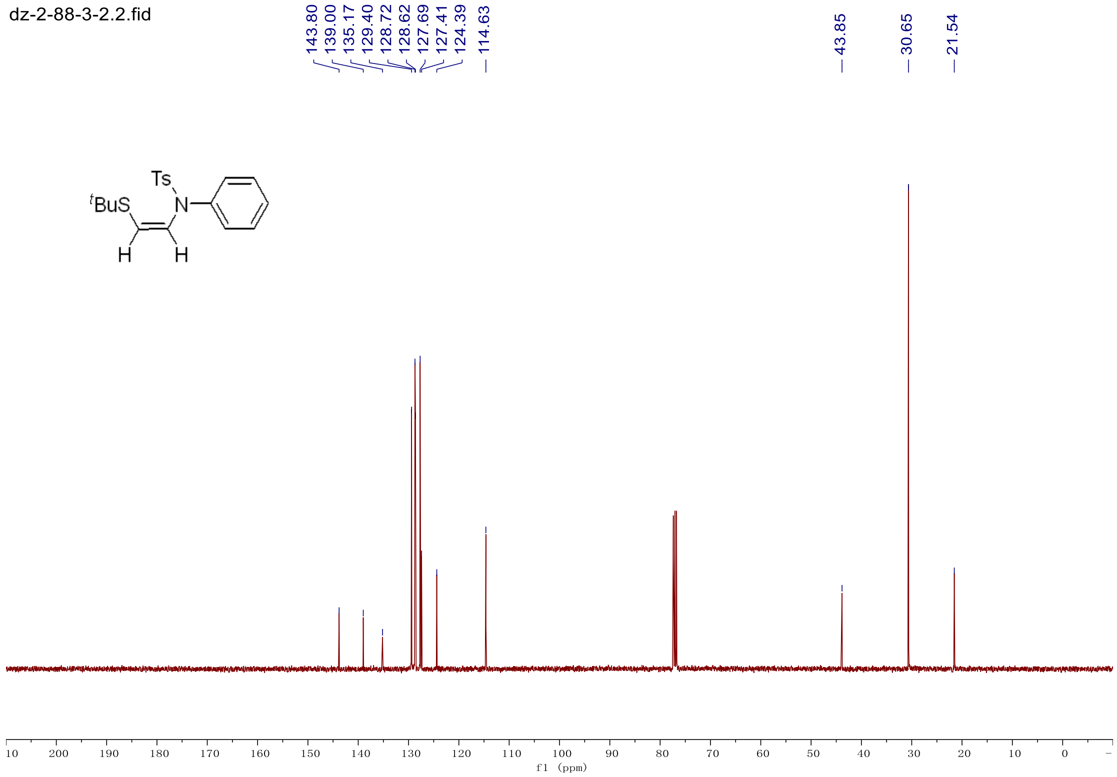


Figure S108. ^{13}C-NMR (100 MHz) spectrum of compound **3ad** in $CDCl_3$

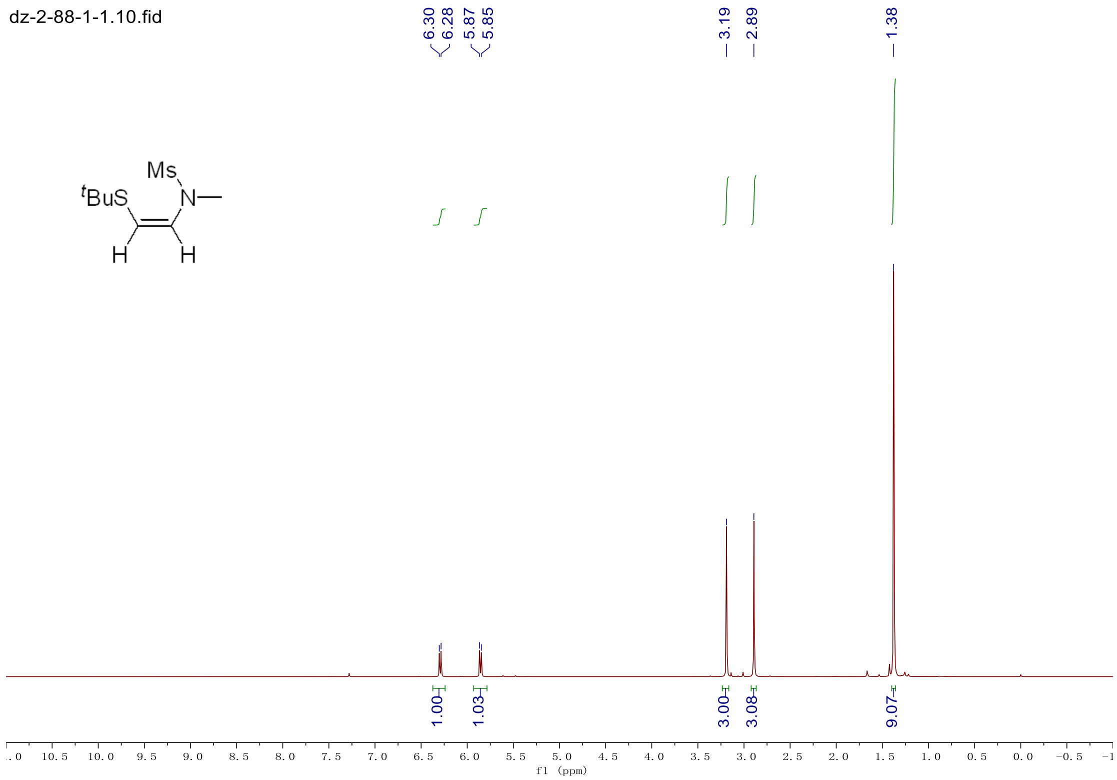


Figure S109. ^{1}H-NMR (400 MHz) spectrum of compound **3ae** in $CDCl_3$

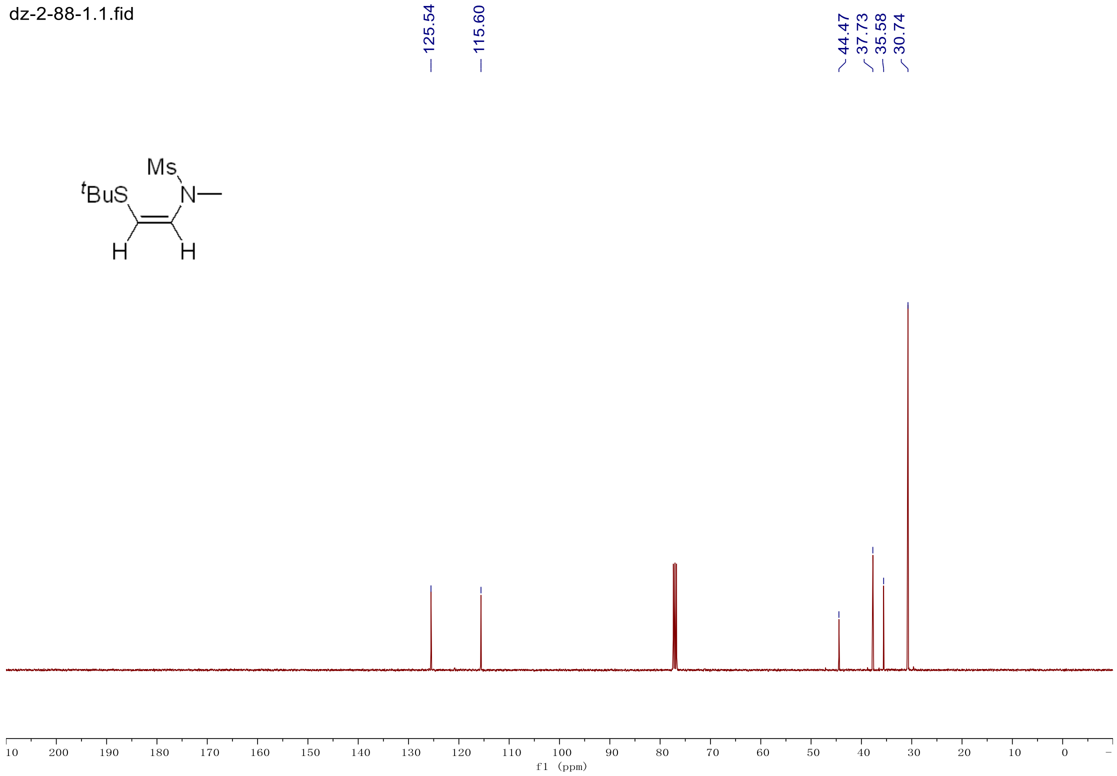


Figure S110. ^{13}C-NMR (100 MHz) spectrum of compound **3ae** in $CDCl_3$

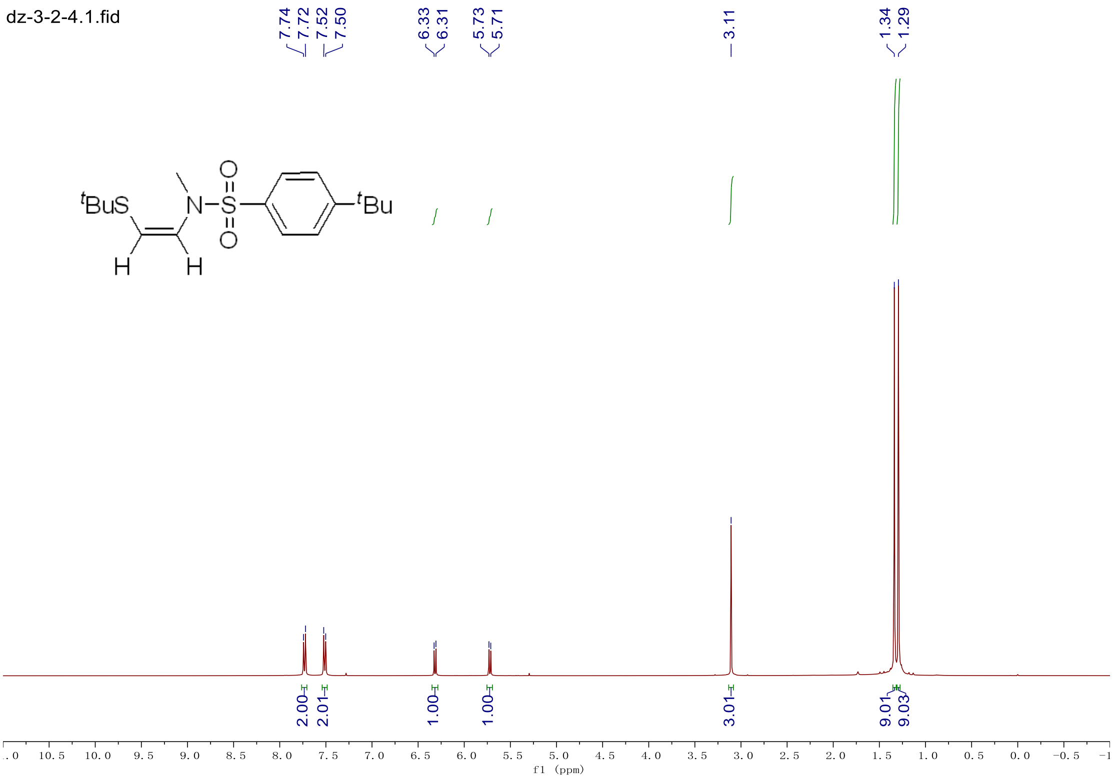


Figure S111. ^{1}H-NMR (400 MHz) spectrum of compound **3af** in CDCl$_3$

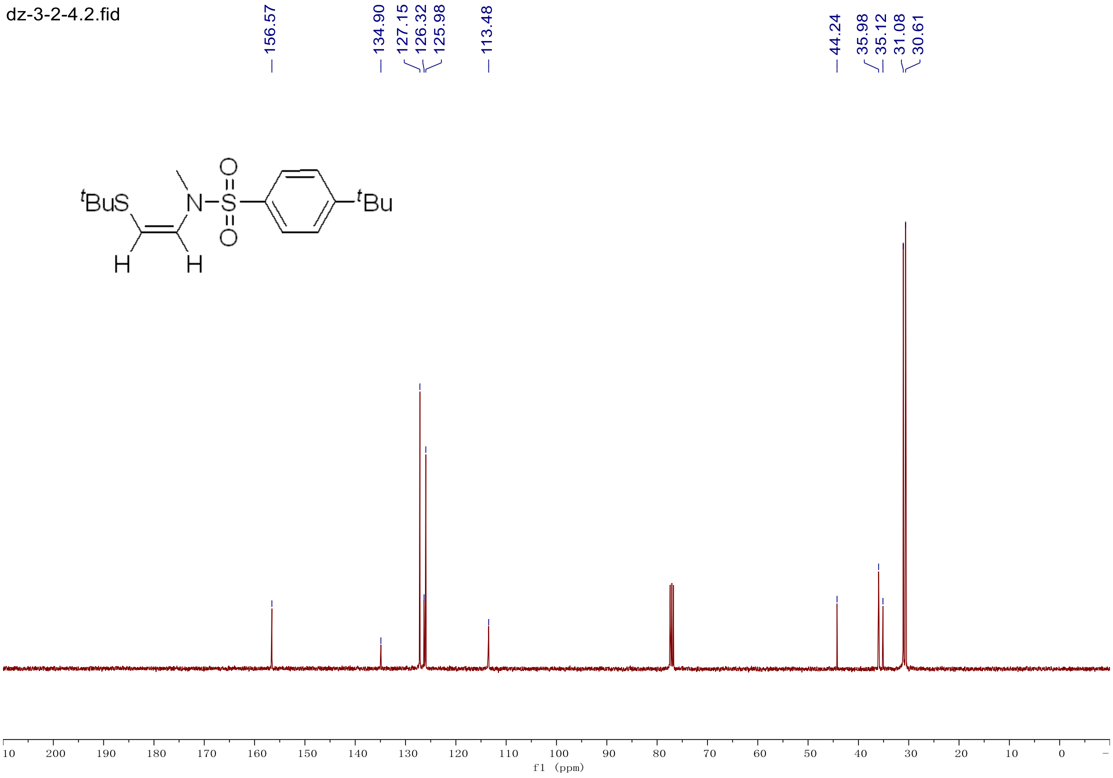


Figure S112. ^{13}C-NMR (100 MHz) spectrum of compound **3af** in CDCl$_3$

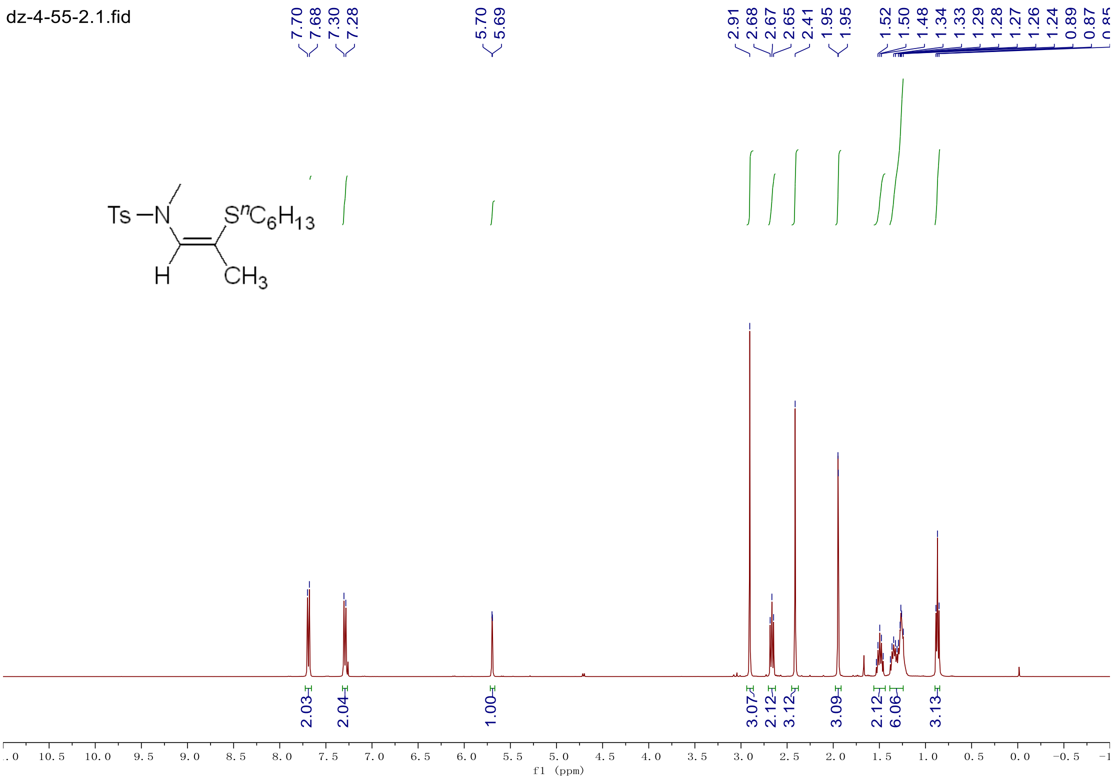


Figure S113. ^{1}H-NMR (400 MHz) spectrum of compound **3ag** in CDCl$_3$

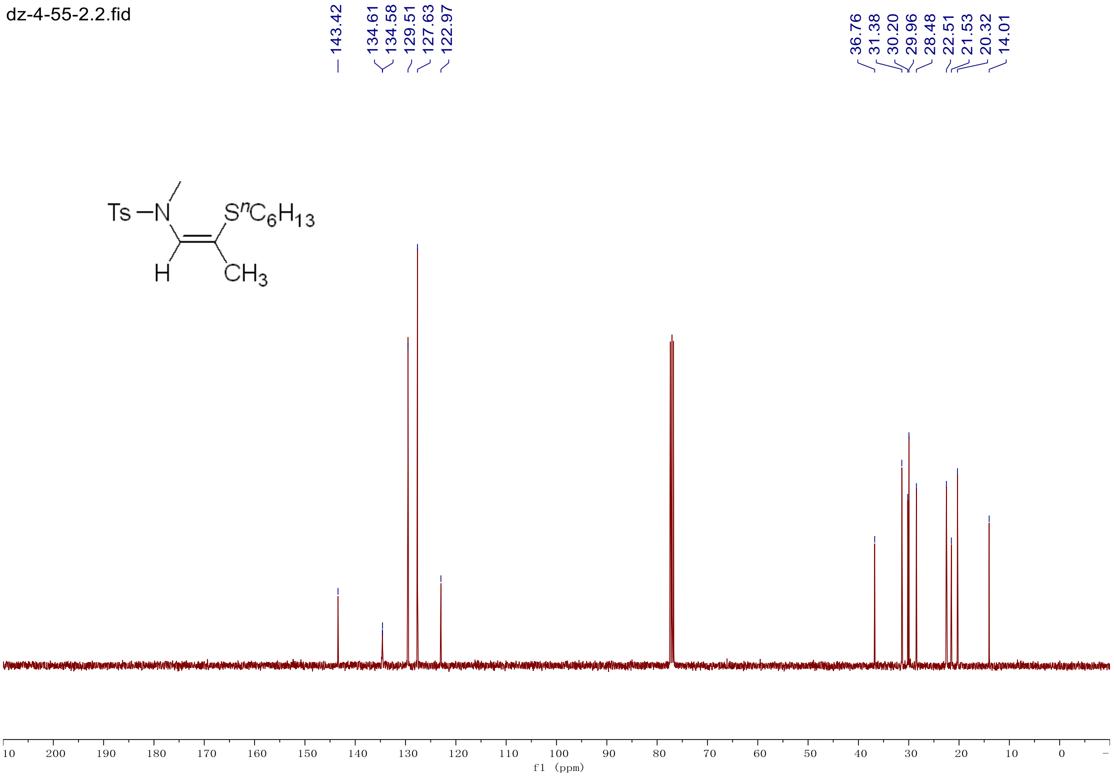


Figure S114. ^{13}C-NMR (100 MHz) spectrum of compound **3ag** in CDCl$_3$

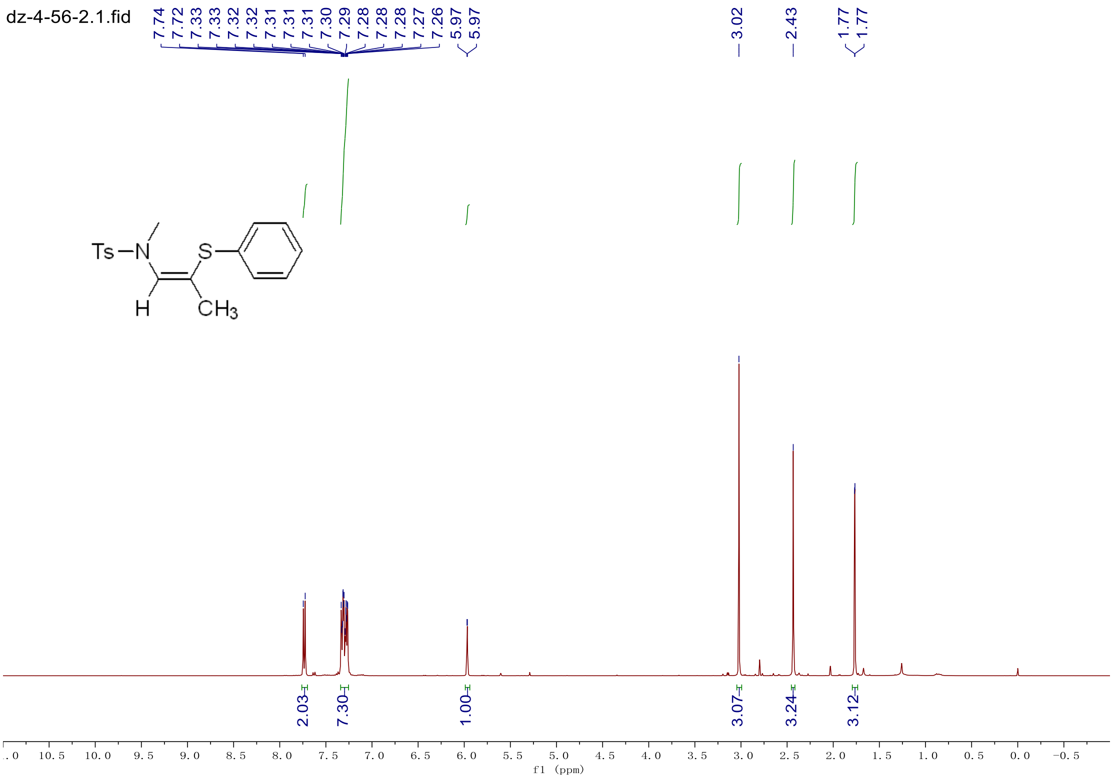


Figure S115. ^{1}H-NMR (400 MHz) spectrum of compound **3bg** in CDCl$_3$

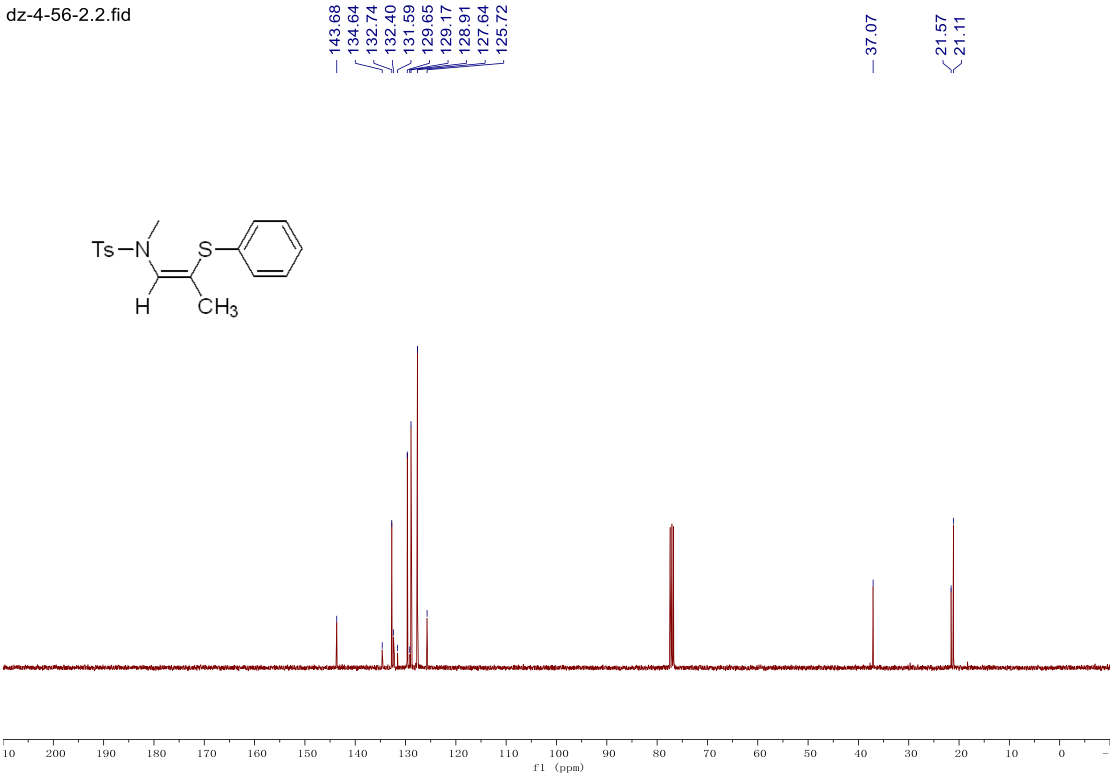


Figure S116. ^{13}C-NMR (100 MHz) spectrum of compound **3bg** in CDCl$_3$

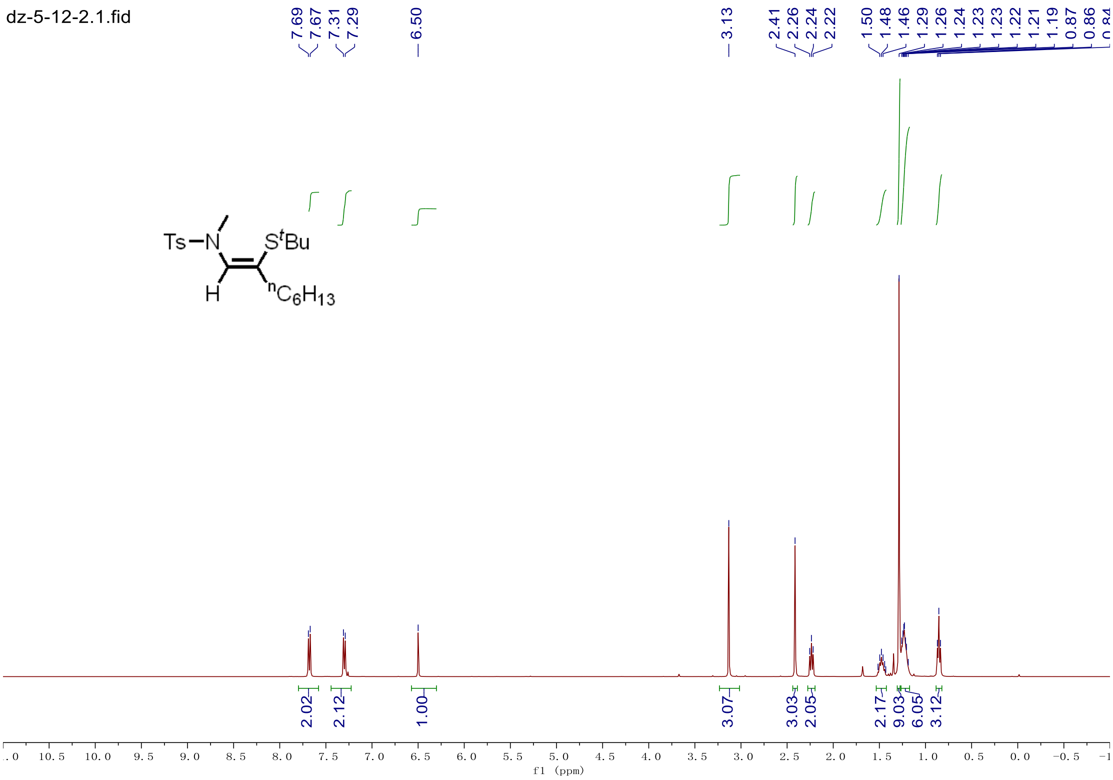


Figure S117. ^{1}H-NMR (400 MHz) spectrum of compound **3cg** in $CDCl_3$

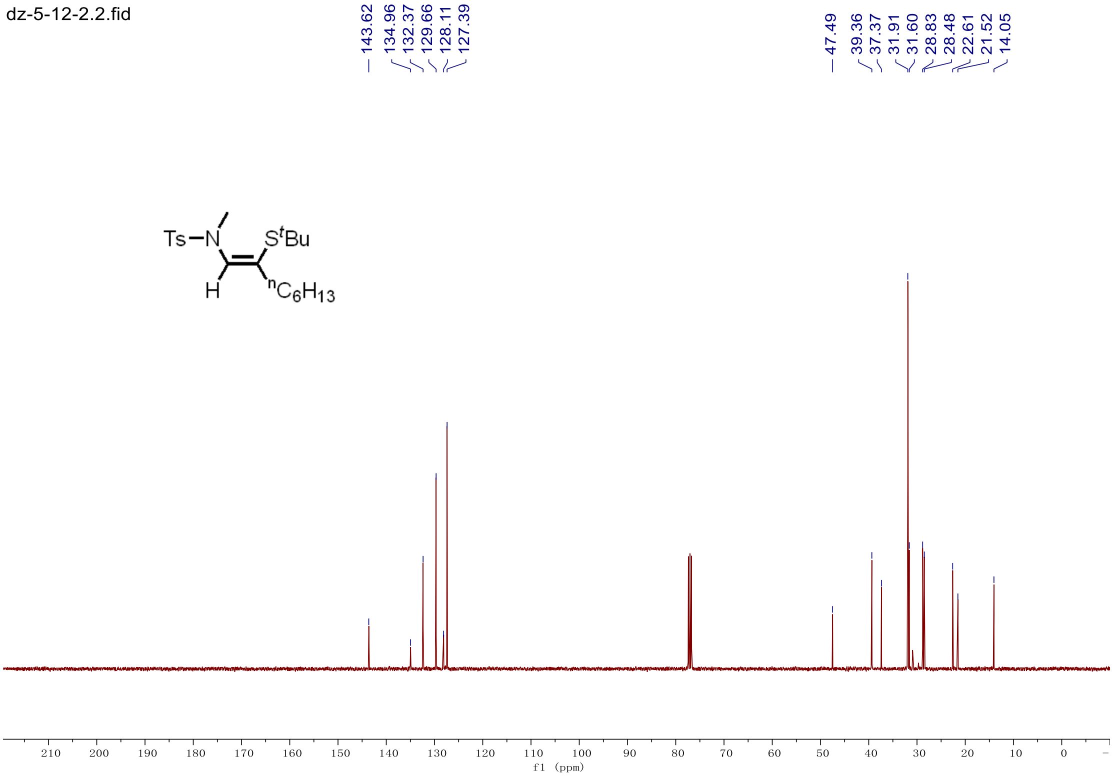


Figure S118. ^{13}C-NMR (100 MHz) spectrum of compound **3cg** in $CDCl_3$

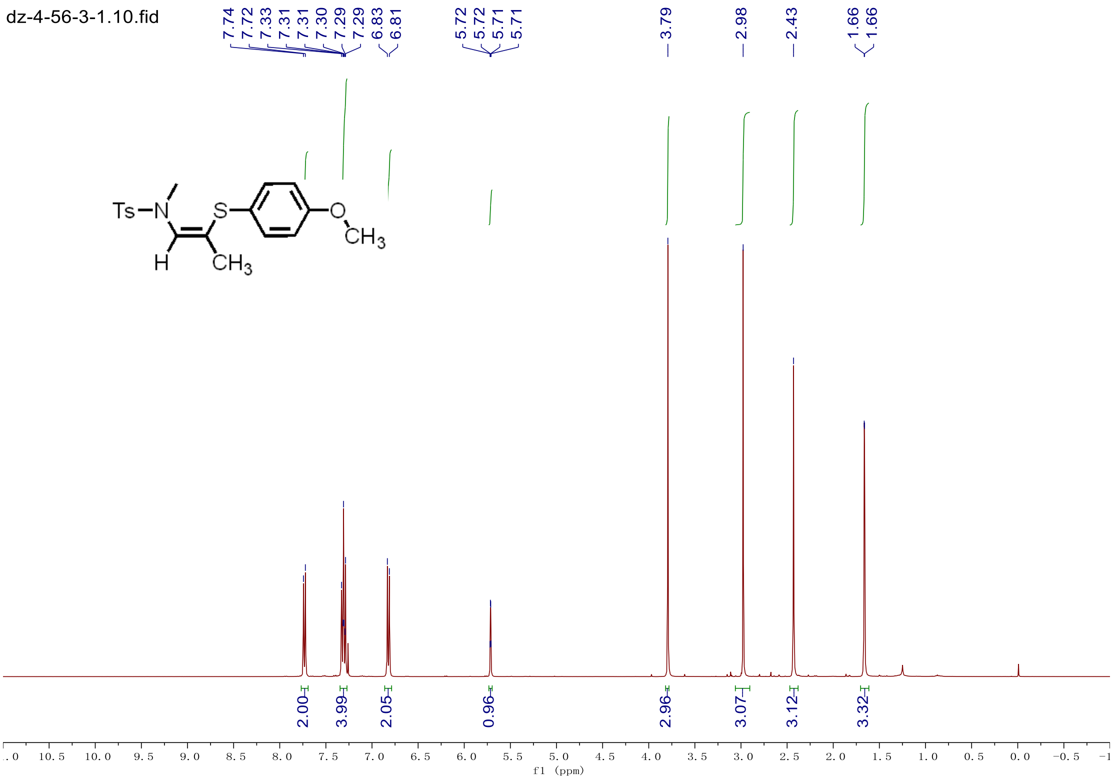


Figure S119. ^{1}H-NMR (400 MHz) spectrum of compound **3dg** in CDCl$_3$

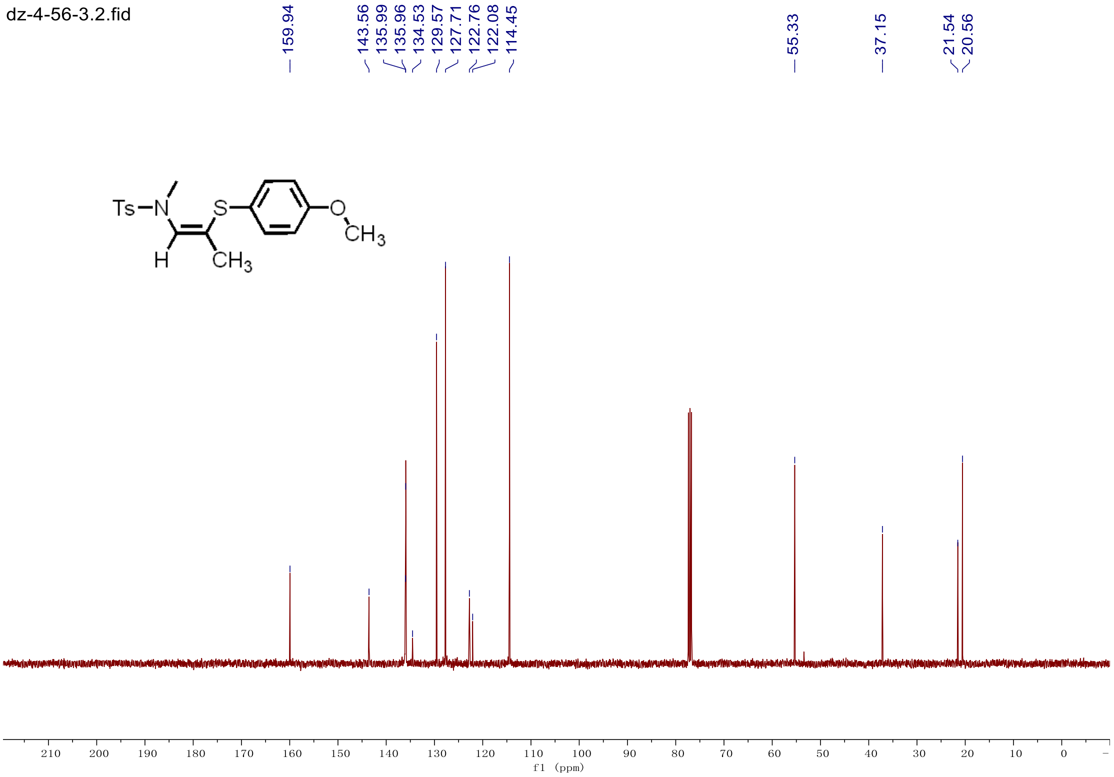


Figure S120. ^{13}C-NMR (100 MHz) spectrum of compound **3dg** in CDCl$_3$

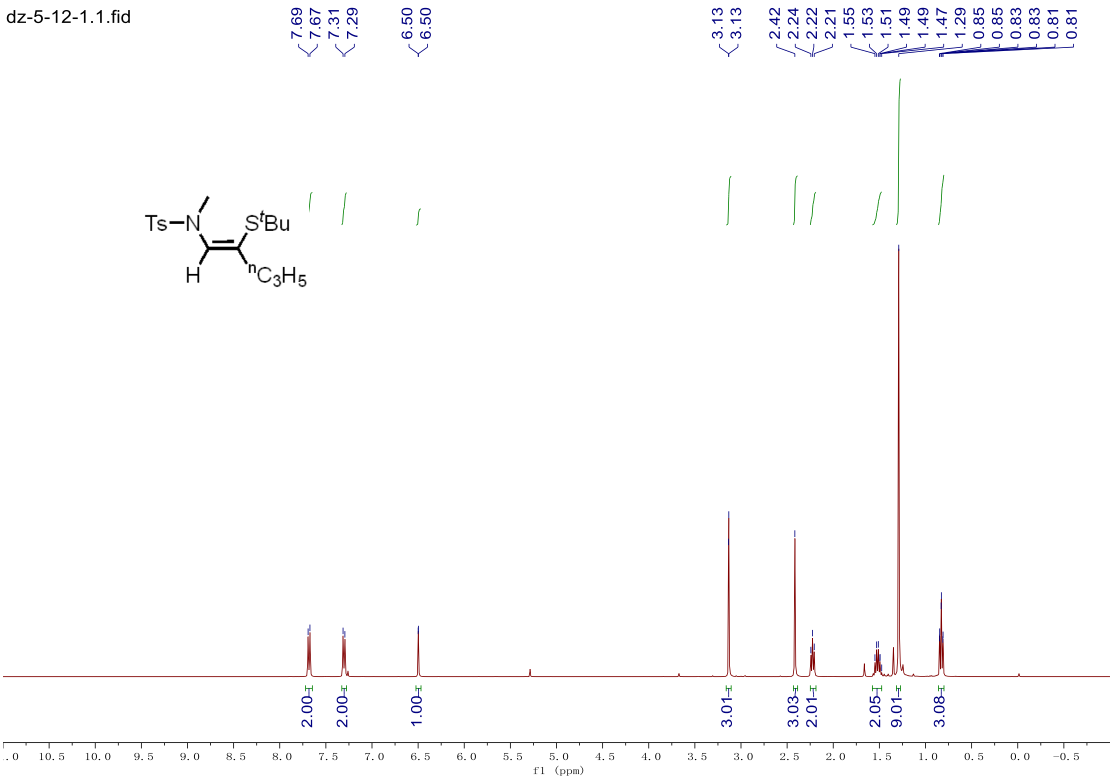


Figure S121. ^{1}H-NMR (400 MHz) spectrum of compound **3ah** in CDCl$_3$

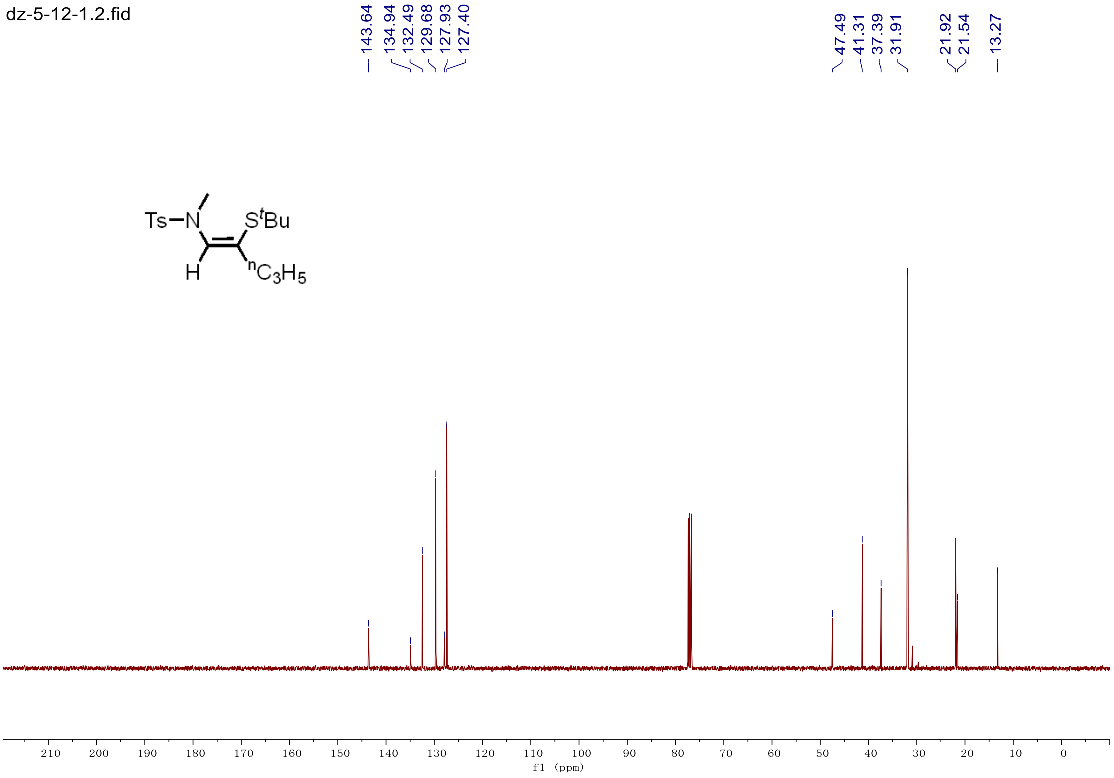


Figure S122. ^{13}C-NMR (100 MHz) spectrum of compound **3ah** in CDCl$_3$

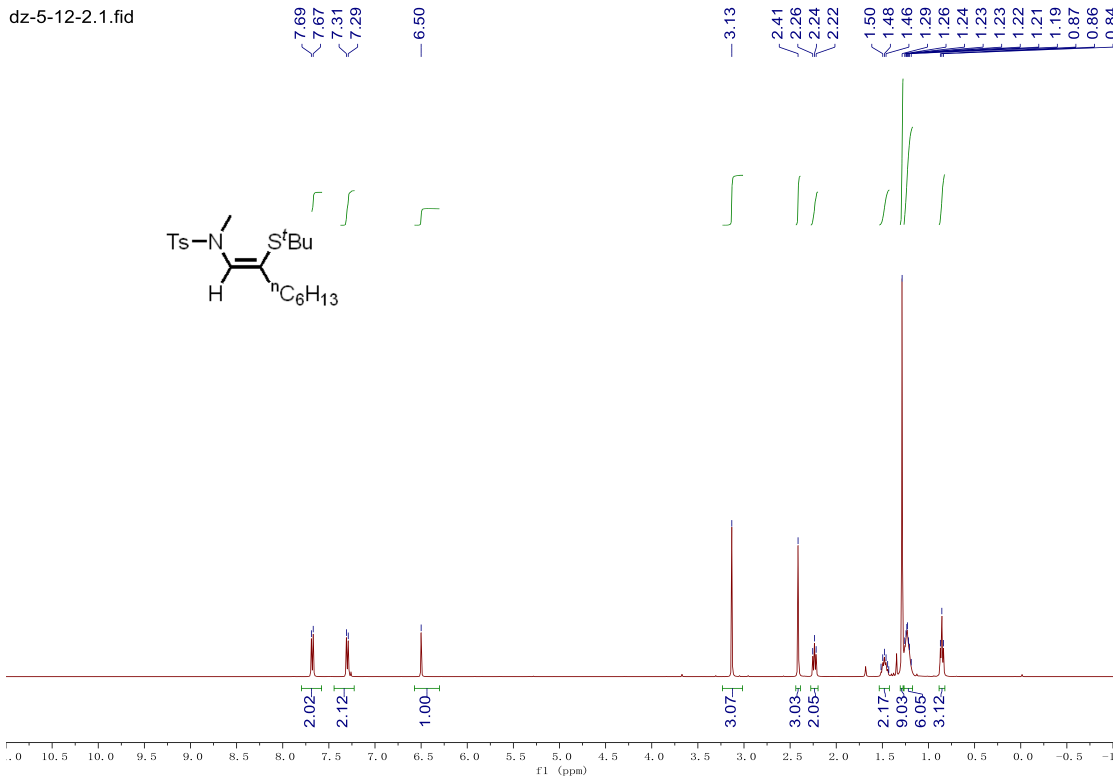


Figure S123. ^{1}H-NMR (400 MHz) spectrum of compound **3ai** in CDCl$_3$

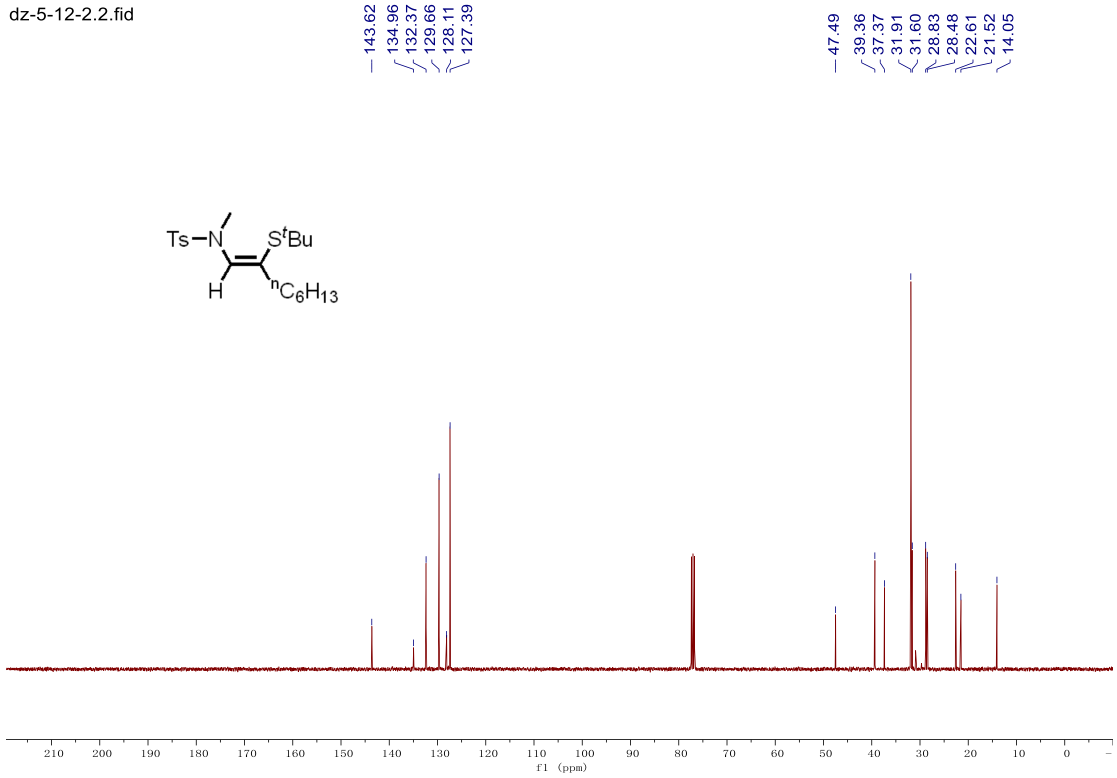


Figure S124. ^{13}C-NMR (100 MHz) spectrum of compound **3ai** in CDCl$_3$

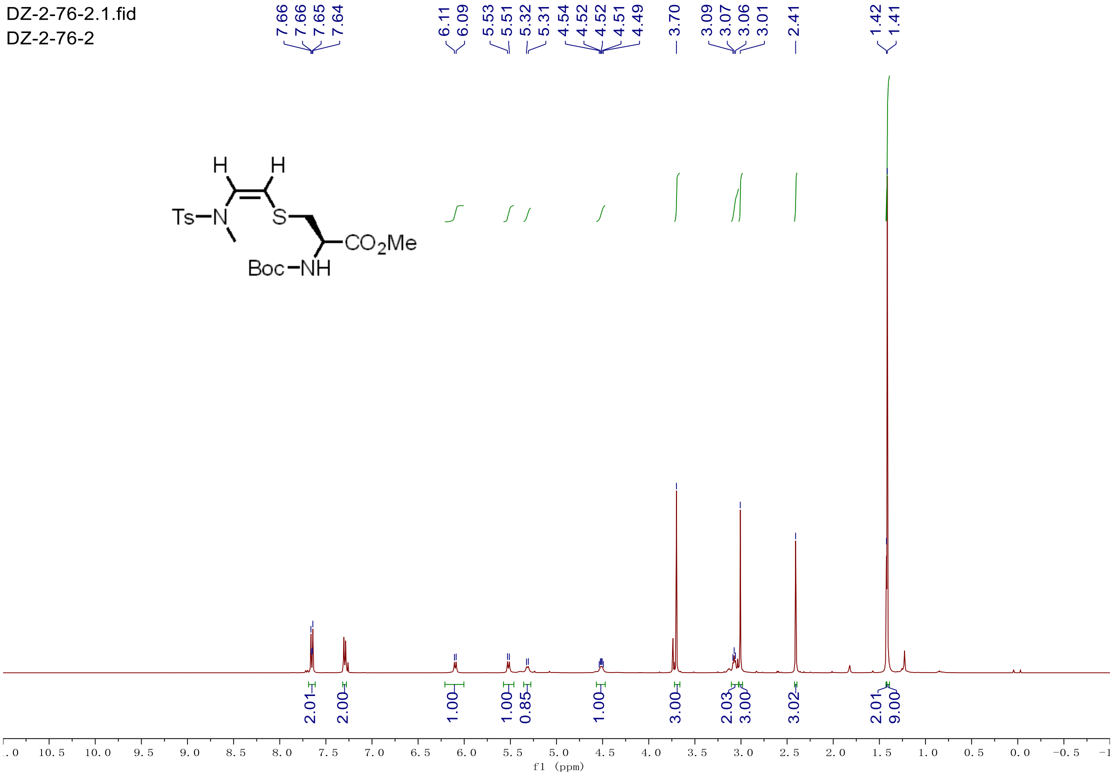


Figure S125. ^{1}H-NMR (400 MHz) spectrum of compound **3ga** in CDCl$_3$

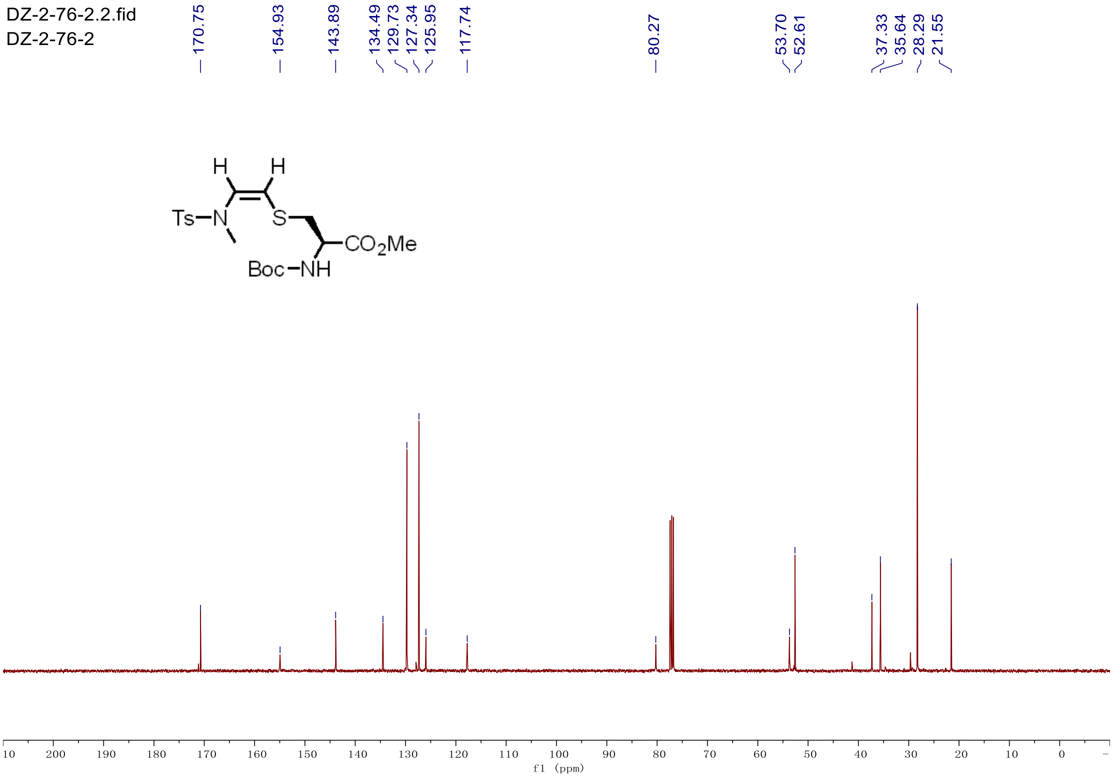


Figure S126. ^{13}C-NMR (100 MHz) spectrum of compound **3ga** in CDCl$_3$

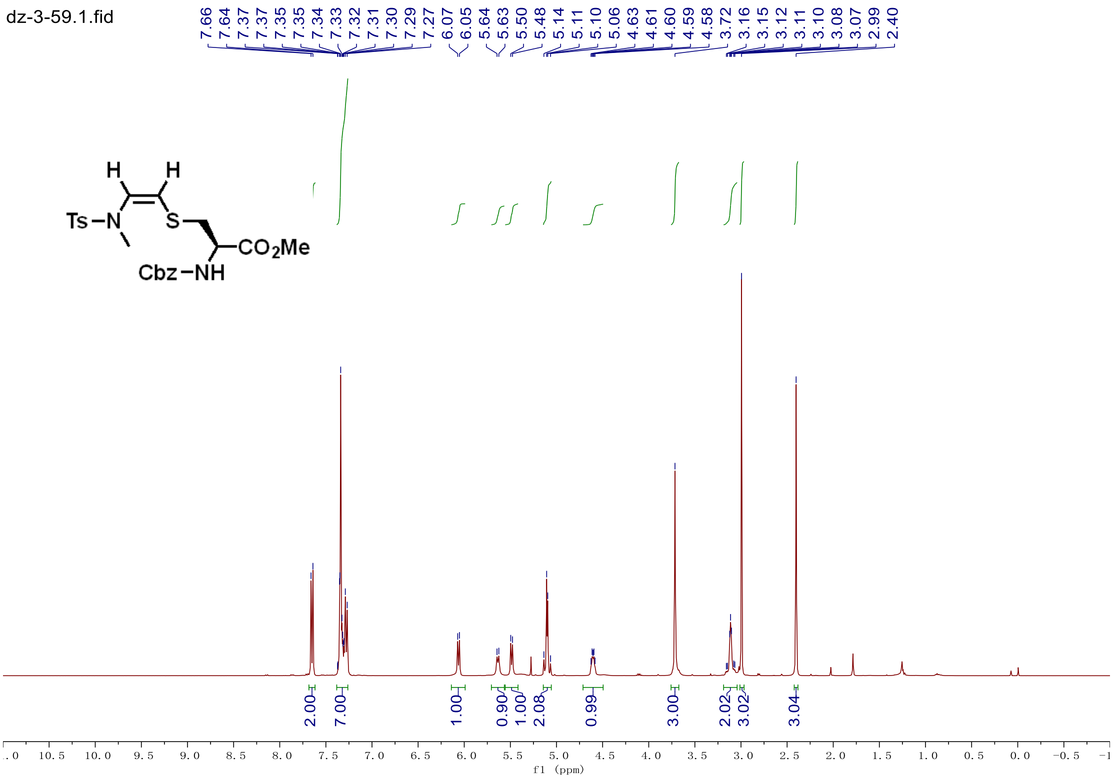


Figure S127. ^{1}H-NMR (400 MHz) spectrum of compound **3ge** in $CDCl_3$

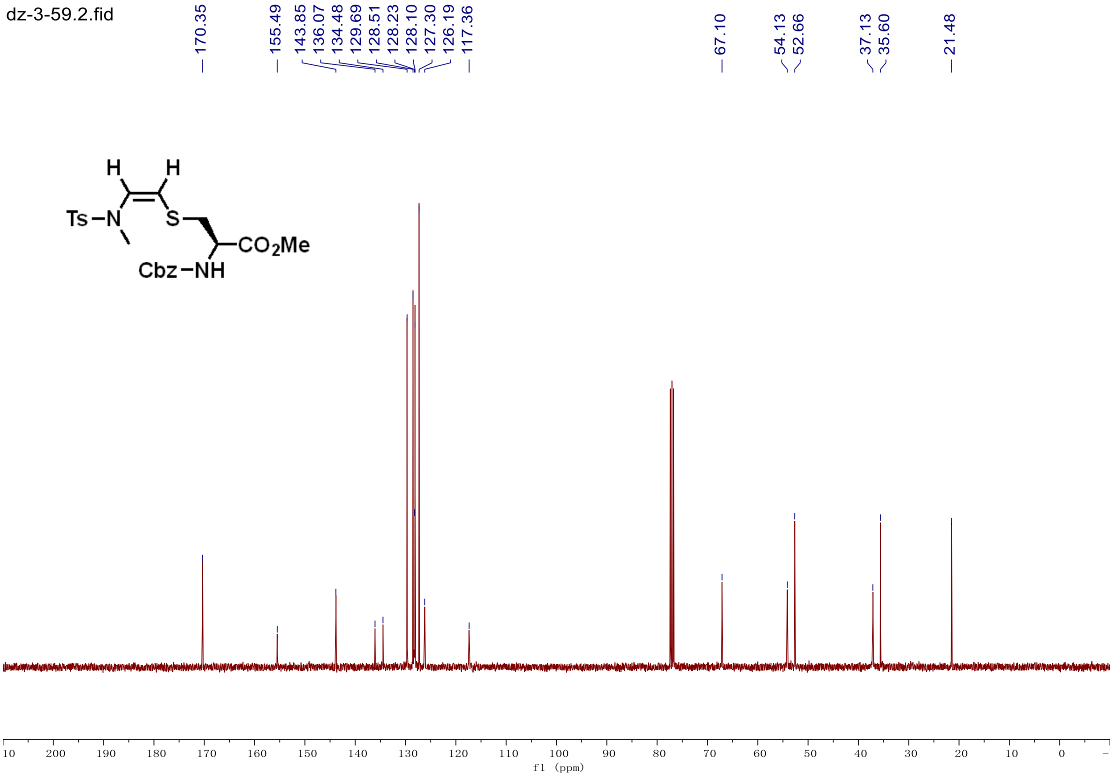


Figure S128. ^{13}C-NMR (100 MHz) spectrum of compound **3ge** in $CDCl_3$

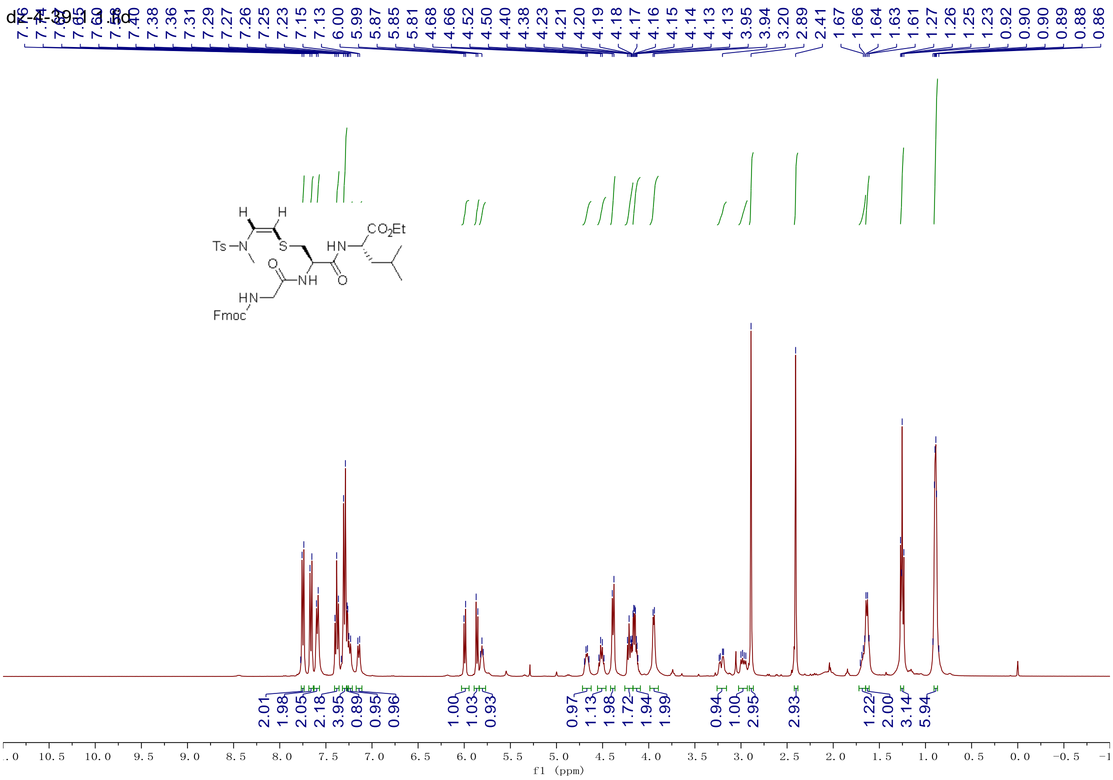


Figure S129. ^{1}H-NMR (400 MHz) spectrum of compound **3ja** in $CDCl_3$

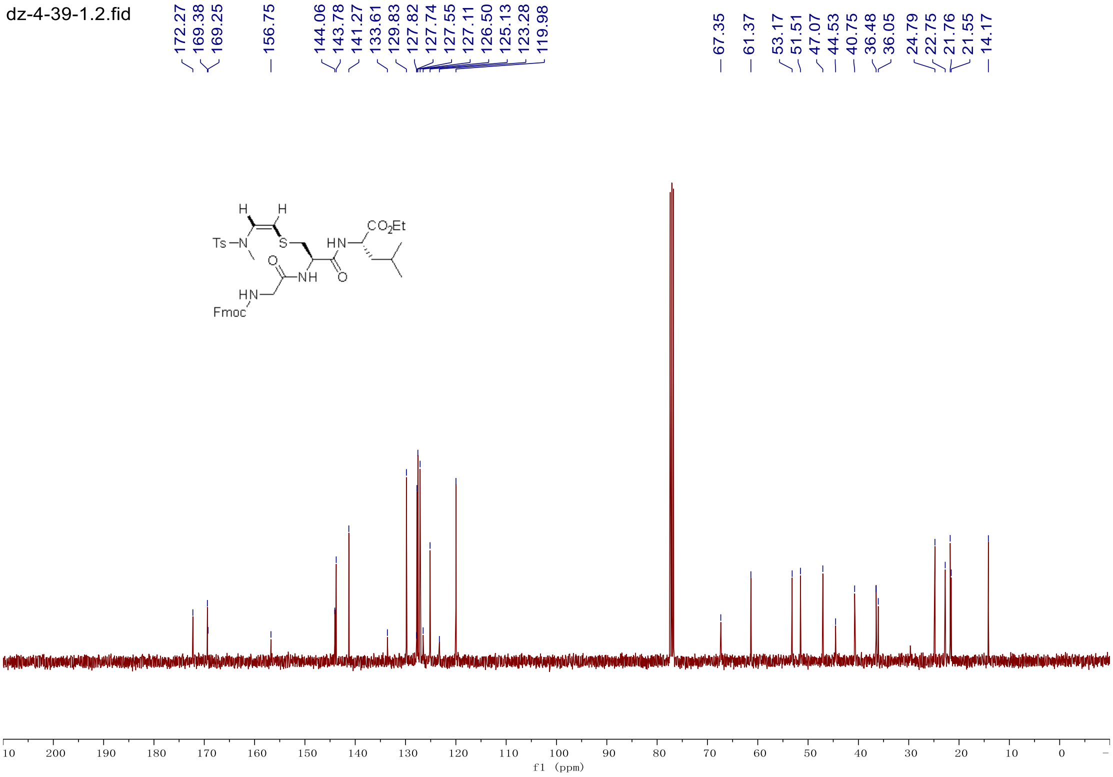


Figure S130. ^{13}C-NMR (100 MHz) spectrum of compound **3ja** in $CDCl_3$

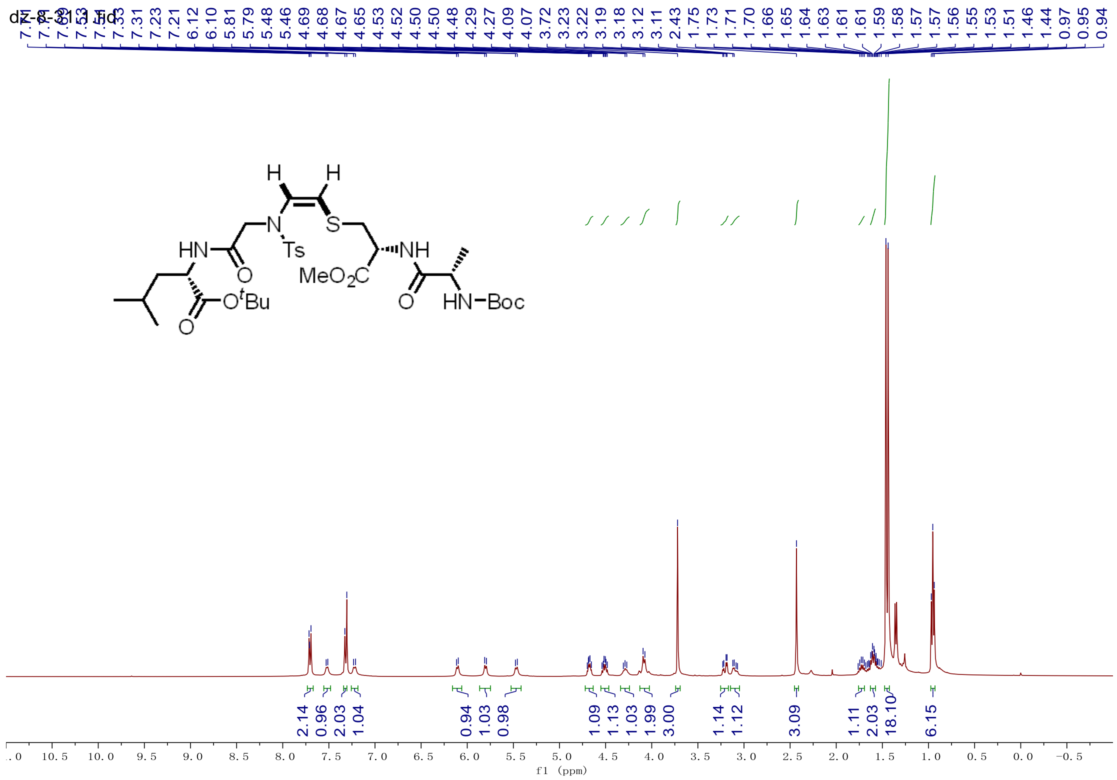


Figure S131. ^{1}H-NMR (400 MHz) spectrum of compound **3kk** in $CDCl_3$

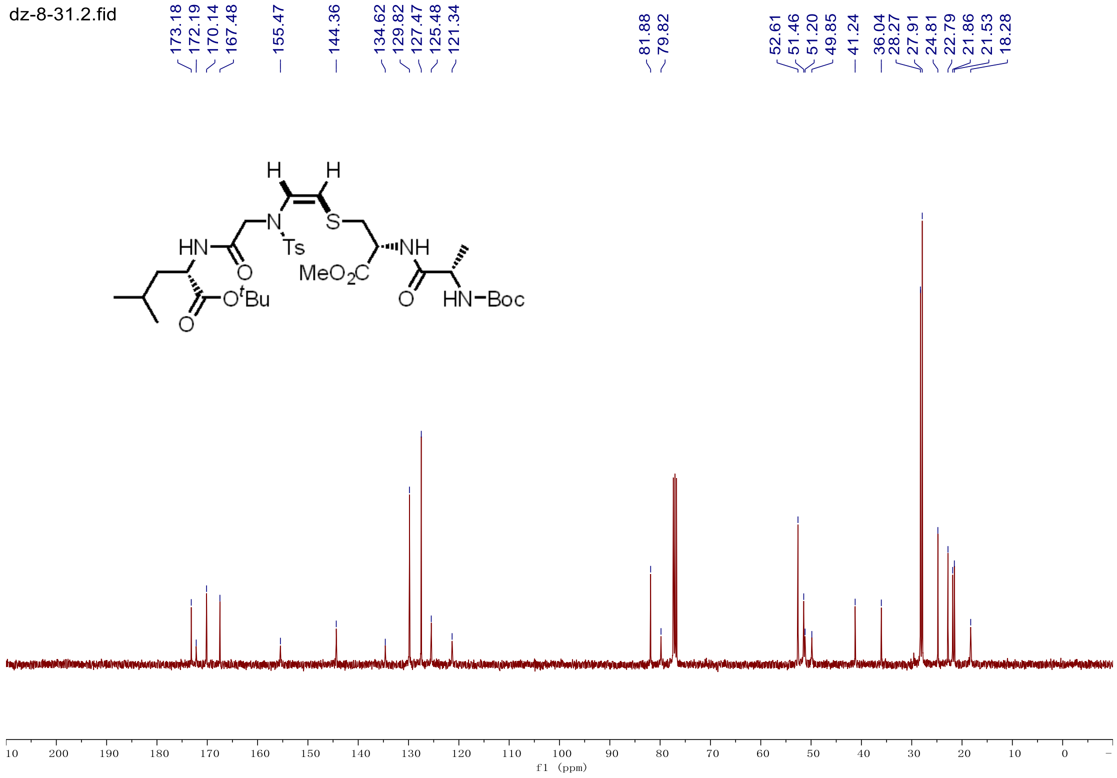


Figure S132. ^{13}C-NMR (100 MHz) spectrum of compound **3kk** in $CDCl_3$

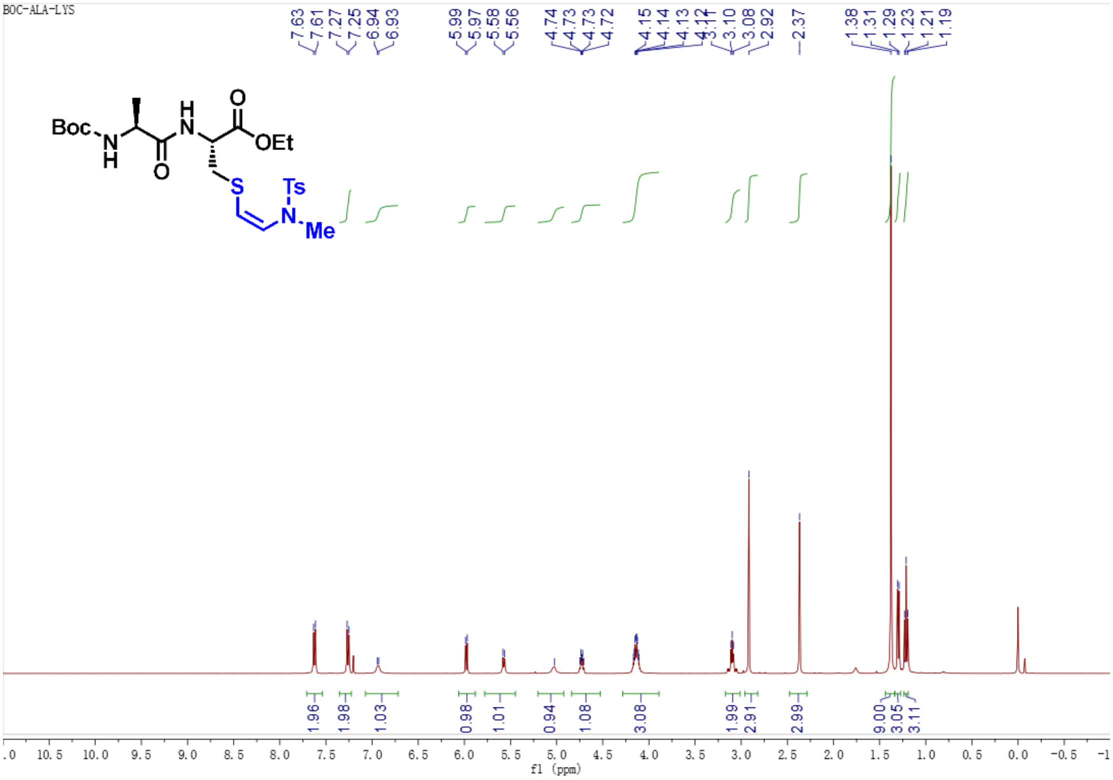


Figure S133. ^{1}H-NMR (400 MHz) spectrum of compound **5aa** in $CDCl_3$

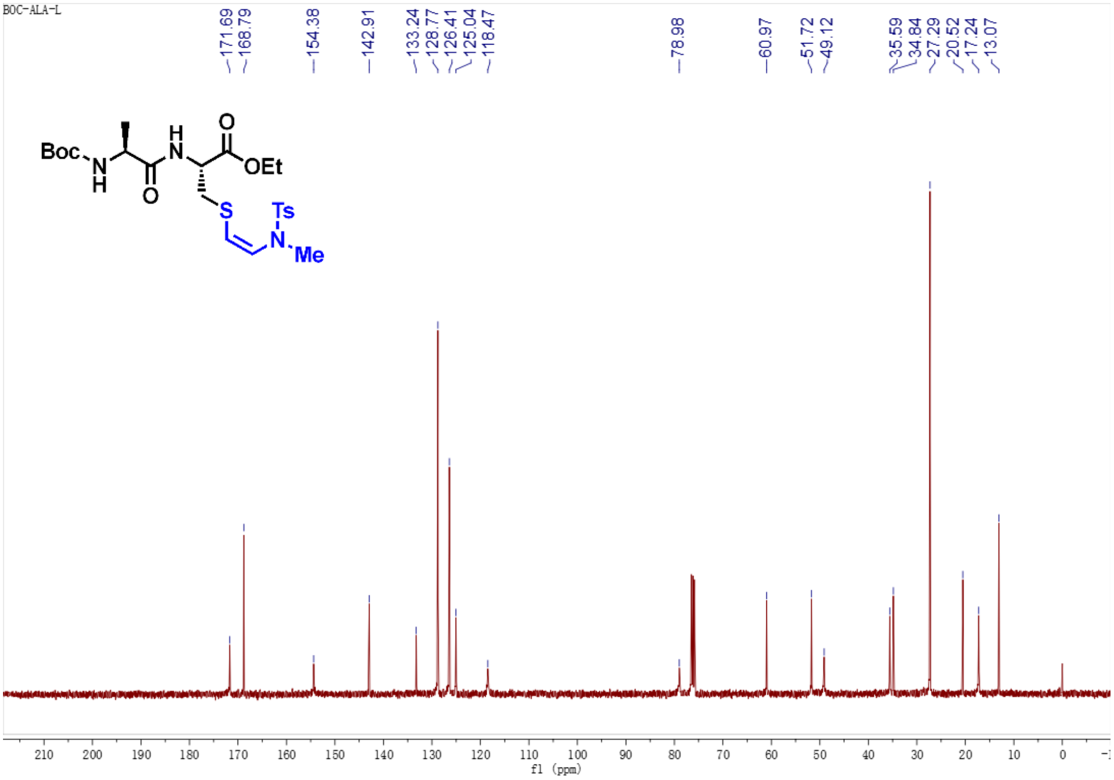


Figure S134. ^{13}C-NMR (100 MHz) spectrum of compound **5aa** in $CDCl_3$

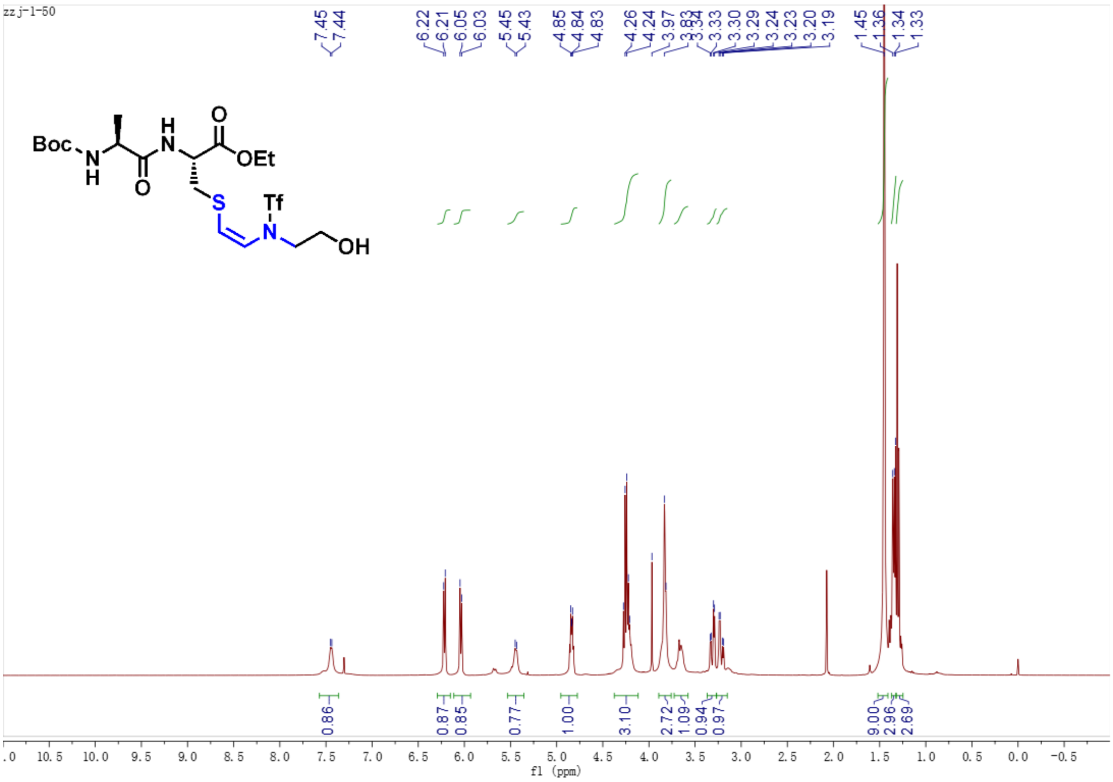


Figure S135. ^{1}H-NMR (400 MHz) spectrum of compound **5an** in $CDCl_3$.

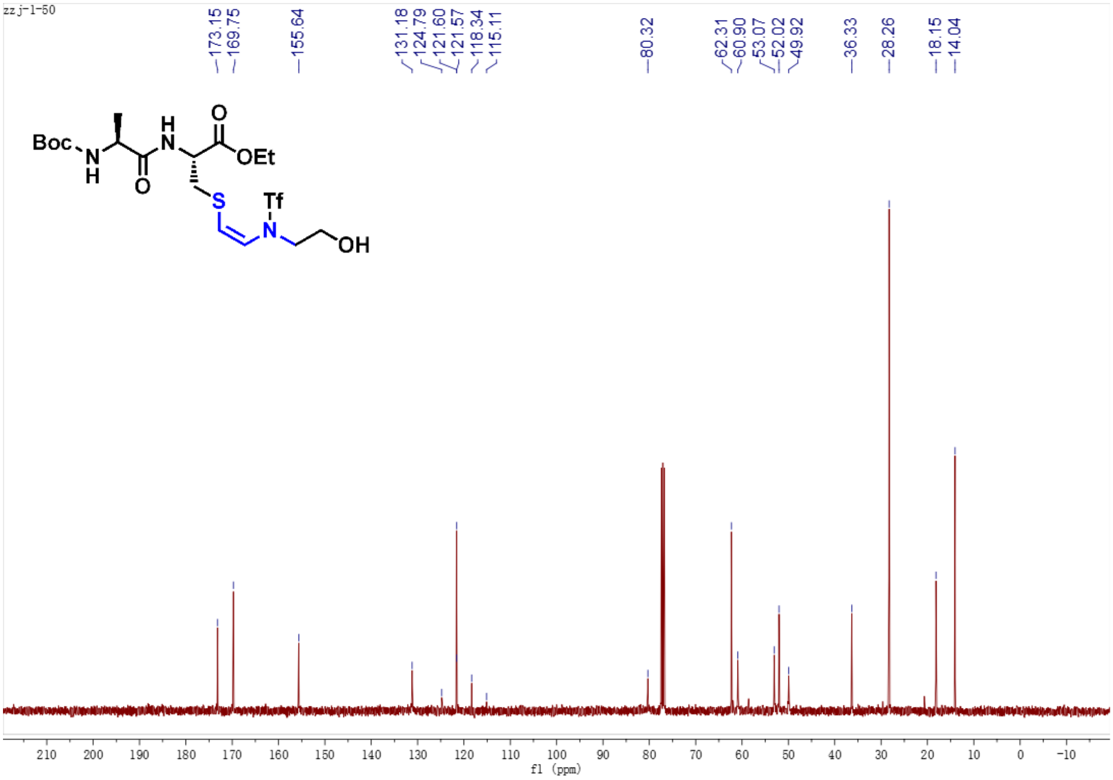


Figure S136. ^{13}C-NMR (100 MHz) spectrum of compound **5an** in $CDCl_3$.

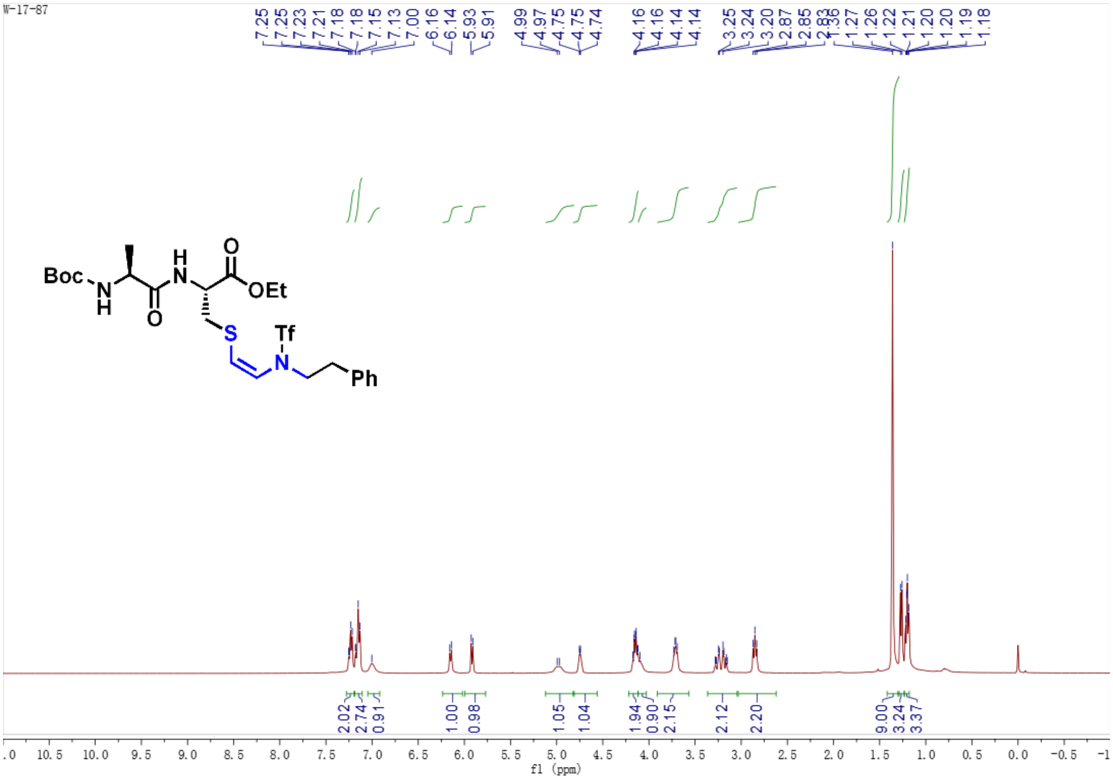


Figure S137. ^{1}H-NMR (400 MHz) spectrum of compound **5ao** in $CDCl_3$.

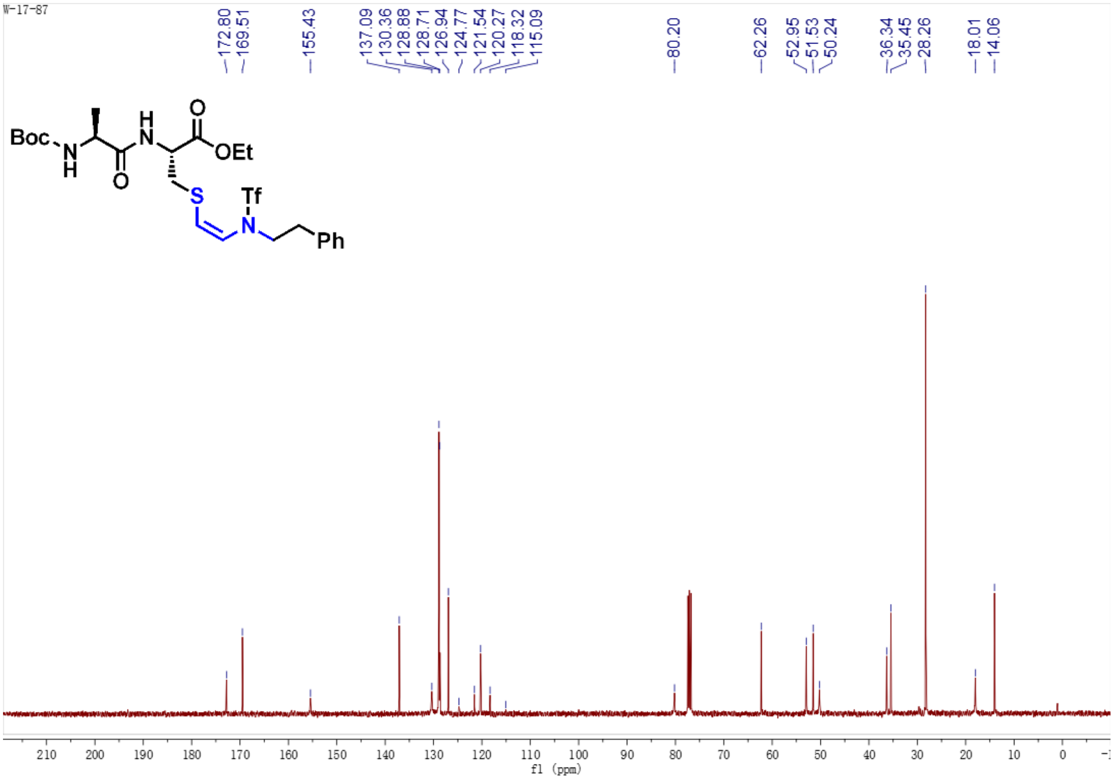


Figure S138. ^{13}C-NMR (100 MHz) spectrum of compound **5ao** in $CDCl_3$.

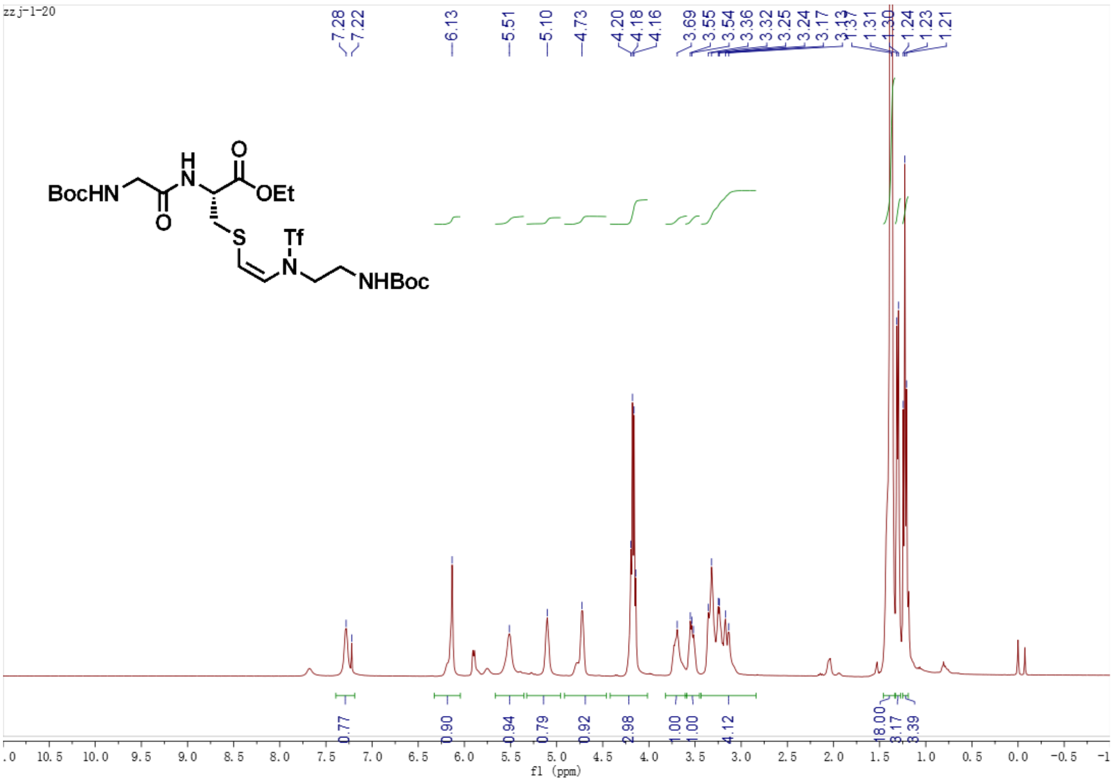


Figure S139. ^{1}H-NMR (400 MHz) spectrum of compound **5ap** in CDCl$_3$

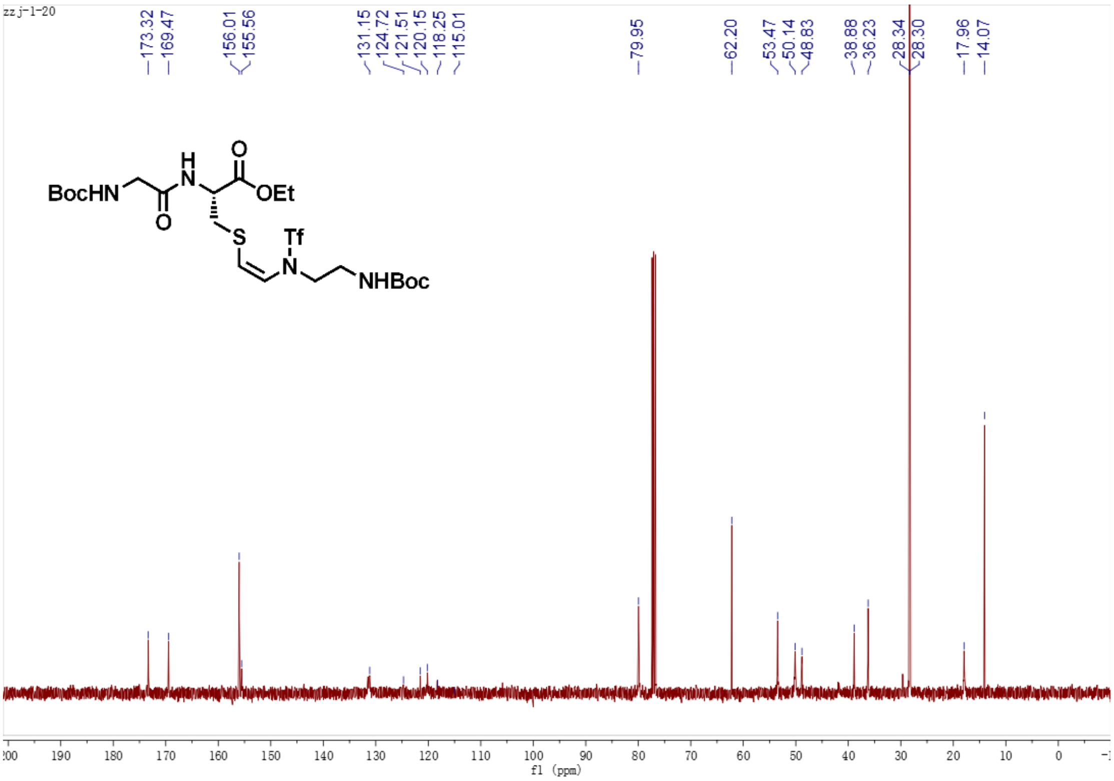


Figure S140. ^{13}C-NMR (100 MHz) spectrum of compound **5ap** in CDCl$_3$

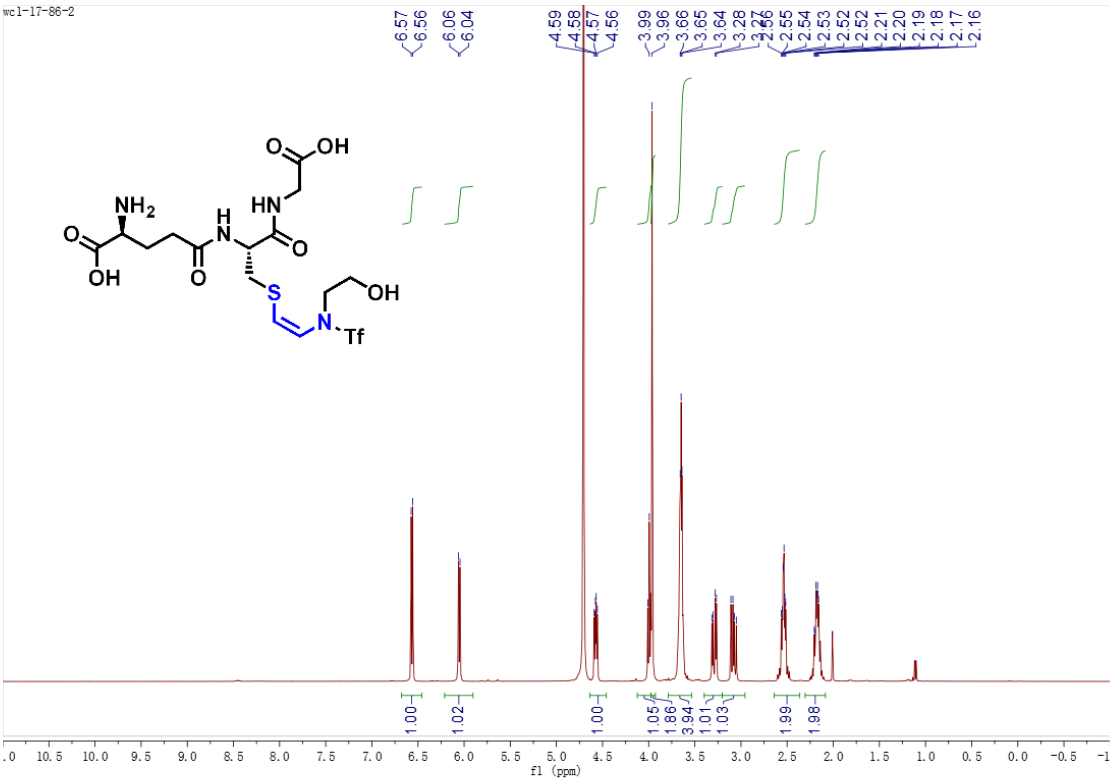


Figure S141. ^{1}H-NMR (400 MHz) spectrum of compound **5mn** in D_2O

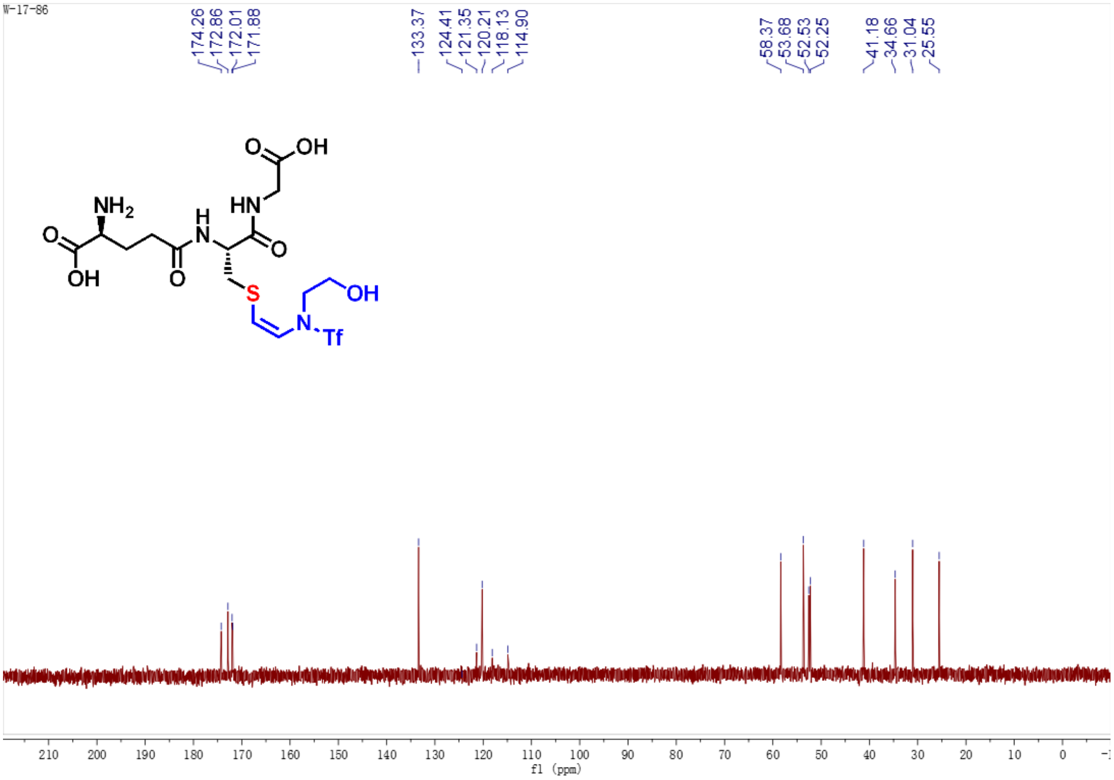


Figure S142. ^{13}C-NMR (100 MHz) spectrum of compound **5mn** in D_2O

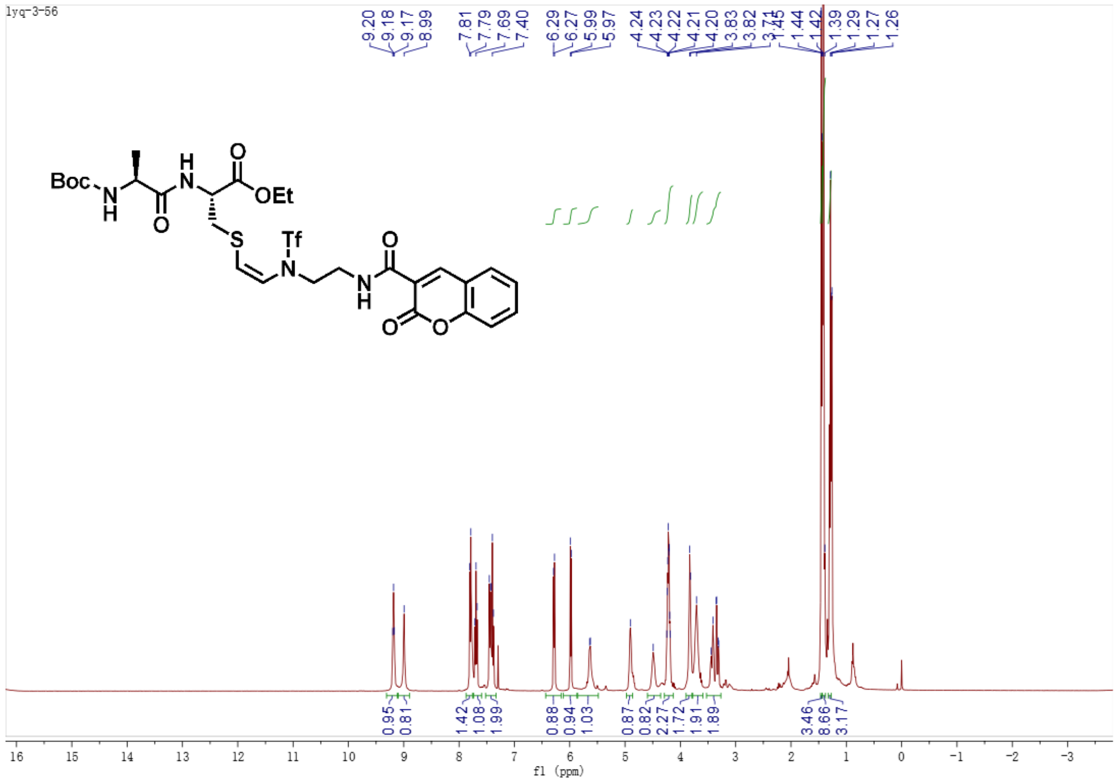


Figure S143. ^{1}H-NMR (400 MHz) spectrum of compound **5aq** in $CDCl_3$

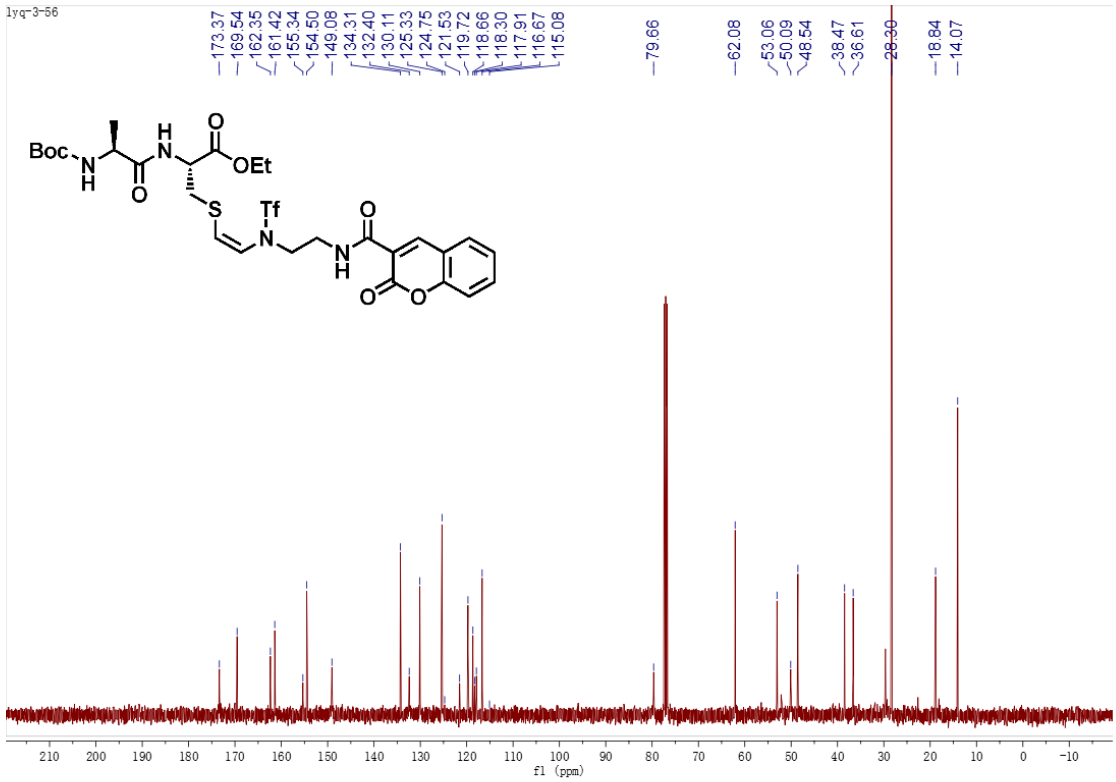


Figure S144. ^{13}C-NMR (100 MHz) spectrum of compound **5aq** in $CDCl_3$

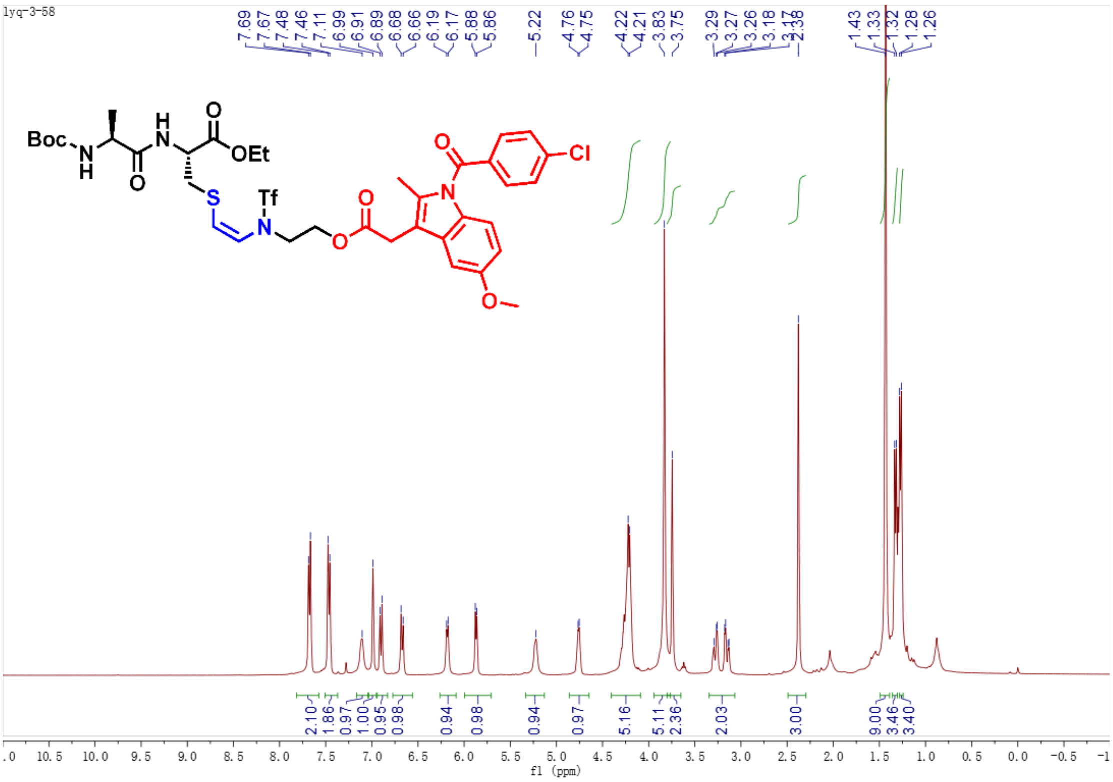


Figure S145. ^{1}H-NMR (400 MHz) spectrum of compound **5ar** in CDCl$_3$

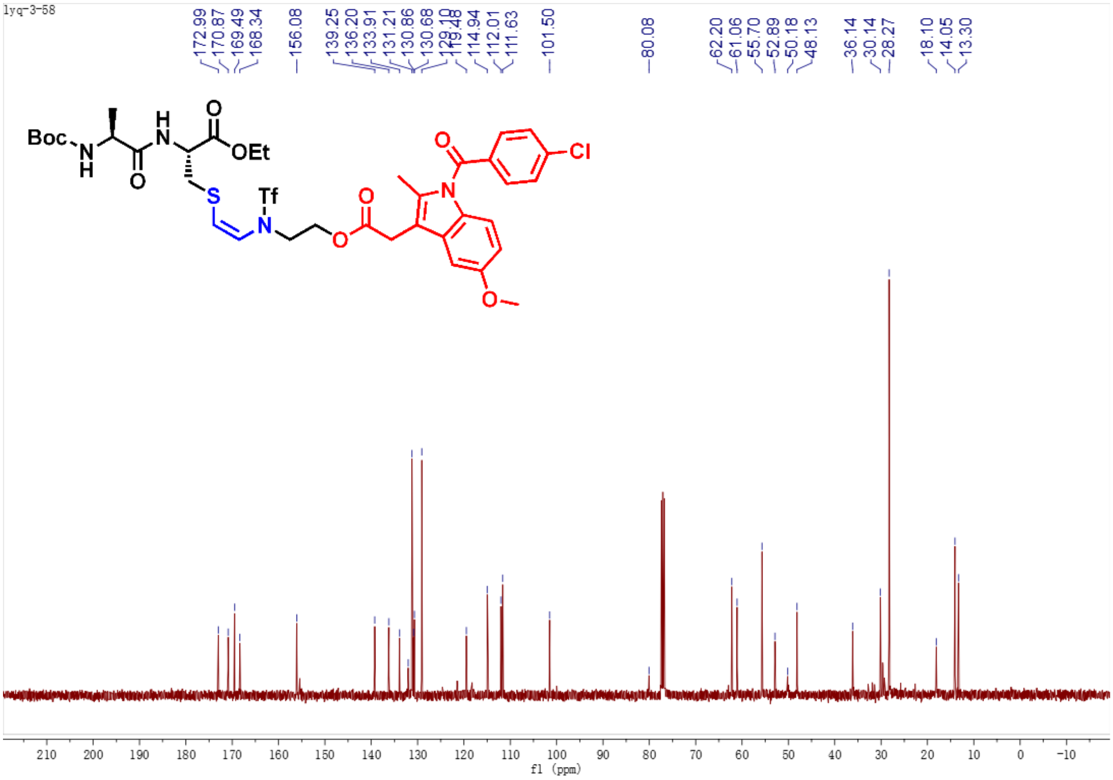


Figure S146. ^{13}C-NMR (100 MHz) spectrum of compound **5ar** in CDCl$_3$

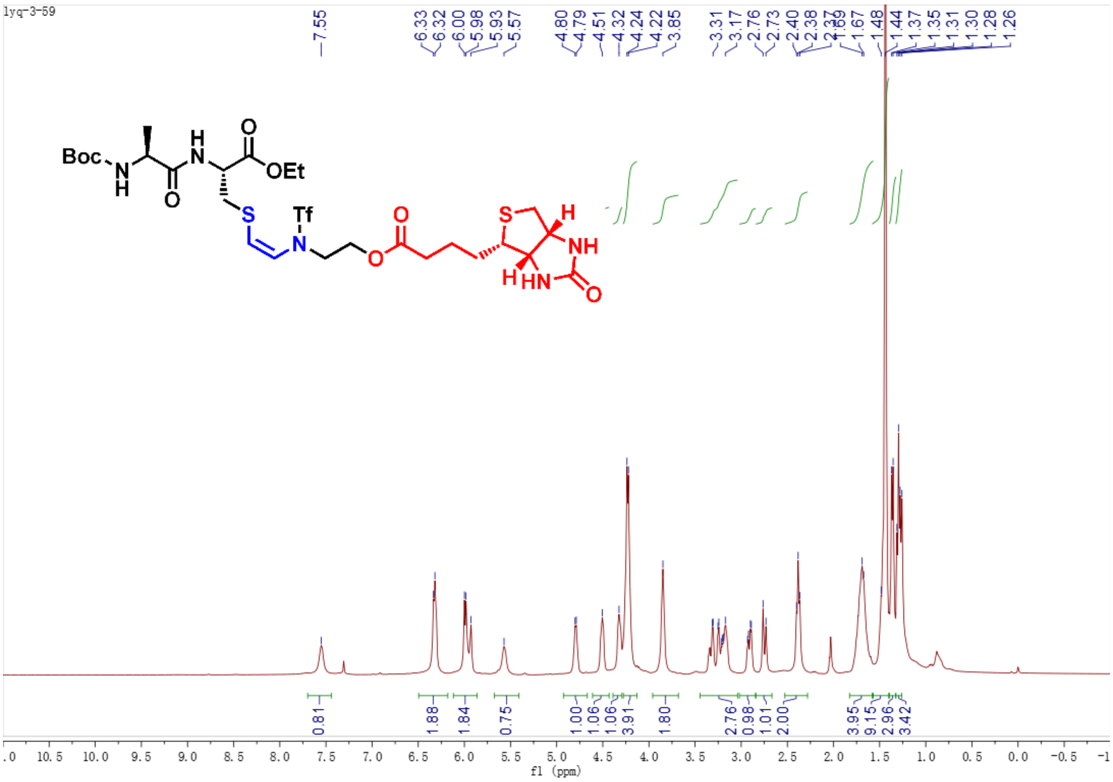


Figure S147. ^{1}H-NMR (400 MHz) spectrum of compound **5as** in $CDCl_3$

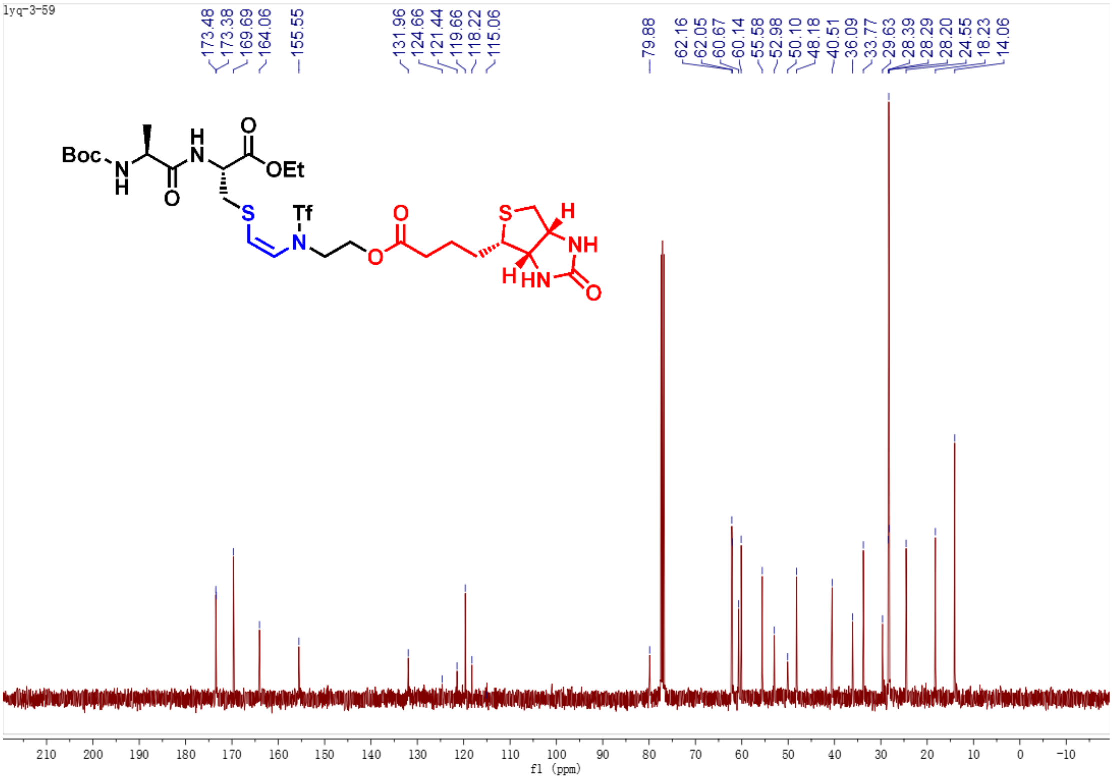


Figure S148. ^{13}C-NMR (100 MHz) spectrum of compound **5as** in $CDCl_3$.

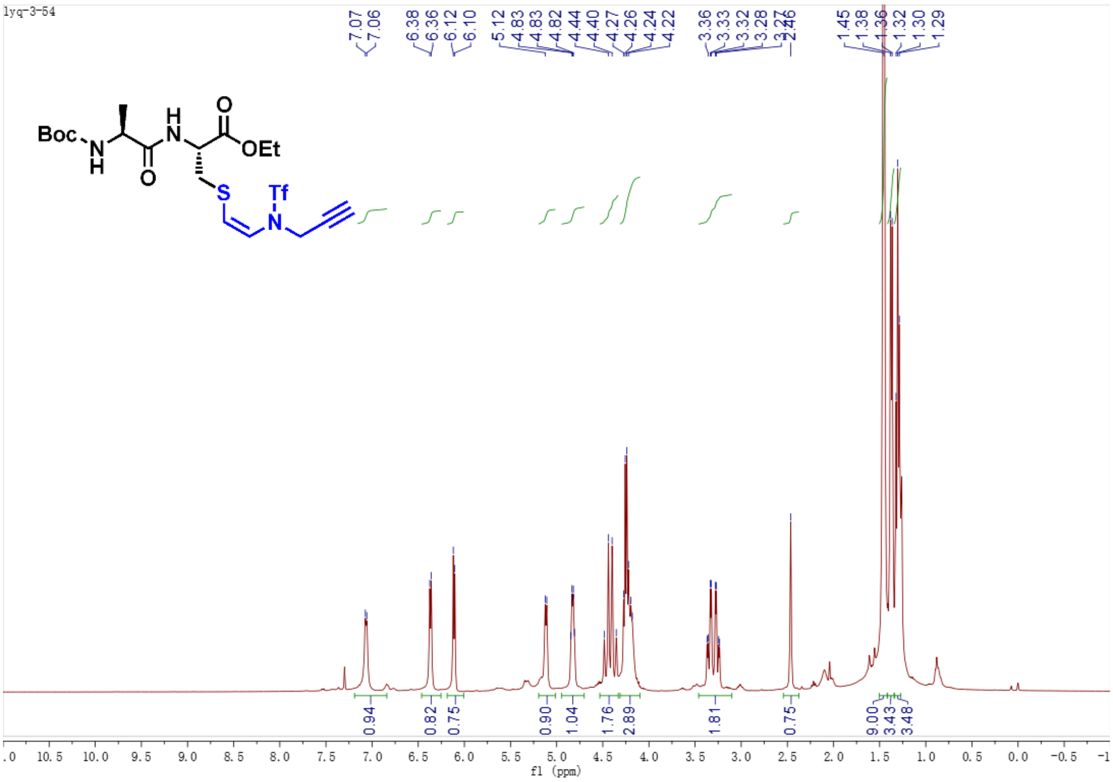


Figure S149. ^{1}H-NMR (400 MHz) spectrum of compound **5at** in CDCl$_3$

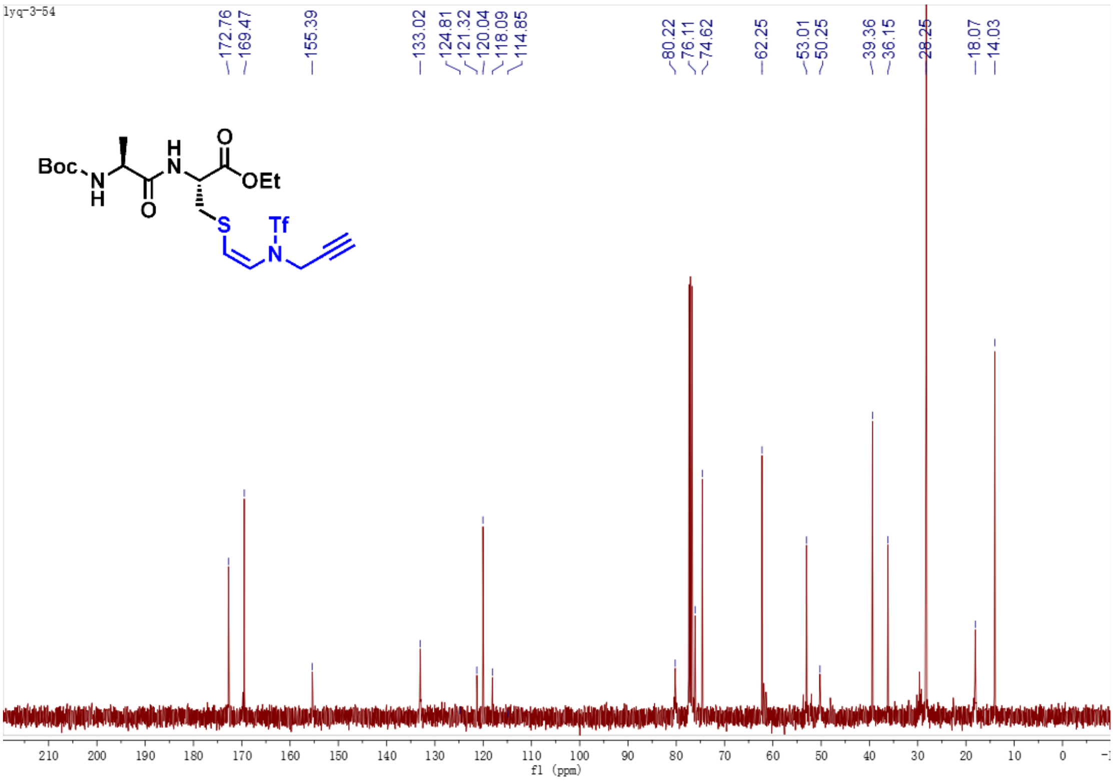


Figure S150. ^{13}C-NMR (100 MHz) spectrum of compound **5at** in CDCl$_3$.

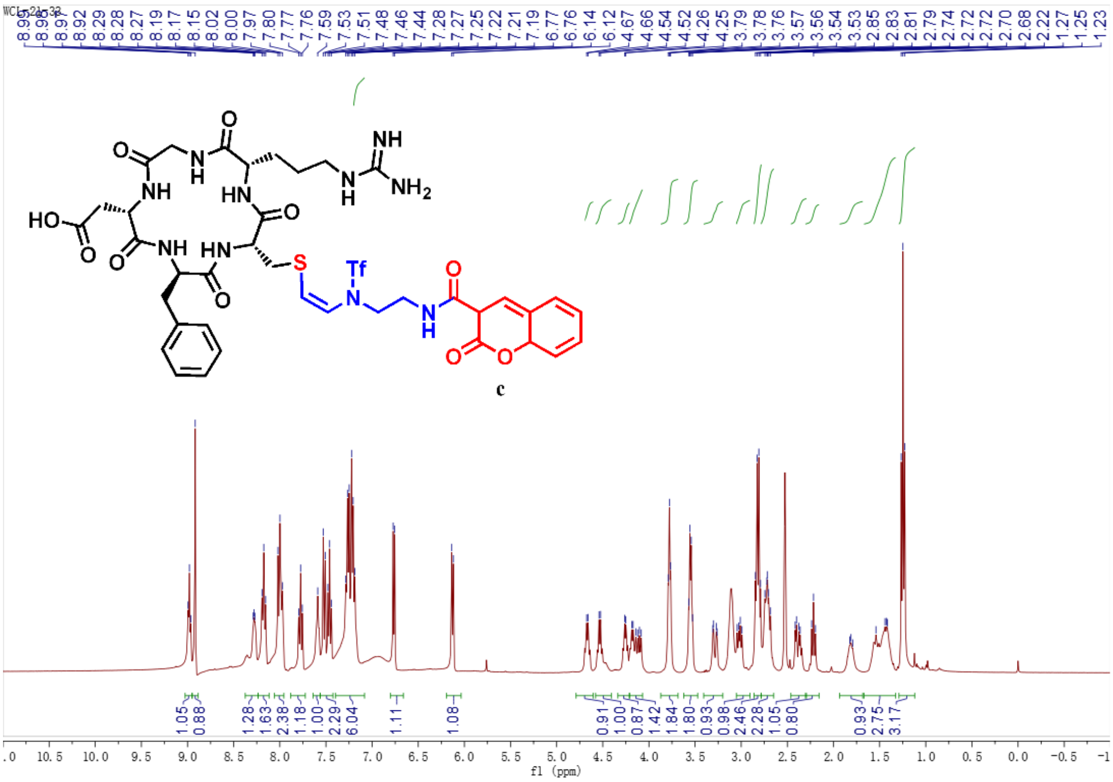


Figure S151. ^{1}H-NMR (400 MHz) spectrum of compound **5oq** in DMSO-*d*$_6$.

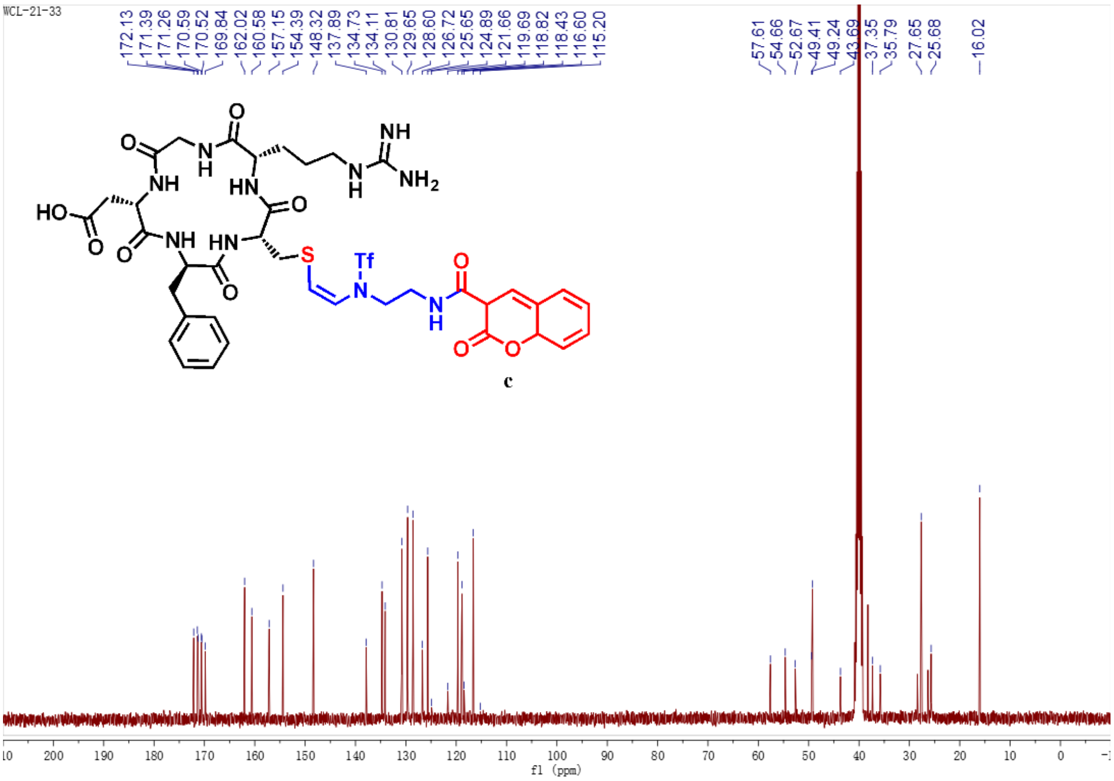


Figure S152. ^{13}C-NMR (100 MHz) spectrum of compound **5oq** in DMSO-*d*$_6$.

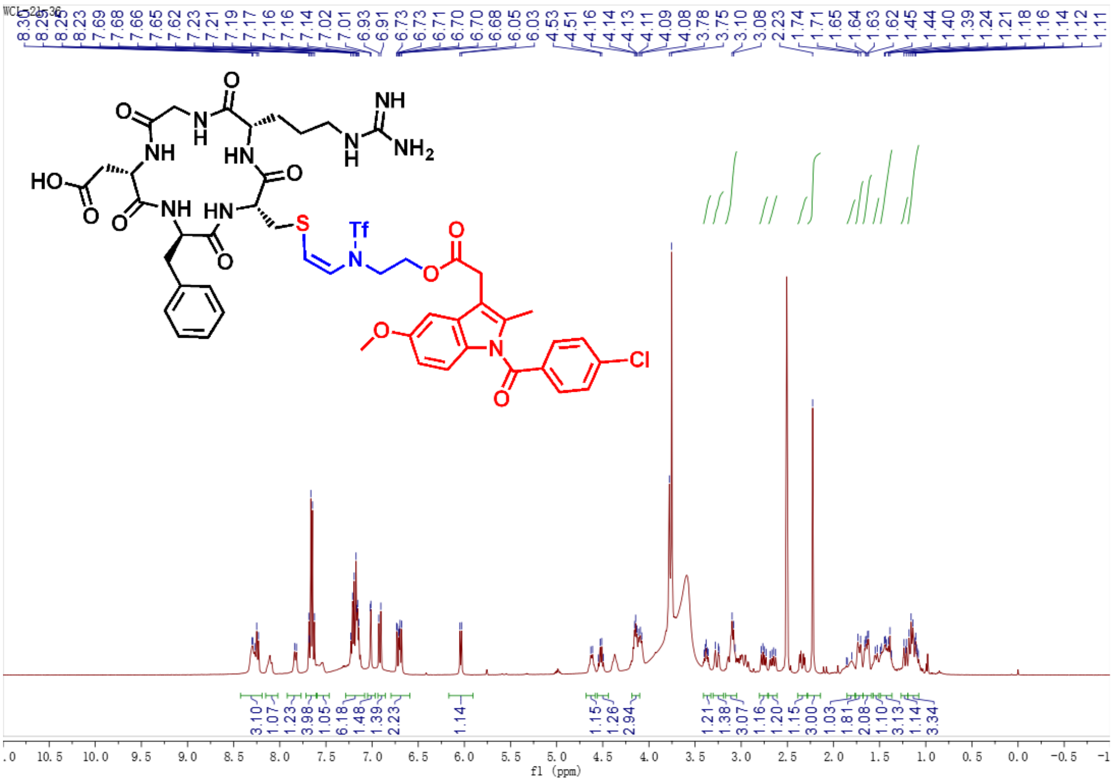


Figure S153. ^{1}H-NMR (400 MHz) spectrum of compound **5or** in DMSO-d_6

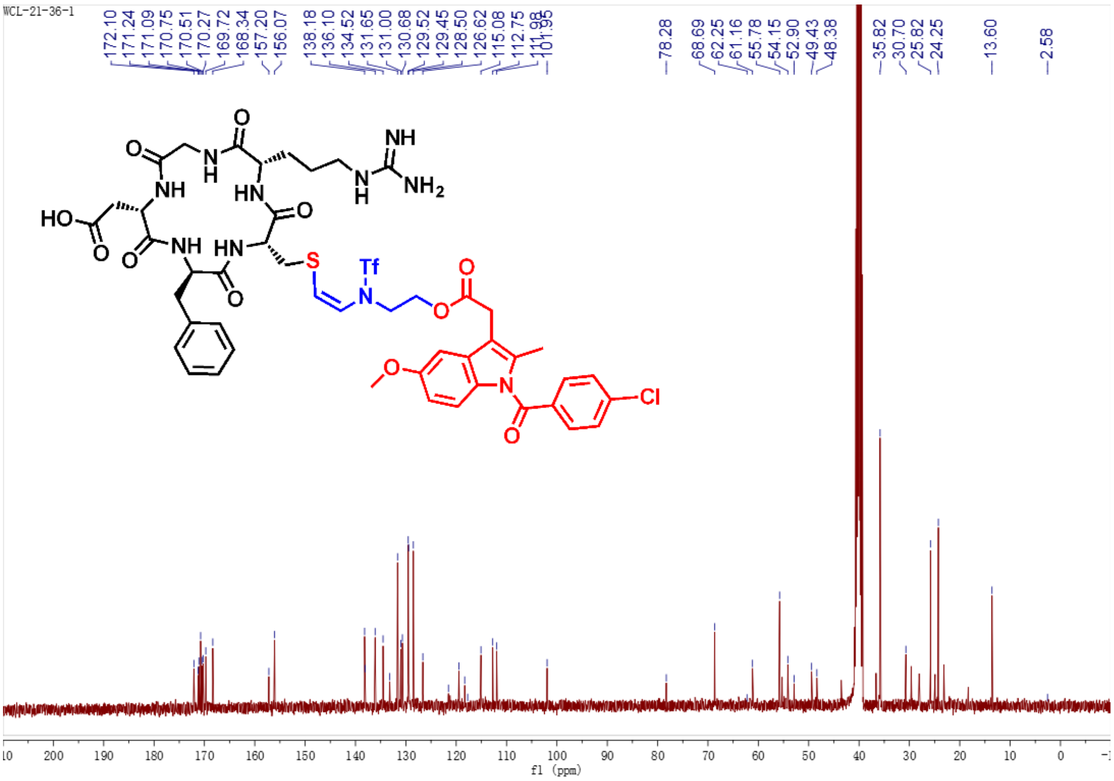


Figure S154. ^{13}C-NMR (100 MHz) spectrum of compound **5or** in DMSO-d_6

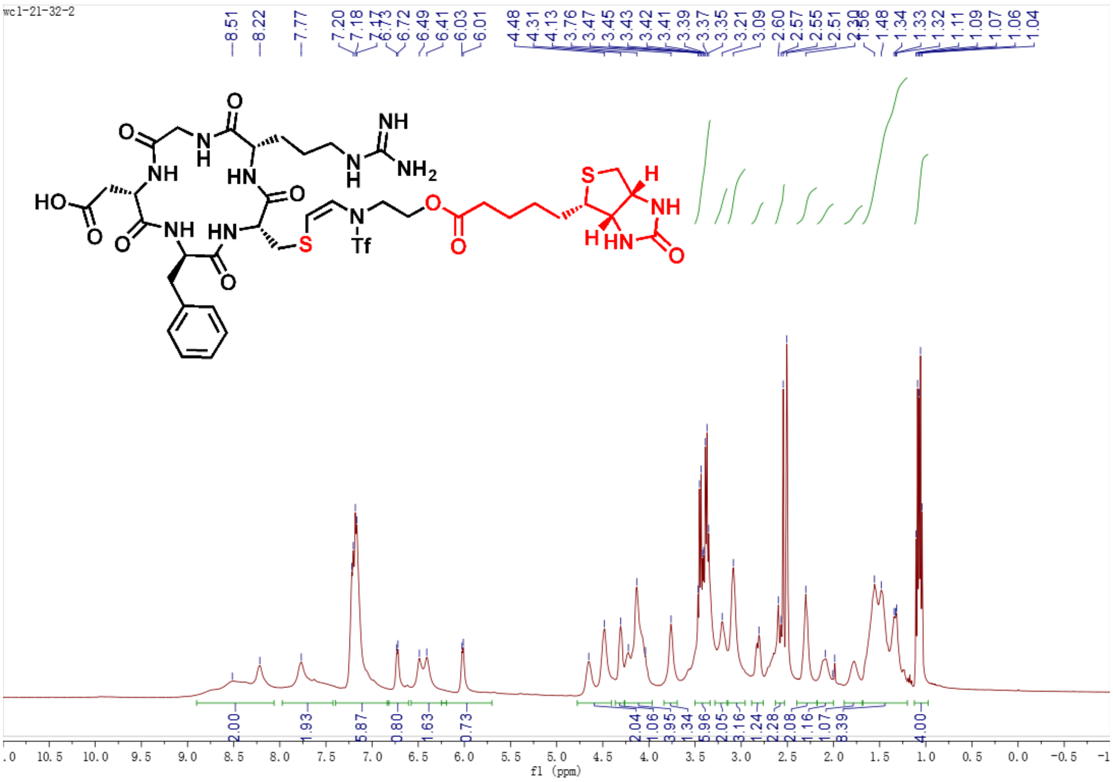


Figure S155. ^{1}H-NMR (400 MHz) spectrum of compound **5os** in DMSO-d_6

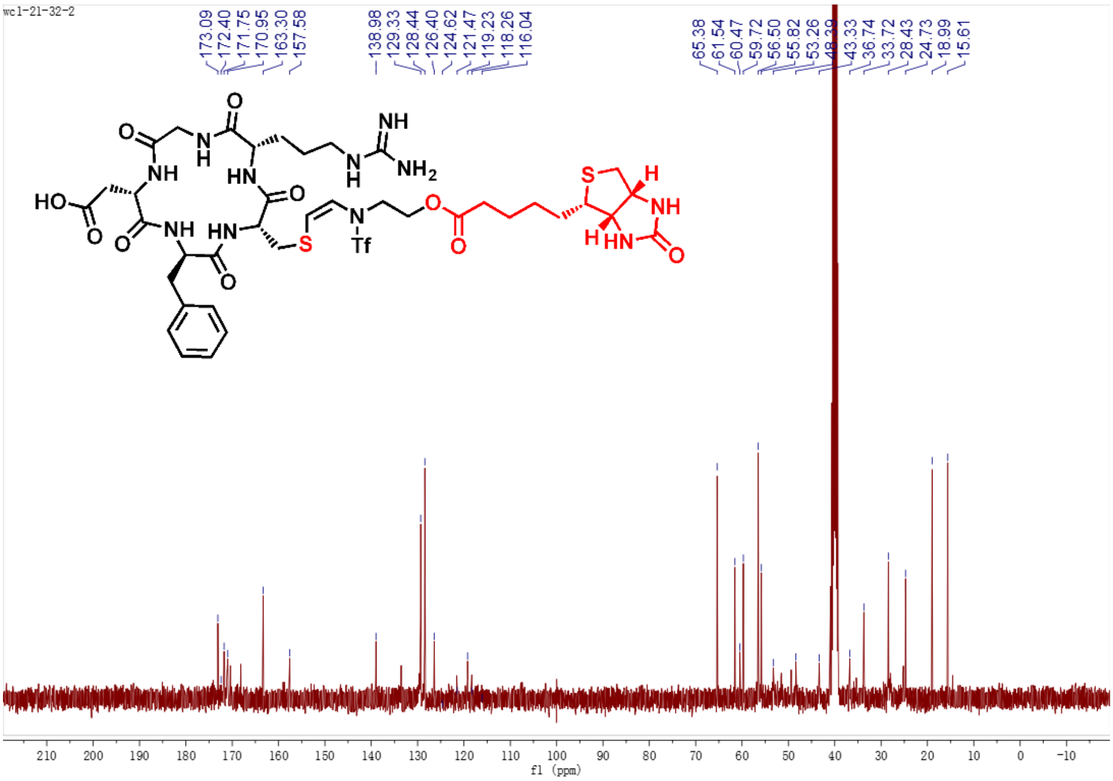


Figure S156. ^{13}C-NMR (100 MHz) spectrum of compound **5os** in DMSO-d_6

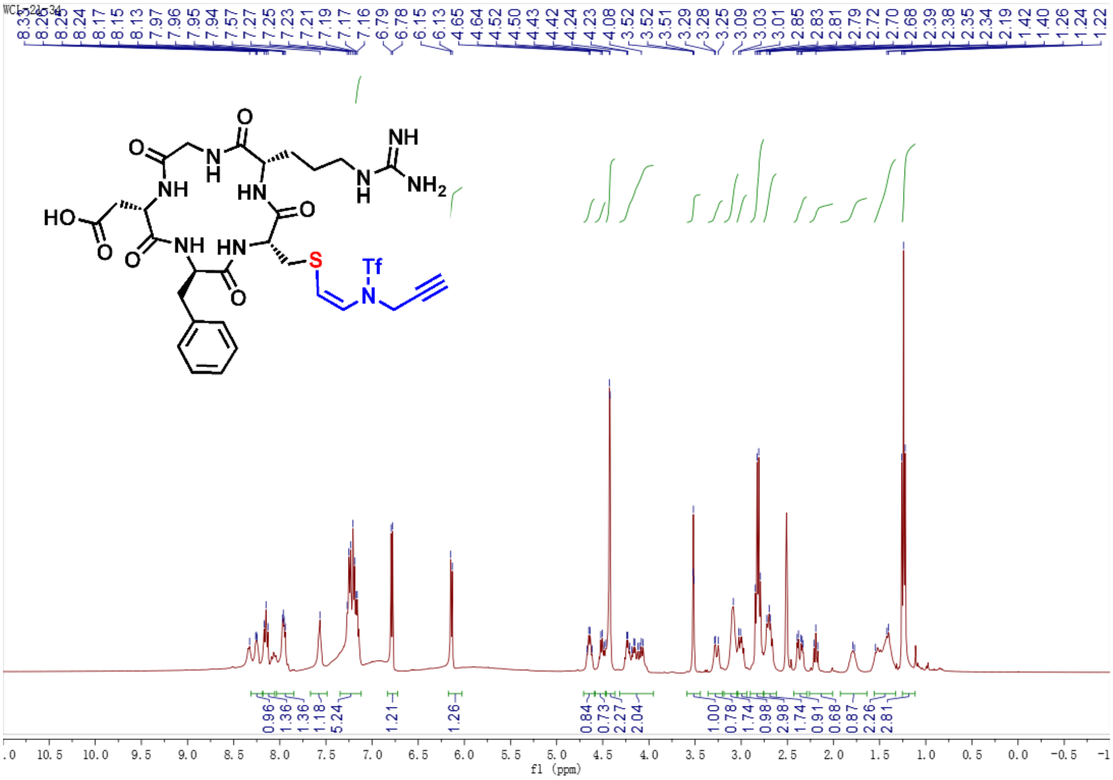


Figure S157. ^{1}H-NMR (400 MHz) spectrum of compound **5ot** in DMSO-d_6

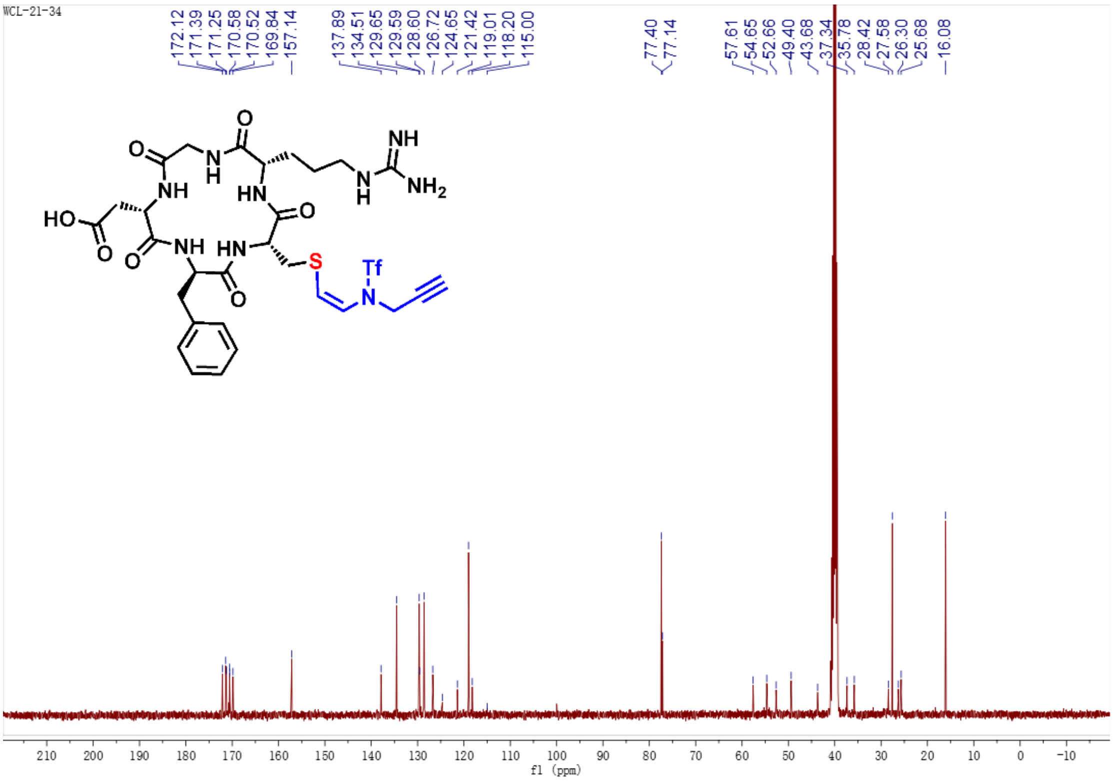


Figure S158. ^{13}C-NMR (100 MHz) spectrum of compound **5ot** in DMSO-d_6

12. Supplementary Figures

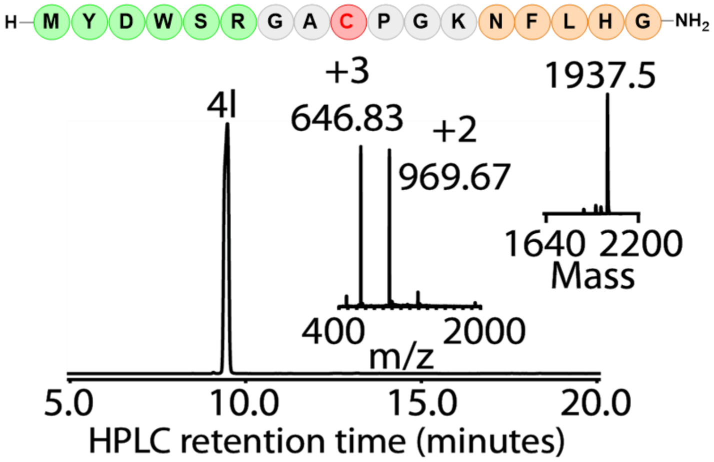


Figure S159. HPLC trace and ESI-MS of **4l** (Obs. 1937.5, Mass calc. 1936.8).

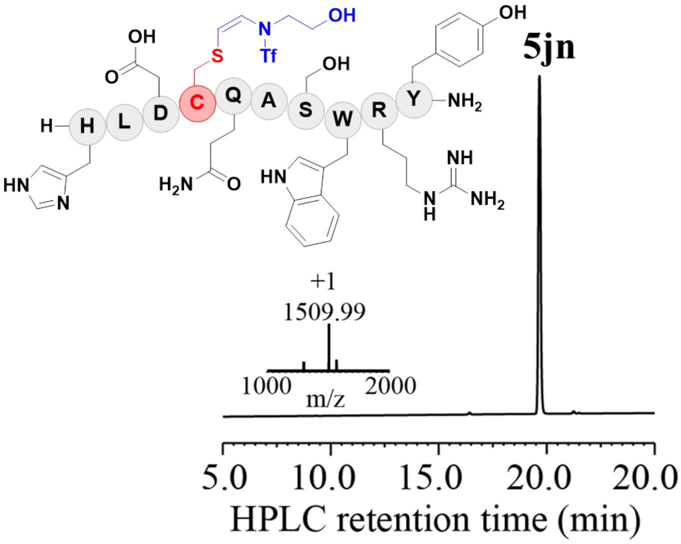


Figure S160. HPLC trace and ESI-MS of of **5jn** ($[M+H]^+$ Obs. 1509.99, Mass calc. 1509.60).

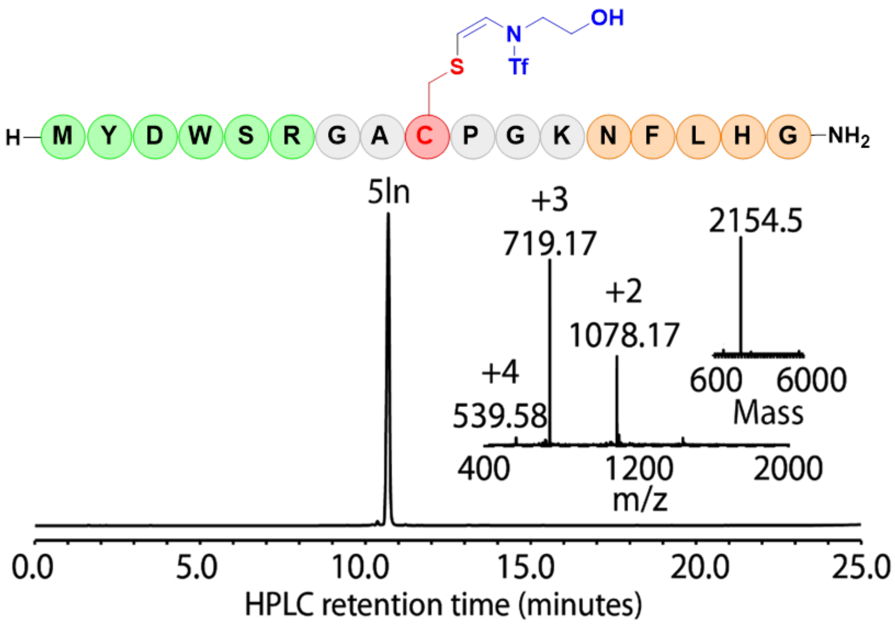


Figure S161. HPLC trace and ESI-MS of **5ln** (Obs. 2154.5 Da, Mass calcd. 2153.9 Da)

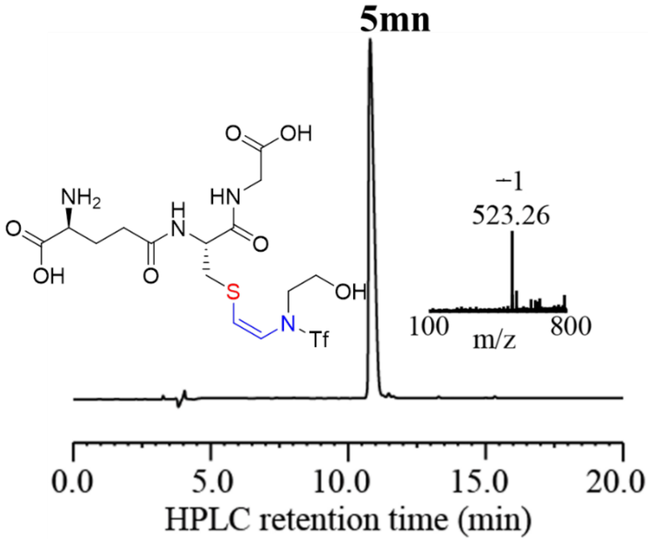


Figure S162. HPLC trace and ESI-MS of **5mn** ($[M-H]^-$ Obs. 523.26, Mass calc. 523.19)

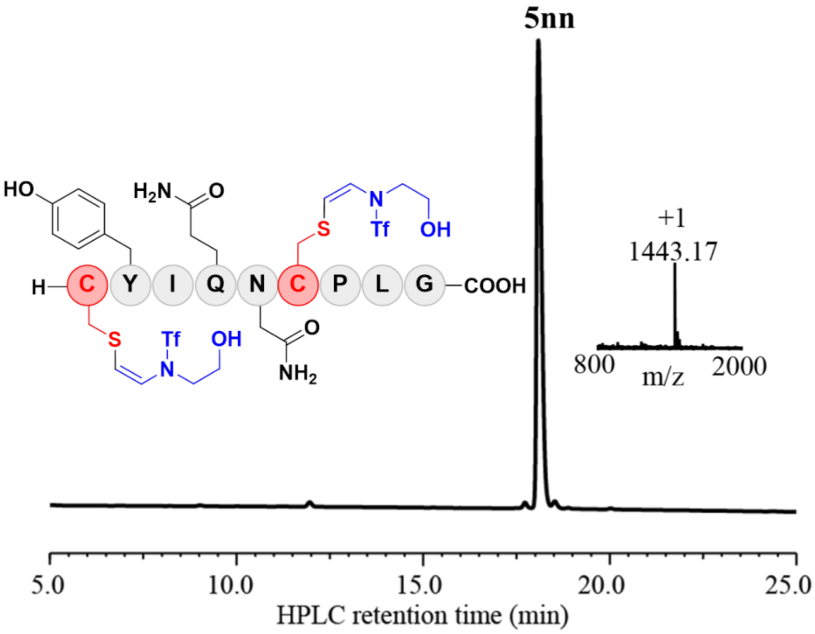


Figure S163. HPLC trace and ESI-MS of of **5nn** ($[M+H]^+$ Obs. 1443.17, Mass calc. 1443.46)

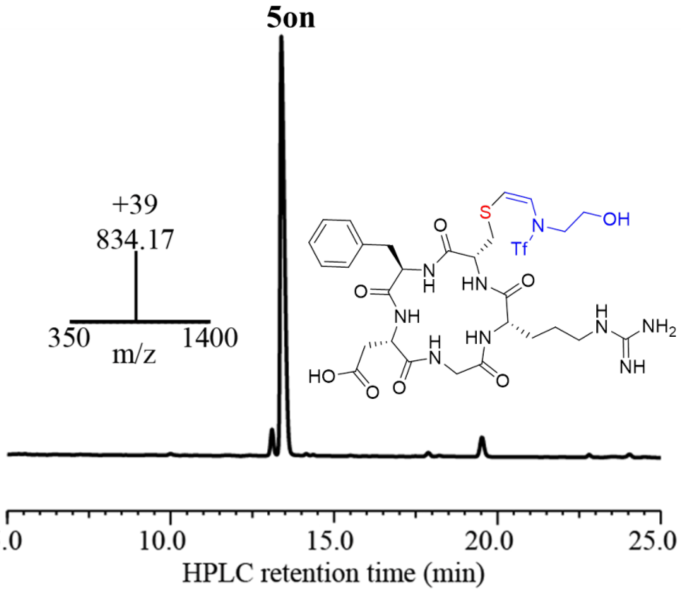


Figure S164. HPLC trace and ESI-MS of **5on** ($[M + K]^+$ Obs. 834.17, Mass calc. 834.19).

5pn

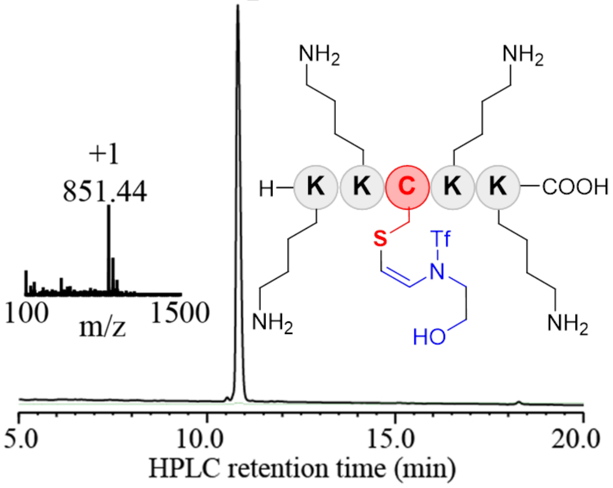


Figure S165. HPLC trace and ESI-MS of **5pn** ([M+H]$^+$ Obs. 851.44, Mass calc. 851.41).

5qn

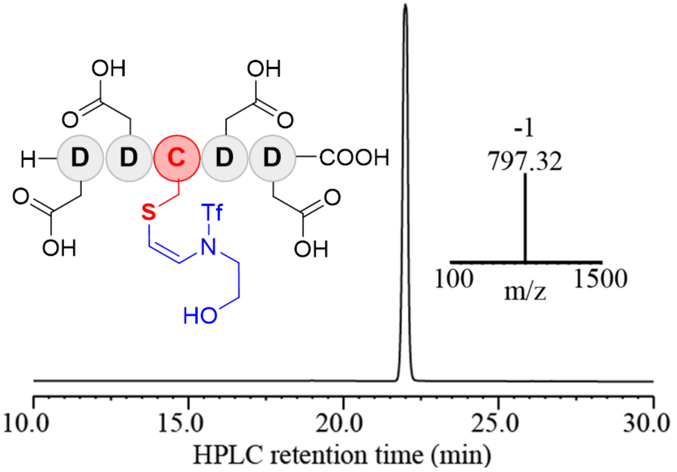


Figure S166. HPLC trace and ESI-MS of **5qn** ([M - H]$^-$ Obs. 797.32, Mass calc. 797.12)

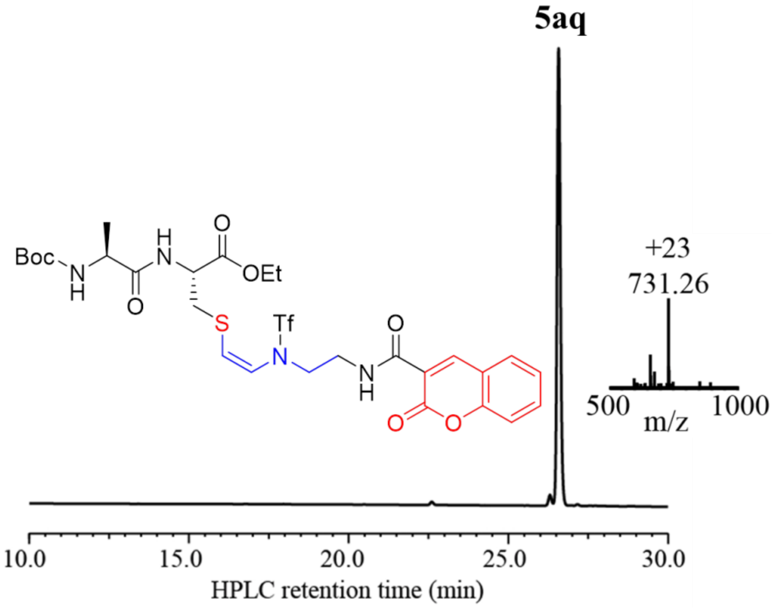


Figure S167. HPLC trace and ESI-MS of **5aq** ([M+Na]$^+$ Obs. 731.26, Mass calc. 731.16).

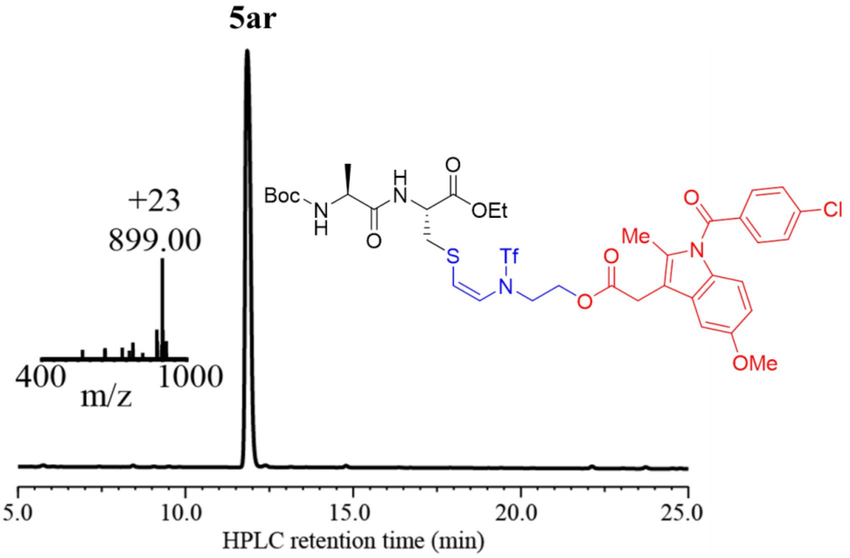


Figure S168. HPLC trace and ESI-MS of **5ar** ([M+Na]$^+$ Obs. 899.00, Mass calc. 899.20).

5as

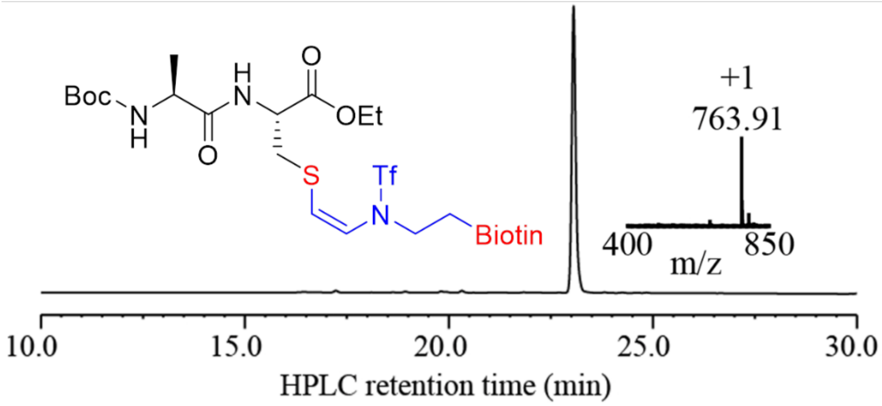


Figure S169. HPLC trace and ESI-MS of **5as** ($[M+H]^+$ Obs. 763.91, Mass calc. 764.23).

5at

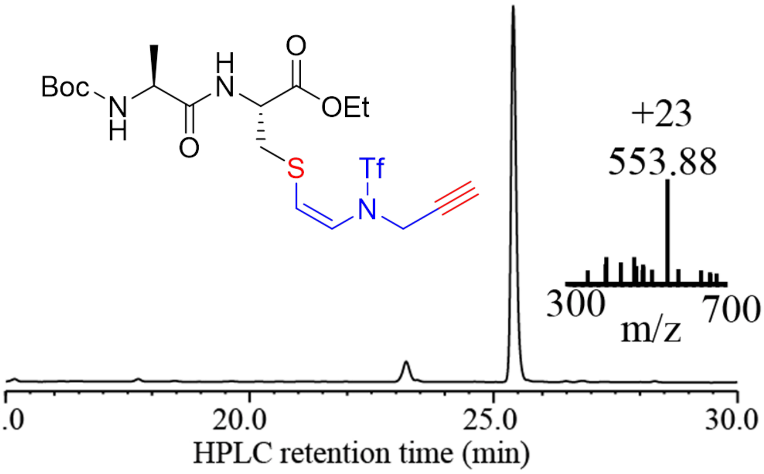


Figure S170. HPLC trace and ESI-MS of **5at** ($[M+Na]^+$ Obs. 553.88, Mass calc. 554.12).

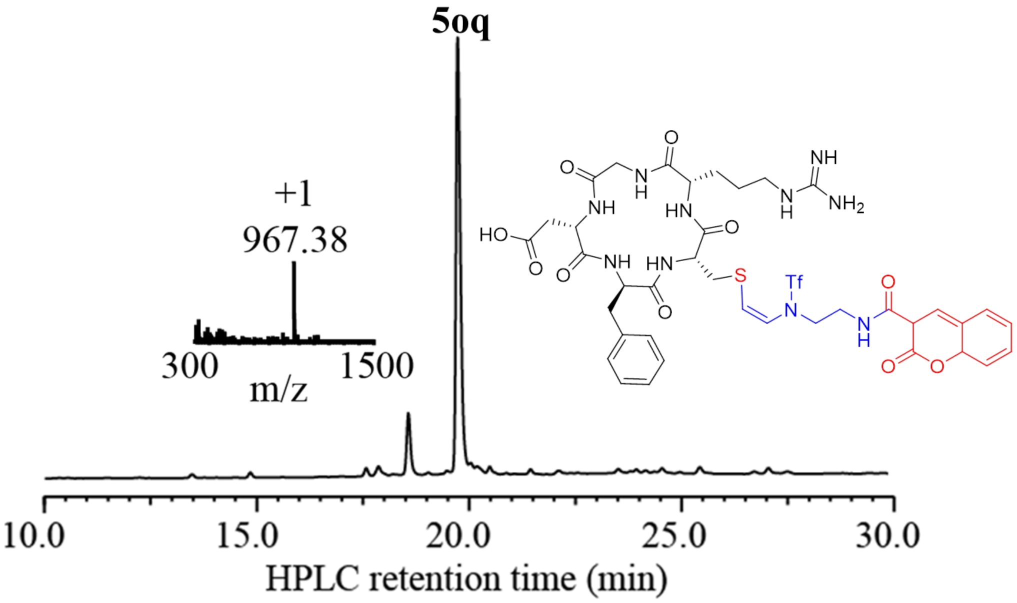


Figure S171. HPLC trace and ESI-MS of **5oq** ([M+H]$^-$ Obs. 967.38, Mass calc. 967.27).

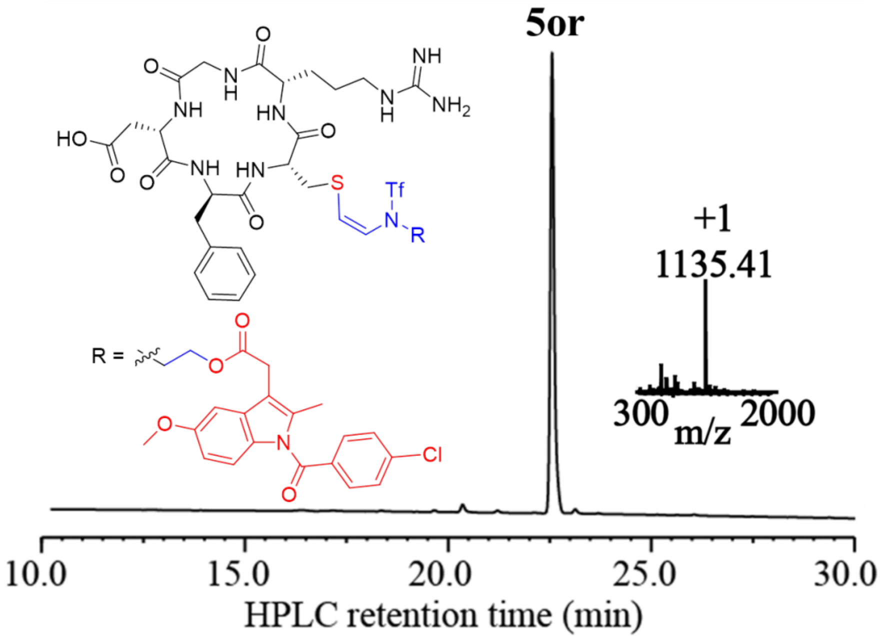


Figure S172. HPLC trace and ESI-MS of **5or** ([M+H]$^+$ Obs. 1135.41, Mass calc. 1135.30)

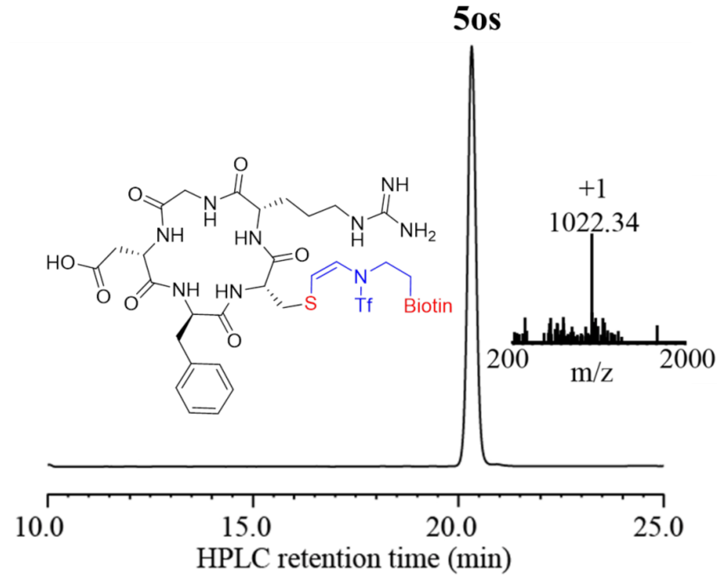


Figure S173. HPLC trace and ESI-MS of **5os** ($[M+H]^+$ Obs. 1022.34, Mass calc. 1022.31)

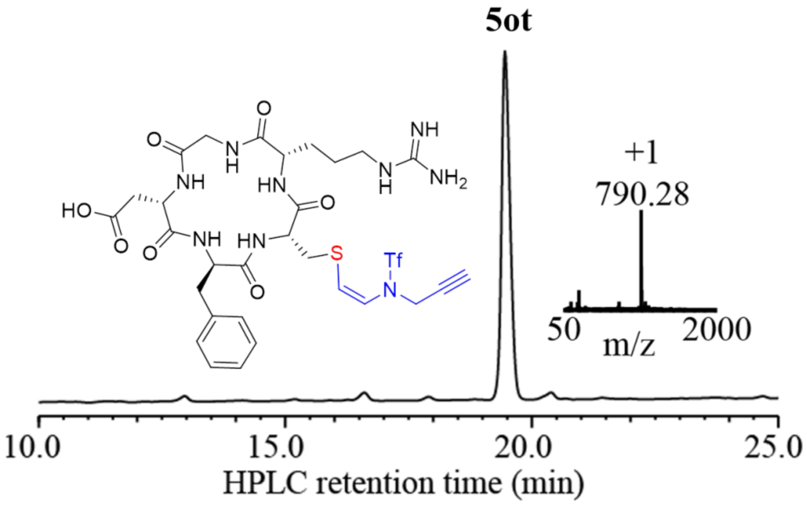


Figure S174. HPLC trace and ESI-MS of **5ot** ($[M+H]^+$ Obs. 790.28, Mass calc. 790.23).

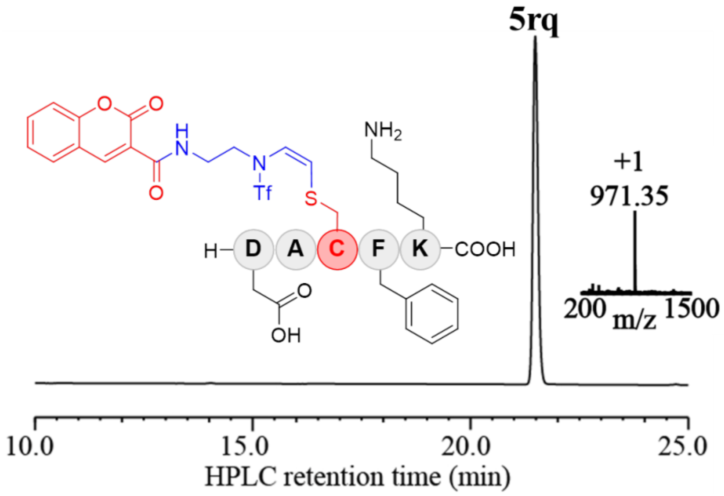


Figure S175. HPLC trace and ESI-MS of **5rq** ([M+H]$^+$ Obs. 971.35, Mass calc. 971.29)

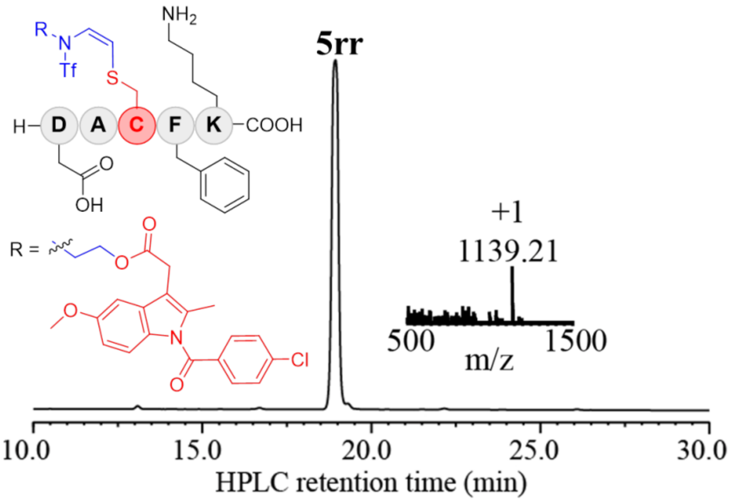


Figure S176. HPLC trace and ESI-MS of **5rr** ([M+H]$^+$ Obs. 1139.21, Mass calc. 1139.32).

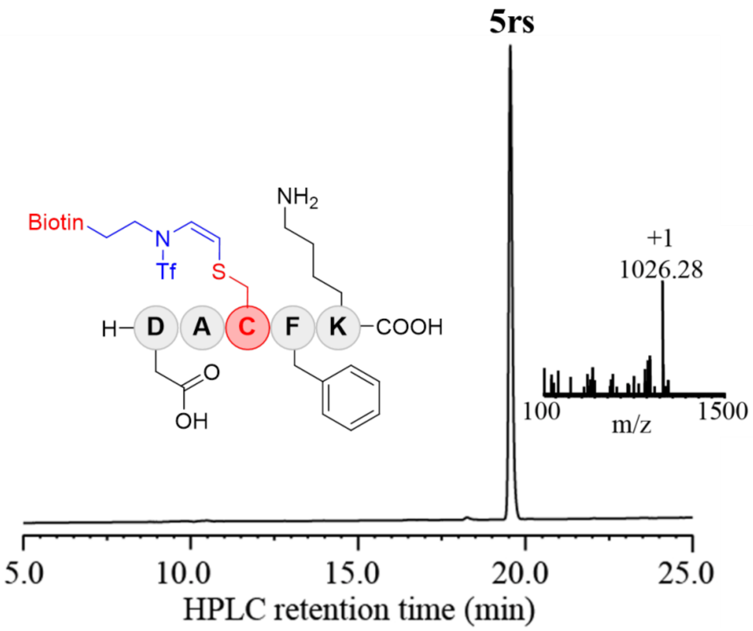


Figure S177. HPLC trace and ESI-MS of **5rs** ([M+H]$^+$ Obs. 1026.28, Mass calc. 1026.33)

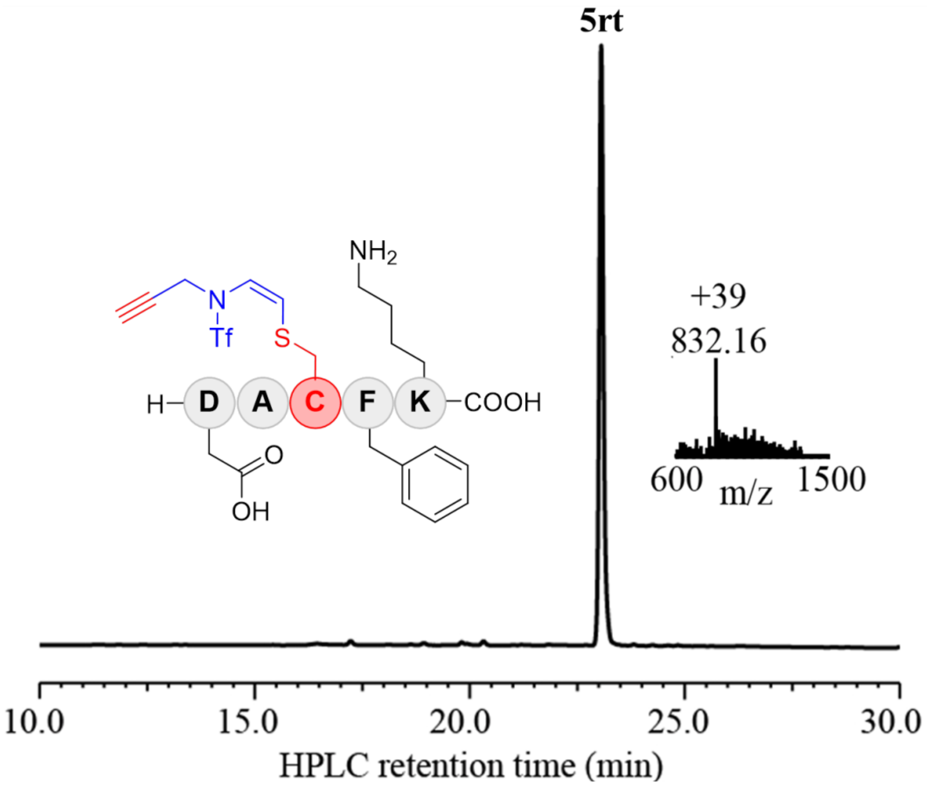


Figure S178. HPLC trace and ESI-MS of **5rt** ([M + K]$^+$ Obs. 832.1, Mass calc. 832.20)

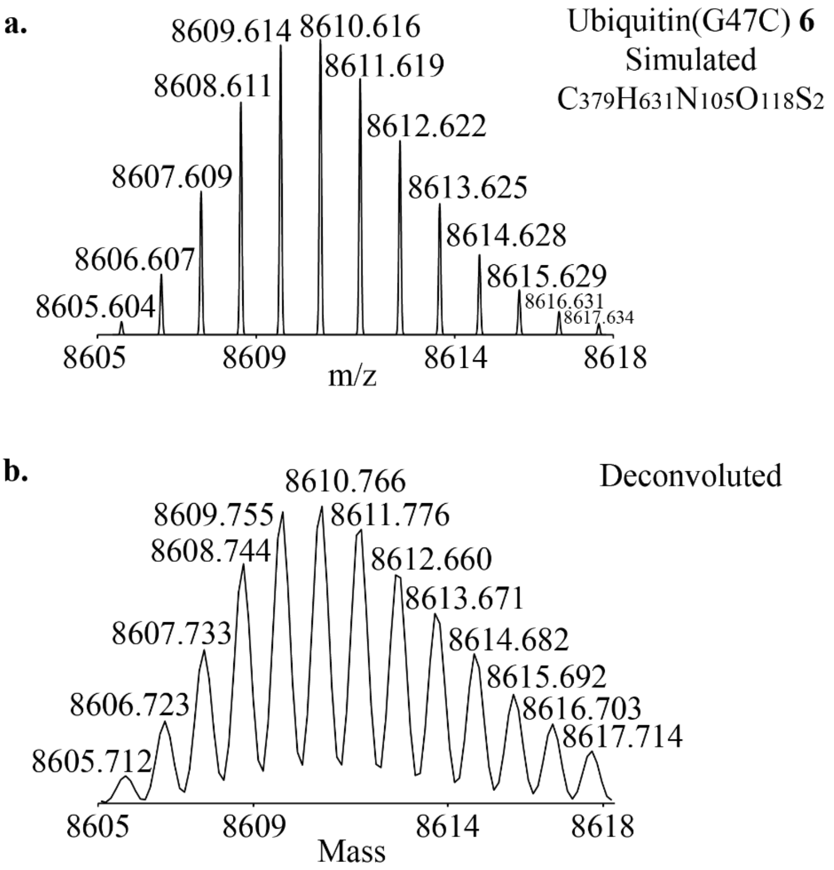


Figure S179. HR-MS analysis of the ubiquitin(G47C) (**6**) **a**. The simulated HR-MS of **6** with chemical formula $C_{379}H_{631}N_{105}O_{118}S_2$ is shown; **b**. The deconvoluted HR-MS of **6**.

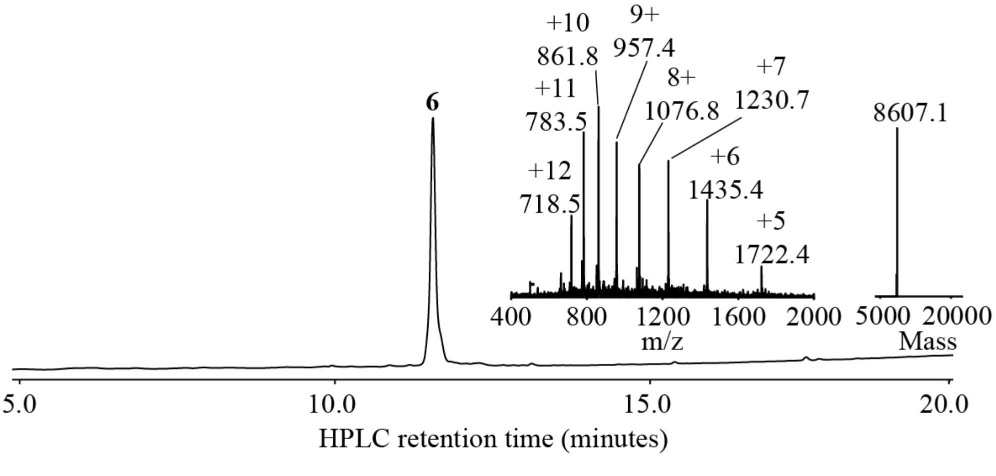


Figure S180. HPLC trace and ESI-MS of ubiquitin(G47C) (**6**)

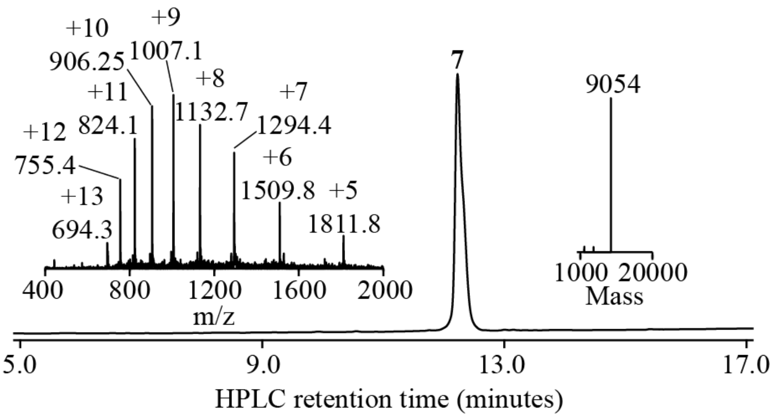


Figure S181. HPLC trace and ESI-MS of modified ubiquitin(G47C*) (**7**)

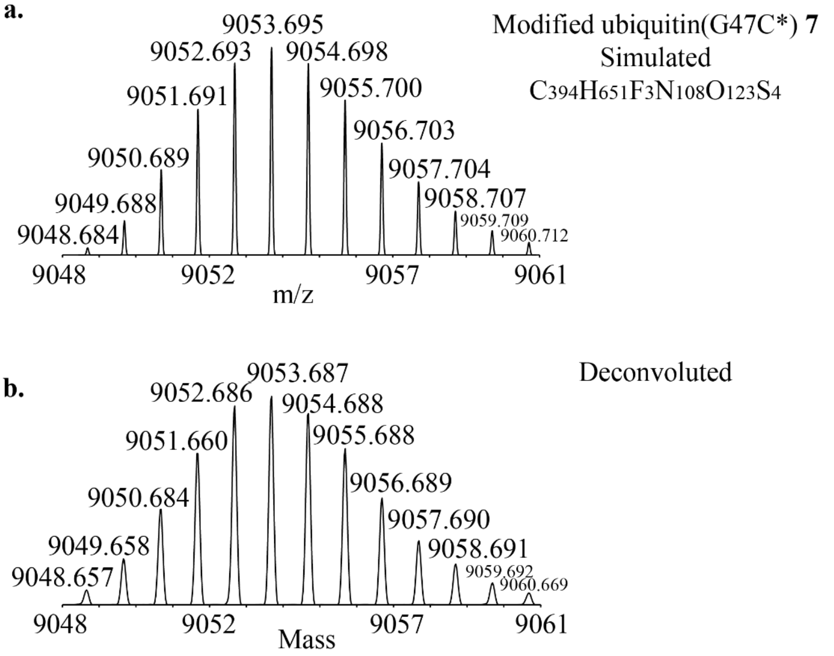


Figure S182. HR-MS analysis of the ubiquitin(G47C*) (**7**) **a**. The simulated HR-MS of **7** with chemical formula $C_{394}H_{651}F_3N_{108}O_{123}S_4$ is shown; **b**. The deconvoluted HR-MS of **7**

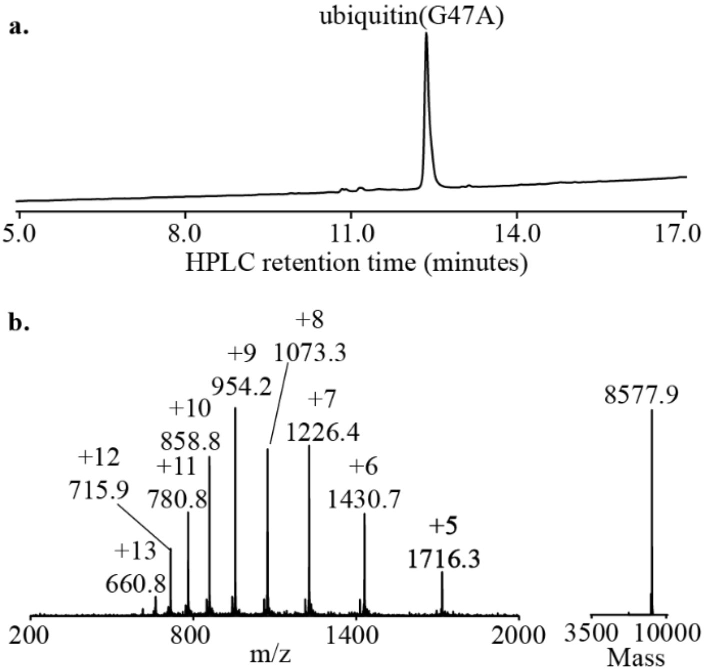


Figure S183. HPLC trace and ESI-MS of ubiquitin(G47A) (Obs. 8577.9 Da, Mass calc. 8578.9 Da)

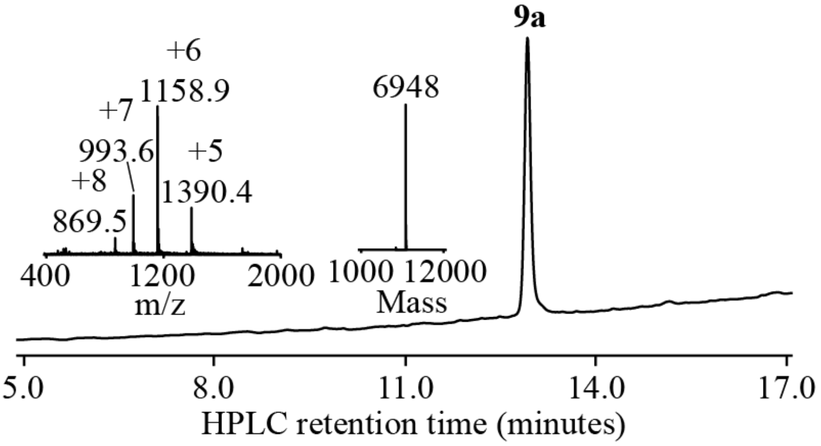


Figure S184. HPLC trace and ESI-MS of doubly modified BPTI **9a** (obs. 6948.0 Da, Mass calc. 6947.8 Da)

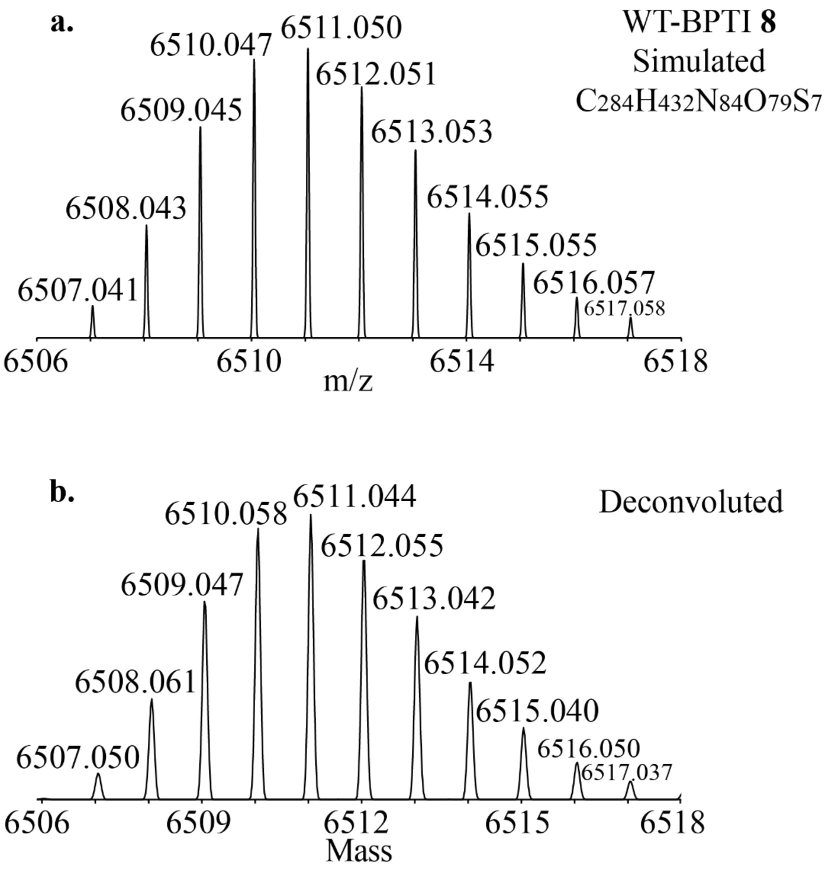


Figure S185. HR-MS analysis of the WT-BPTI (**8**) **a**. The simulated HR-MS of **8** with chemical formula $C_{284}H_{432}N_{84}O_{79}S_7$ is shown; **b**. The deconvoluted HR-MS of **8**

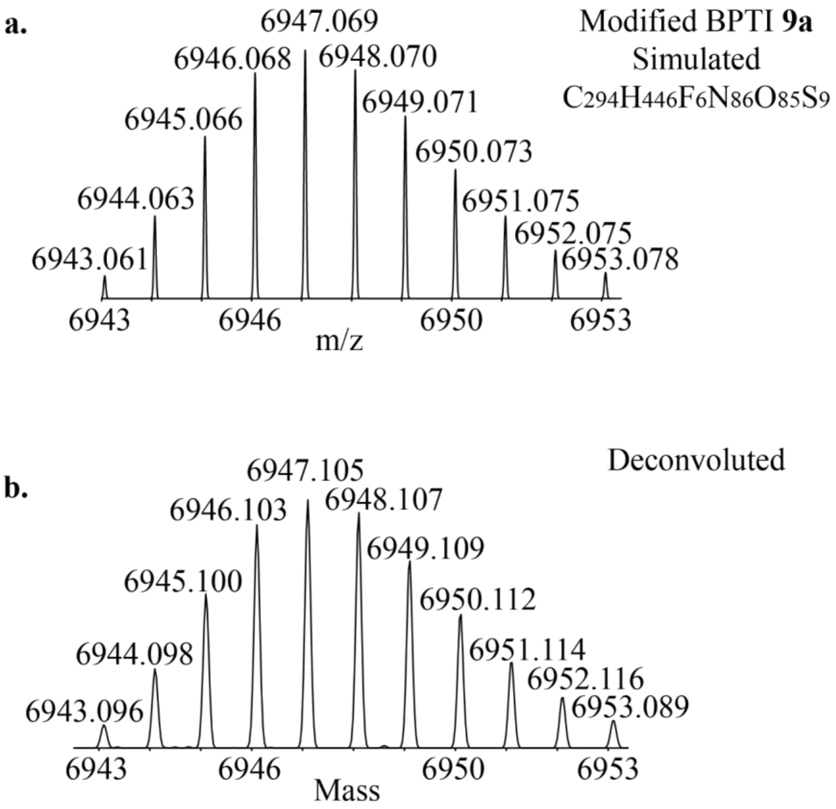


Figure S186. HR-MS analysis of the BPTI (**9a**) **a**. The simulated HR-MS of **9a** with chemical formula $C_{294}H_{446}F_6N_{86}O_{85}S_9$ is shown; **b**. The deconvoluted HR-MS of **9a**

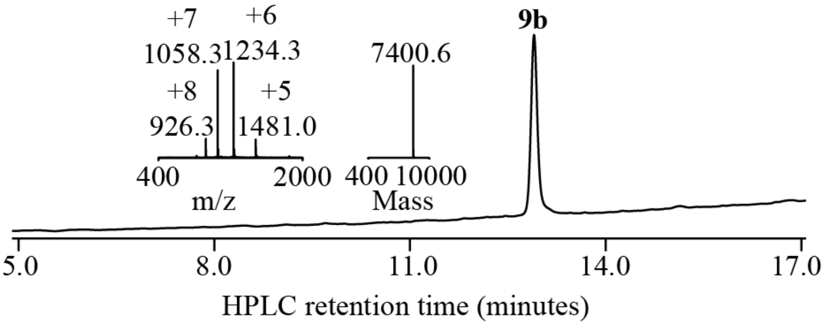


Figure S187. HPLC trace and ESI-MS of the doubly modified BPTI (**9b**) (Obs. 7400.6 Da, Mass calc. 7400.4 Da).

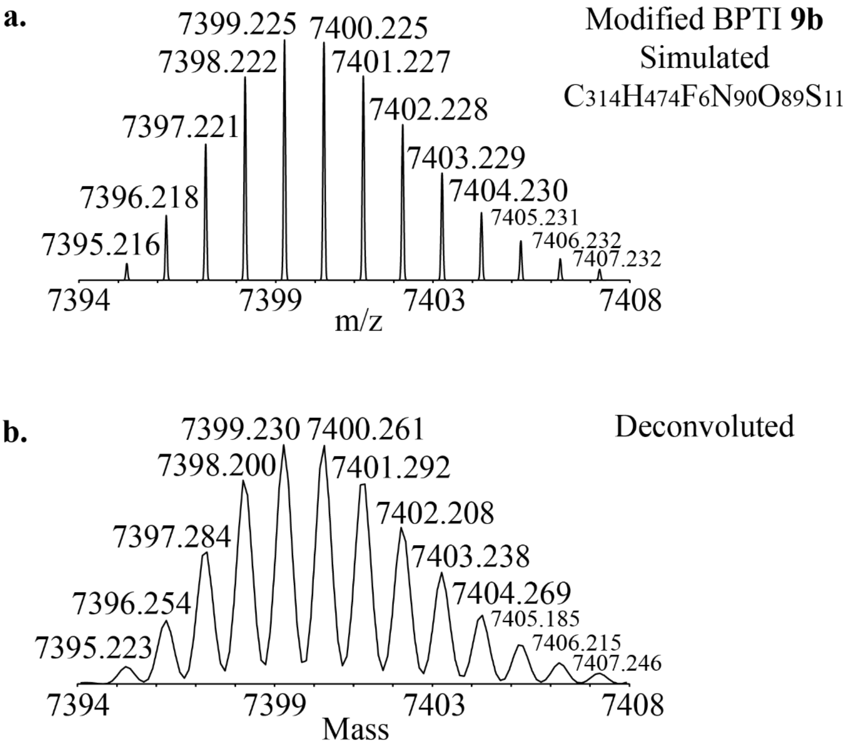


Figure S188. HR-MS analysis of the doubly modified BPTI (**9b**) **a**. The simulated HR-MS of **9b** with chemical formula $C_{314}H_{474}F_6N_{90}O_{89}S_{11}$ is shown; **b**. The deconvoluted HR-MS of **9b**

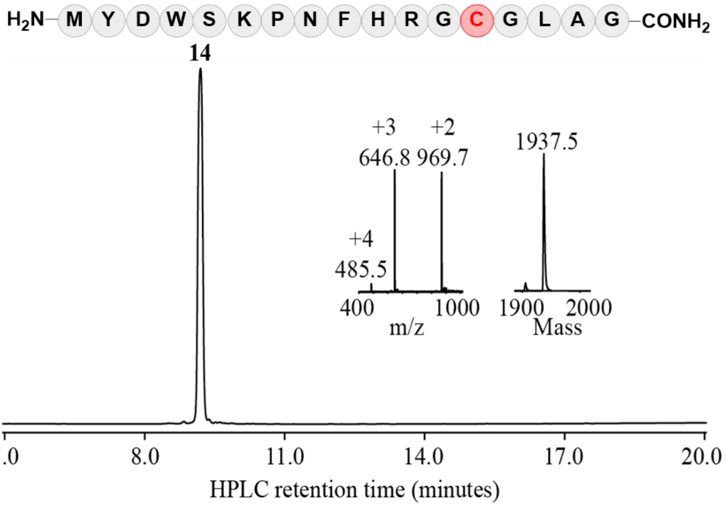


Figure S189. HPLC trace and ESI-MS of peptide **14** (Obs. 1937.5 Da, Mass calc. 1936.8 Da).

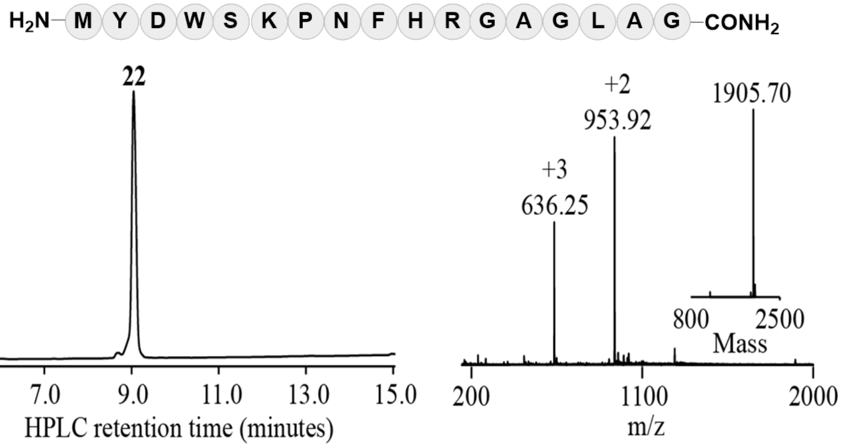


Figure S190. HPLC trace and ESI-MS of peptide **22** (Obs. 1905.7 Da, Mass calc. 1905.9 Da)

13. References

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