Supporting information

Cleavable Cys labeling directed Lys site-selective stapling and single-site modification

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1. General information

1.1 Reagents

Commercially available reagents and solvents were purchased from Energy Chemical, Bidepharm or Sigma and all these reagents were used directly without further purification unless otherwise noted. RP-HPLC solvents were purchased as HPLC grade from Energy Chemical.

Fmoc-amino acids for solid phase peptide synthesis (SPPS) were used with the following side-chain protection: Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(*t*Bu)-OH, FmocThr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH.

1.2 Instruments

NMR spectra were recorded on a Bruker AVANCE AV 400 instrument and all NMR experiments were reported in units, parts per million (ppm), using TMS as internal reference. Data for ^{1}H NMR was recorded as follows: chemical shift (δ, ppm), multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, q = quartet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, and br = broad signal), Coupling constants (*J*) were reported as Hertz (Hz). HRMS spectra were recorded with a LCMS-IT-TOF mass spectrometer, equipped with an ESI source. LC-MS spectra were performed on an Agilent Technologies 1260 Infinity Ⅱ HPLC system, connected to an Agilent G6125B LC/MSD. Preparative RP-HPLC was performed on Hanbon Sci. &Tech. Newstyle HPLC systems equipped with NP7000 serials pump, NU3000 serials UV/VIS detector and DM-A dynamic mixer, using the following column: Dubhe C18 D18122010.25 column, 12 nm, 10 μm, 20 x 250 mm. TLC analysis was visualized by fluorescence quenching under UV light (254 nm and 365 nm), or developing the plates with I_2, permanganate. Flash chromatography purification was performed on silica gel GF254 (200-300 mesh) purchased from Qingdao Haiyang Chemical.

2. LC-MS and preparative HPLC information

2.1 LC-MS analysis

LC-MS measurement was performed on an Agilent Technologies 1260 Infinity Ⅱ HPLC system with a G7129A 1260 Vislsampler, a G7111B 1260 Quat Pump and a G7114A 1260 VWD detector, connected to an Agilent G6125B LC/MSD. Water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid, were used as the mobile phase. Low-resolution mass spectrometric measurement was acquired using the following parameters: positive electrospray ionization (ESI), temperature of drying gas = 350 °C, flow rate of drying gas = 12 L/min, pressure of nebulizer gas = 60 psi, capillary voltage = 4000 V and fragmentor voltage = 70 V.

Following LC methods were used:

Method A (Column: Agilent Poroshell SB-C18 column, 3 × 100 mm, 2.7 μm, flow rate 0.6 mL/min)

Time (min)	H_2O (%)	Acetonitrile (%)
0	95	5
15	50	50
18	5	95
24	5	95

Method B (Column: Agilent Poroshell SB-C18 column, 3 × 100 mm, 2.7 μm, flow rate 0.6 mL/min)

Time (min)	H_2O (%)	Acetonitrile (%)
0	100	0
15	50	50
18	5	95
24	5	95

Method C (Column: Agilent Poroshell SB-C18 column, 3 × 100 mm, 2.7 μm, flow rate 0.6 mL/min)

Time (min)	H_2O (%)	Acetonitrile (%)
0	95	5
2	90	10
18	75	25
20	5	95
24	5	95

Method D (Column: Agilent Poroshell SB-C18 column, 3 × 100 mm, 2.7 μm, flow rate 0.6 mL/min)

Time (min)	H_2O (%)	Acetonitrile (%)
0	95	5
2	80	20
18	70	30
20	5	95
24	5	95

Method E (Column: Agilent TC-C18 (2) column, 4.6 × 150 mm, 5.0 μm, flow rate 1.0 mL/min)

Time (min)	H_2O (%)	Acetonitrile (%)
0	95	5
2	85	15
18	80	20
20	5	95
24	5	95

All reported LC-MS yields were determined by integrating UV spectra at 220 nm. The peak areas for all relevant peptide-containing species on the chromatogram were integrated using Agilent software package. The yields were determined as follows: %yield = $S_{product}/S_{total}$, where $S_{product}$ is the peak area of the product and S_{total} is the peak area of combined peptide-containing species (product, starting material and byproduct).

2.2 Preparative HPLC

Preparative RP-HPLC was performed on a Hanbon Sci.&Tech. Newstyle HPLC system with a NU3000 serials UV/VIS detector, a NP7000 serials pump, a DM-A dynamic mixer and a collector, coupled with a Dubhe C18 column (30 x 250 mm, 10 μm). Water (solvent A) and acetonitrile (solvent B), each containing 0.1% TFA, were used as the mobile phase.

Following LC method was used:

Method F (Column: Dubhe C18 column, 30 x 250 mm, 10 μm, flow rate 20 mL/min)

Time (min)	H_2O (%)	Acetonitrile (%)
0	95	5
5	95	5
40	60	50
45	0	100
60	0	100

3. Solid phase peptide synthesis (SPPS) and purification

Peptide synthesis was carried out manually using standard Fmoc SPPS-chemistry and 2-chloro-trityl chloride (2-CTC) resin (0.98 mmol/g resin, 0.2 mmol) or Rink amide resin (0.606 mmol/g resin, 0.2 mmol).

Preloading of 2-chloro-trityl chloride resin: 2-CTC resin was swollen in anhydrous DCM for 10 min, then washed with DCM (3 × 5 mL). A solution of Fmoc-AA-OH (4 eq.) and DIPEA (8 eq.) in anhydrous DCM (4 mL) was added to the resin and the suspension was shaken at room temperature for 2 h. The resin was washed with DCM (3 × 5 mL), DMF (3 × 5 mL) and DCM (3 × 5 mL). Then, the resin was capped with a solution of DCM/MeOH/DIPEA (17/2/1, v/v/v, 4 mL) for 30 min and washed with DMF (3 × 5 mL), MeOH (3 × 5 mL) and DCM (3 × 5 mL).

Preloading of Rink amide resin: Rink amide resin was swollen in anhydrous DCM for 10 min, then washed with DCM (3 × 5 mL) and DMF (3 × 5 mL). Then the resin was treated with piperidine/DMF (1/4, v/v, 2 × 4 mL, 2 × 5 min) at room temperature and washed with DMF (3 × 5 mL), MeOH (3 × 5 mL) and DCM (3 × 5 mL). A solution of Fmoc-AA-OH (4 eq.), HATU (4 eq.) and collidine (8 eq.) in anhydrous DMF (4 mL) was added to the resin and the suspension was shaken at room temperature for 2 h. The resin was washed with DMF (3 × 5 mL), MeOH (3 × 5 mL) and DCM (3 × 5 mL). Then,

the resin was capped with acetic anhydride/pyridine (1/9, v/v) (30 min) and washed with DMF (3 × 5 mL), MeOH (3 × 5 mL) and DCM (3 × 5 mL).

Fmoc-deprotection: The washed resin was treated with piperidine/DMF (1/4, v/v, 2 × 4 mL, 2 × 5 min) at room temperature and then washed with DMF (3 × 5 mL), MeOH (3 × 5 mL) and DCM (3 × 5 mL).

Amino acid coupling and Fmoc-deprotection: A solution of protected amino acid (4 eq.), HATU (4 eq.) and collidine (8 eq.) in DMF (4 mL) was added to the resin. The reaction mixture was agitated at room temperature for 2 h. After the reaction completion, the resin-bound peptide was washed with DMF (3 × 5 mL), MeOH (3 × 5 mL) and DCM (3 × 5 mL). Then the washed resin was treated with piperidine/DMF (1/4, v/v, 2 × 4 mL, 2 × 5 min) at room temperature and washed with DMF (3 × 5 mL), MeOH (3 × 5 mL) and DCM (3 × 5 mL).

Acetylation of *N*-terminal (optional): DCM (4 mL) and DIPEA (8 eq.) was added to the resin, then acetyl chloride (2 eq.) was added to the resin slowly. The reaction mixture was agitated at room temperature for 10min. Then the resin was washed with DMF (3 × 5 mL), MeOH (3 × 5 mL) and DCM (3 × 5 mL) for cleavage.

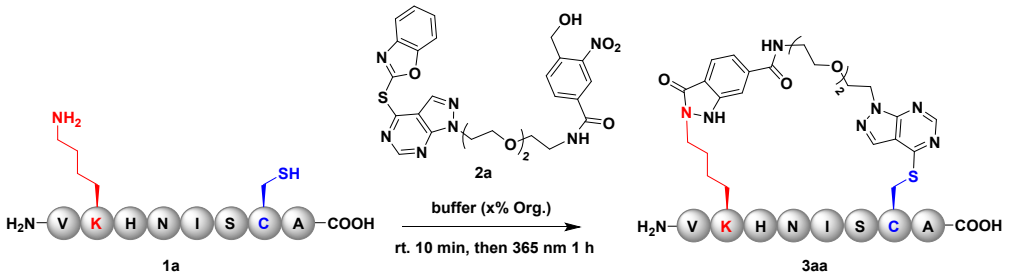
Peptide cleavage and deprotection: Peptide was deprotected and cleaved from the resin under reducing conditions, by treatment with 2.5% v/v water, 2.5% v/v *i*-Pr_3SiH and 2.5% v/v thioanisole in neat trifluoroacetic acid (4 mL). The resulting mixture was shaken at room temperature for 1 h. Then, the resin was removed by filtration and washed with TFA (2 × 3 mL). The combined cleavage solutions were concentrated in vacuum.

Peptide purification and analysis: Peptide was dissolved in water with a minimum amount of organic co-solvent (acetonitrile). Then purified by preparative RP-HPLC (Method F). Fractions containing the desired peptide were lyophilized. The purity was assessed by analyzing a peptide solution by LC-MS. At the same time, low-resolution mass spectrometric measurement was also acquired.

The synthesis of Dde-protected peptides was in section 8, and Fmoc-Lys(Dde)-OH was used in corresponding position.

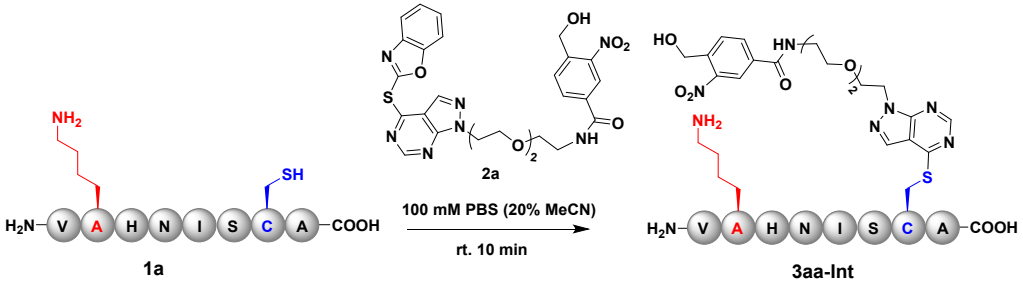
4. Optimization of Cys directed Cys-Lys stapling

Table S1: Optimization of reaction conditions for Cys-Lys Stapling[a]

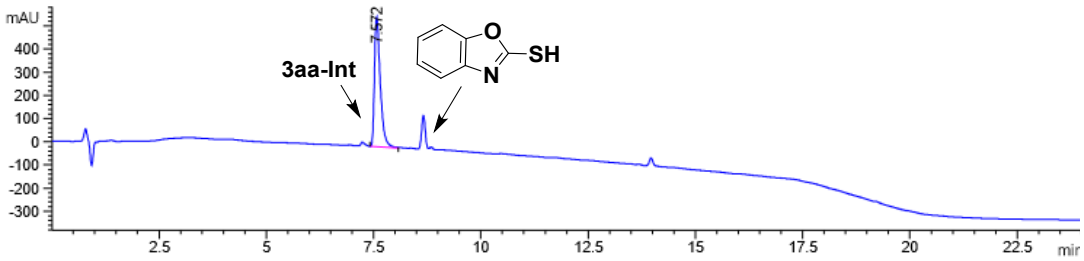


Entry	Buffer	pH	eq. (**2a**)	Org.	Yield (%)[b]
1	100 mM PBS	7.4	1.2	10% MeCN	56
2	100 mM PBS	7.6	1.2	10% MeCN	65
3	100 mM PBS	7.8	1.2	10% MeCN	77
4	100 mM PBS	8.0	1.2	10% MeCN	94
5	100 mM PBS	8.2	1.2	10% MeCN	85
6	100 mM PBS	8.4	1.2	10% MeCN	84
7	100 mM HEPES	8.0	1.2	10% MeCN	94
8	100 mM PBS	8.0	1.5	10% MeCN	94
9	100 mM PBS	8.0	2.0	10% MeCN	94
10[c]	100 mM PBS	8.0	1.2	10% MeCN	**3aa-Int**
11	100 mM PBS	8.0	1.2	5% MeCN	87
12	100 mM PBS	8.0	1.2	10% MeOH	93
13	100 mM PBS	8.0	1.2	10% DMSO	94
14[d]	100 mM PBS	8.0	1.2	20% MeCN	90
15	100 mM PBS	8.0	1.2	20% MeCN	95 (84[e])

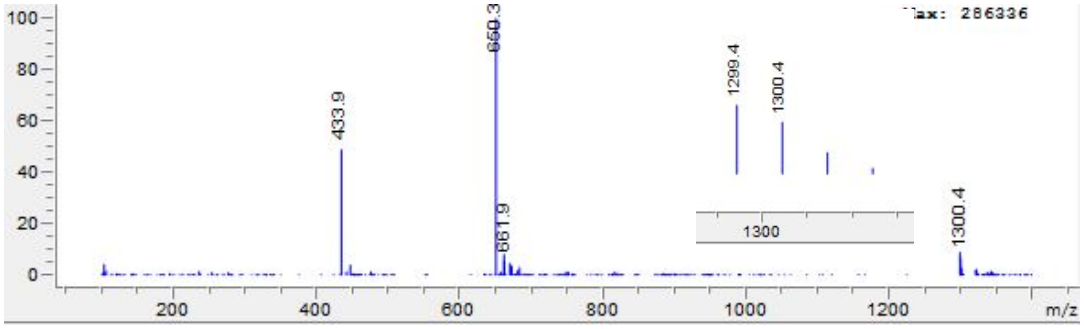
a) Reaction conditions: 0.5 μmol **1a** and **2a** (eq.) in 500 uL non-degassed buffer was stirred at room temperature for 10 min, then stirred at room temperature for another 1 h under irradiation of blue LED (10 W). b) Reported yields were LC-MS yields. c) Without 365 nm irradiation. d) Stirred for 30 min under 365 nm irradiation. e) Isolated yield based on 5 μmol scale.



Without irradiation of blue LED (10 W), only Cys reacted intermediate **3aa-Int** was detected.



LC-MS UV spectrum of reaction mixture on 220 nm without irradiation. Gradient used：Method A



ESI Mass spectrum of **3aa-Int**. Calculated Mass $[M+H]^{+}$: 1299.6, $[M+2H]^{2+}$: 650.3, $[M+3H]^{3+}$: 433.9; Mass Found (ESI+) $[M+H]^{+}$: 1299.4, $[M+2H]^{2+}$: 650.3, $[M+3H]^{3+}$: 433.9.

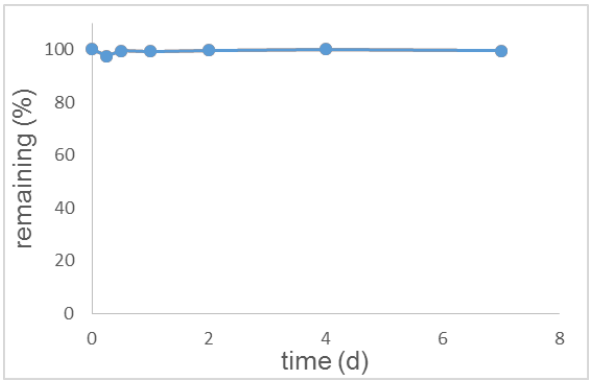
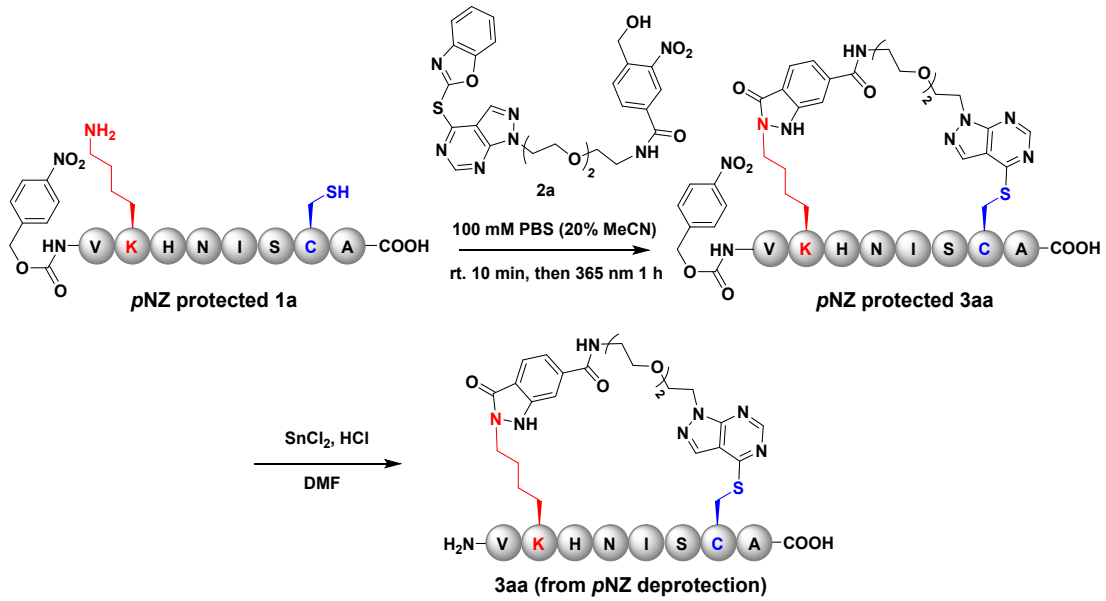


Figure S1. Stability of **3aa** in PBS buffer. **3aa** (1 mM) was stable in PBS buffer (10 mM, pH = 7.4) at room temperature after 7 days and no conversion was observed.

Procedure for **3aa** in Table S1 (entry 15): 0.5 μmol peptide (**1a**) was dissolved in 400 μL non-degassed PBS buffer (100 mM, pH 8.0) and **2a** (0.6 μmol) in 100μL MeCN was added. The resulting solution was stirred at room temperature for 10 min, followed by irradiating at 365 nm for another 1 h. After this time, the reaction was analyzed by HPLC-MS and purified by preparative HPLC to afford **3aa** as a white solid. 95% HPLC yield (84% isolated yield based on 5 μmol scale). ^{1}H NMR (400 MHz, DMSO-d_6) δ 14.36 (s, 1H), 14.25 (s, 1H), 10.61 (s, 1H), 8.95 (s, 1H), 8.73 (s, 1H), 8.58 (t, *J* = 5.6 Hz, 1H), 8.48 (t, *J* = 8.8 Hz, 2H), 8.35 (d, *J* = 8.4 Hz, 1H), 8.27 (d, *J* = 6.8 Hz, 1H), 8.19 (s, 1H), 8.20-8.09 (m, 4H), 7.99 (d, *J* = 8.4 Hz, 1H), 7.71 – 7.65 (m, 2H), 7.54 (s, 1H), 7.52 – 7.47 (m, 1H), 7.33 (s, 1H), 7.06 (s, 1H), 4.77 – 4.67 (m, 1H), 4.67 – 4.54 (m,

2H), 4.52 – 4.46 (m, 2H), 4.35 – 4.14 (m, 5H), 3.99 (dd, *J* = 13.2, 4.4 Hz, 1H), 3.93 – 3.84 (m, 3H), 3.83 – 3.74 (m, 1H), 3.62 (br, 1H), 3.56 (d, *J* = 6.0 Hz, 2H), 3.54 – 3.41 (m, 6H), 3.41 – 3.34 (m, 2H), 3.03 (dd, *J* = 14.8, 4.8 Hz, 1H), 2.89 (dd, *J* = 14.8, 8.0 Hz, 1H), 2.59 – 2.51 (m, 1H), 2.49 – 2.41 (m, 1H), 2.09 – 1.93 (m, 1H), 1.82 – 1.51 (m, 5H), 1.42 – 1.18 (m, 5H), 1.06 – 0.92 (m, 1H), 0.90 – 0.84 (m, 6H), 0.72 (d, *J* = 6.4 Hz, 3H), 0.67 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.09, 172.17, 171.68, 171.44, 171.30, 170.60, 170.12, 169.73, 168.17, 166.42, 164.52, 160.19, 158.85, 158.52, 154.35, 151.23, 145.40, 137.73, 134.22, 131.75, 129.51, 123.33, 120.06, 119.07, 118.40, 117.35, 115.46, 111.83, 111.51, 69.96, 69.89, 69.19, 68.52, 61.99, 57.63, 57.48, 55.58, 53.05, 51.94, 51.70, 50.38, 48.25, 46.98, 43.30, 37.35, 37.19, 31.93, 30.69, 30.33, 28.01, 27.63, 24.44, 22.63, 18.67, 18.16, 17.43, 15.76, 11.66 .



Procedure for **3aa** prepared from ***p*NZ proteted 1a**: 10 μmol *p*-nitrobenzyloxycarbonyl (*p*NZ) protected **1a** was dissolved in 8.0 mL non-degassed PBS buffer (100 mM, pH 8.0). Then, corresponding stapling reagent (**2a**, 12.0 μmol) in 2.0 mL MeCN was added. The resulting solution was stirred at room temperature for 10 min followed by irradiating at 365 nm for another 1 h. After this time, the reaction purified by HPLC to afford the ***p*NZ protected 3aa**. Then ***p*NZ protected 3aa** was dissolved in 2.0 mL DMF, added with $SnCl_2$ (2 eq.) and HCl (aq. 0.2 eq.). The reaction mixture stirred at room temperature until reaction finished and purified by HPLC to afford **3aa (from *p*NZ deprotection)**.

Chemoselectivity determination: Performing ^{1}H NMR analysis of the product **3aa (from *p*NZ deprotection)** prepared from ***p*NZ protected 1a** (above procedure) and compared with the ^{1}H NMR spectrum of **3aa** prepared from directly stapling of unprotected peptide **1a** (Table S1, entry 15). The results showed that **3aa (from *p*NZ deprotection)** and **3aa** have same chemical signal in ^{1}H NMR (Figure S2), demonstrating that the reaction occurred on ε-amino of Lys rather than α-amino of *N*-terminal.

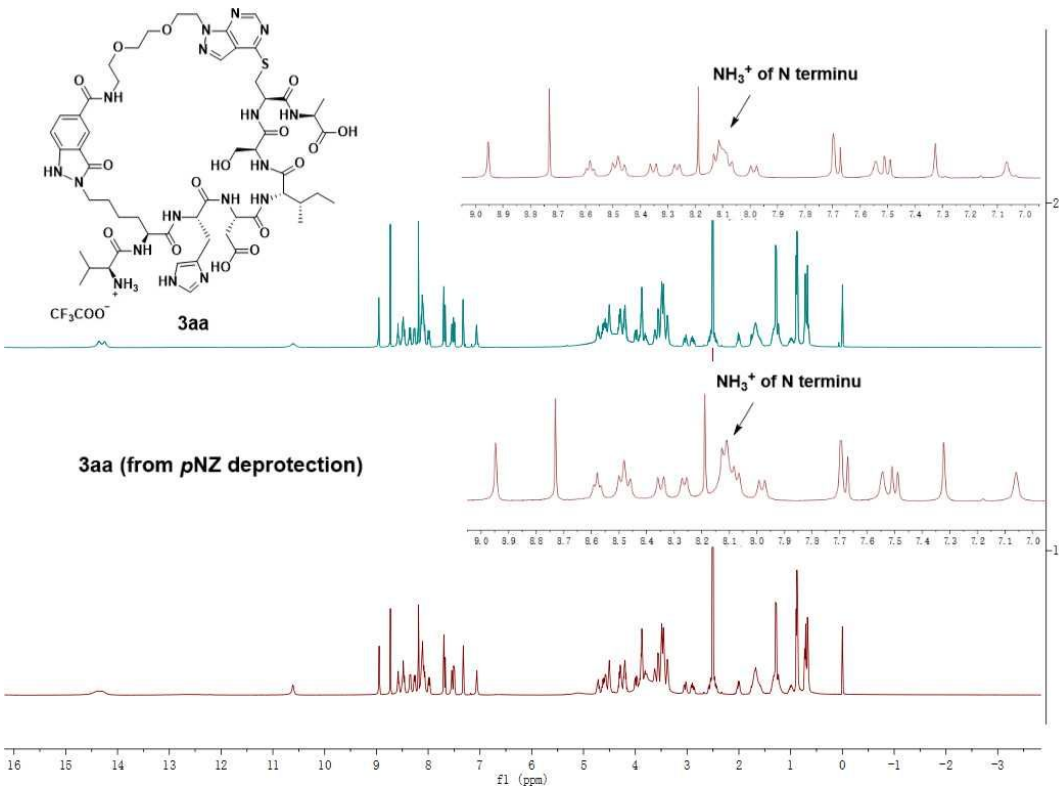
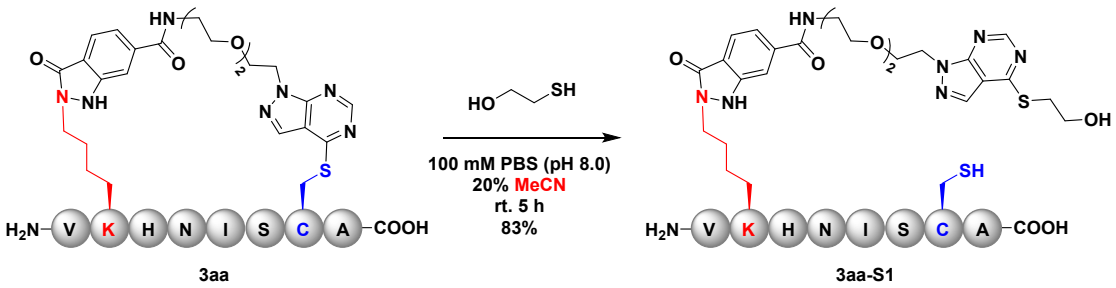


Figure S2. Comparison of ^{1}H NMR of **3aa** from fully deprotected **1a** and **3aa** from ***p*NZ protected 1a**

5. Cys regeneration and multi-functionalization

General procedure for the following Lys single-site modification by Cys regeneration with thiol: 0.5 μmol stapled peptide (**3**) was dissolved in 400 μL non-degassed PBS buffer (100 mM, pH 8.0) and 100 μL MeCN/DMSO/DMF. Then, mercaptoethanol (BME, 50 μmol) was added and the resulting solution was stirred at room temperature for 5 h. After that, the reaction was analyzed by HPLC-MS.



When MeCN was used as co-solvent, regenerated native Cys containing product **3aa-S1** was detected as product.

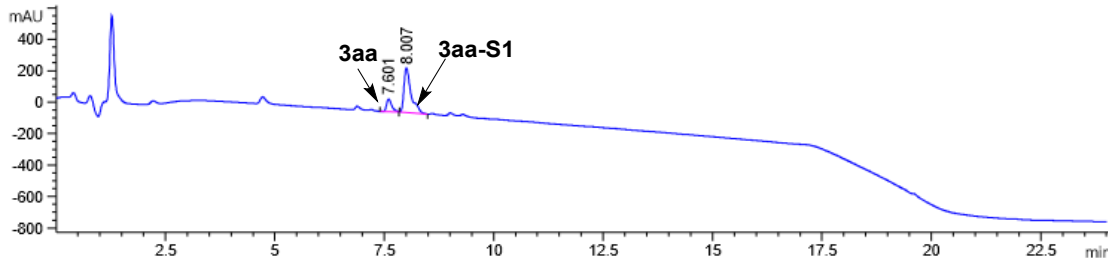


Figure S3. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

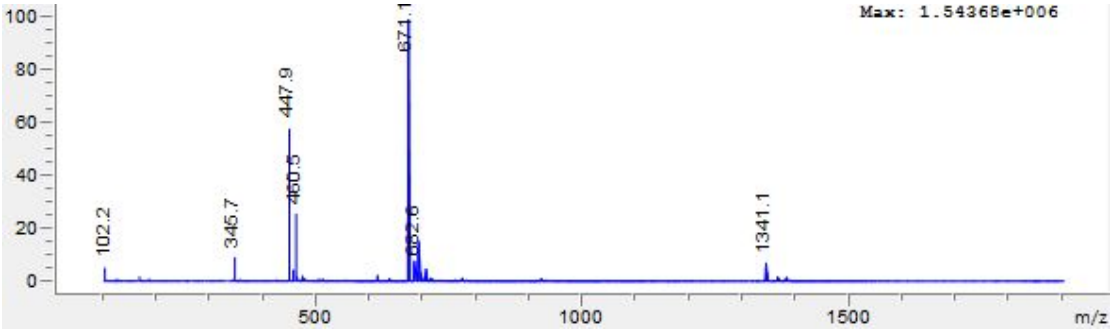
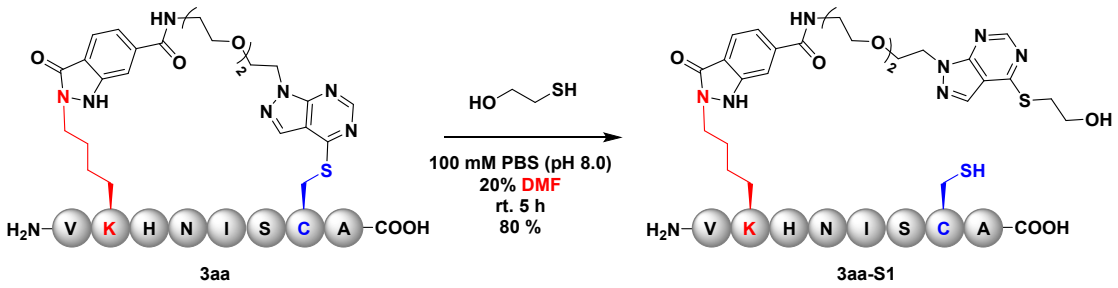


Figure S4. ESI Mass spectrum of product **3aa-S1**. Calculated Mass $[M+H]^{+}$: 1341.6, $[M+2H]^{2+}$: 671.3, $[M+3H]^{3+}$: 447.9; Mass Found (ESI+) $[M+H]^{+}$: 1341.1, $[M+2H]^{2+}$: 671.1, $[M+3H]^{3+}$: 447.9.



When DMF was used as co-solvent, regenerated native Cys containing product **3aa-S1** was detected as product.

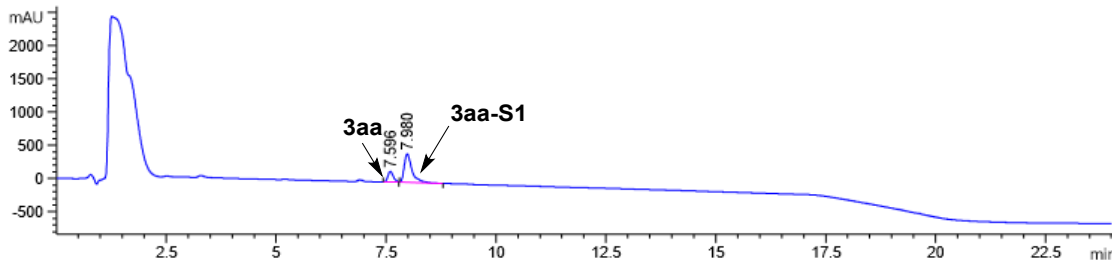


Figure S5. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

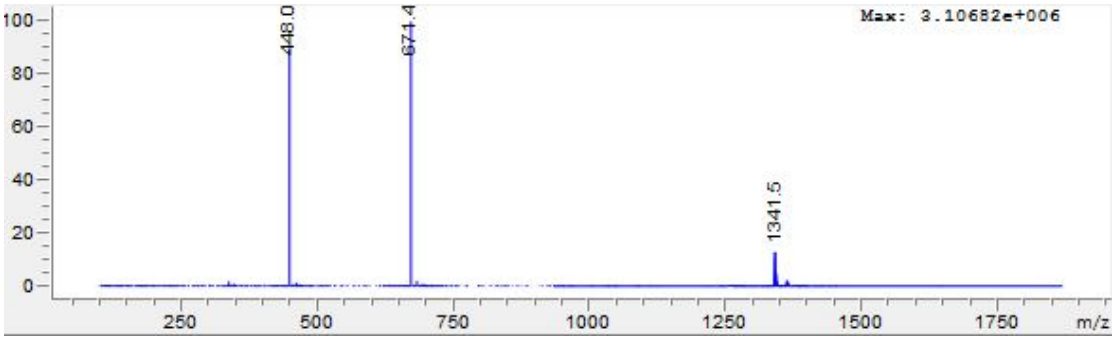
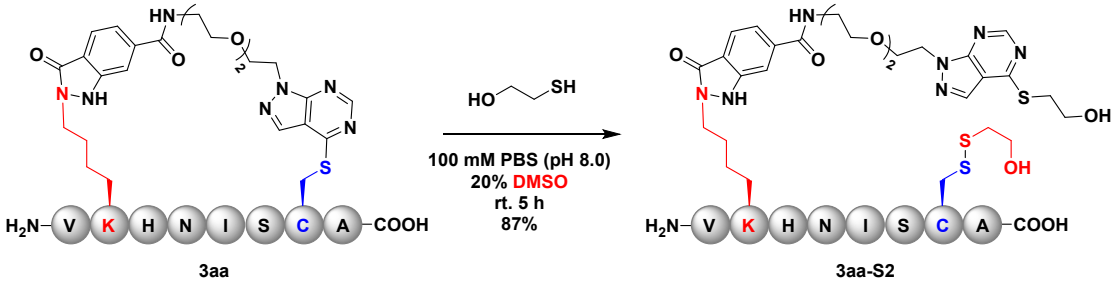


Figure S6. ESI Mass spectrum of product **3aa-S1**. Calculated Mass $[M+H]^{+}$: 1341.6, $[M+2H]^{2+}$: 671.3, $[M+3H]^{3+}$: 447.9; Mass Found (ESI+) $[M+H]^{+}$: 1341.5, $[M+2H]^{2+}$: 671.4, $[M+3H]^{3+}$: 448.0.



3aa 3aa-S2

When DMSO was used as co-solvent, **3aa-S2** that regenerated native Cys dimerized with BME was detected as product.

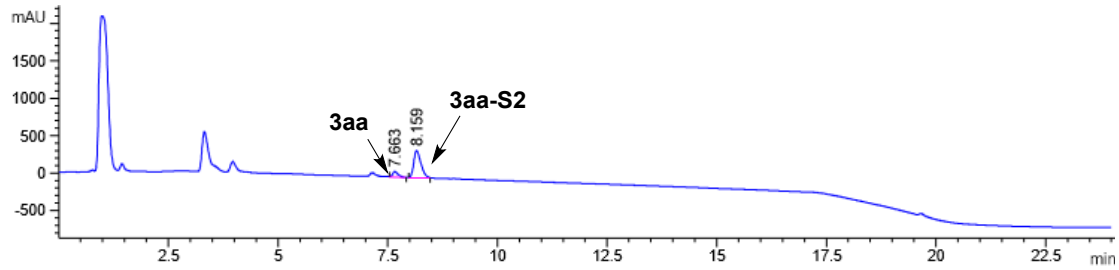


Figure S7. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

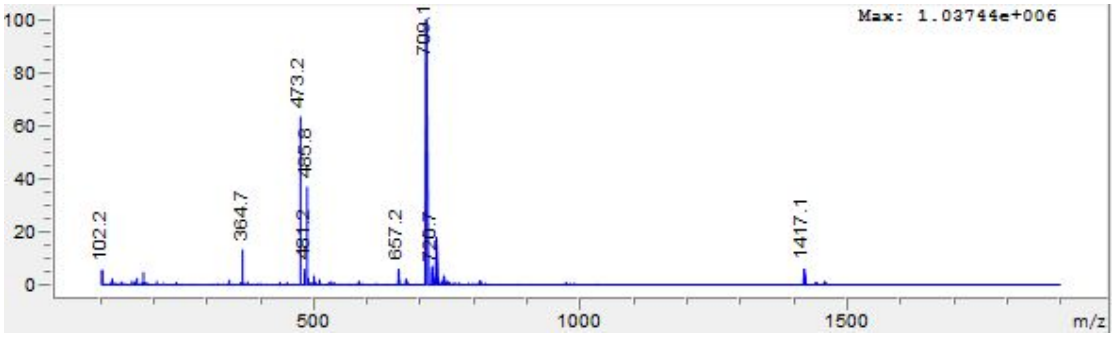
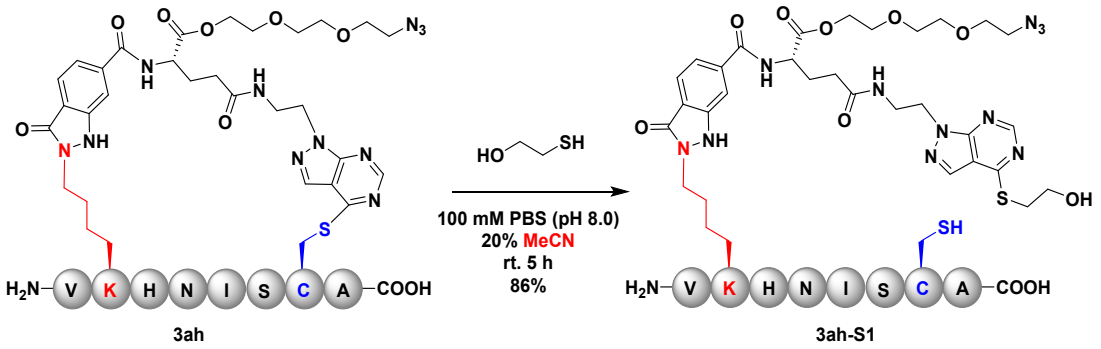


Figure S8. ESI Mass spectrum of product **3aa-S2**. Calculated Mass $[M+H]^{+}$: 1417.6, $[M+2H]^{2+}$: 709.3, $[M+3H]^{3+}$: 473.2; Mass Found (ESI+) $[M+H]^{+}$: 1417.1, $[M+2H]^{2+}$: 709.1, $[M+3H]^{3+}$: 473.2.



When MeCN was used as co-solvent, regenerated native Cys containing product **3ah-S1** was detected as product.

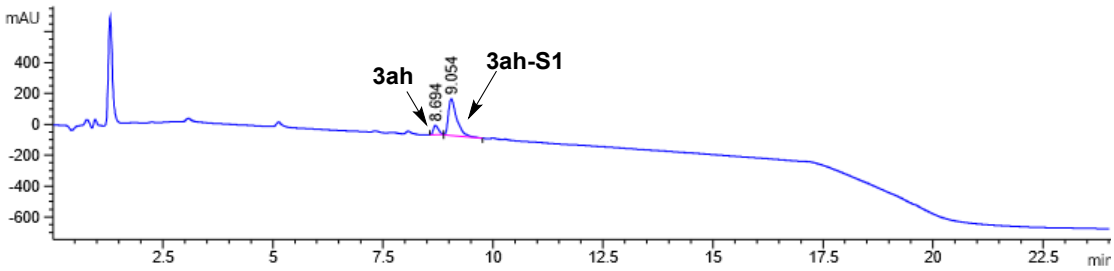


Figure S9. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

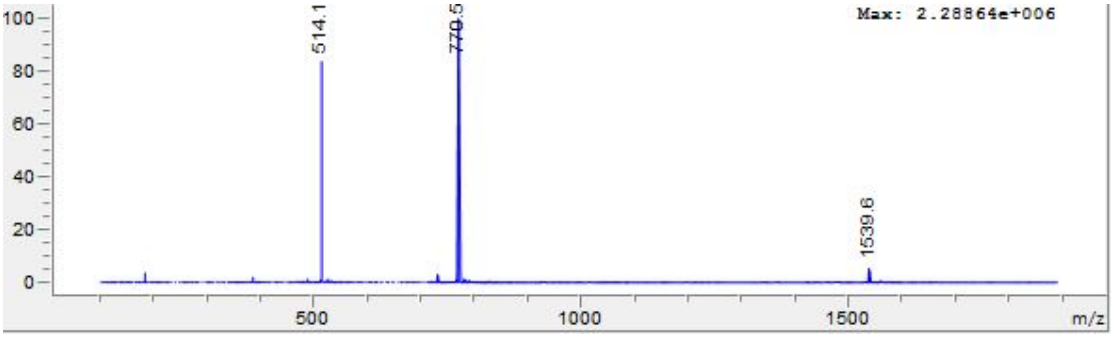
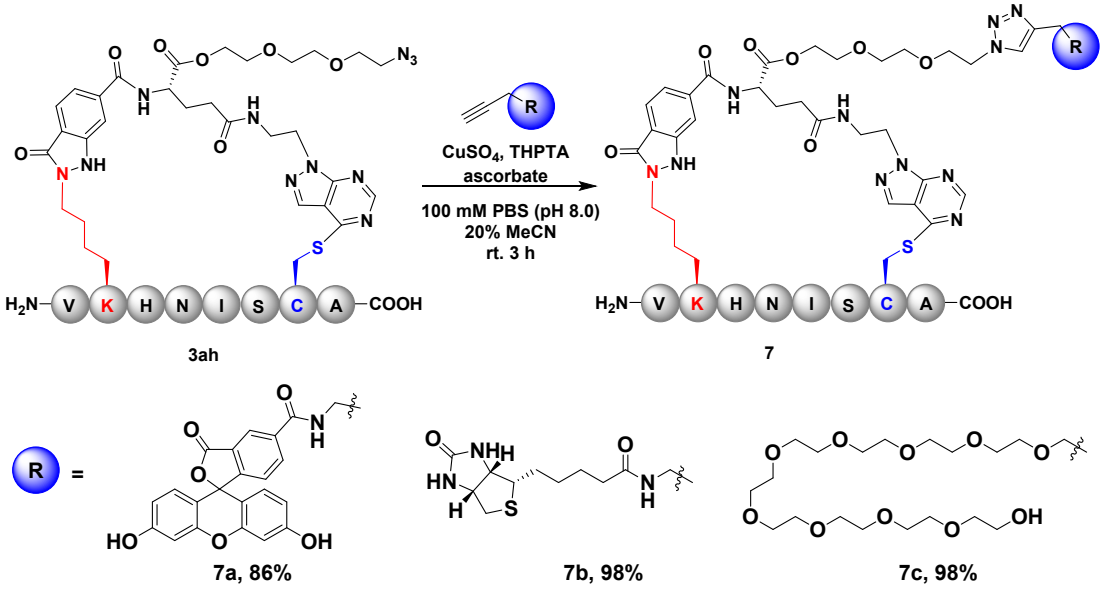


Figure S10. ESI Mass spectrum of product **3ah-S1**. Calculated Mass $[M+H]^{+}$: 1539.7, $[M+2H]^{2+}$: 770.3, $[M+3H]^{3+}$: 513.9; Mass Found (ESI+) $[M+H]^{+}$: 1539.6, $[M+2H]^{2+}$: 770.5, $[M+3H]^{3+}$: 514.1.



General procedure for the following multi-functionalization via click reaction: **3ah** (0.5 μmol, 1 eq.) and corresponding alkyne (5 eq.) were dissolved in 500 μL 100 mM PBS buffer (pH 8.0, 20% MeCN, v/v). $CuSO_4$ (20 mol%) and THPTA (100 mol%) in water (10 μL) was added, followed by sodium ascorbate (200 mol%) in water (10 μL) was added to the reaction mixture. The mixture was stirred at room temperature for 3 h. After that, the reaction was analyzed by HPLC-MS.

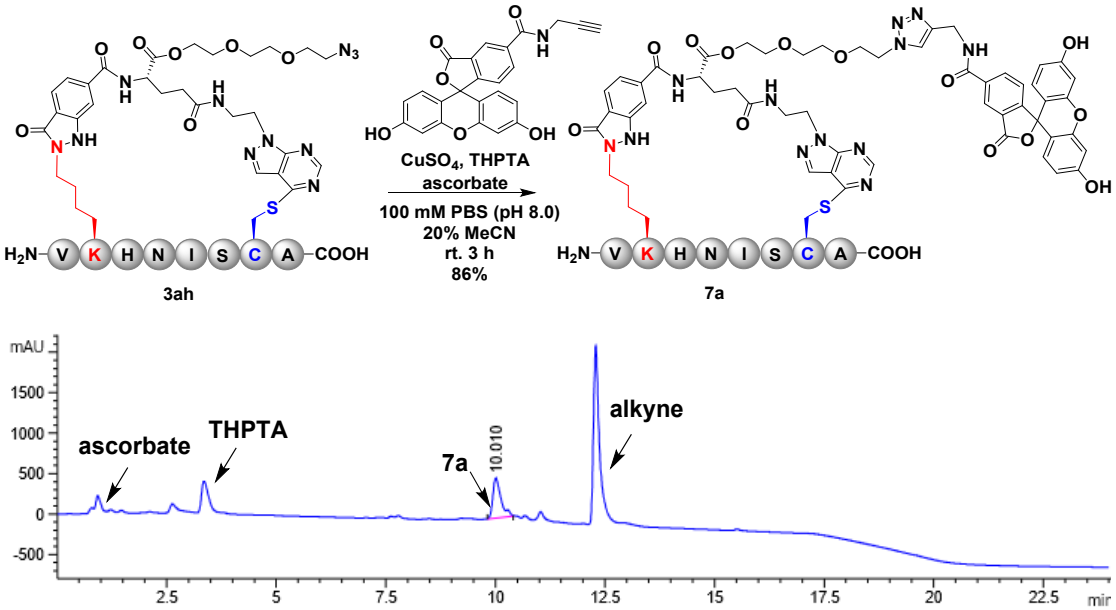


Figure S11. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

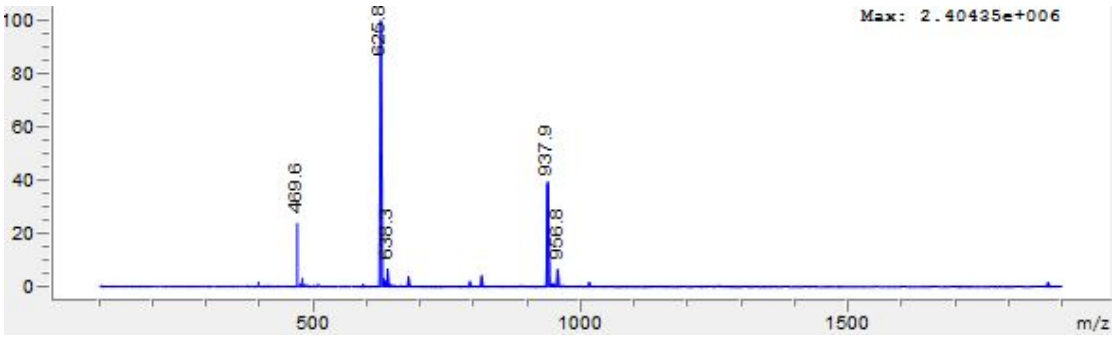


Figure S12. ESI Mass spectrum of product **7a**. Calculated Mass $[M+2H]^{2+}$: 937.9, $[M+3H]^{3+}$: 625.6, $[M+4H]^{4+}$: 469.4; Mass Found (ESI+) $[M+2H]^{2+}$: 937.9, $[M+3H]^{3+}$: 625.8, $[M+4H]^{4+}$: 469.6.

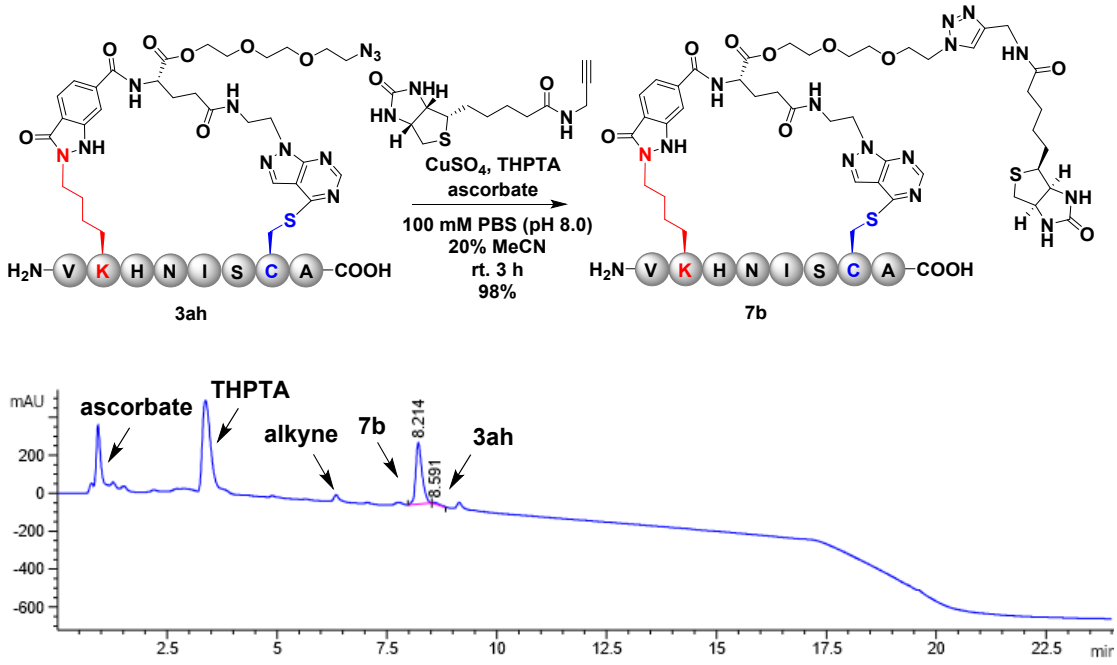


Figure S13. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

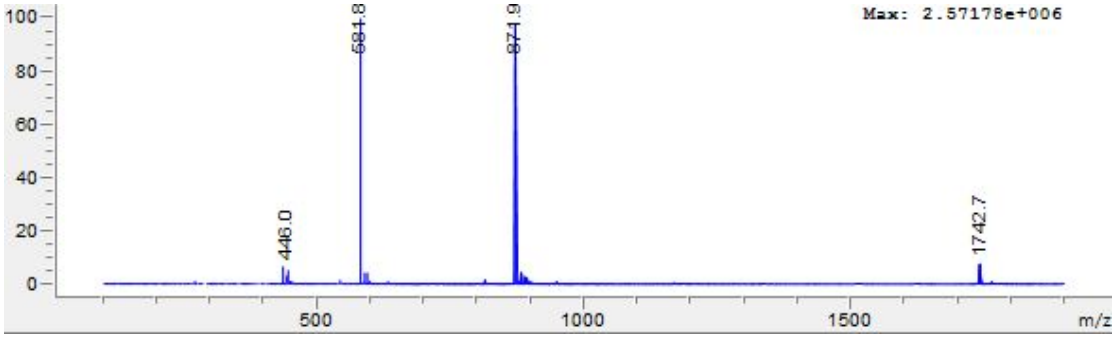


Figure S14. ESI Mass spectrum of product **7b**. Calculated Mass $[M+H]^{+}$: 1742.8, $[M+2H]^{2+}$: 871.9, $[M+3H]^{3+}$: 581.6; Mass Found (ESI+) $[M+H]^{+}$: 1742.7, $[M+2H]^{2+}$: 871.9, $[M+3H]^{3+}$: 581.8.

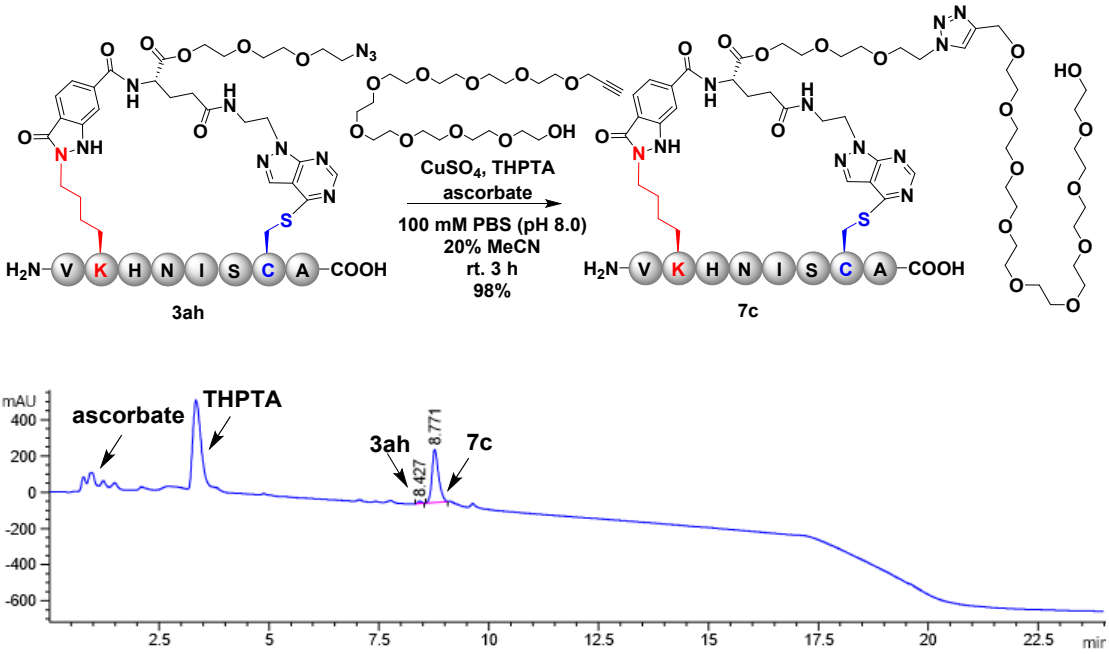


Figure S15. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

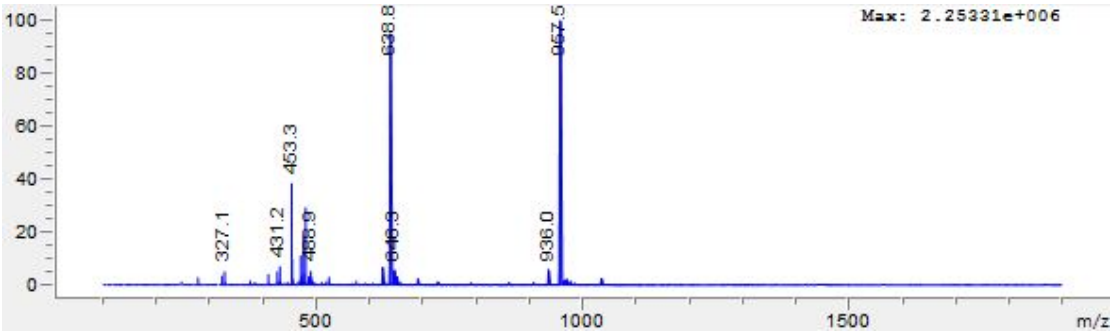


Figure S16. ESI Mass spectrum of product **7c**. Calculated Mass $[M+2H]^{2+}$: 957.5, $[M+3H]^{3+}$: 638.6; Mass Found (ESI+) $[M+2H]^{2+}$: 957.5, $[M+3H]^{3+}$: 638.6.

6. Synthesis and characterization of stapling reagents

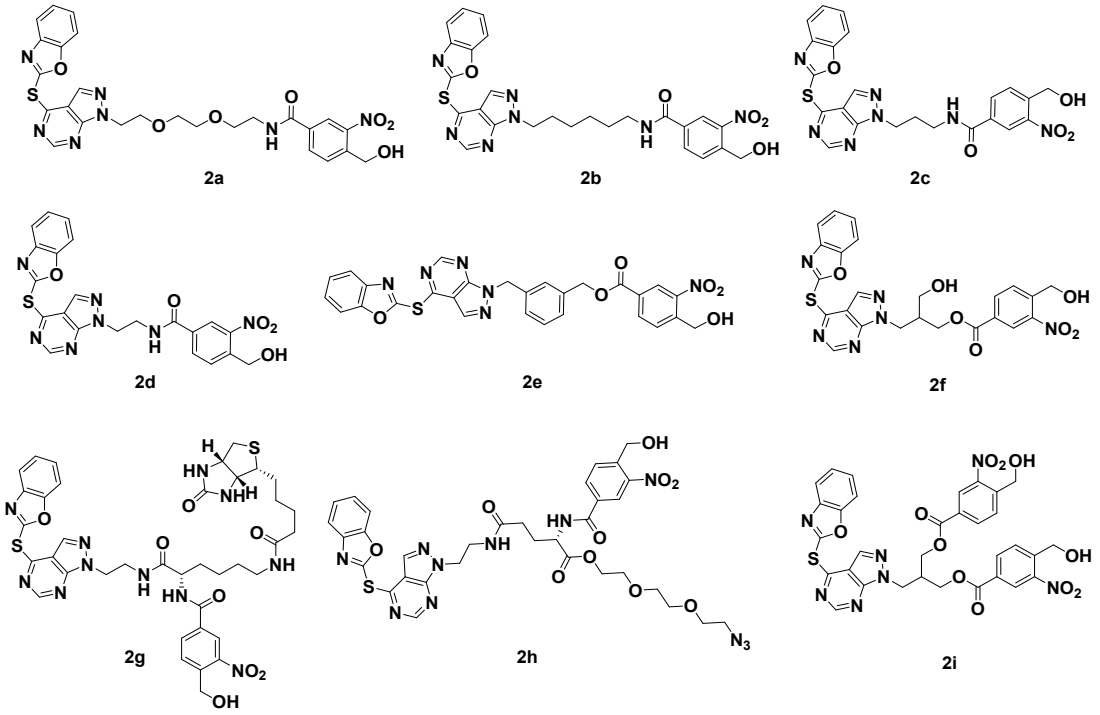
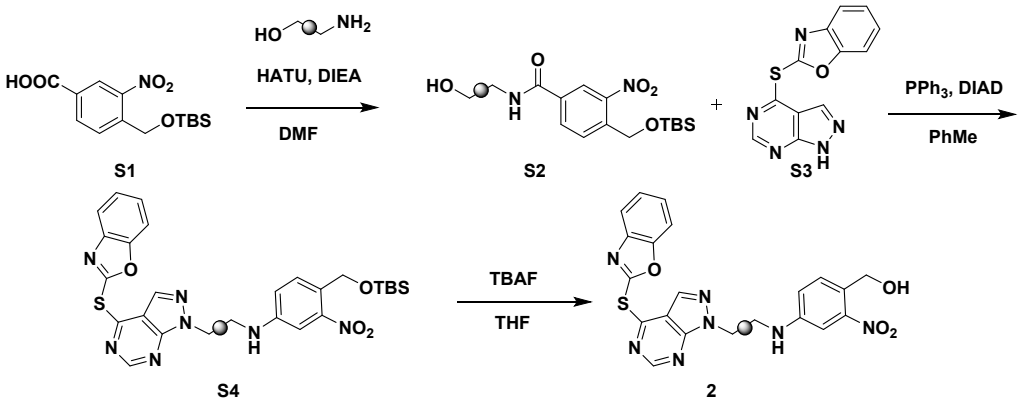


Figure S17. Structure of stapling reagents

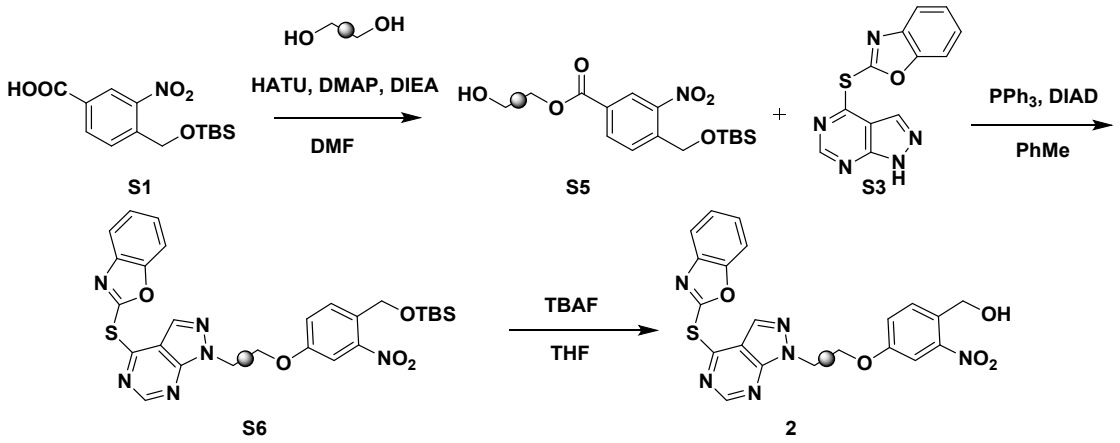
General procedure Ⅰ



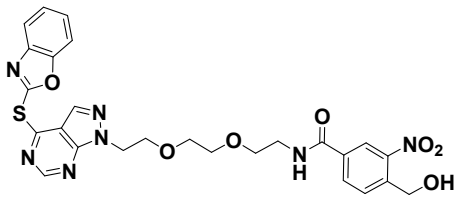
S1[1] (1.0 mmol), HATU (1.1 mmol) and DIPEA (2.0 mmol) were dissolved in DMF (5 mL), corresponding amino alcohol (1.1 mmol) was added and the mixture was stirred at room temperature until reaction completion. The suspension was diluted with EtOAc (20 mL) and washed successively with saturated aqueous $NaHCO_3$ (10 mL), water (10 mL) and brine (10 mL). The organic extract was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude residue was purified by silica gel (200−300 mesh) column chromatography to afford the intermediate **S2**. Then **S2** (1.2 eq.), **S3**[2] (1.0 eq.) and triphenylphosphine (PPh_3, 1.5 eq.) were dissolved in dry PhMe (5 mL), followed by dropwise addition of diisopropyl azodiformate (DIAD, 1.5 eq.) at 0 °C. After reaction completion, the reaction mixture was evaporated in vacuo and

purified by silica gel (200−300 mesh) column chromatography to afford the desired compound **S4**. Finally, **S4** was dissolved in THF (3 mL), TBAF (1 M in THF, 1.5 eq.) was added and the mixture was stirred at 0 °C until reaction completion. The reaction mixture was concentrated under reduced pressure and the crude residue was purified by silica gel (200−300 mesh) column chromatography to afford the desired product **2**.

General procedure Ⅱ

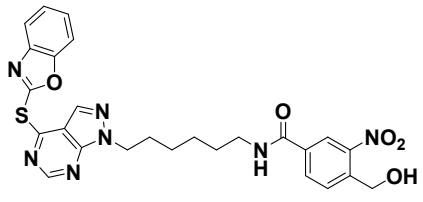


S1 (1.0 mmol), HATU (1.1 mmol), DMAP (0.1 mmol) and DIPEA (2.0 mmol) were dissolved in DMF (5 mL), corresponding alcohol (1.1 mmol) was added and stirred at room temperature until reaction completion. Performing the following procedure as procedure Ⅰ to afford the corresponding product **2**.



2a

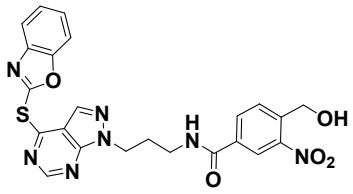
General procedure Ⅰ: **2a**, white solid, 29% yield (3 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (s, 1H), 8.43 (d, *J* = 1.2 Hz, 1H), 8.11 – 8.02 (m, 1H), 7.89 – 7.79 (m, 2H), 7.65 – 7.56 (m, 2H), 7.52 – 7.40 (m, 2H), 7.29 (br, 1H), 5.01 (s, 2H), 4.66 (t, *J* = 5.2 Hz, 2H), 4.01 (t, *J* = 5.2 Hz, 2H), 3.66 – 3.52 (m, 8H), 2.86 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.09, 160.84, 154.75, 154.19, 152.62, 152.23, 146.81, 141.52, 140.51, 134.62, 132.44, 131.38, 129.49, 126.80, 125.34, 123.37, 120.63, 112.38, 111.08, 70.40, 70.11, 69.52, 69.00, 61.85, 47.27, 40.11. HRMS (ESI) m/z calcd for $C_{26}H_{26}N_7O_7S^+$ $[M+H]^+$ 580.1609, found 580.1639.



2b

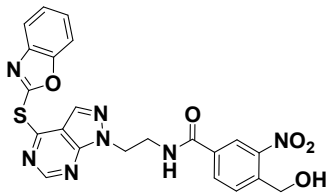
General procedure Ⅰ: **2b**, white solid, 8% yield (3 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.69 (s, 1H), 8.38 (d, *J* = 1.6 Hz, 1H), 8.01 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.89 – 7.80 (m, 2H), 7.68 (s, 1H), 7.59 (dd, *J* = 6.8, 1.6 Hz, 1H), 7.50 – 7.38 (m, 2H), 6.70 (t, *J* = 5.2 Hz, 1H), 5.02 (s, 2H), 4.48 (t, *J* = 6.8 Hz, 2H), 3.50 – 3.35 (m, 2H), 2.01 – 1.88 (m, 2H), 1.71 – 1.53 (m, 2H), 1.48 – 1.38 (m, 2H), 1.37 – 1.29 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.45, 160.70, 154.99, 154.03, 152.57, 151.68, 146.95, 141.44, 140.46, 134.58, 132.19, 131.18, 129.66, 126.79, 125.35,

123.25, 120.58, 112.51, 111.07, 61.83, 47.33, 40.22, 29.20, 29.12, 26.16, 25.99. HRMS (ESI) m/z calcd for $C_{26}H_{25}N_7O_5SNa^+$ $[M+H]^+$ 570.1530, found 570.1529.



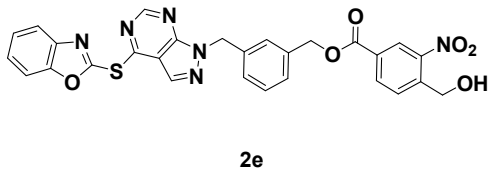
2c

General procedure Ⅰ: **2c**, white solid, 14% yield (3 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.73 (s, 1H), 8.48 (s, 1H), 8.02 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.91 (d, *J* = 8.0 Hz, 1H), 7.84 (dd, *J* = 6.8, 2.0 Hz, 1H), 7.75 (s, 1H), 7.60 (dd, *J* = 6.8, 2.0 Hz, 1H), 7.51 – 7.40 (m, 3H), 5.06 (s, 2H), 4.63 (t, *J* = 6.0 Hz, 2H), 3.40 (q, *J* = 6.0 Hz, 2H), 2.32 – 2.18 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.70 161.62, 154.41, 154.33, 152.67, 151.88, 146.86, 141.52, 140.96, 134.17, 132.24, 131.71, 129.55, 126.88, 125.37, 122.99, 120.68, 112.29, 111.12, 61.82, 45.07, 37.04, 28.32. HRMS (ESI) m/z calcd for $C_{23}H_{20}N_7O_5S^+$ $[M+H]^+$ 506.1241, found 5061237.



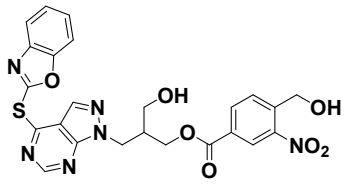
2d

General procedure Ⅰ: **2d**, white solid, 54% yield (3 steps). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.84 (t, *J* = 6.0 Hz, 1H), 8.67 (s, 1H), 8.34 (d, *J* = 2.0 Hz, 1H), 8.16 (s, 1H), 8.05 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.94 – 7.87 (m, 2H), 7.83 (d, *J* = 7.6 Hz, 1H), 7.60 – 7.46 (m, 2H), 5.65 (t, *J* = 5.6 Hz, 1H), 4.87 (d, *J* = 5.6 Hz, 2H), 4.67 (t, *J* = 5.6 Hz, 2H), 3.82 – 3.69 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.92, 160.40, 154.91, 154.60, 152.61, 152.29, 146.89, 141.94, 141.66, 134.17, 132.46, 132.03, 128.92, 127.35, 125.81, 123.46, 120.80, 112.37, 111.75, 60.38, 47.29. HRMS (ESI) m/z calcd for $C_{22}H_{18}N_7O_5S^+$ $[M+H]^+$ 492.1085, found 492.1088.



2e

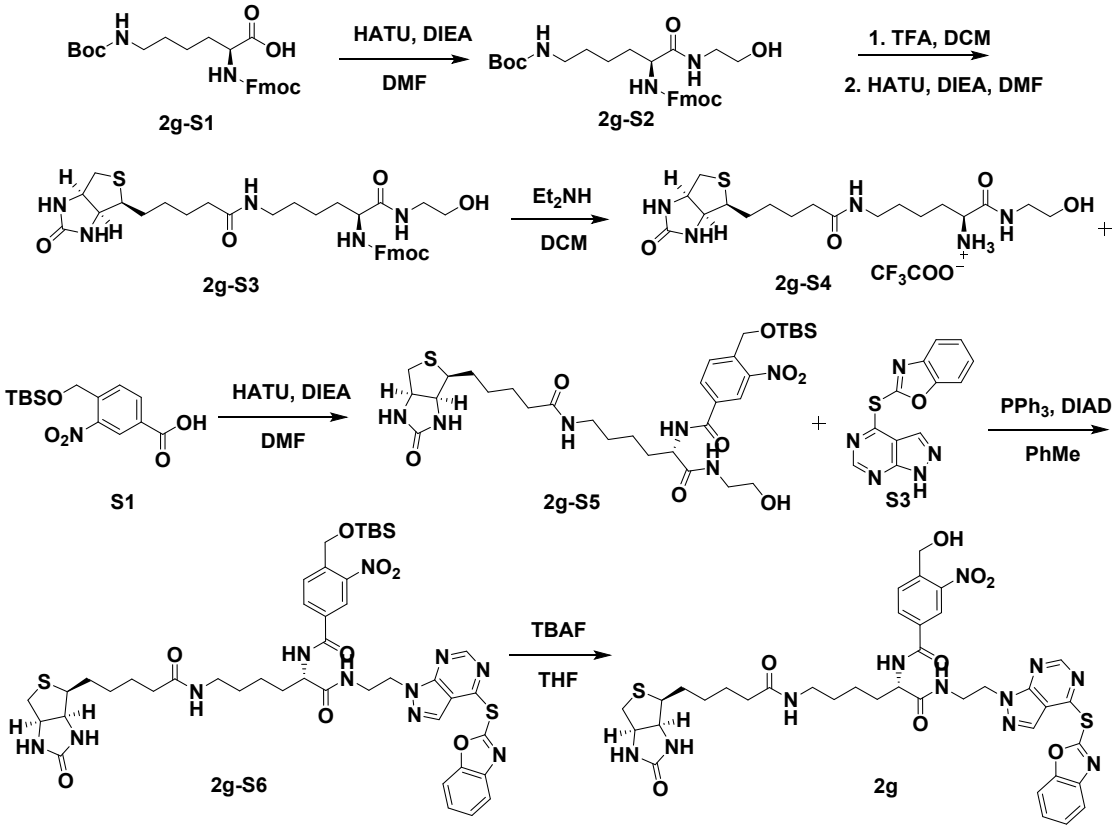
General procedure Ⅱ: **2e**, white solid, 20% yield (3 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.69 (s, 1H), 8.67 (d, *J* = 1.6 Hz, 1H), 8.19 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.93 (d, *J* = 8.0 Hz, 1H), 7.83 (dd, *J* = 7.2, 1.6 Hz, 1H), 7.67 (s, 1H), 7.58 (dd, *J* = 7.2, 2.4 Hz, 1H), 7.49 – 7.40 (m, 2H), 7.37 – 7.32 (4, 5H), 5.66 (s, 2H), 5.35 (s, 2H), 5.09 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.11, 160.82, 154.82, 154.42, 152.61, 151.97, 147.13, 142.27, 141.52, 136.33, 136.15, 134.34, 131.72, 130.23, 129.41, 129.25, 128.22, 128.00, 127.50, 126.81, 126.02, 125.34, 120.66, 112.46, 111.09, 66.93, 61.93, 50.98. HRMS (ESI) m/z calcd for $C_{28}H_{21}N_6O_6S^+$ $[M+H]^+$ 569.1238, found 569.1253.



2f

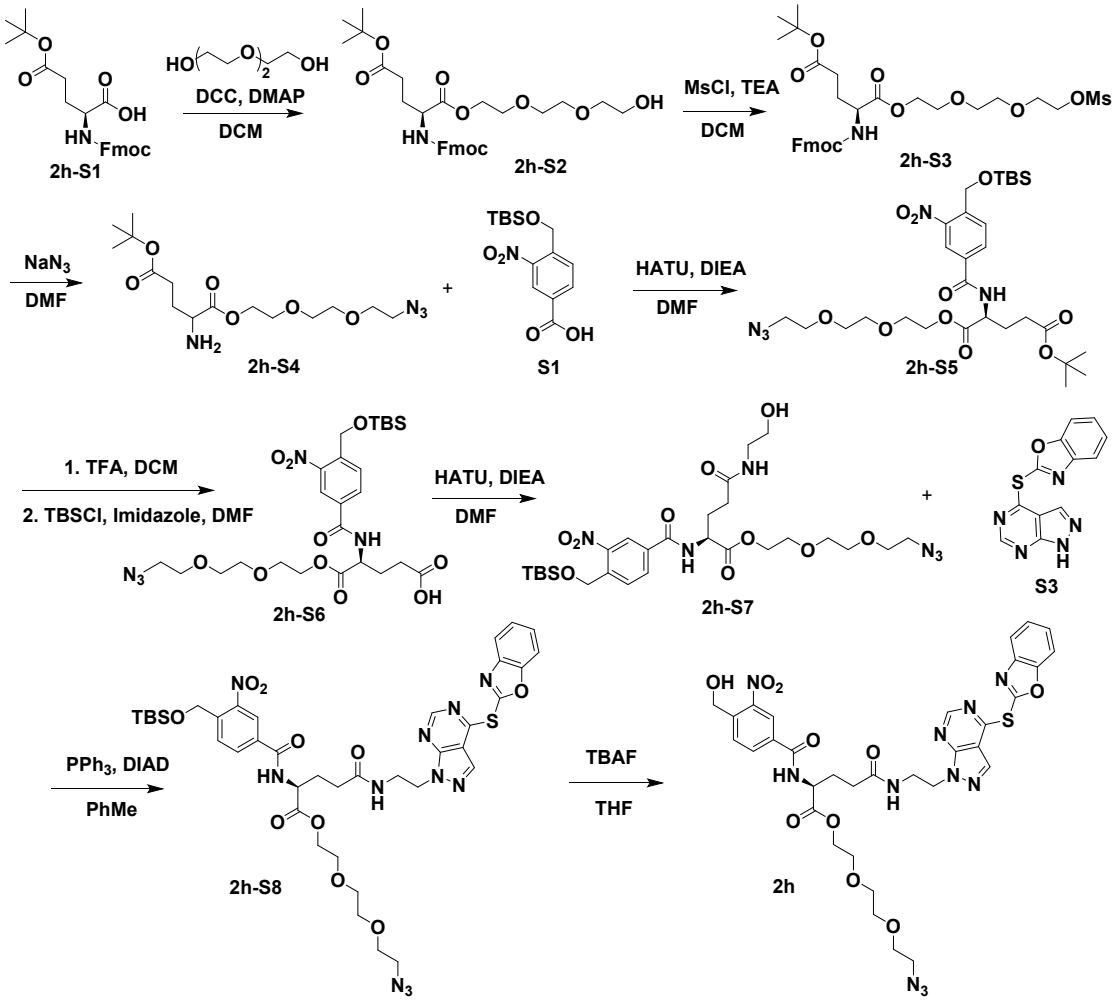
General procedure Ⅱ: **2f**, white solid, 11% yield (3 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (s, 1H), 8.43 (d, *J* = 1.2 Hz, 1H), 7.98 – 7.91 (m, 1H), 7.89 – 7.81 (m, 2H), 7.74 (s, 1H), 7.65 – 7.58 (m, 1H), 7.54 – 7.40 (m, 2H), 5.13 (s, 2H), 4.84 – 4.67 (m, 2H), 4.56 – 4.38 (m, 2H), 3.73 (d, *J* = 5.6 Hz, 2H), 3.12 (br, 1H), 2.79 – 2.64 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.95, 161.29, 154.24, 154.15, 152.74, 152.10, 146.93, 142.88,

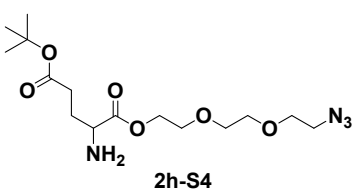
141.33, 133.74, 131.43, 129.60, 129.17, 127.01, 125.61, 125.46, 120.62, 112.00, 111.19, 64.94, 61.85, 60.35, 46.59, 40.92. HRMS (ESI) m/z calcd for $C_{24}H_{20}N_6O_7SNa^+$ $[M+H]^+$ 559.1006, found 559.1022.



Fmoc-Lys(Boc)-OH (**2g-S1**, 4 mmol, 1.0 eq.), HATU (1.1 eq.) and DIEA (2.0 eq.) were dissolved in 10 mL DMF, ethanolamine (1.2 eq.) was added and stirred at room temperature until reaction completion. The suspension was diluted with EtOAc (30 mL) and washed successively with saturated aqueous $NaHCO_3$ (15 mL), water (15 mL) and brine (15 mL). The organic extract was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford the crude product **2g-S2**. The crude **2g-S2** was dissolved in 10 mL DCM, 5 mL TFA was added and the reaction mixture was stirred at room temperature for 2 h. Then, the mixture was concentrated under reduced pressure, added to a mixture of biotin (4 mmol), HATU (4 mmol) and DIEA (12 mmol) in 10 mL DMF, and stirred at room temperature until reaction completion. The suspension was diluted with EtOAc (30 mL) and washed successively with saturated aqueous $NaHCO_3$ (15 mL), water (15 mL) and brine (15 mL). The organic extract was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford the crude product **2g-S3**. Then **2g-S3** was dissolved in 10 mL DCM, 5 mL Et_2NH was added and stirred at room temperature until reaction completion. The mixture was concentrated under reduced pressure and

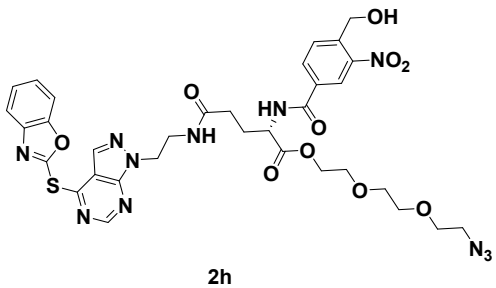
purified by RP-HPLC to afford the key intermediate **2g-S4**. Then according to the general procedure Ⅰ to afford the **2g** as a white solid. 7% total yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.70 (s, 1H), 8.67 (d, *J* = 8.0 Hz, 1H), 8.55 (s, 1H), 8.23 (d, *J* = 8.0 Hz, 1H), 8.13 (s, 1H), 8.09 (t, *J* = 5.6 Hz, 1H), 7.92 (t, *J* = 8.4 Hz, 2H), 7.84 (d, *J* = 8.0 Hz, 1H), 7.74 (t, *J* = 5.2 Hz, 1H), 7.61 – 7.46 (m, 2H), 6.42 (s, 1H), 6.37 (s, 1H), 4.89 (s, 2H), 4.52 (t, *J* = 5.2 Hz, 2H), 4.37 – 4.18 (m, 2H), 4.17 – 3.99 (m, 1H), 3.72 – 3.62 (m, 1H), 3.58 – 3.44 (m, 1H), 3.13 – 2.93 (m, 3H), 2.81 (dd, *J* = 12.4, 5.2 Hz, 1H), 2.57 (d, *J* = 12.4 Hz, 1H), 2.02 (t, *J* = 7.2 Hz, 2H), 1.68 – 1.39 (m, 6H), 1.35 – 1.08 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.33, 164.54, 163.19, 160.35, 154.92, 154.59, 152.62, 152.22, 146.96, 141.86, 141.66, 133.90, 132.97, 128.76, 127.38, 125.84, 123.85, 120.81, 112.21, 111.76, 61.49, 60.39, 59.66, 55.87, 54.05, 47.12, 40.32, 38.60, 38.55, 35.69, 31.46, 29.33, 28.67, 28.46, 25.78, 23.58. HRMS (ESI) m/z calcd for $C_{38}H_{43}N_{11}O_8S_2Na^+$ $[M+H]^+$ 868.2630, found 868.2631.





Fmoc-Glu(*t*Bu)-OH (**2h-S1**, 4 mmol, 1.0 eq.), DCC (1.1 eq.) and DMAP (0.1 eq.) were dissolved in 20 mL DCM, triglycol (1.5 eq.) was added and stirred at room temperature until reaction completion. The suspension was filtration and washed with DCM. The filtrate was

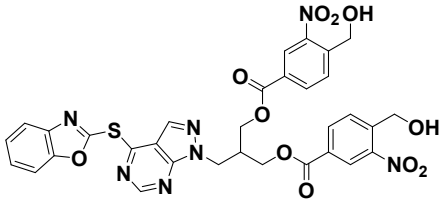
concentrated under reduced pressure and purified by silica gel (200−300 mesh) column chromatography to afford the desired intermediate **2h-S2**. **2h-S2** (1.0 eq.) and TEA (1.5 eq.) were dissolved in 20 mL DCM, MsCl (1.2 eq.) was added by dropwise at 0 °C and stirred at room temperature until reaction finished. The reaction mixture was washed with brine and the organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford the crude product **2h-S3** without further purification. Then **2h-S3** and NaN_3 (2.0 eq.) in DMF (5 mL) was stirred at 50 °C until reaction completion. The suspension was diluted with EtOAc (20 mL) and washed successively with saturated aqueous $NaHCO_3$ (10 mL), water (10 mL) and brine (10 mL). The organic extract was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude residue was purified by silica gel (200−300 mesh) column chromatography to afford the desired compound **2h-S4** as a colorless oil. 40% yield (3 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.35 – 4.19 (m, 1H), 3.78 – 3.62 (m, 4H), 3.50 (dd, *J* = 8.1, 5.3 Hz, 1H), 3.43 – 3.35 (m, 1H), 2.37 (t, *J* = 7.6 Hz, 1H), 2.10 – 1.96 (m, 1H), 1.88 – 1.73 (m, 1H), 1.44 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.67, 172.42, 80.40, 70.70, 70.62, 70.13, 69.12, 63.94, 53.78, 50.67, 31.77, 29.86, 28.09. HRMS (ESI) m/z calcd for $C_{15}H_{29}N_4O_6^+$ $[M+H]^+$ 361.2082, found 361.2069.



2h

S1 (2.2 mmol), HATU (2.2 mmol) and DIEA (4.0 mmol) were dissolved in 5 mL DMF, **2h-S4** (2.0 mmol) was added and stirred at room temperature until reaction completion. The suspension was diluted with EtOAc (20 mL) and washed successively with saturated aqueous $NaHCO_3$ (10 mL), water (10 mL) and brine (10 mL). The organic extract was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure, followed by silica gel column chromatography affording the product **2h-S5**. Then **2h-S5** (1.6 mmol) was dissolved in 10 mL DCM, 5 mL TFA was added and stirred at room temperature until reaction finished. The mixture was concentrated under reduced pressure to remove DCM and TFA. The residue dissolved in 5 mL DMF, added with imidazole (3.2 mmol) and TBSCl (3.2 mmol), stirred at room temperature until reaction completion. The suspension was diluted with EtOAc (20 mL) and washed by 2 M HCl (aq.), the organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure, followed by silica gel column chromatography affording the key intermediate **2h-S6**. Then according to the general procedure Ⅰ affording the **2h** as a white solid. 8% total yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.63 (s, 1H), 8.49 (d, *J* = 6.4 Hz, 1H), 8.43 (d, *J* = 1.2 Hz, 1H), 8.01 (d, *J* = 7.2 Hz, 1H), 7.86 (d, *J* = 8.4 Hz, 1H), 7.81 (dd, *J* = 6.8, 2.0 Hz, 1H), 7.67 (s, 1H), 7.58 (dd, *J* = 6.8, 2.0 Hz, 1H), 7.51 – 7.38 (m, 2H), 6.94 (t, *J* = 5.6 Hz, 1H), 5.00 (s, 2H), 4.65 – 4.57 (m, 3H), 4.48 (brz, 1H), 4.38 – 4.21 (m, 2H), 3.87 – 3.74 (m, 2H), 3.71 (t, *J* = 4.8 Hz, 2H), 3.67 – 3.55 (m, 6H), 3.36 (t, *J* = 4.8 Hz, 2H), 2.46 – 2.29 (m, 2H), 2.28 – 2.07 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.74, 171.43, 165.09, 160.81, 154.70, 154.31, 152.56, 152.20, 146.77,

141.38, 141.25, 133.26, 132.25, 131.62, 129.30, 126.85, 125.37, 123.69, 120.53, 112.39, 111.11, 70.56, 70.40, 69.91, 68.93, 64.44, 61.67, 53.19, 50.62, 46.86, 39.48, 32.18, 26.38. HRMS (ESI) m/z calcd for $C_{33}H_{35}N_{11}O_{10}SNa^+$ $[M+H]^+$ 800.2181, found 800.2173.

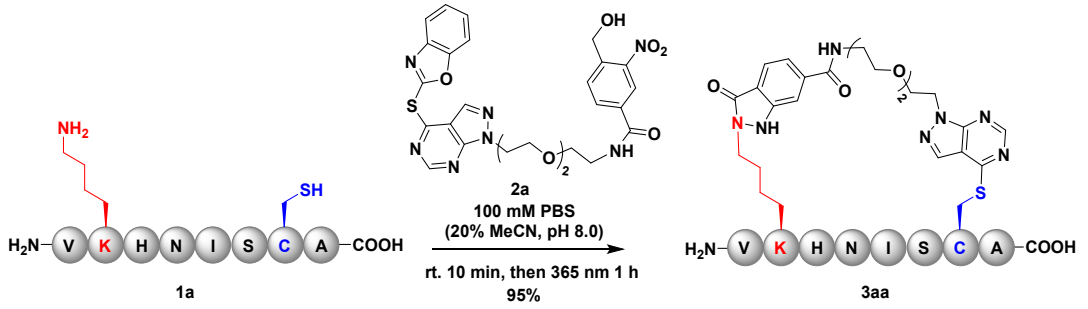


2i

General procedure Ⅱ, **2i**, white solid, 4% total yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.60 (s, 1H), 8.34 (d, J = 1.2 Hz, 2H), 8.17 (dd, J = 8.0, 0.8 Hz, 2H), 8.15 (s, 1H), 7.97 (d, J = 8.4 Hz, 2H), 7.88 (d, J = 8.0 Hz, 1H), 7.81 (d, J = 8.0 Hz, 1H), 4.58 – 4.47 (m, 2H), 5.72 (br, 2H), 4.92 (s, 4H), 4.84 (d, J = 6.4 Hz, 2H), 4.61 – 4.53 (m, 2H), 4.52 – 4.43 (m, 2H), 3.19 – 3.04 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.18, 160.78, 154.66, 154.59, 152.61, 152.20, 146.91, 144.33, 141.63, 134.14, 132.30, 129.28, 129.21, 127.37, 125.80, 125.18, 120.80, 112.01, 111.72, 64.60, 60.47, 46.65, 38.08. HRMS (ESI) m/z calcd for $C_{32}H_{26}N_7O_{11}S^+$ $[M+H]^+$ 716.1406, found 716.1415.

7. Cys directed peptides Cys-Lys stapling and bicyclization

General procedure for peptides Cys-Lys stapling: 0.5 μmol peptide (**1**) was dissolved in 400 μL non-degassed PBS buffer (100 mM, pH 8.0). Then, corresponding stapling reagent (**2**, 0.6 μmol) in 100 μL MeCN or DMSO was added. The resulting solution was stirred at room temperature for 10 min, followed by irradiating at 365 nm for another 1 h. After this time, the reaction was analyzed by HPLC-MS.



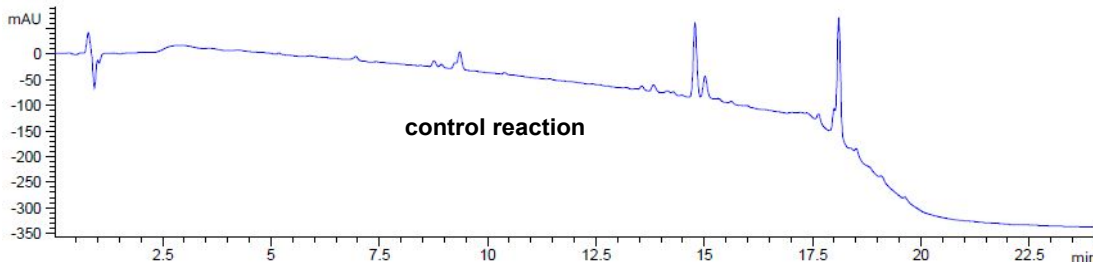


Figure S18. LC-MS UV spectrum of control reaction (without peptide) on 220 nm. Gradient used：Method A

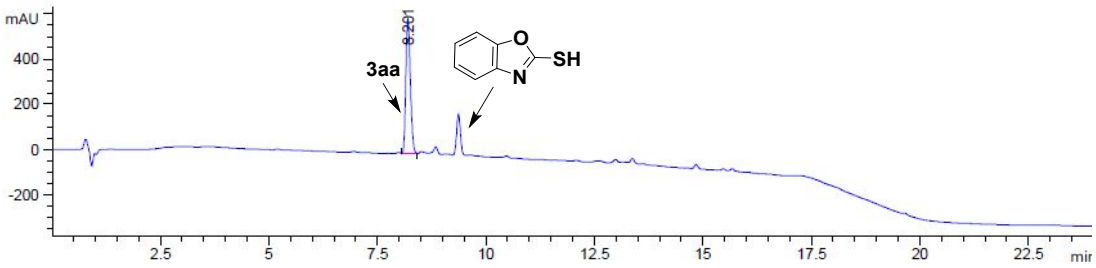


Figure S19. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

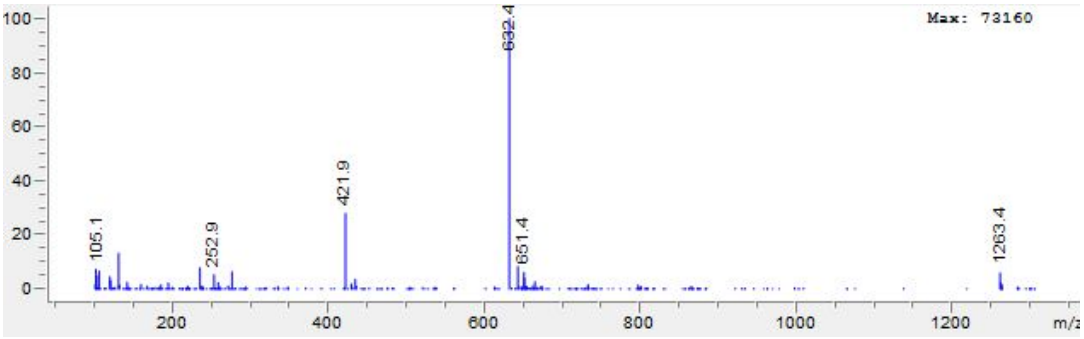


Figure S20. ESI Mass spectrum of product **3aa**. Calculated Mass $[M+H]^+$: 1263.6, $[M+2H]^{2+}$: 632.3; Mass Found (ESI+) $[M+H]^+$: 1263.4, $[M+2H]^{2+}$: 632.4.

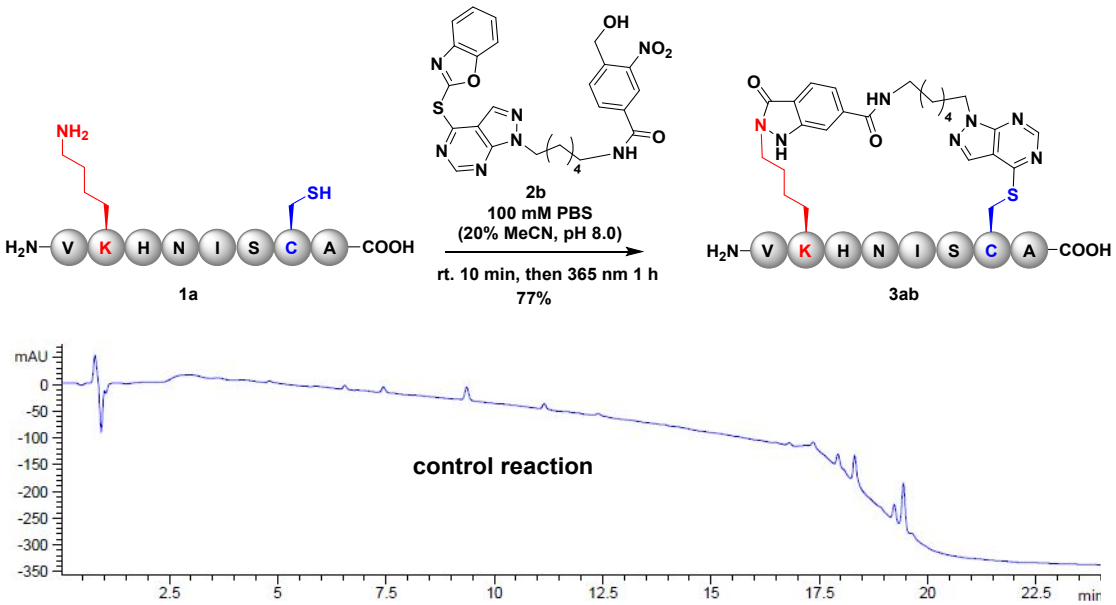


Figure S21. LC-MS UV spectrum of control reaction (without peptide) on 220 nm. Gradient used：Method A

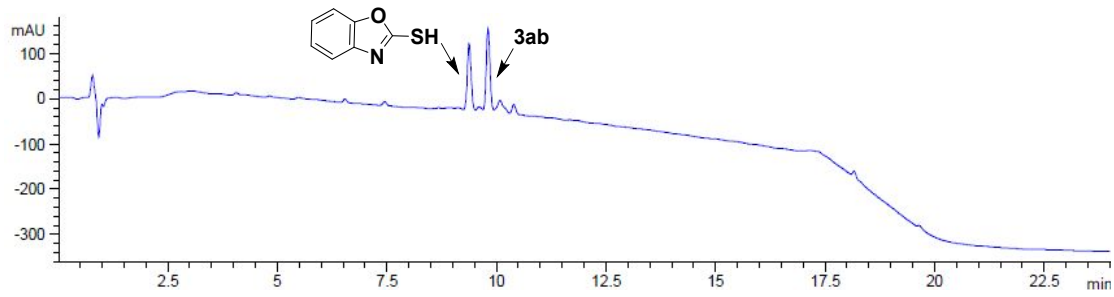


Figure S22. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

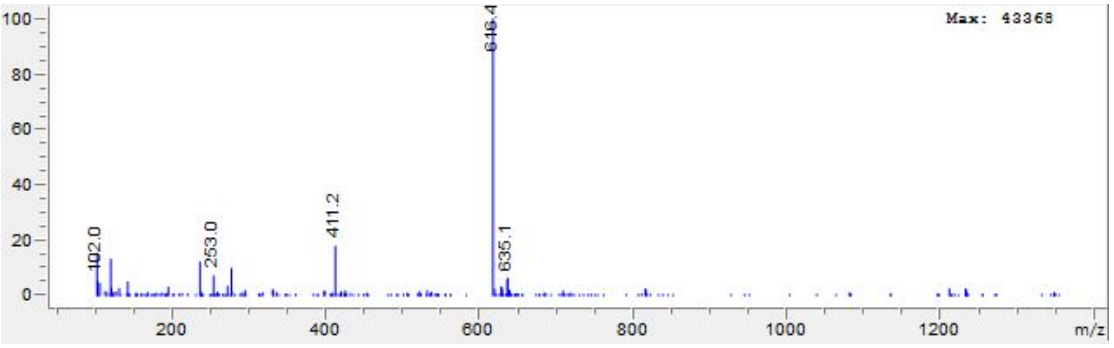


Figure S23. ESI Mass spectrum of product **3ab**. Calculated Mass $[M+2H]^{2+}$: 616.3; Mass Found (ESI+) $[M+2H]^{2+}$: 616.4.

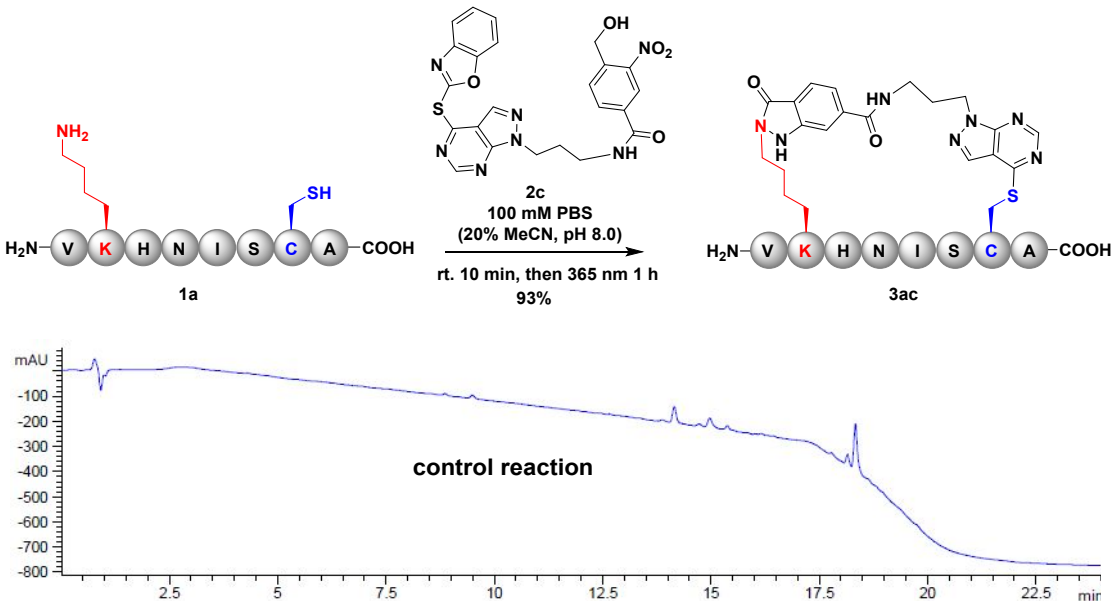


Figure S24. LC-MS UV spectrum of control reaction (without peptide) on 220 nm. Gradient used：Method A

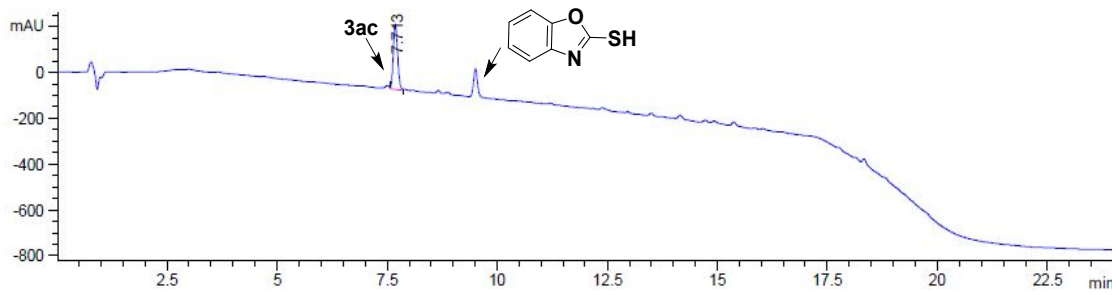


Figure S25. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

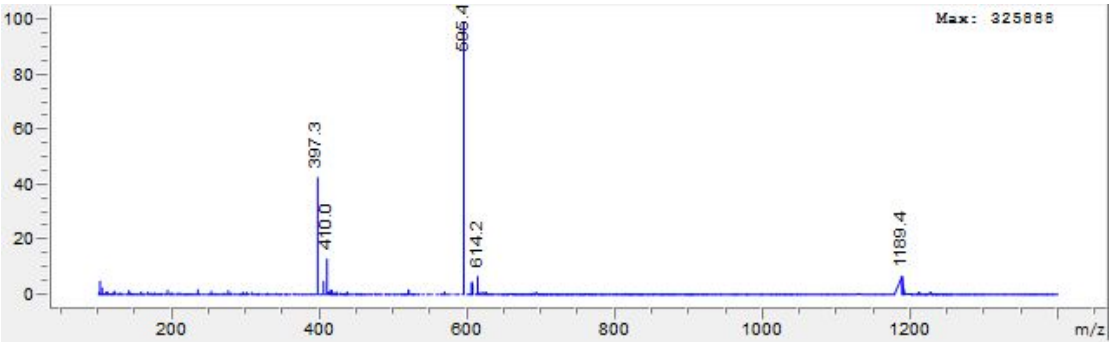


Figure S26. ESI Mass spectrum of product **3ac**. Calculated Mass $[M+H]^{+}$: 1189.5, $[M+2H]^{2+}$: 595.3, $[M+3H]^{3+}$: 397.2; Mass Found (ESI+) $[M+H]^{+}$: 1189.4, $[M+2H]^{2+}$: 595.4, $[M+3H]^{3+}$: 397.3.

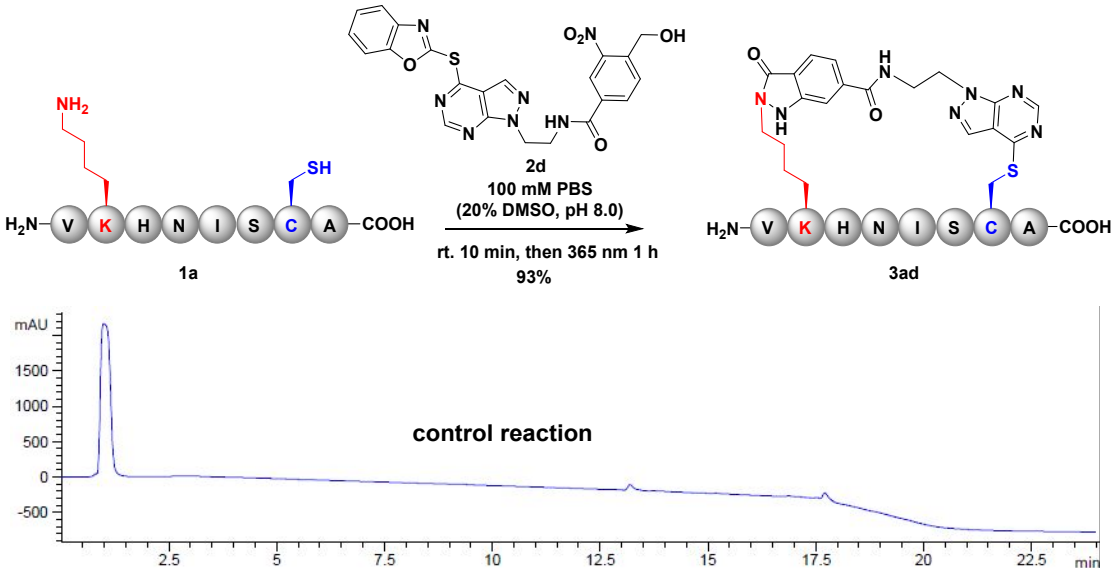


Figure S27. LC-MS UV spectrum of control reaction (without peptide) on 220 nm. Gradient used：Method A

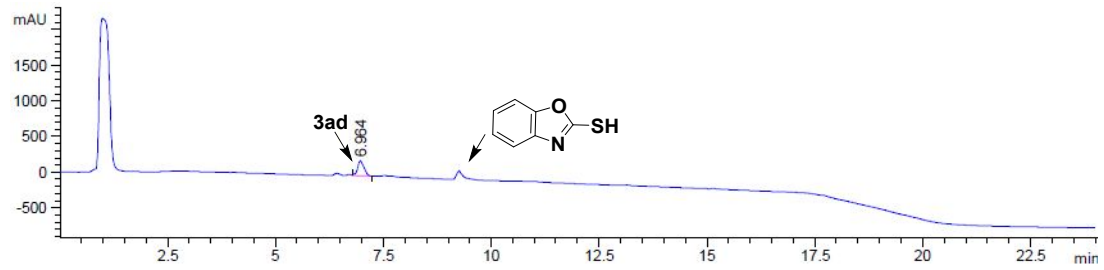


Figure S28. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

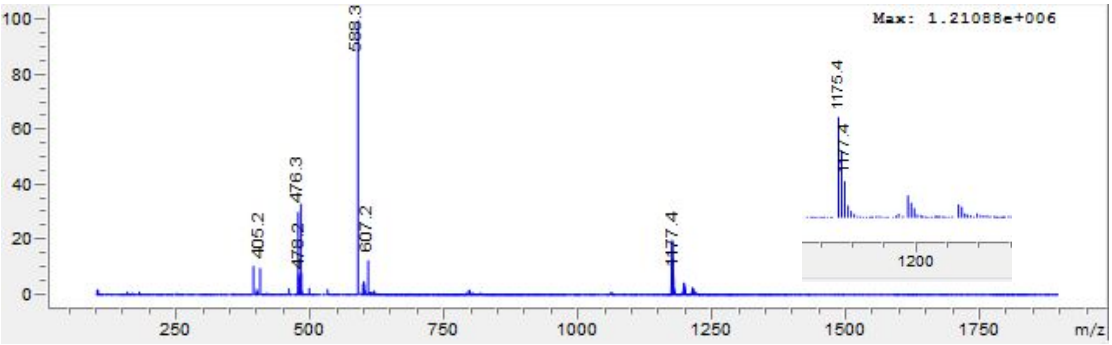


Figure S29. ESI Mass spectrum of product **3ad**. Calculated Mass $[M+H]^+$: 1175.5, $[M+2H]^{2+}$: 588.3; Mass Found (ESI+) $[M+H]^+$: 1175.4, $[M+2H]^{2+}$: 588.3.

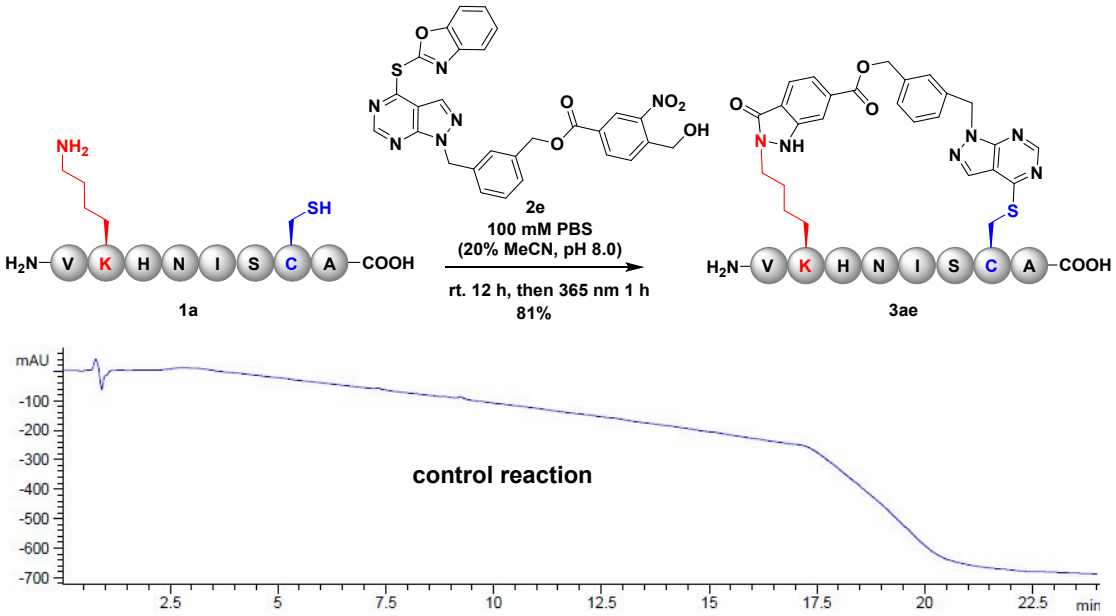


Figure S30. LC-MS UV spectrum of control reaction (without peptide) on 220 nm. Gradient used：Method A

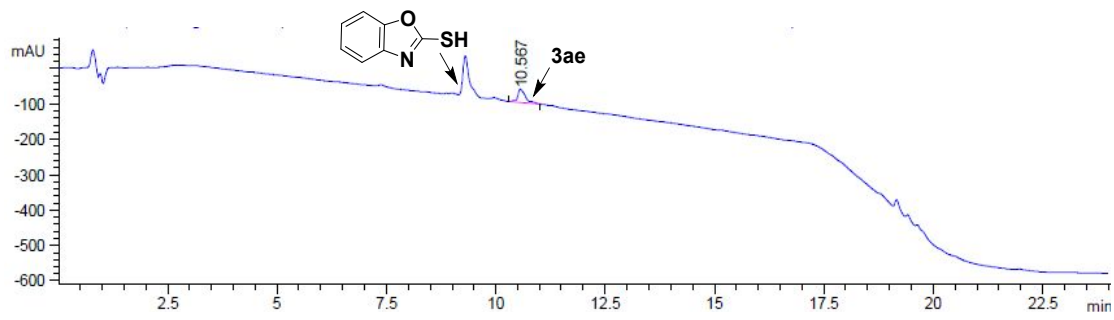


Figure S31. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

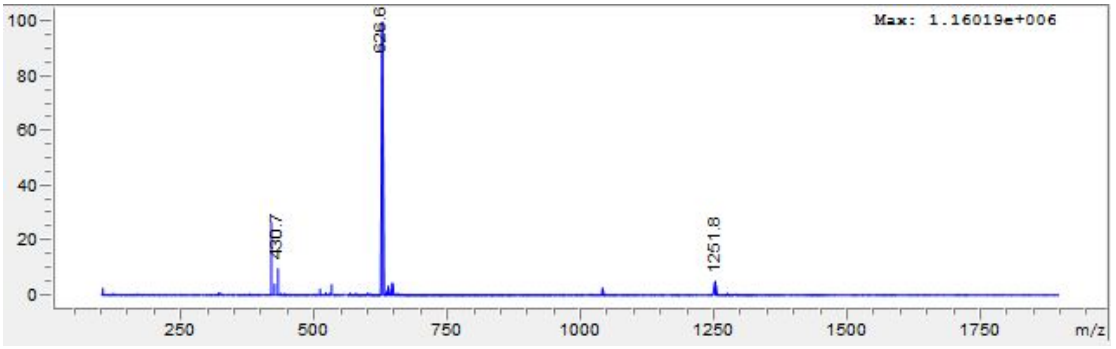


Figure S32. ESI Mass spectrum of product **3ae**. Calculated Mass $[M+H]^+$: 1252.5, $[M+2H]^{2+}$: 626.6; Mass Found (ESI+) $[M+H]^+$: 1251.8, $[M+2H]^{2+}$: 626.6.

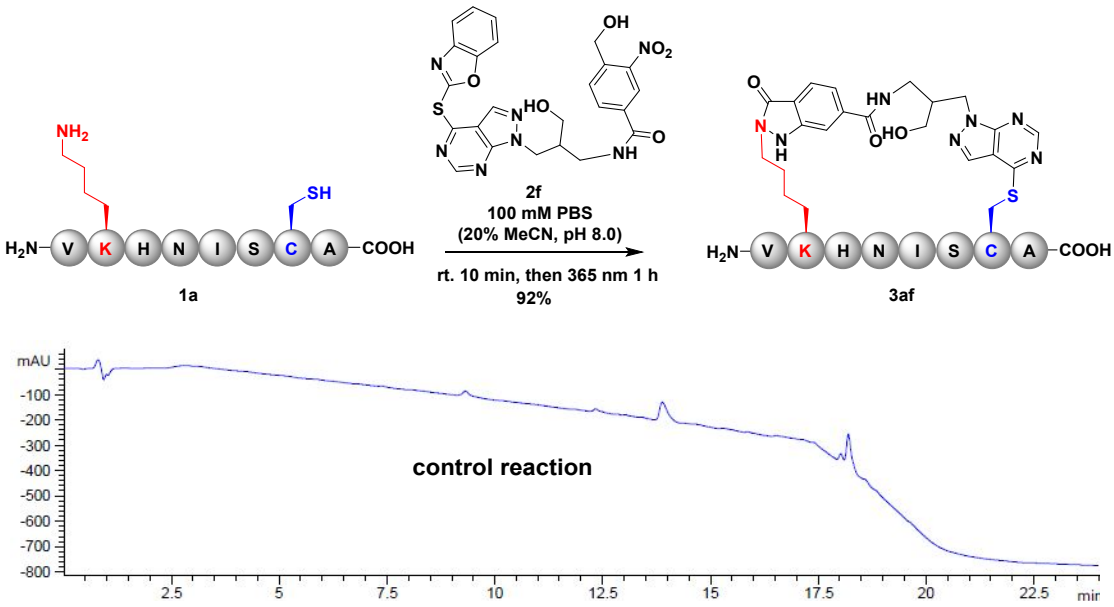


Figure S33. LC-MS UV spectrum of control reaction (without peptide) on 220 nm. Gradient used：Method A

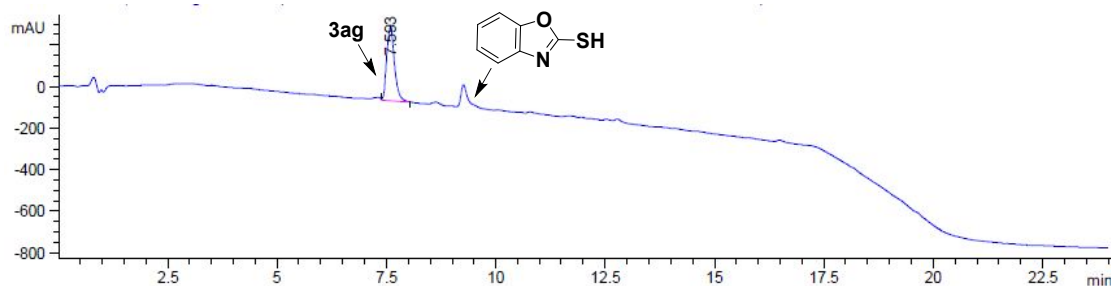


Figure S34. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

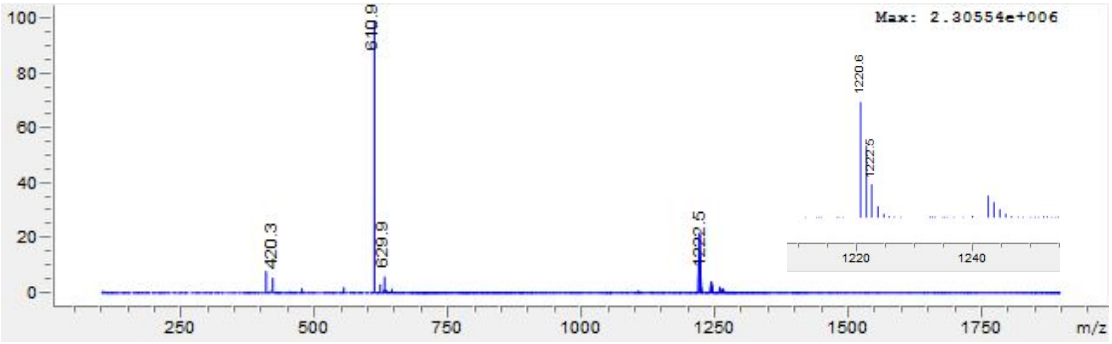
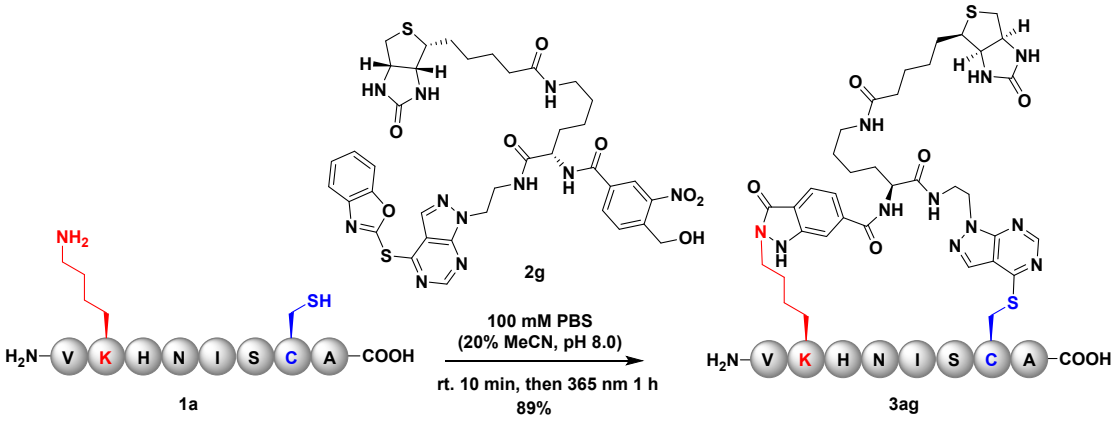


Figure S35. ESI Mass spectrum of product **3af**. Calculated Mass $[M+H]^+$: 1219.5, $[M+2H]^{2+}$: 610.3; Mass Found (ESI+) $[M+H]^+$: 1220.6, $[M+2H]^{2+}$: 610.9.



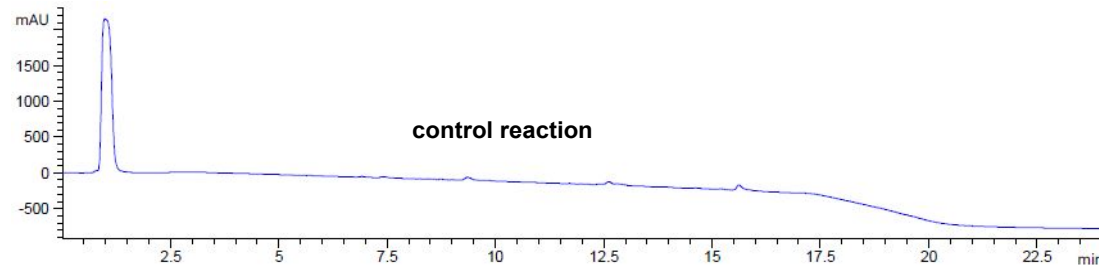


Figure S36. LC-MS UV spectrum of control reaction (without peptide) on 220 nm. Gradient used：Method A

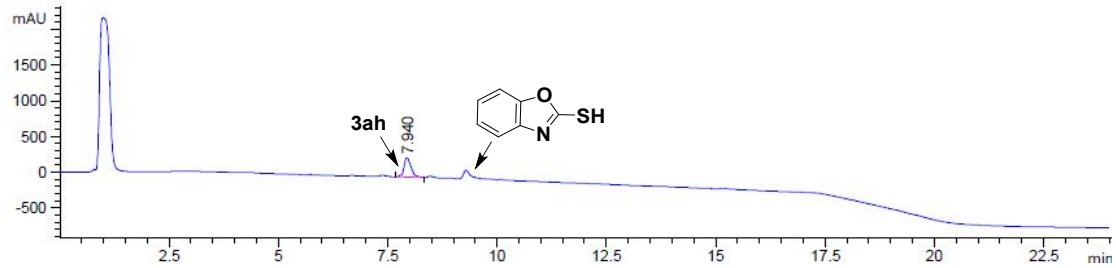


Figure S37. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

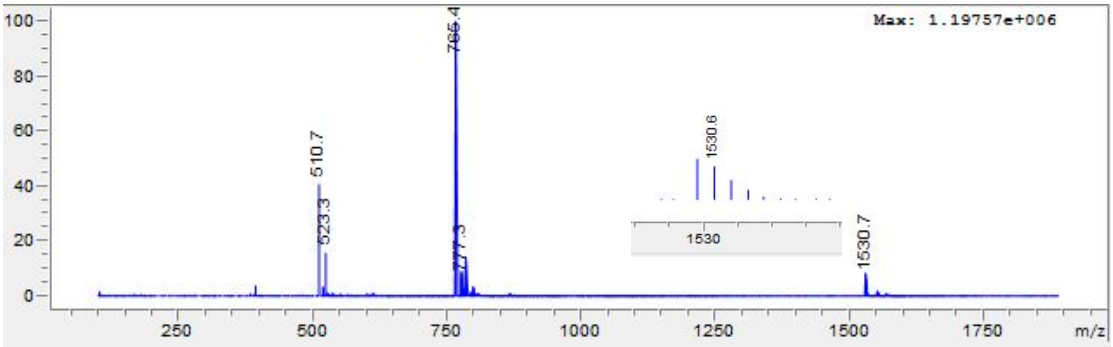
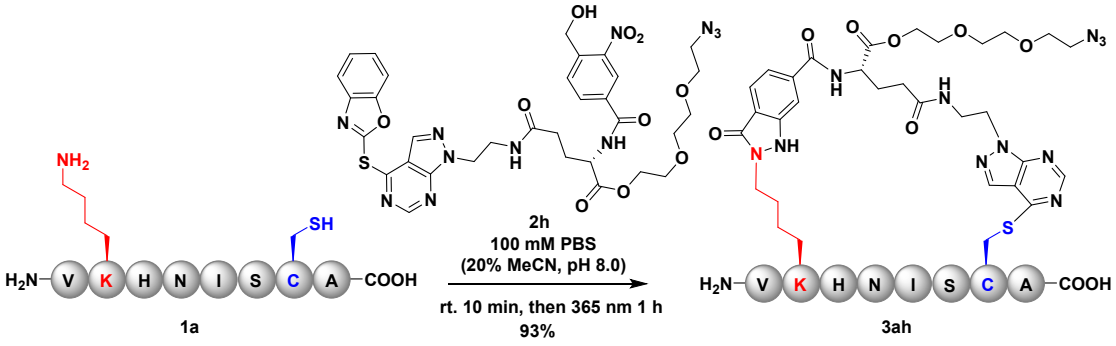


Figure S38. ESI Mass spectrum of product **3ag**. Calculated Mass $[M+H]^{+}$: 1529.7, $[M+2H]^{2+}$: 765.3, $[M+3H]^{3+}$: 510.6; Mass Found (ESI+) $[M+H]^{+}$: 1529.6, $[M+2H]^{2+}$: 765.4, $[M+3H]^{3+}$: 510.7.



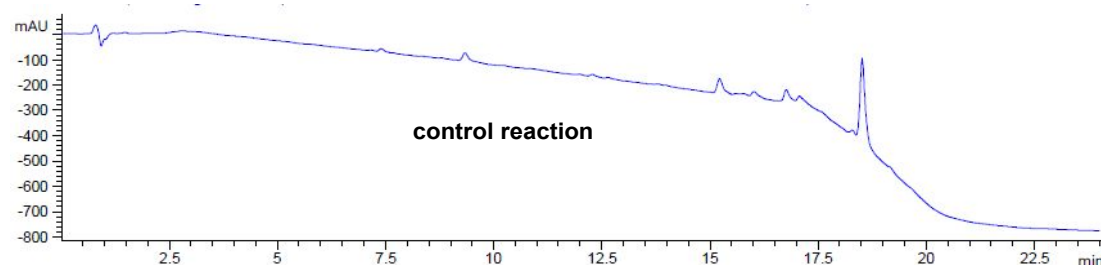


Figure S39. LC-MS UV spectrum of control reaction (without peptide) on 220 nm. Gradient used：Method A

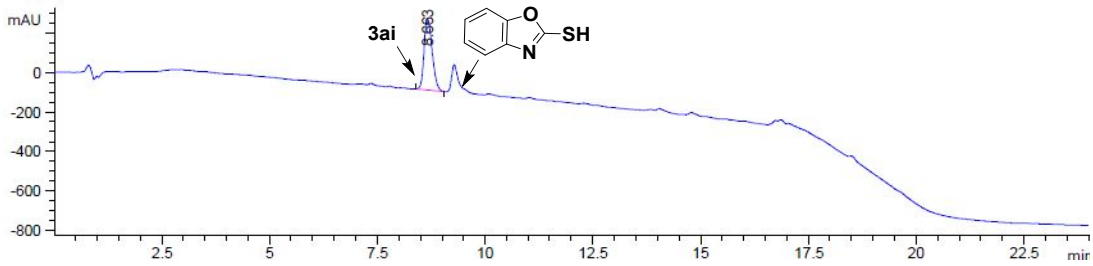


Figure S40. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

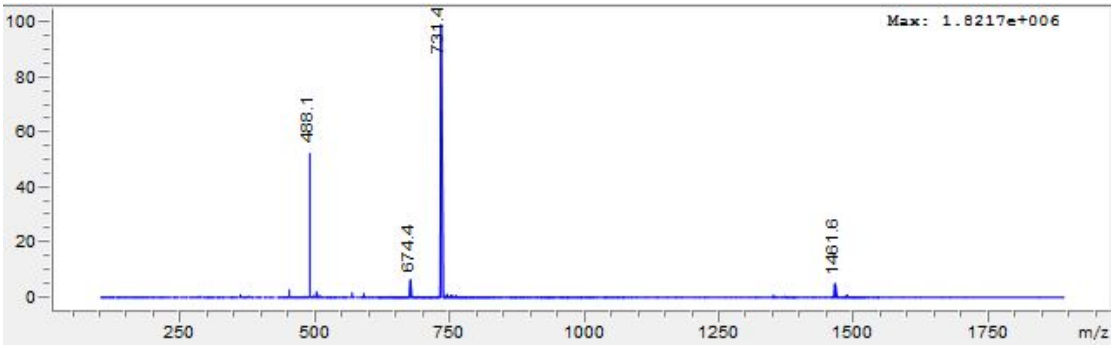


Figure S41. ESI Mass spectrum of product **3ah**. Calculated Mass $[M+H]^{+}$: 1461.6, $[M+2H]^{2+}$: 731.3, $[M+3H]^{3+}$: 487.9; Mass Found (ESI+) $[M+H]^{+}$: 1461.6, $[M+2H]^{2+}$: 731.4, $[M+3H]^{3+}$: 488.1.

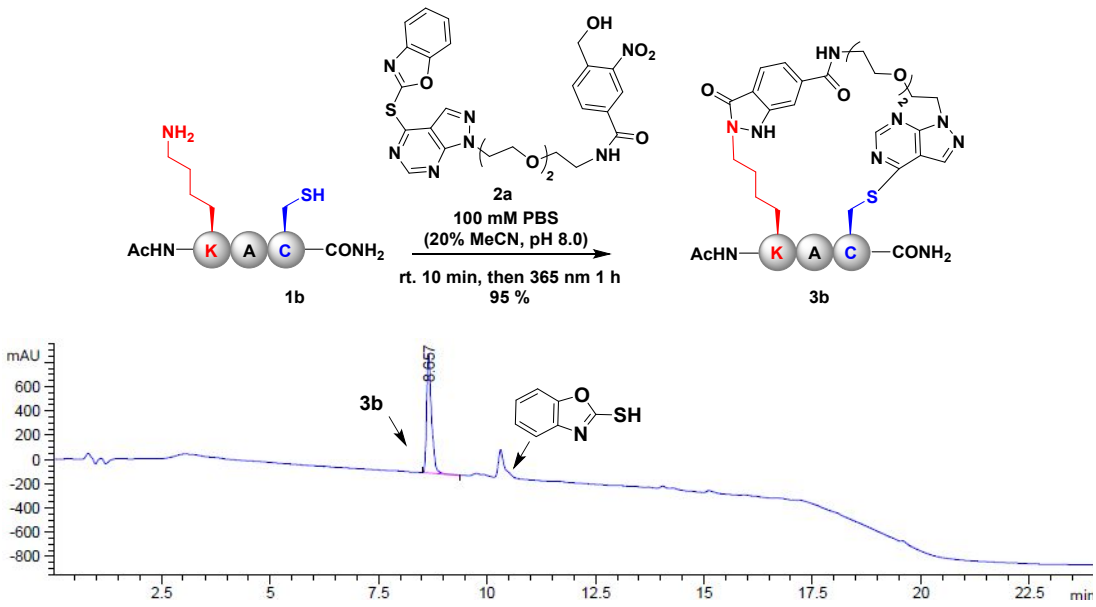


Figure S42. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method B

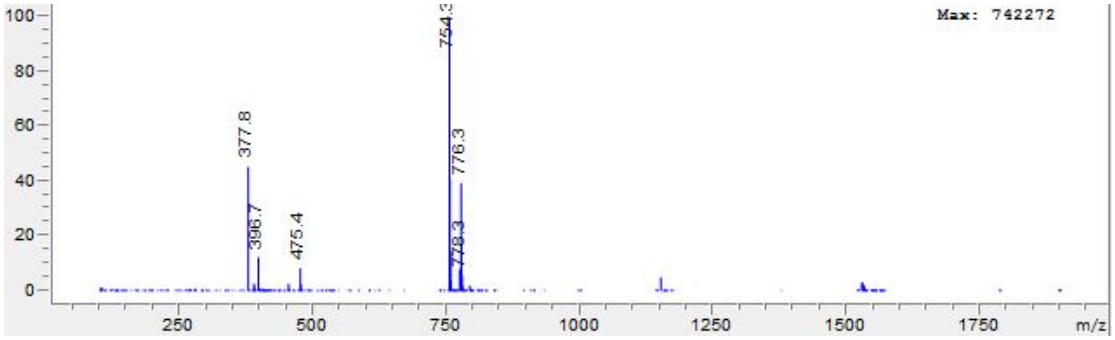
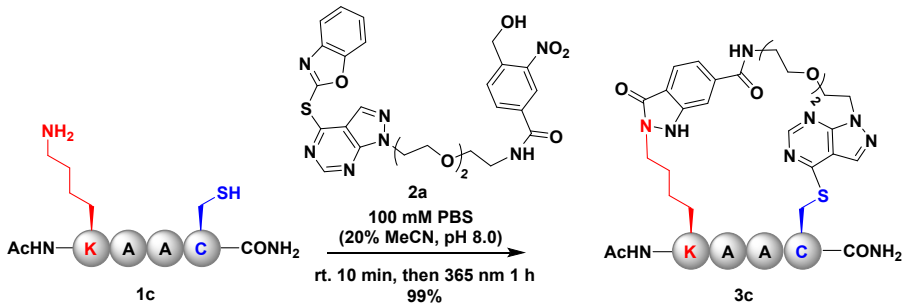


Figure S43. ESI Mass spectrum of product **3b**. Calculated Mass $[M+H]^+$: 754.3, $[M+2H]^{2+}$: 377.7; Mass Found (ESI+) $[M+H]^+$: 754.3, $[M+2H]^{2+}$: 377.7.



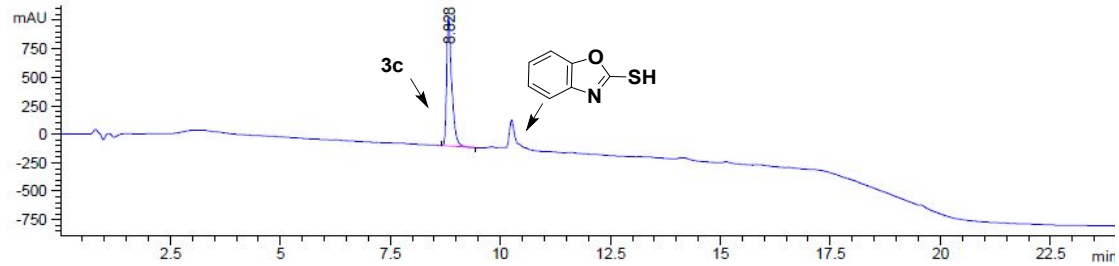


Figure S44. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method B

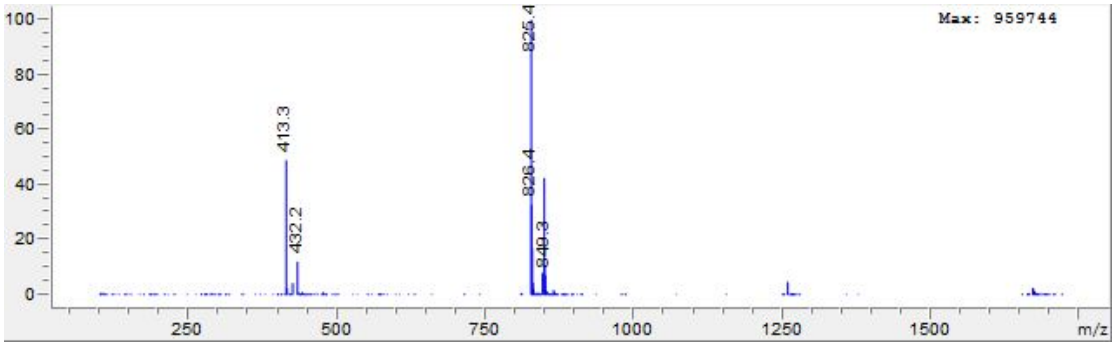


Figure S45. ESI Mass spectrum of product **3c**. Calculated Mass $[M+H]^+$: 825.3, $[M+2H]^{2+}$: 413.2; Mass Found (ESI+) $[M+H]^+$: 825.4, $[M+2H]^{2+}$:413.3.

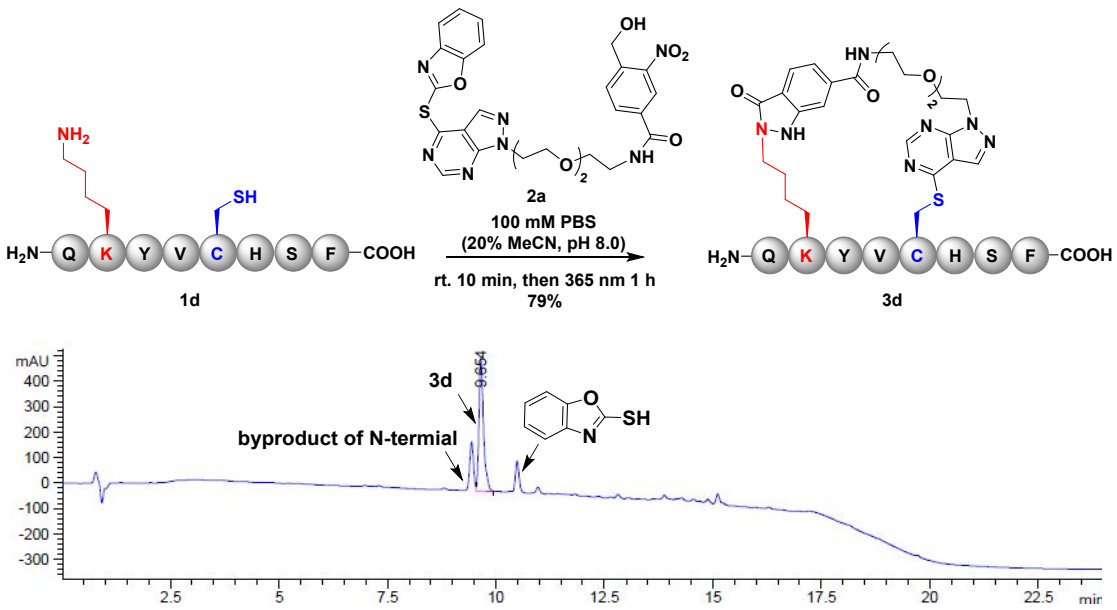


Figure S46. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

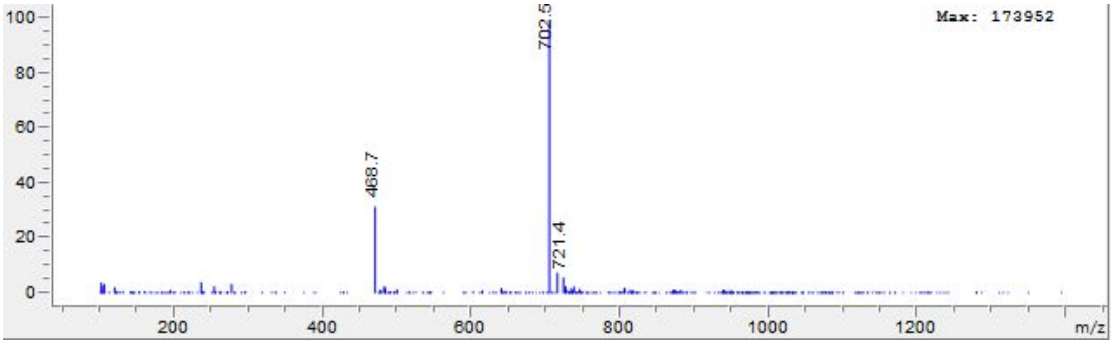
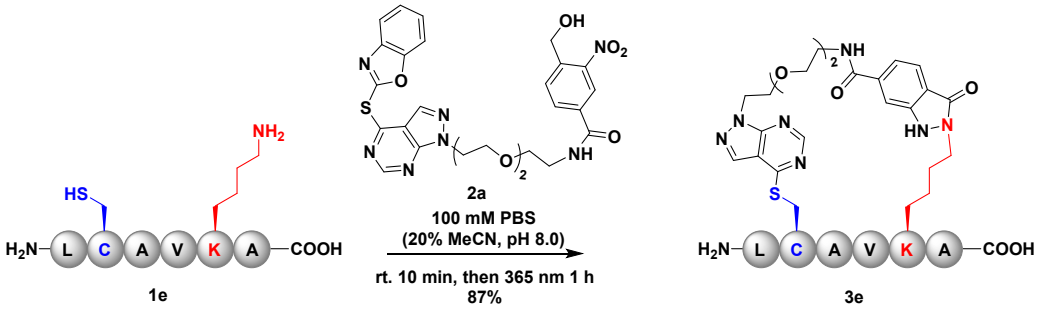


Figure S47. ESI Mass spectrum of product **3d**. Calculated Mass $[M+2H]^{2+}$: 702.3, $[M+3H]^{3+}$: 468.5; Mass Found (ESI+) $[M+2H]^{2+}$: 702.5, $[M+3H]^{3+}$: 468.7.



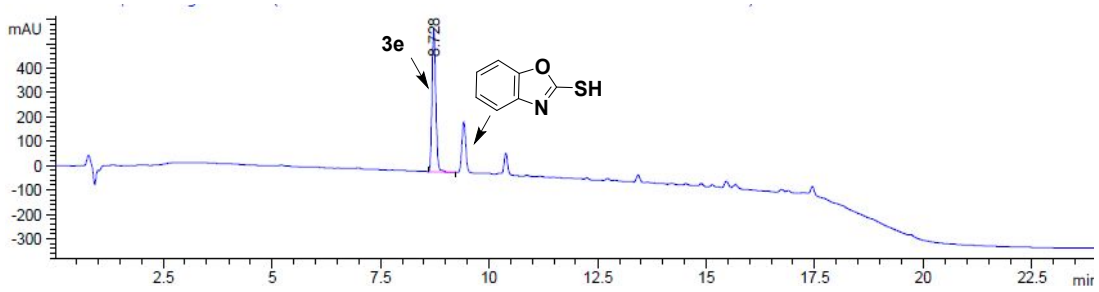


Figure S48. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

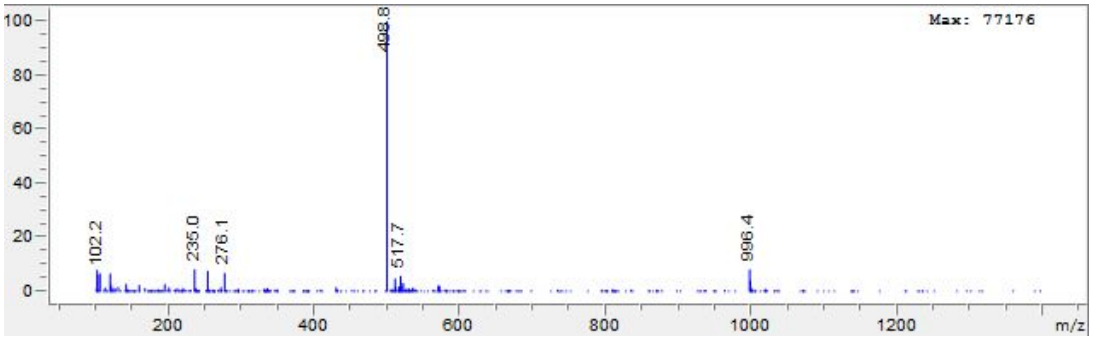
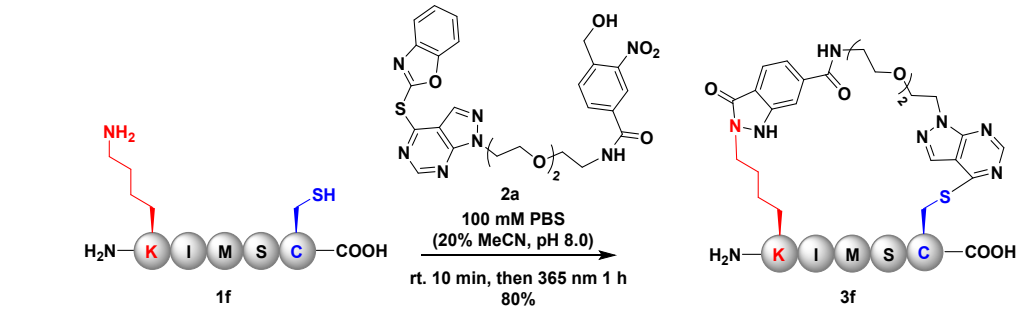


Figure S49. ESI Mass spectrum of product **3e**. Calculated Mass $[M+H]^{+}$: 996.5, $[M+2H]^{2+}$: 498.7; Mass Found (ESI+) $[M+H]^{+}$: 996.4, $[M+2H]^{2+}$: 498.8.



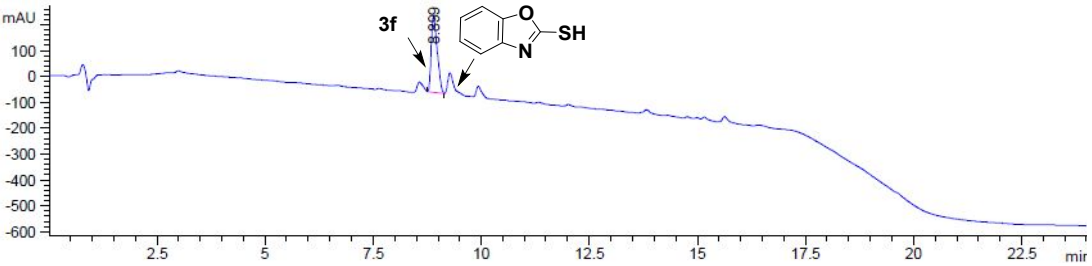


Figure S50. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

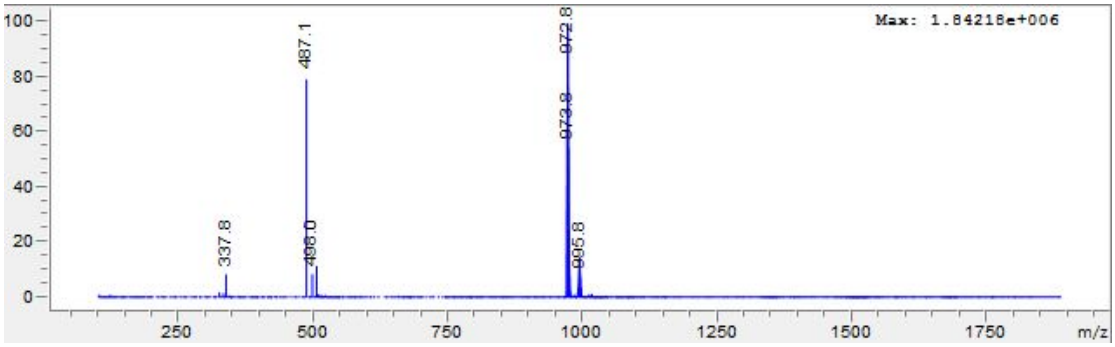
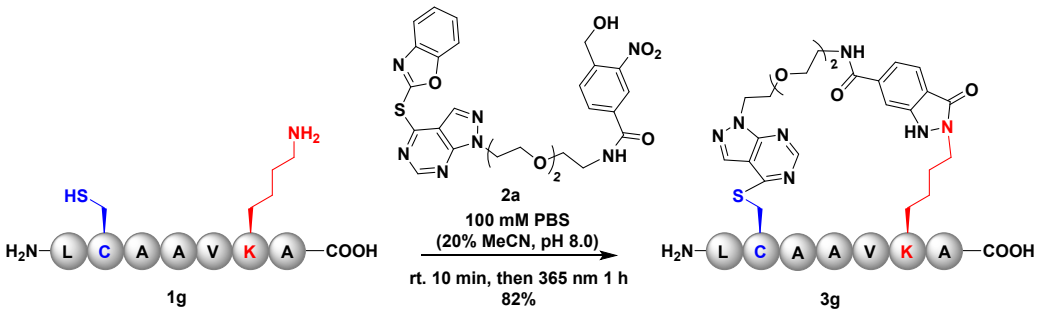


Figure S51. ESI Mass spectrum of product **3f**. Calculated Mass $[M+H]^+$: 973.4, $[M+2H]^{2+}$: 487.2; Mass Found (ESI+) $[M+H]^+$: 972.8, $[M+2H]^{2+}$: 487.1.



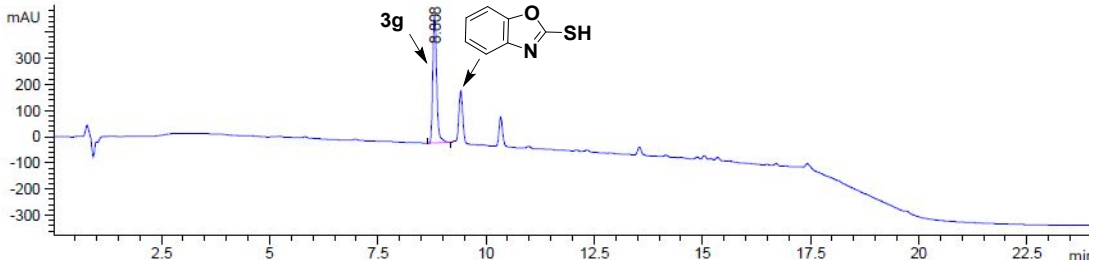


Figure S52. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

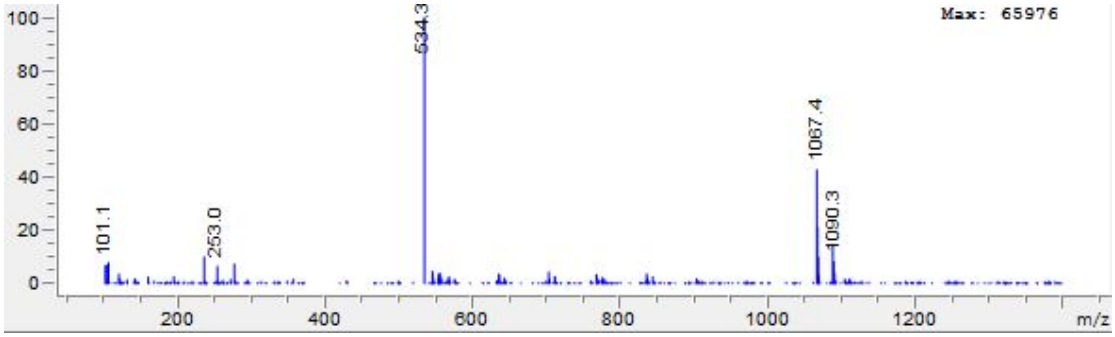


Figure S53. ESI Mass spectrum of product **3g**. Calculated Mass $[M+H]^+$: 1067.5, $[M+2H]^{2+}$: 534.3; Mass Found (ESI+) $[M+H]^+$: 1067.4, $[M+2H]^{2+}$: 534.3.

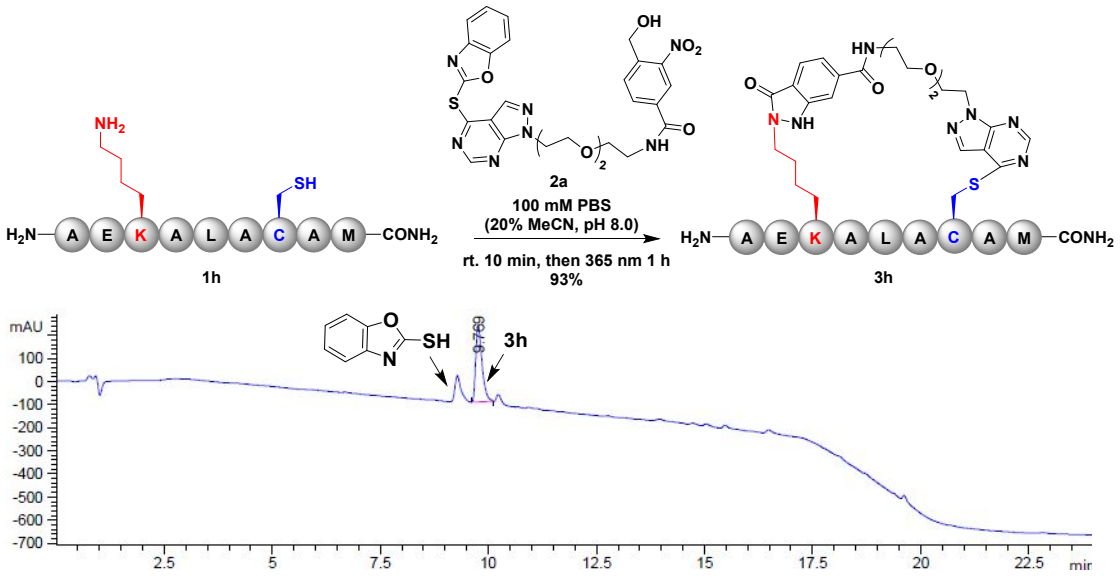


Figure S54. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

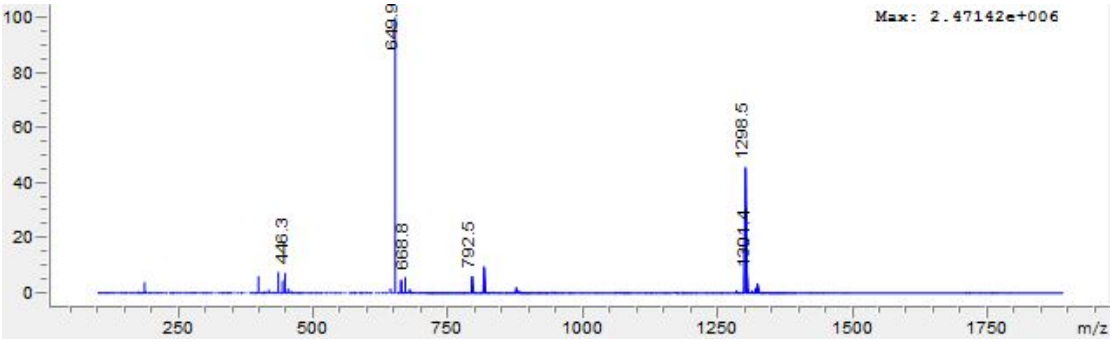


Figure S55. ESI Mass spectrum of product **3h**. Calculated Mass $[M+H]^+$: 1298.6, $[M+2H]^{2+}$: 649.8; Mass Found (ESI+) $[M+H]^+$: 1298.5, $[M+2H]^{2+}$: 649.9.

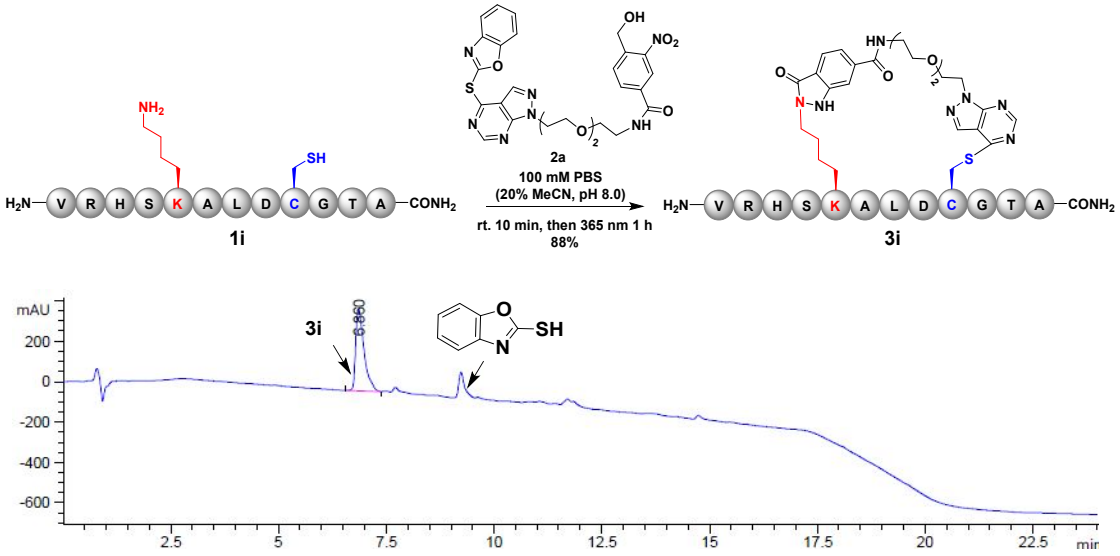


Figure S56. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

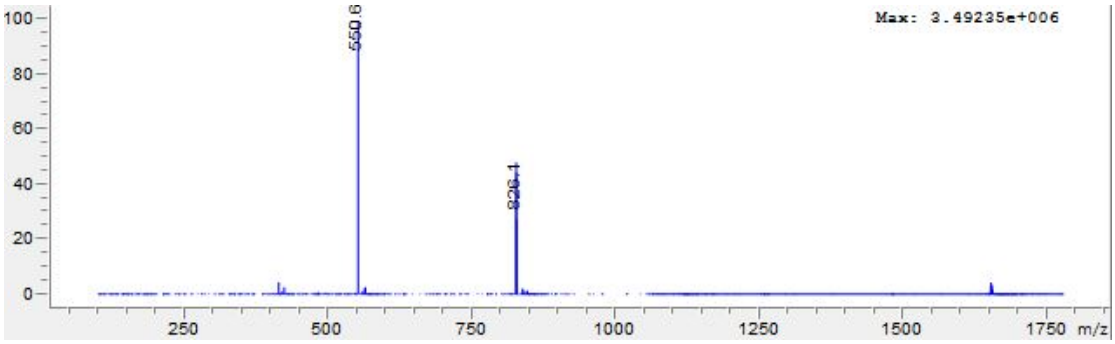
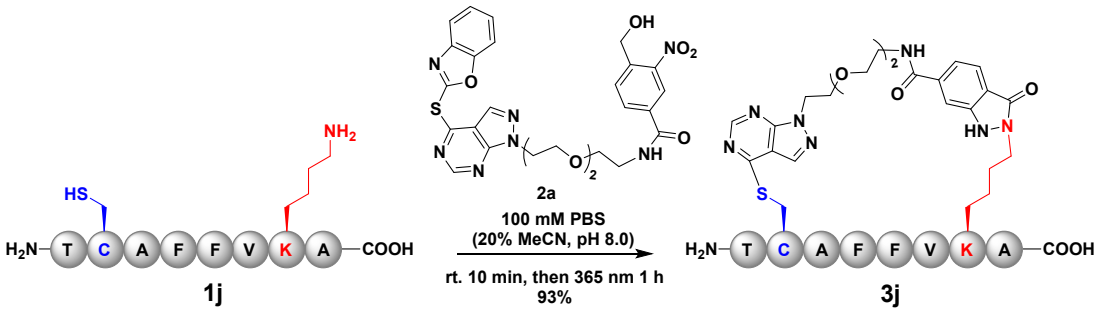


Figure S57. ESI Mass spectrum of product **3i**. Calculated Mass $[M+2H]^{2+}$: 825.9, $[M+3H]^{3+}$: 550.3; Mass Found (ESI+) $[M+2H]^{2+}$: 826.1, $[M+3H]^{3+}$: 550.6.



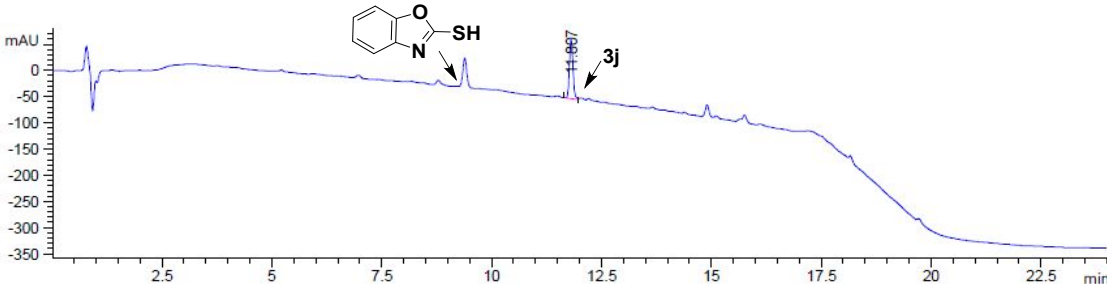


Figure S58. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

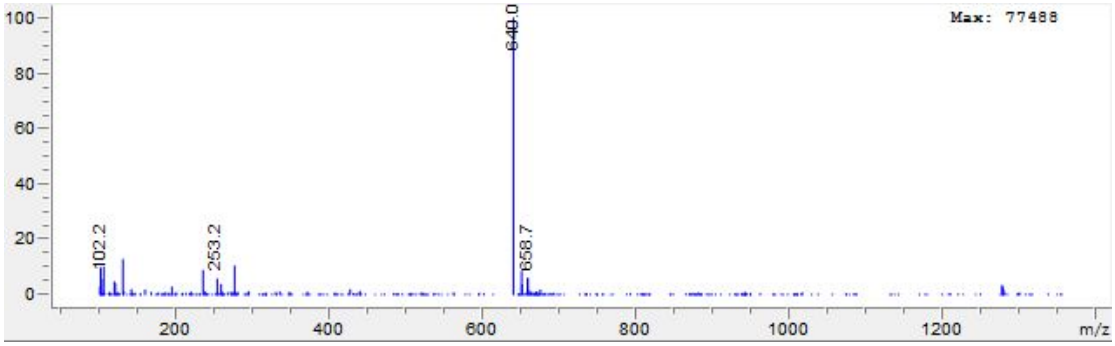


Figure S59. ESI Mass spectrum of product **3j**. Calculated Mass $[M+2H]^{2+}$: 639.8; Mass Found (ESI+) $[M+2H]^{2+}$: 640.0.

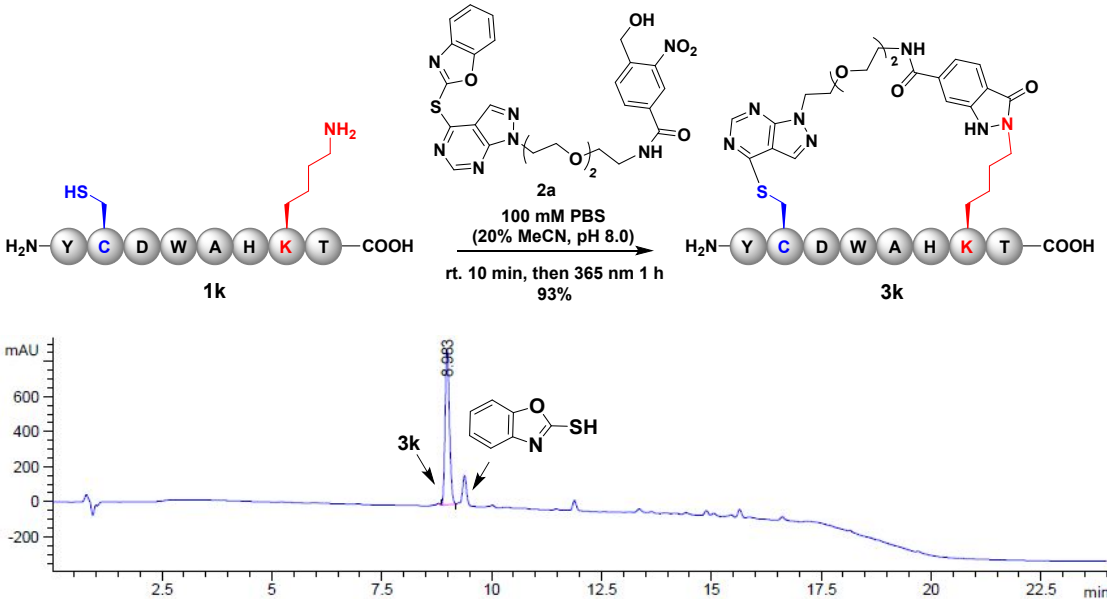


Figure S60. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

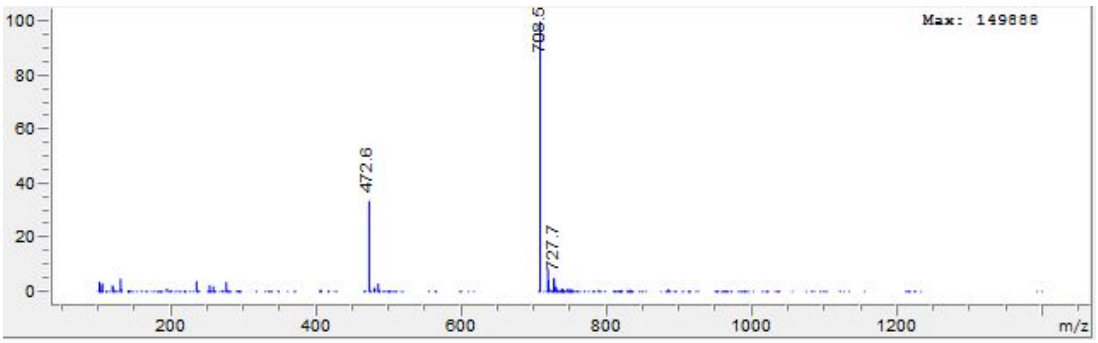


Figure S61. ESI Mass spectrum of product **3k**. Calculated Mass $[M+2H]^{2+}$: 708.3, $[M+3H]^{3+}$: 472.5; Mass Found (ESI+) $[M+2H]^{2+}$: 708.5, $[M+3H]^{3+}$: 472.6.

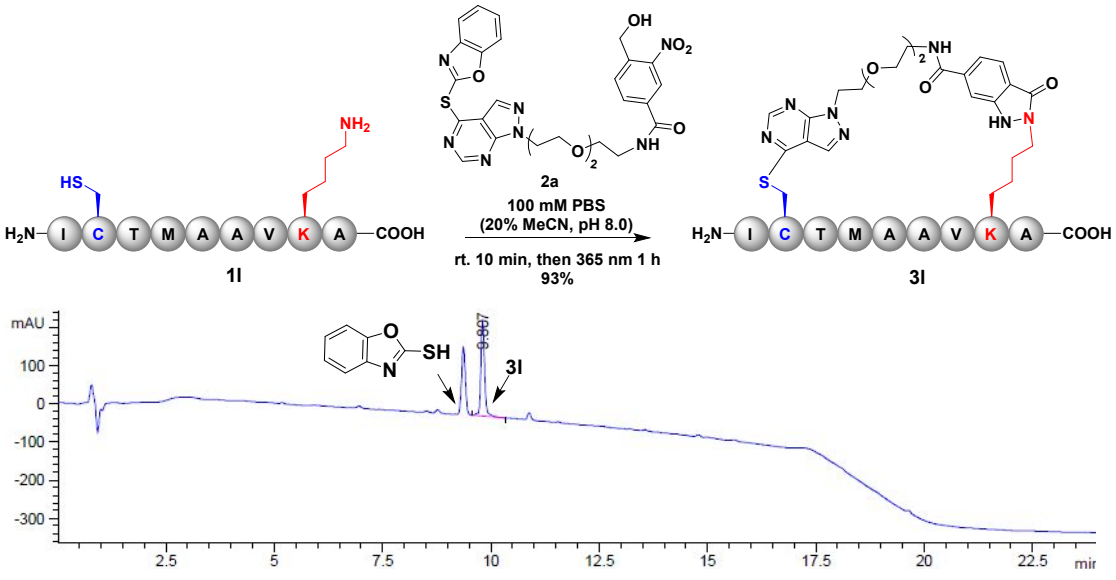


Figure S62. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

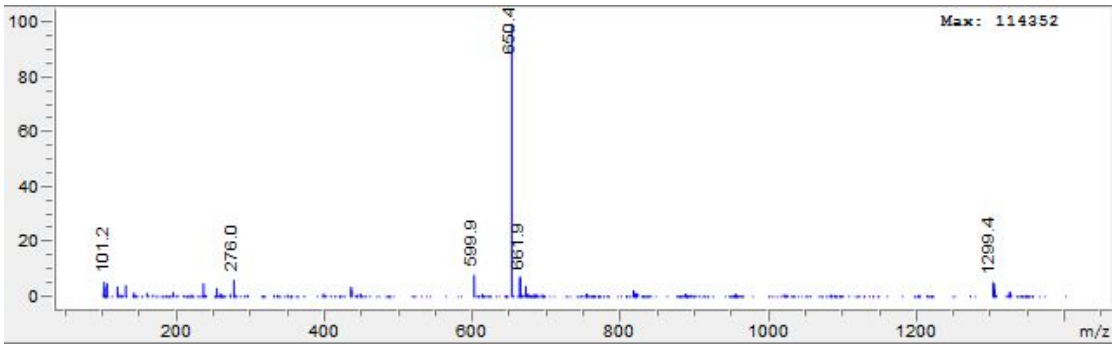


Figure S63. ESI Mass spectrum of product **3l**. Calculated Mass $[M+H]^{+}$: 1299.6, $[M+2H]^{2+}$: 650.3; Mass Found (ESI+) $[M+H]^{+}$: 1299.4, $[M+2H]^{2+}$: 650.4.

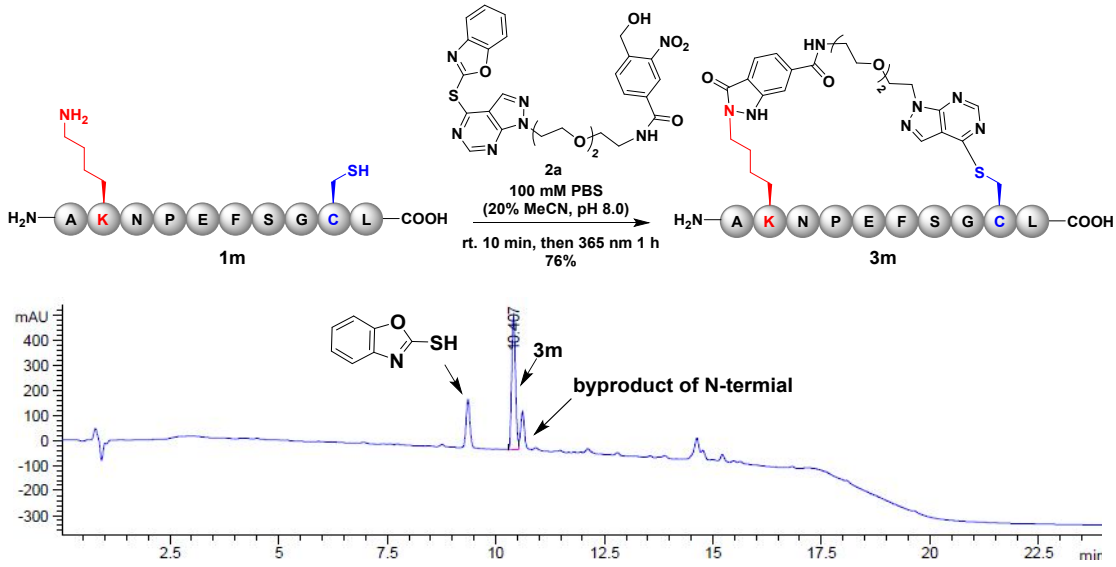


Figure S64. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

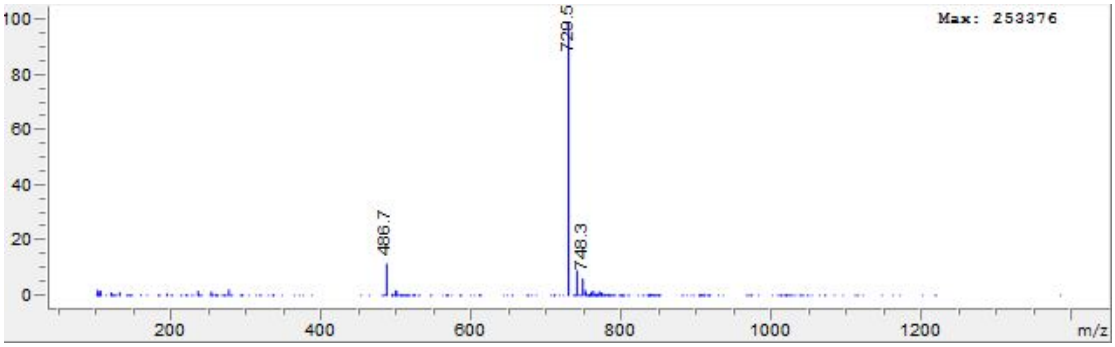


Figure S65. ESI Mass spectrum of product **3m**. Calculated Mass $[M+2H]^{2+}$: 729.3, $[M+3H]^{3+}$: 486.5; Mass Found (ESI+) $[M+2H]^{2+}$: 729.5, $[M+3H]^{3+}$: 486.7.

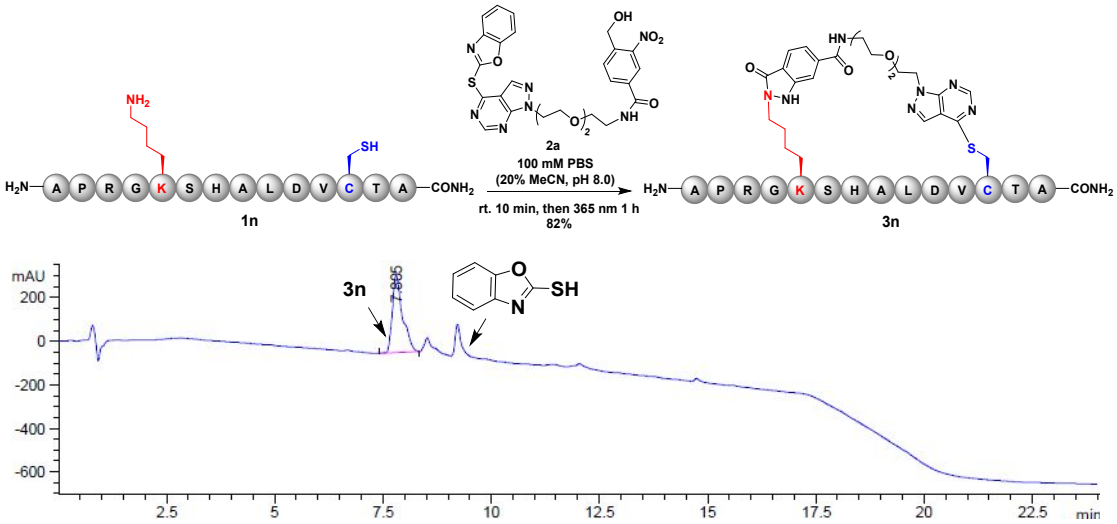


Figure S66. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

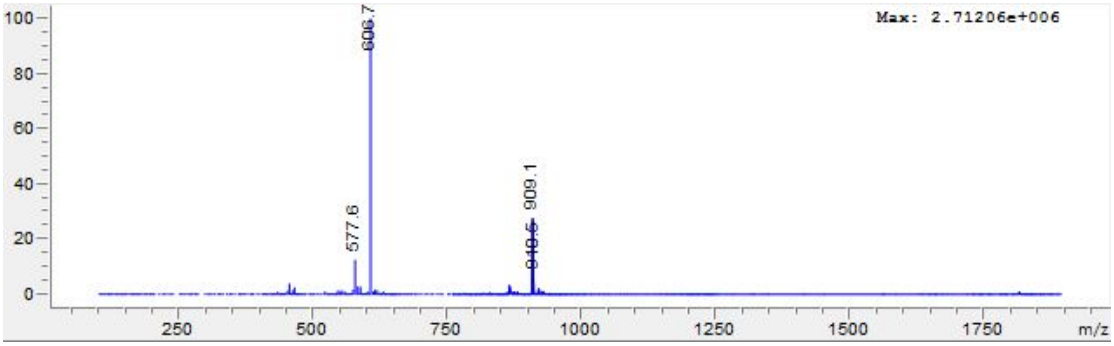


Figure S67. ESI Mass spectrum of product **3n**. Calculated Mass $[M+2H]^{2+}$: 909.4, $[M+3H]^{3+}$: 606.6; Mass Found (ESI+) $[M+2H]^{2+}$: 909.1, $[M+3H]^{3+}$: 606.7.

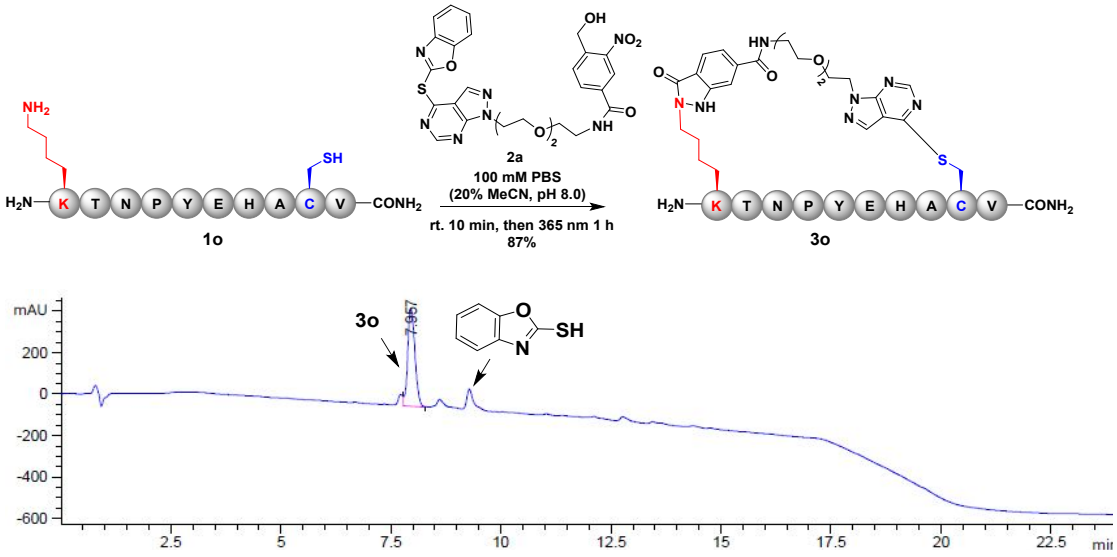


Figure S68. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method C

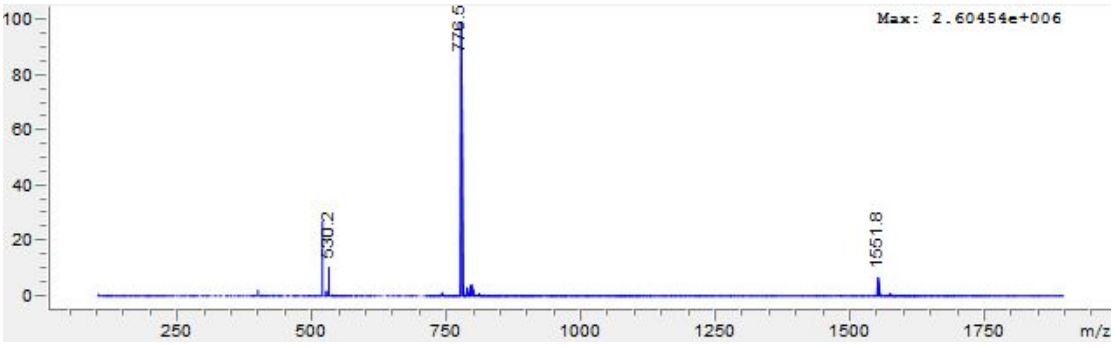
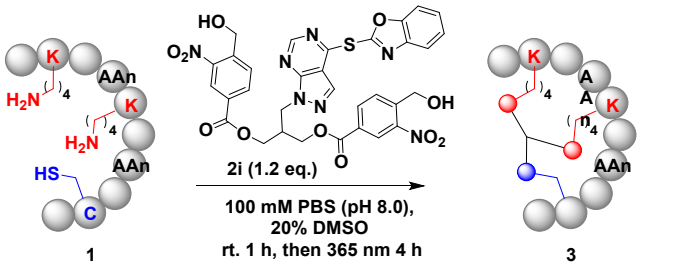


Figure S69. ESI Mass spectrum of product **3o**. Calculated Mass $[M+H]^+$: 1552.7, $[M+2H]^{2+}$: 776.9; Mass Found (ESI+) $[M+H]^+$: 1551.8, $[M+2H]^{2+}$: 776.5.



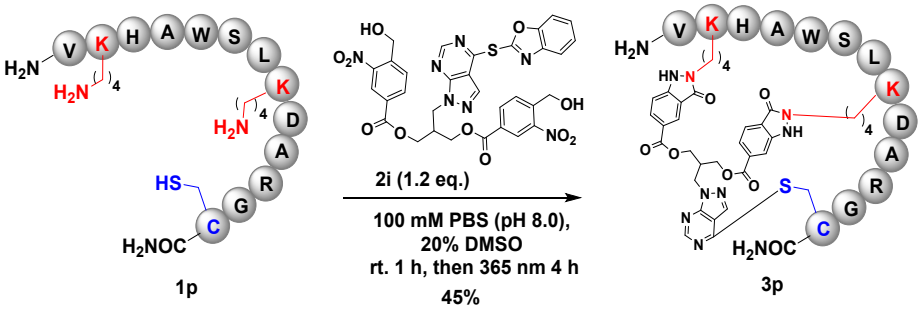
3p, NH_2-VKHAWSLKDARGC-$CONH_2$, 45%

3q, NH_2-VKLTNPFKAYEGCA-$CONH_2$, 71%

3r, NH_2-VLKSAPVCDATGKA-$CONH_2$, 71%

3s, NH_2-VKLSAPCGDATGKA-$CONH_2$, 69%

General procedure for peptides Cys directed bicyclization: 0.5 μmol peptide (**1**) was dissolved in 400 μL non-degassed PBS buffer (100 mM, pH 8.0). Then, corresponding stapling reagent (**2i**, 0.6 μmol) in 100μL DMSO was added. The resulting solution was stirred at room temperature for 1 h, followed by irradiating at 365nm for another 4 h. After this time, the reaction was analyzed by HPLC-MS.



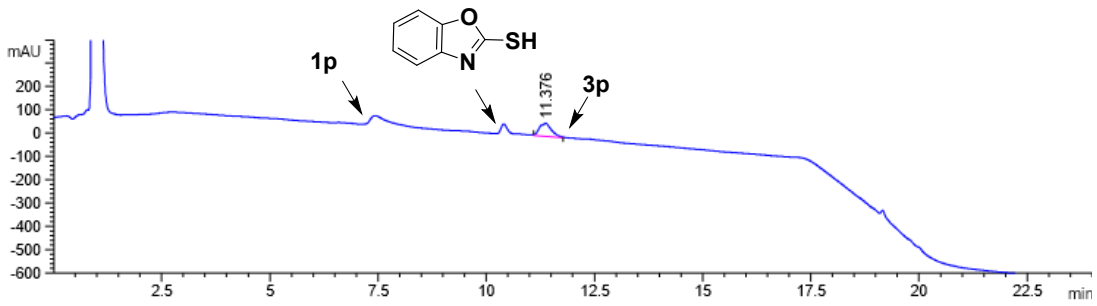


Figure S70. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A but 40% acetonitrile in 15 min.

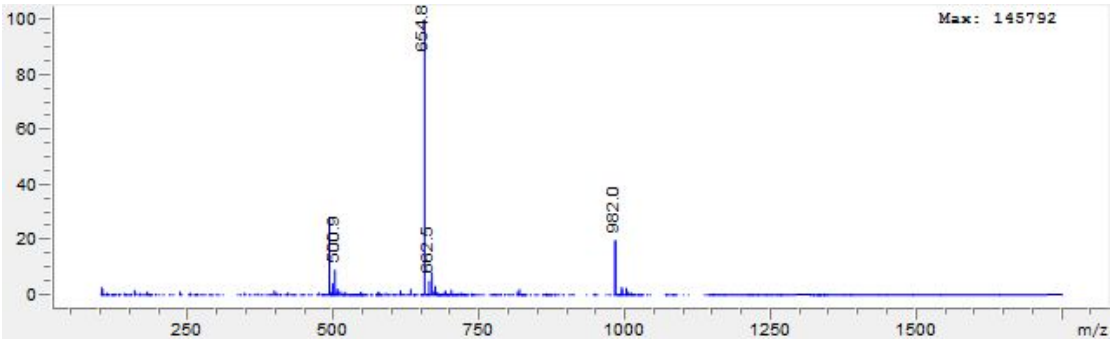
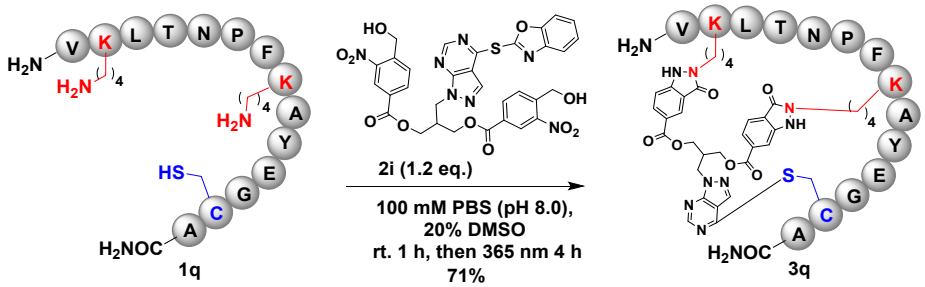


Figure S71. ESI Mass spectrum of product **3p**. Calculated Mass $[M+2H]^{2+}$: 981.4, $[M+3H]^{3+}$: 654.6; Mass Found (ESI+) $[M+2H]^{2+}$: 982.0, $[M+3H]^{3+}$: 654.8.



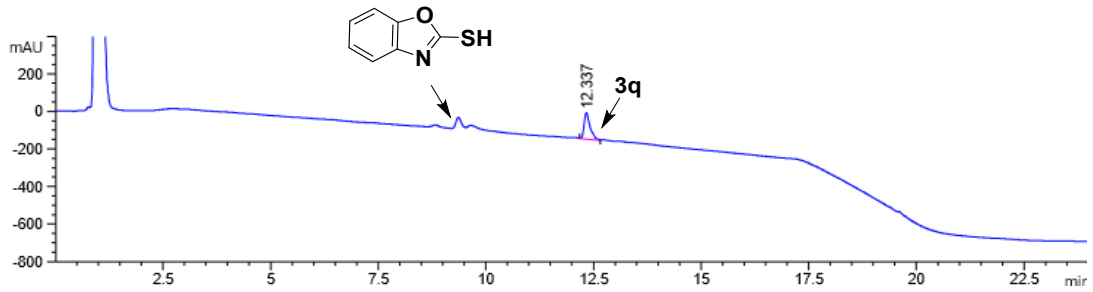


Figure S72. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

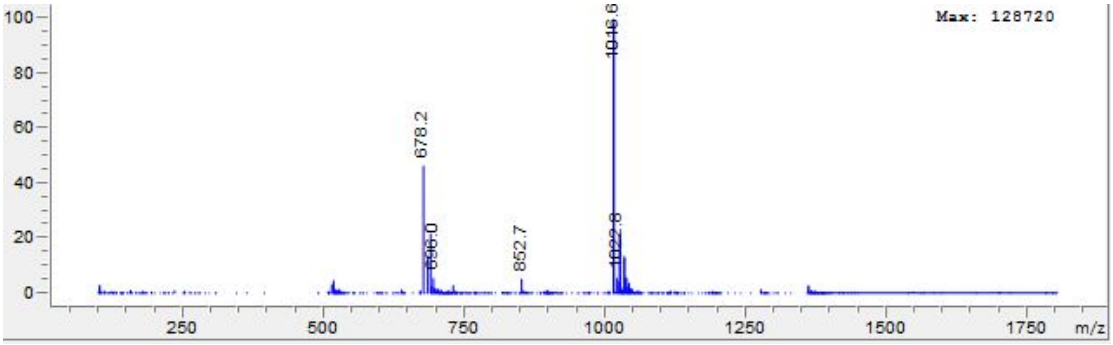


Figure S73. ESI Mass spectrum of product **3q**. Calculated Mass $[M+2H]^{2+}$: 1016.4, $[M+3H]^{3+}$: 678.0; Mass Found (ESI+) $[M+2H]^{2+}$: 1016.6, $[M+3H]^{3+}$: 678.2.

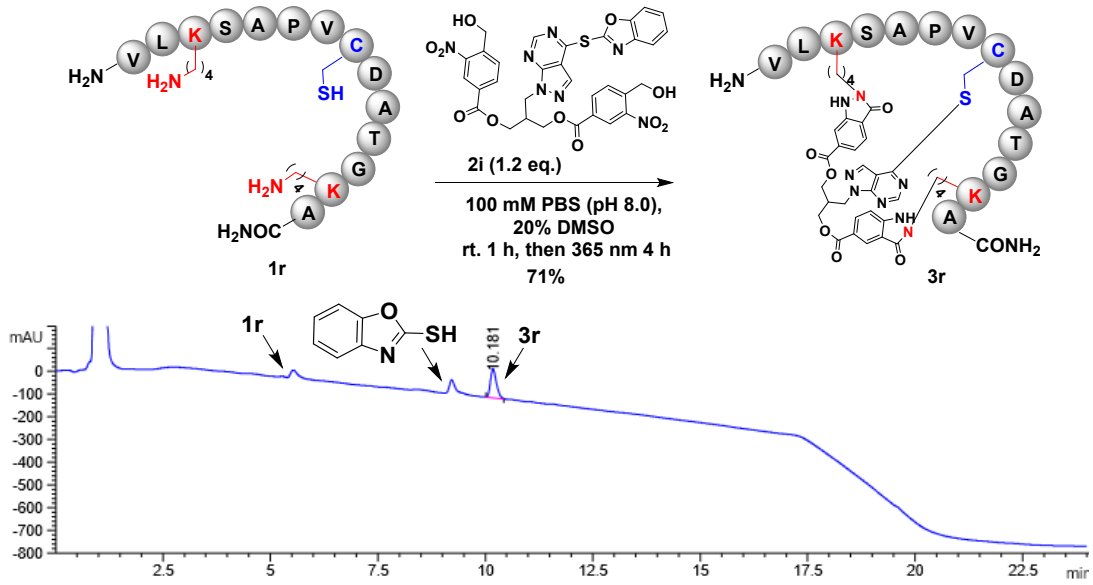


Figure S74. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

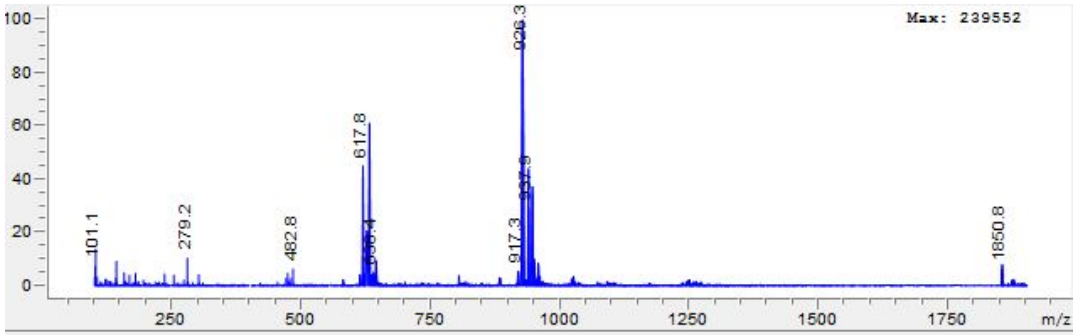


Figure S75. ESI Mass spectrum of product **3r**. Calculated Mass $[M+H]^{+}$: 1850.8, $[M+2H]^{2+}$: 925.9, $[M+3H]^{3+}$: 617.6; Mass Found (ESI+) $[M+H]^{+}$: 1850.8, $[M+2H]^{2+}$: 926.3, $[M+3H]^{3+}$: 617.3.

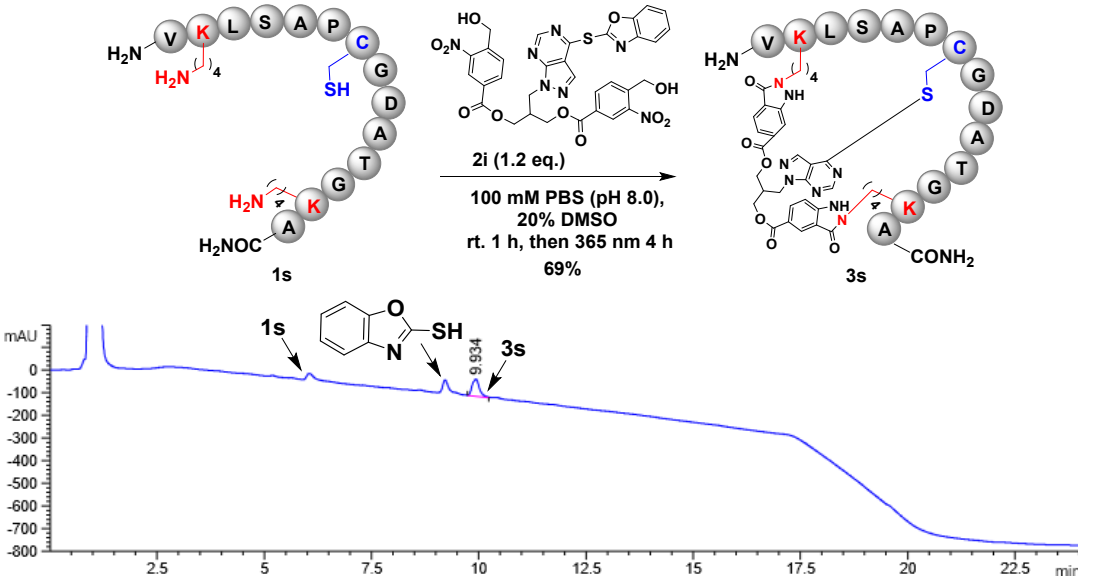


Figure S76. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

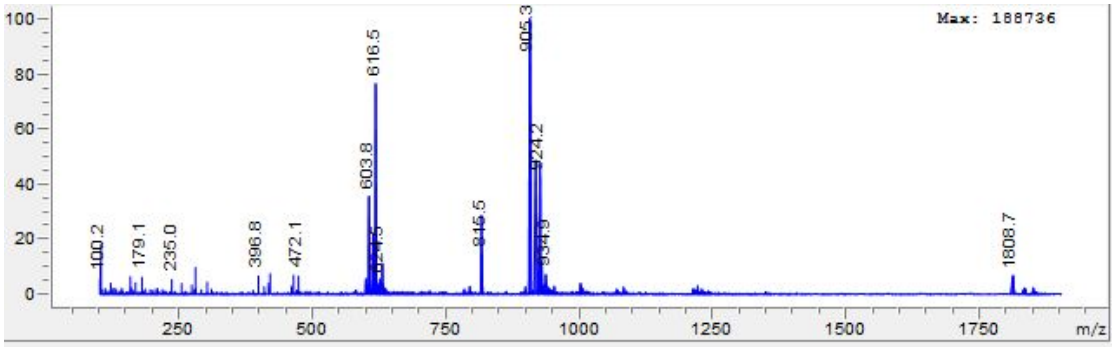


Figure S77. ESI Mass spectrum of product **3s**. Calculated Mass $[M+H]^{+}$: 1808.8, $[M+2H]^{2+}$: 904.9, $[M+3H]^{3+}$: 603.6; Mass Found (ESI+) $[M+H]^{+}$: 1808.7, $[M+2H]^{2+}$: 905.3, $[M+3H]^{3+}$: 603.8.

8. One-pot Lys single-site modification and multi-functionalization

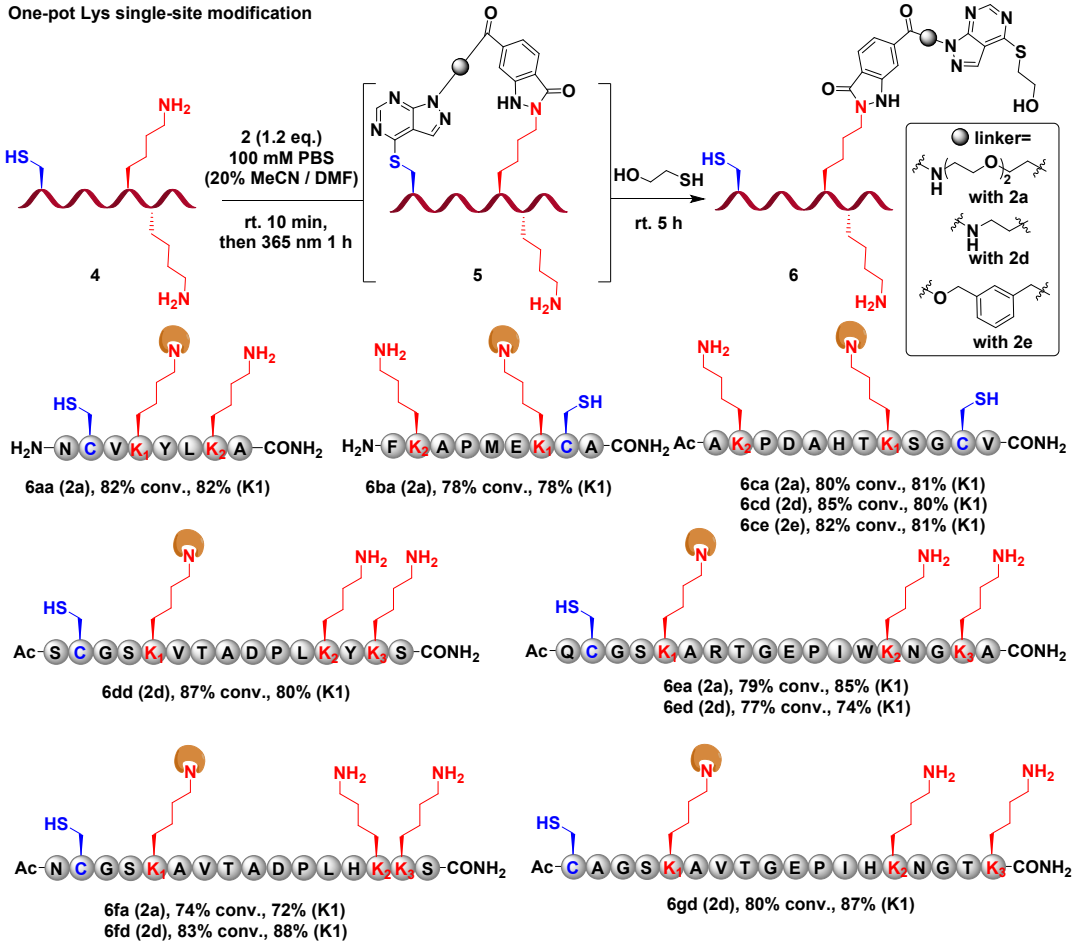


Figure S78. One-pot Lys single-site modification of multiple Lys containing peptides.

General procedure for one-pot Lys selective single-site modification of multiple Lys containing peptides: 0.5 μmol peptide (**4**) was dissolved in 400 μL non-degassed PBS buffer (100 mM, pH 8.0). Then, corresponding stapling reagent (**2**, 0.6 μmol) in 100μL DMF or MeCN was added. The resulting solution was stirred at room temperature for

10 min followed by irradiating at 365nm for another 1 h. Then, mercaptoethanol (BME, 50 μmol) was added to the reaction mixture and stirred at room temperature for 5 h. After this time, the reaction was analyzed by HPLC-MS. The reported conversion was the whole conversion for Cys-Lys stapling and BME cleavage (**4** to **6**). The selectivity was the percent (%) reacted at Lys1 over other Lys residues.

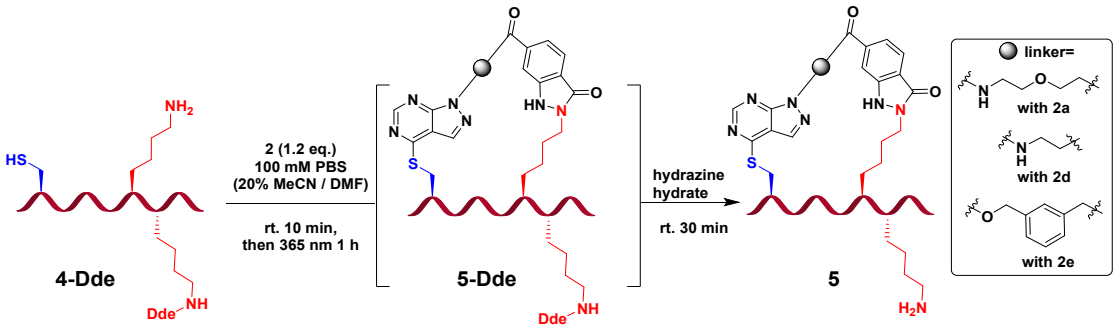


Figure S79. Position selectivity determination for one-pot Lys single-site modification.

Position selectivity determination for one-pot Lys single-site modification of multiple Lys containing peptides: 0.5 μmol Dde-protected peptide (**4-Dde**) that containing only one free Lys and other Dde-protected Lys was dissolved in 400 μL non-degassed PBS buffer (100 mM, pH 8.0). Then, corresponding stapling reagent (**2**, 0.6 μmol) in 100μL DMF or MeCN was added. The resulting solution was stirred at room temperature for 10 min followed by irradiating at 365nm for another 1 h. Then, hydrazine hydrate (80%, 12.5 μL) was added to the reaction mixture and stirred at room temperature for 30 min to remove the Dde group. After this time, the reaction was analyzed by HPLC-MS and compared the retention time of **5** with the reaction mixture by unprotected peptide **4** (Fig. S78), thereby determining the stapled Lys position.

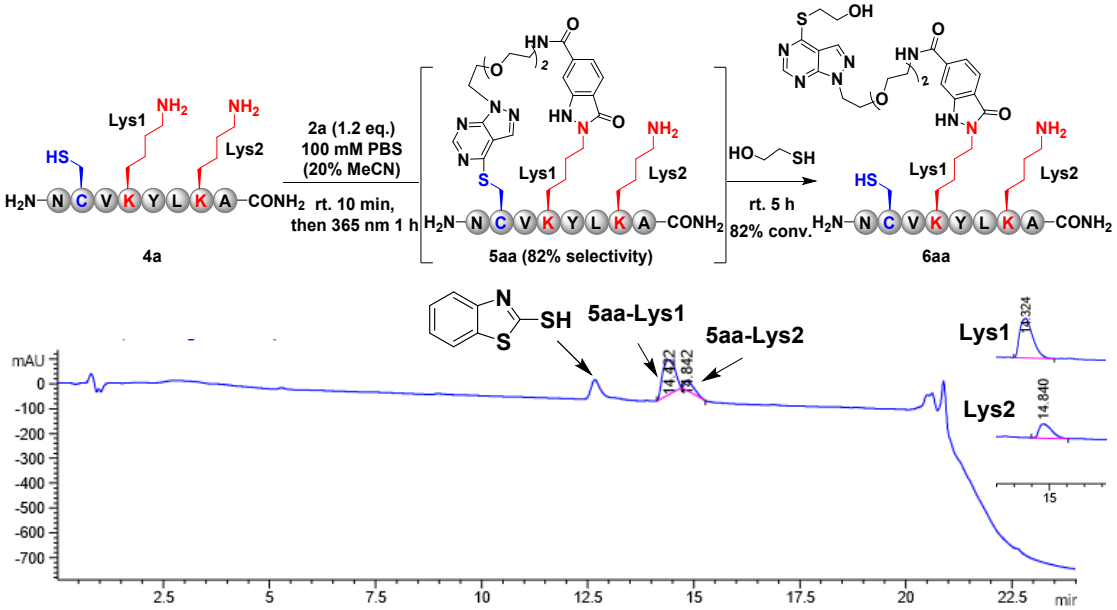


Figure S80. LC-MS UV spectrum of the first step on 220 nm. Selectivity to Lys1: 82%. Gradient used：Method C

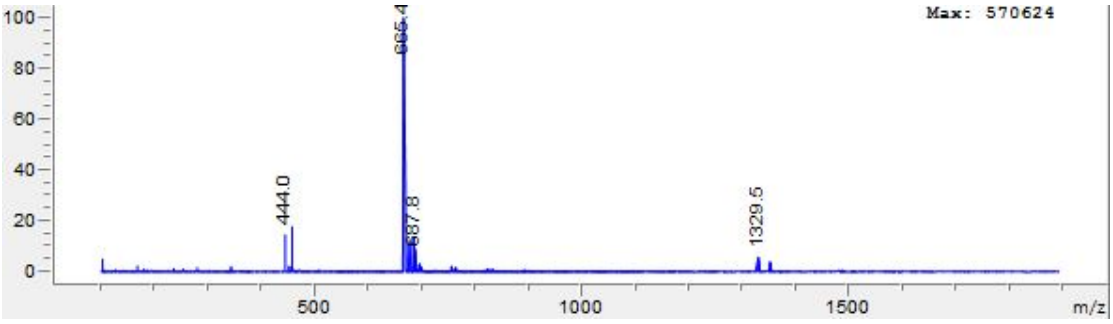


Figure S81. ESI Mass spectrum of product **5aa**. Calculated Mass $[M+H]^{+}$: 1329.7, $[M+2H]^{2+}$: 665.3, $[M+3H]^{3+}$: 443.9; Mass Found (ESI+) $[M+H]^{+}$: 1329.5, $[M+2H]^{2+}$: 665.4, $[M+3H]^{3+}$: 444.0.

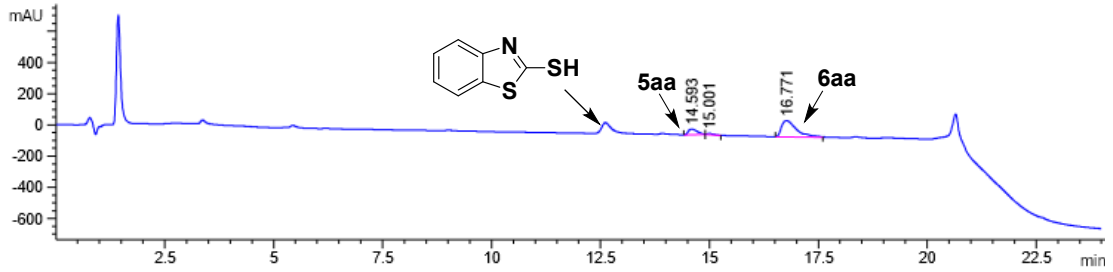


Figure S82. LC-MS UV spectrum of the whole process for **6aa** on 220 nm. Gradient used：Method C

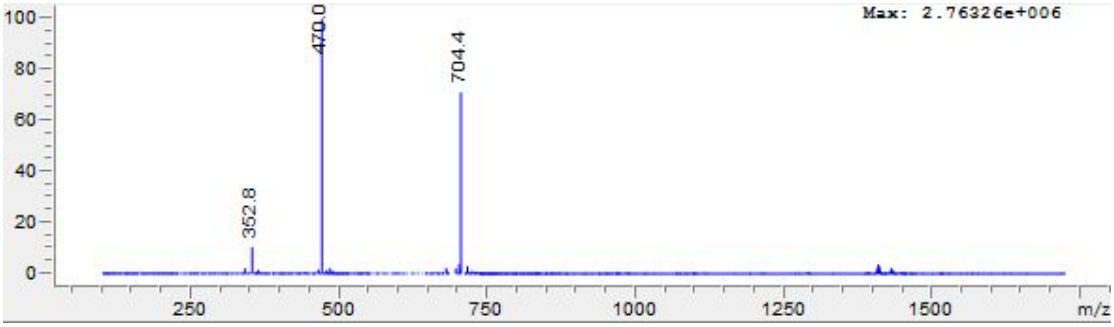
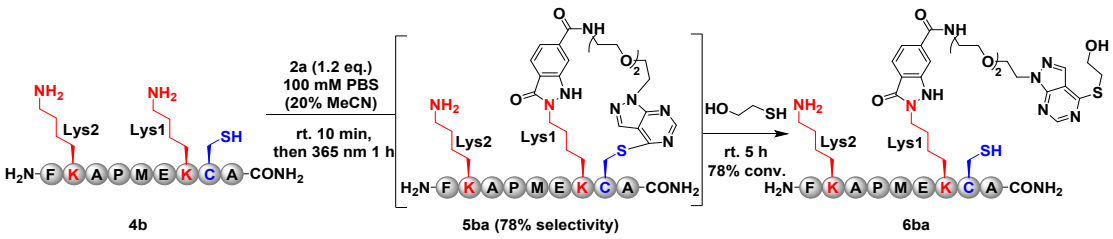


Figure S83. ESI Mass spectrum of the product **6aa**. Calculated Mass $[M+2H]^{2+}$: 704.3, $[M+3H]^{3+}$: 469.9; Mass Found (ESI+) $[M+2H]^{2+}$: 704.4, $[M+3H]^{3+}$: 470.0.



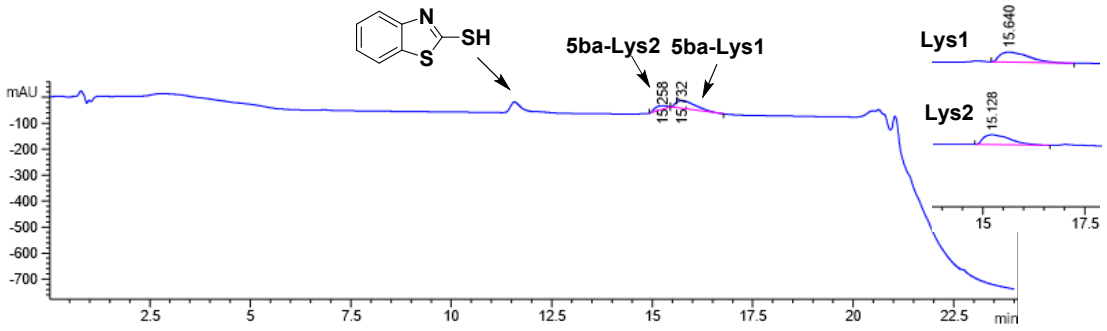


Figure S84. LC-MS UV spectrum of the first step on 220 nm. Selectivity to Lys1: 78%. Gradient used：Method C but 15% MeCN in 2 min.

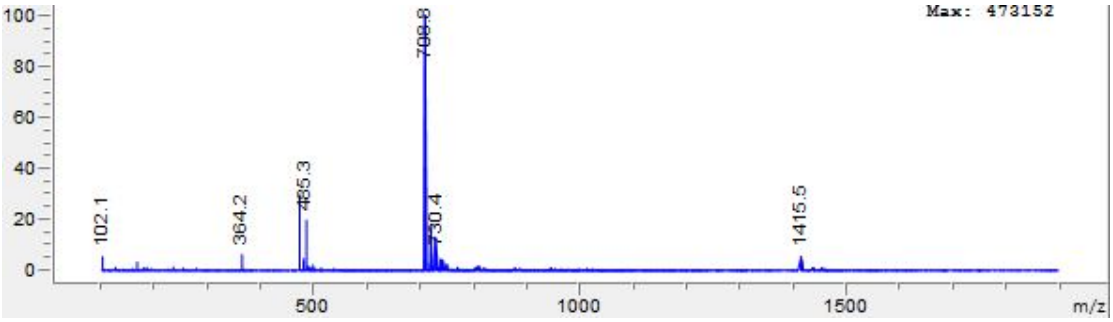


Figure S85. ESI Mass spectrum of the product **5ba**. Calculated Mass $[M+H]^+$: 1415.6, $[M+2H]^{2+}$: 708.3; Mass Found (ESI+) $[M+H]^+$: 1415.5, $[M+2H]^{2+}$: 708.8.

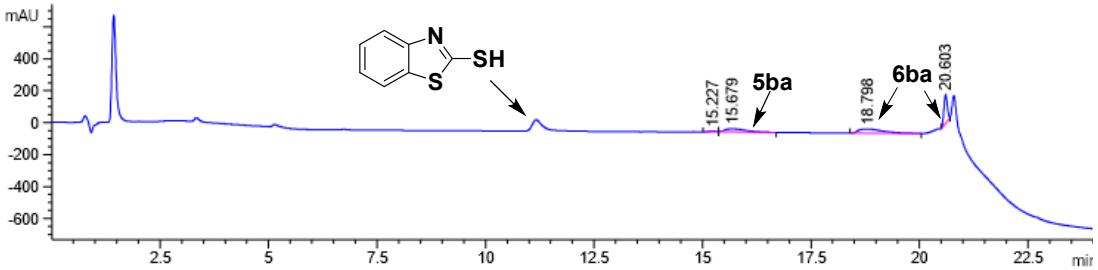


Figure S86. LC-MS UV spectrum of the whole process for **6ba** on 220 nm. Gradient used：Method C but 15% MeCN in 2 min.

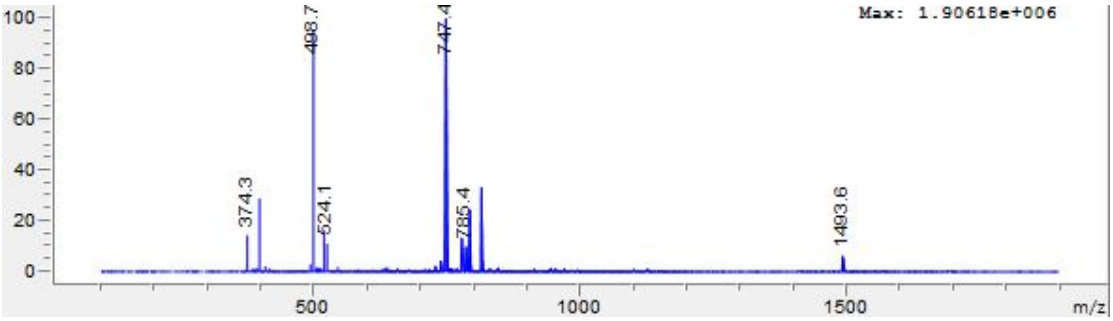


Figure S87. ESI Mass spectrum of the product **6ba**. Calculated Mass $[M+H]^+$: 1493.6, $[M+2H]^{2+}$: 747.3, $[M+3H]^{3+}$: 498.6; Mass Found (ESI+) $[M+H]^+$: 1493.6, $[M+2H]^{2+}$: 747.4, $[M+3H]^{3+}$: 498.7.

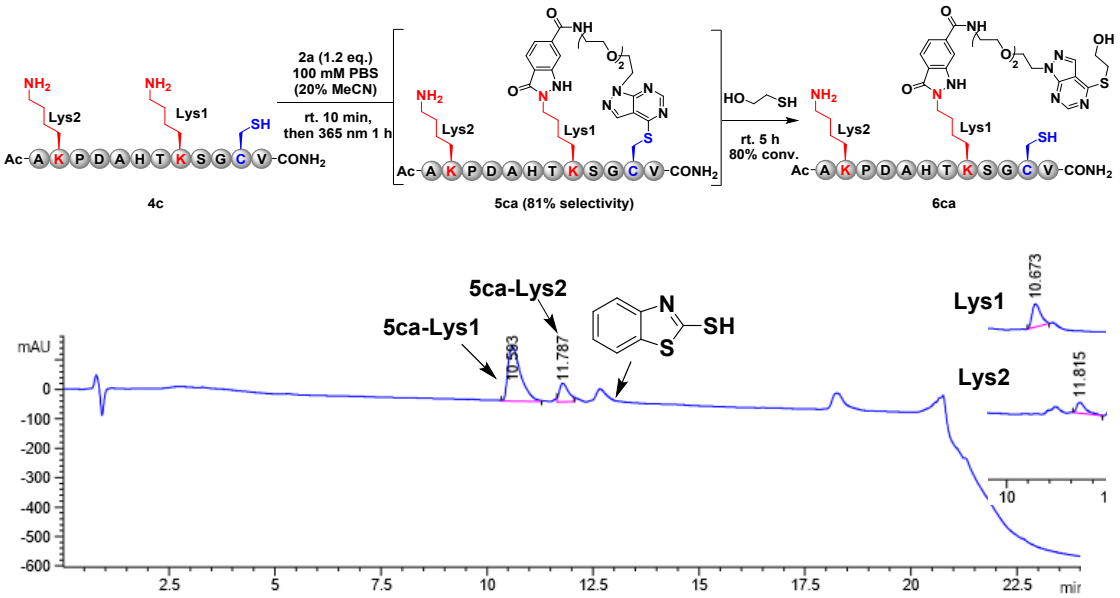


Figure S88. LC-MS UV spectrum of the first step on 220 nm. Selectivity to Lys1: 81%. Gradient used：Method C.

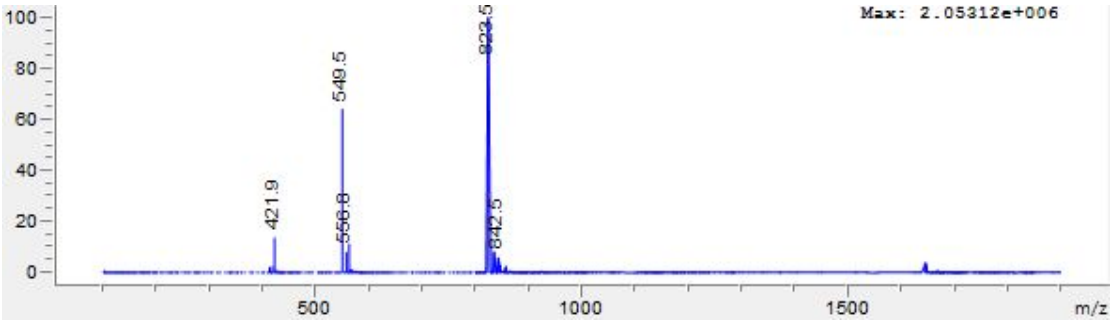


Figure S89. ESI Mass spectrum of the product **5ca**. Calculated Mass $[M+2H]^{2+}$: 823.9, $[M+3H]^{3+}$: 549.6; Mass Found (ESI+) $[M+2H]^{2+}$: 823.5, $[M+3H]^{3+}$: 549.5.

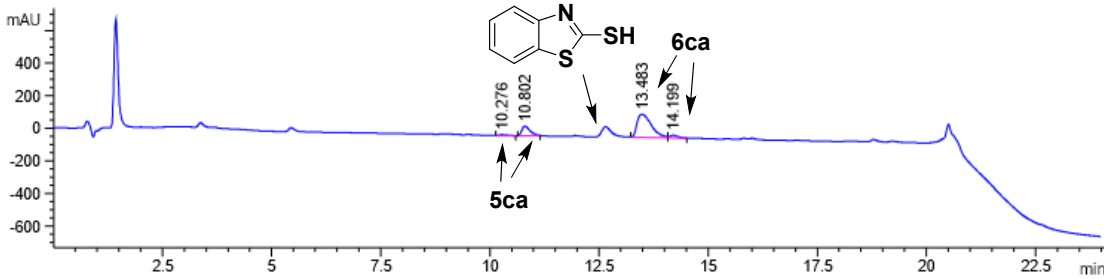


Figure S90. LC-MS UV spectrum of the whole process for **6ca** on 220 nm. Gradient used：Method C.

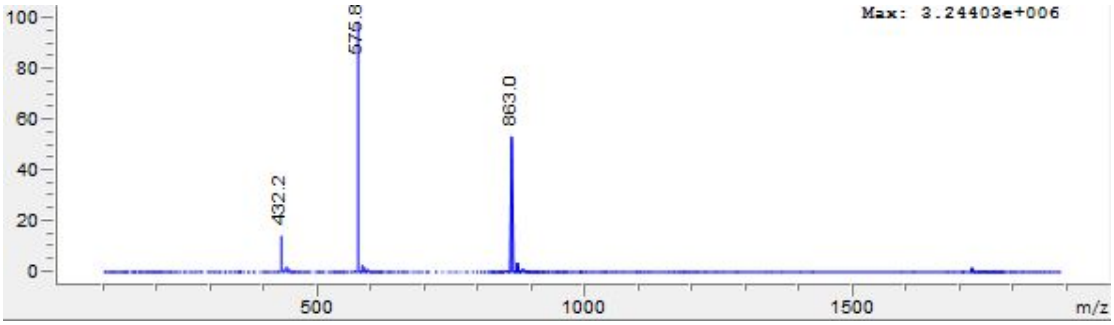
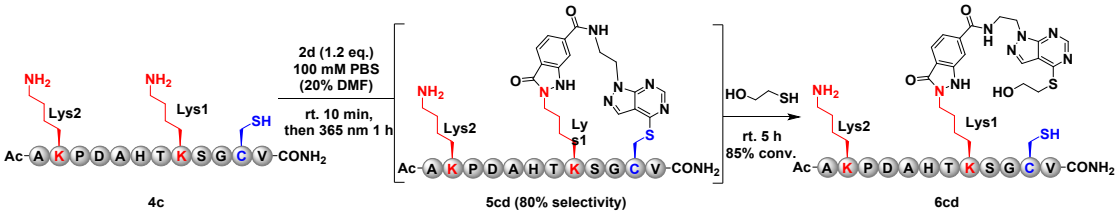


Figure S91. ESI Mass spectrum of the product **6ca**. Calculated Mass $[M+2H]^{2+}$: 862.9, $[M+3H]^{3+}$: 575.6, $[M+4H]^{4+}$: 432.0; Mass Found (ESI+) $[M+2H]^{2+}$: 863.0, $[M+3H]^{3+}$: 575.8, $[M+4H]^{4+}$: 432.2.



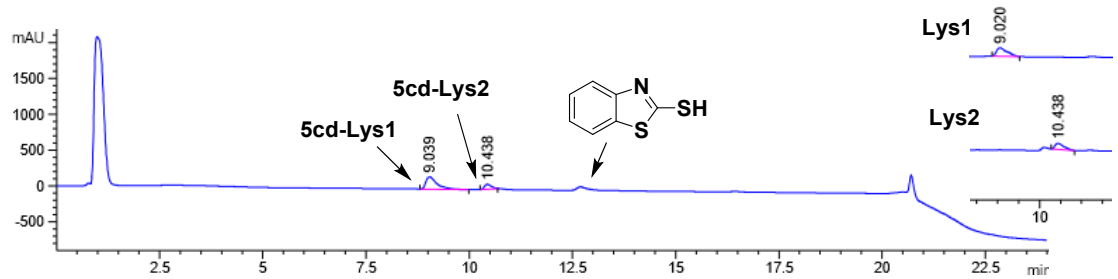


Figure S92. LC-MS UV spectrum of the first step on 220 nm. Selectivity to Lys1: 80%. Gradient used：Method C.

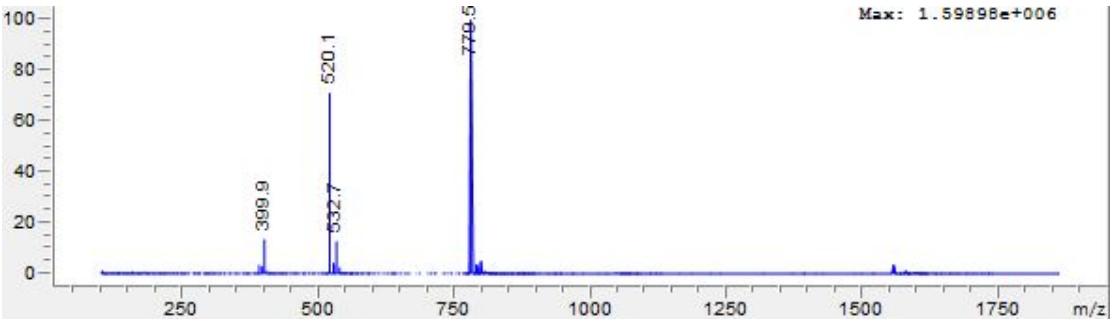


Figure S93. ESI Mass spectrum of the product **5cd**. Calculated Mass $[M+2H]^{2+}$: 779.9, $[M+3H]^{3+}$: 520.2; Mass Found (ESI+) $[M+2H]^{2+}$: 779.5, $[M+3H]^{3+}$: 520.1.

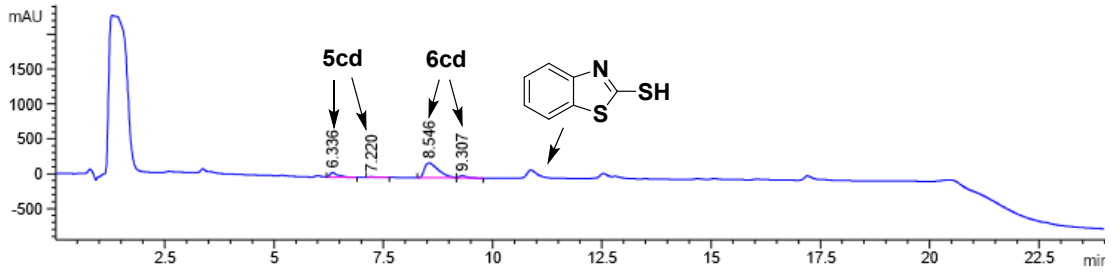


Figure S94. LC-MS UV spectrum of the whole process for **6cd** on 220 nm. Gradient used：Method C but 15% MeCN in 2 min.

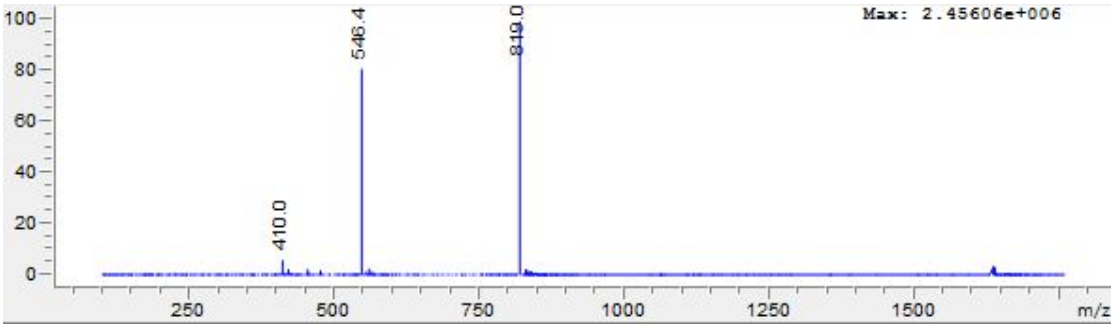
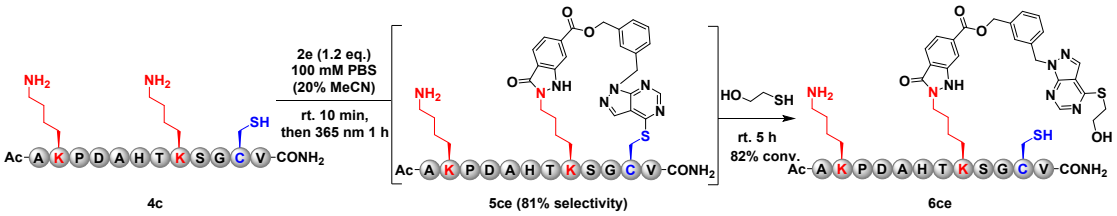


Figure S95. ESI Mass spectrum of the product **6cd**. Calculated Mass $[M+2H]^{2+}$: 818.9, $[M+3H]^{3+}$: 546.2; Mass Found (ESI+) $[M+2H]^{2+}$: 819.0, $[M+3H]^{3+}$: 546.4.



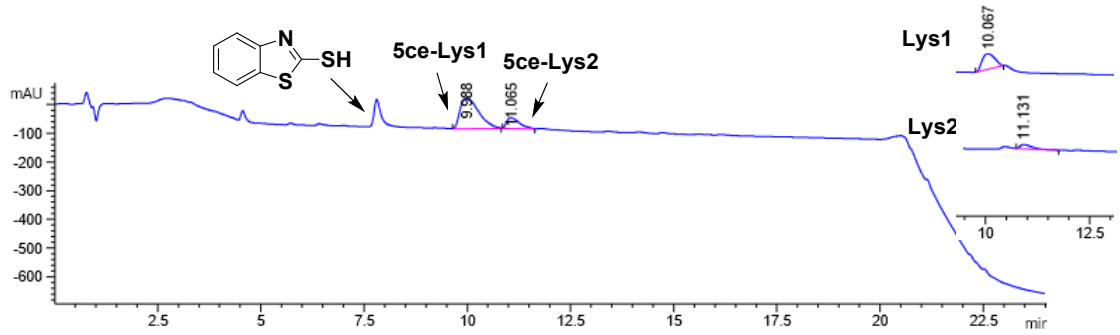


Figure S96. LC-MS UV spectrum of the first step on 220 nm. Selectivity to Lys1: 81%. Gradient used：Method D.

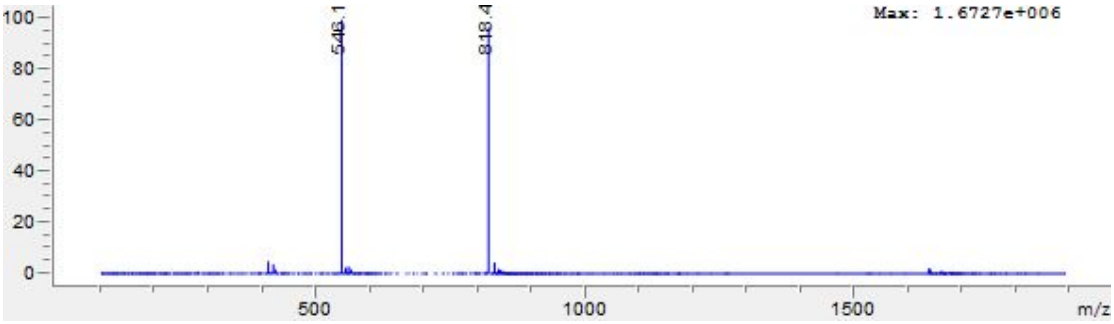


Figure S97. ESI Mass spectrum of the product **5ce**. Calculated Mass $[M+2H]^{2+}$: 818.4, $[M+3H]^{3+}$: 545.9; Mass Found (ESI+) $[M+2H]^{2+}$: 818.4, $[M+3H]^{3+}$: 546.1.

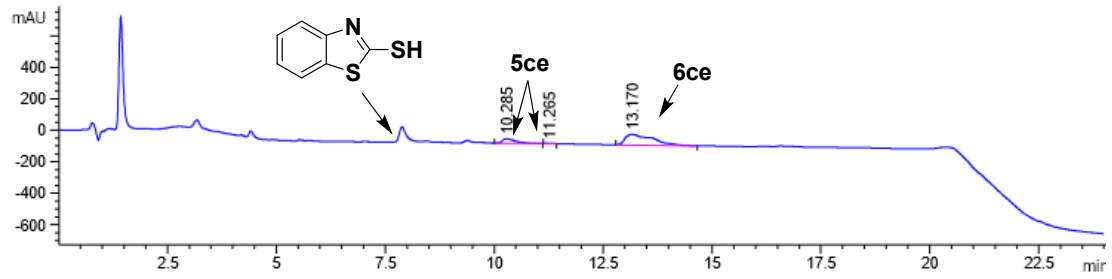


Figure S98. LC-MS UV spectrum of the whole process for **6ce** on 220 nm. Gradient used：Method D.

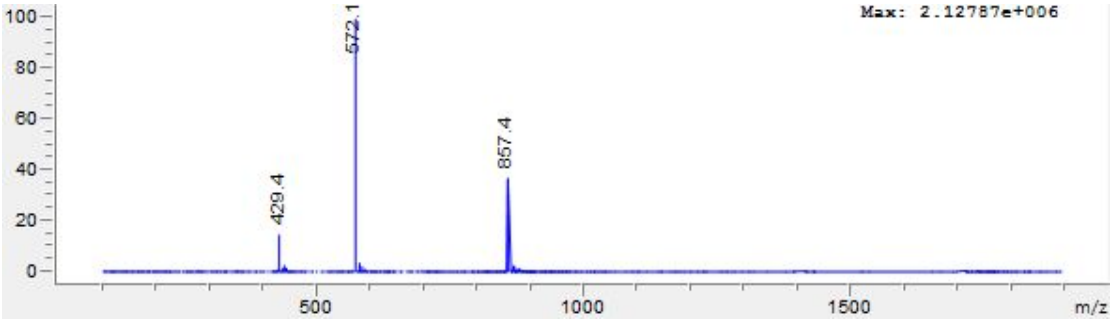
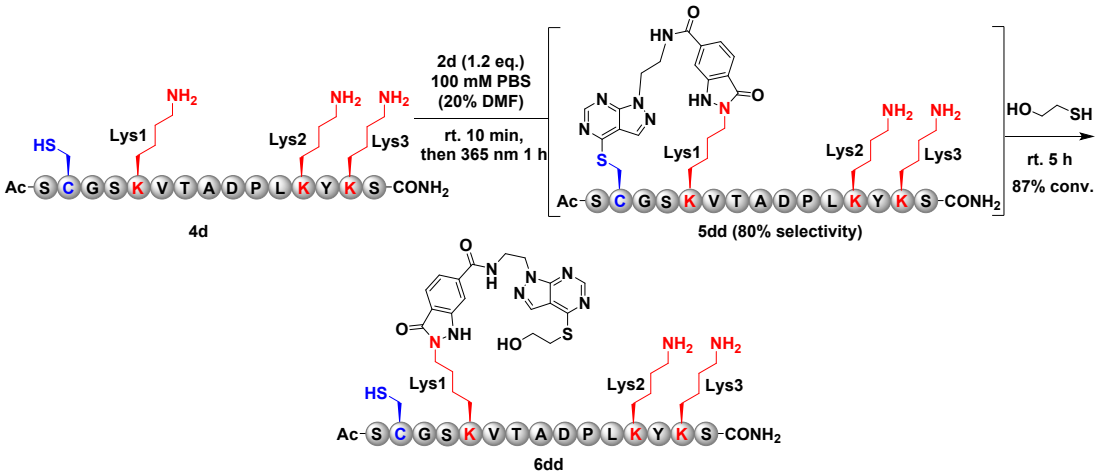


Figure S99. ESI Mass spectrum of the product **6ce**. Calculated Mass $[M+2H]^{2+}$: 857.4, $[M+3H]^{3+}$: 571.9, $[M+4H]^{4+}$: 429.2; Mass Found (ESI+) $[M+2H]^{2+}$: 857.4, $[M+3H]^{3+}$: 572.1, $[M+4H]^{4+}$: 429.4.



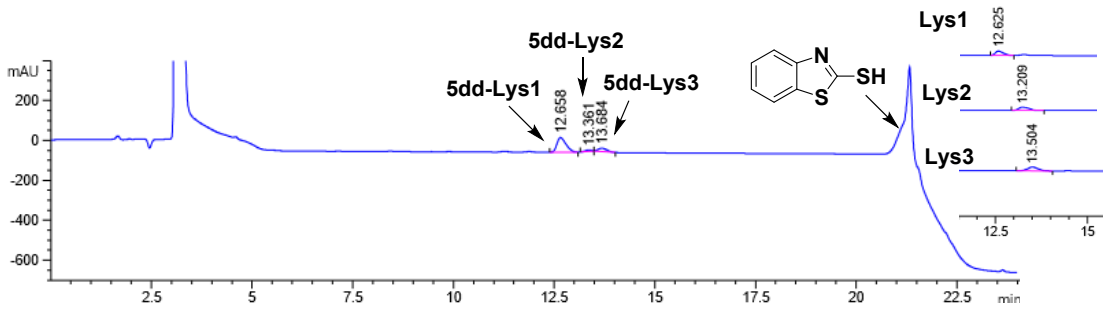


Figure S100. LC-MS UV spectrum of the first step on 220 nm. Selectivity to Lys1: 80%. Gradient used：Method E but 17% MeCN in 2 min.

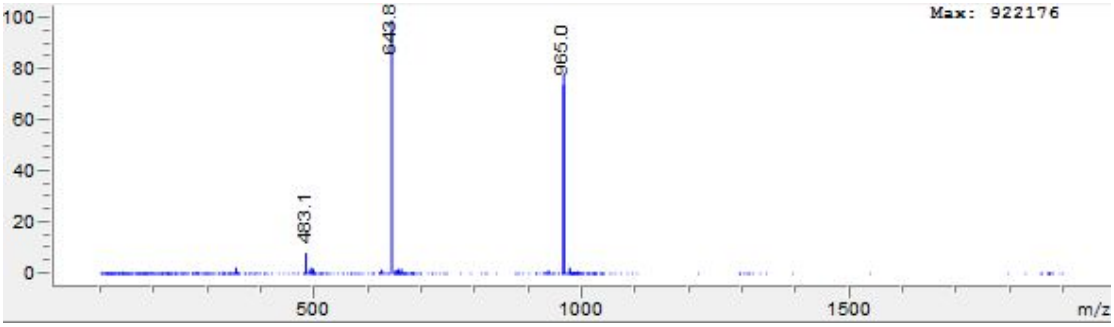


Figure S101. ESI Mass spectrum of the product **5dd**. Calculated Mass $[M+2H]^{2+}$: 965.0, $[M+3H]^{3+}$: 963.4, $[M+4H]^{4+}$: 483.0; Mass Found (ESI+) $[M+2H]^{2+}$: 965.0, $[M+3H]^{3+}$: 643.8, $[M+4H]^{4+}$: 481.3.

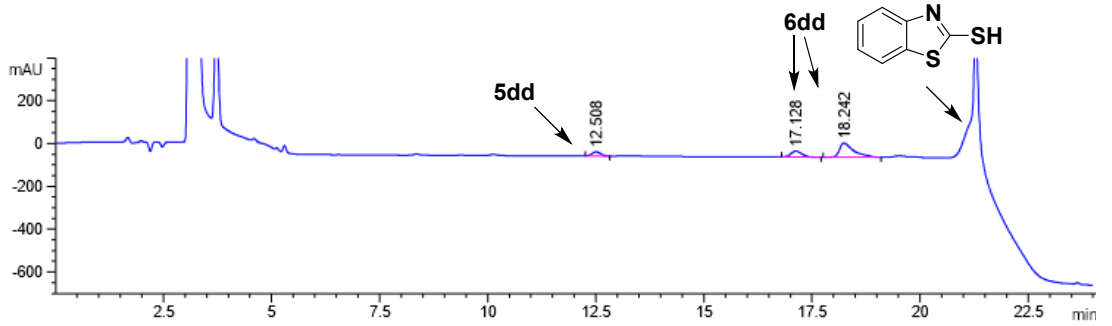


Figure S102. LC-MS UV spectrum of the whole process for **6dd** on 220 nm. Gradient used：Method E but 17% MeCN in 2 min.

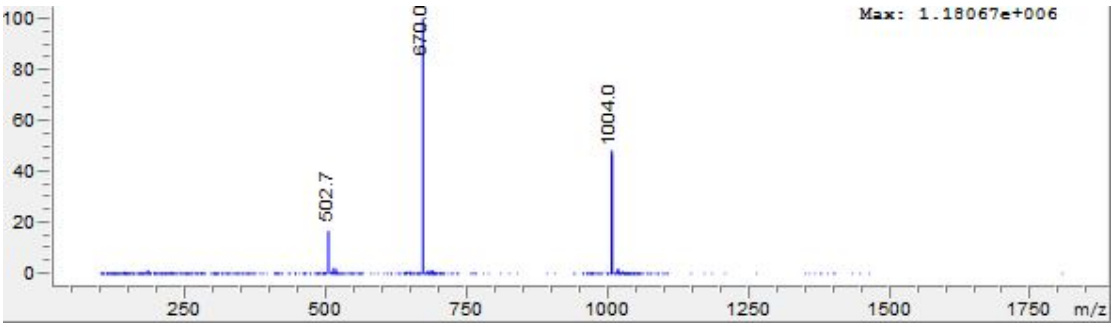


Figure S103. ESI Mass spectrum of the product **6dd**. Calculated Mass $[M+2H]^{2+}$: 1004.0, $[M+3H]^{3+}$: 669.6, $[M+4H]^{4+}$: 502.5; Mass Found (ESI+) $[M+2H]^{2+}$: 1004.0, $[M+3H]^{3+}$: 670.0, $[M+4H]^{4+}$: 502.7.

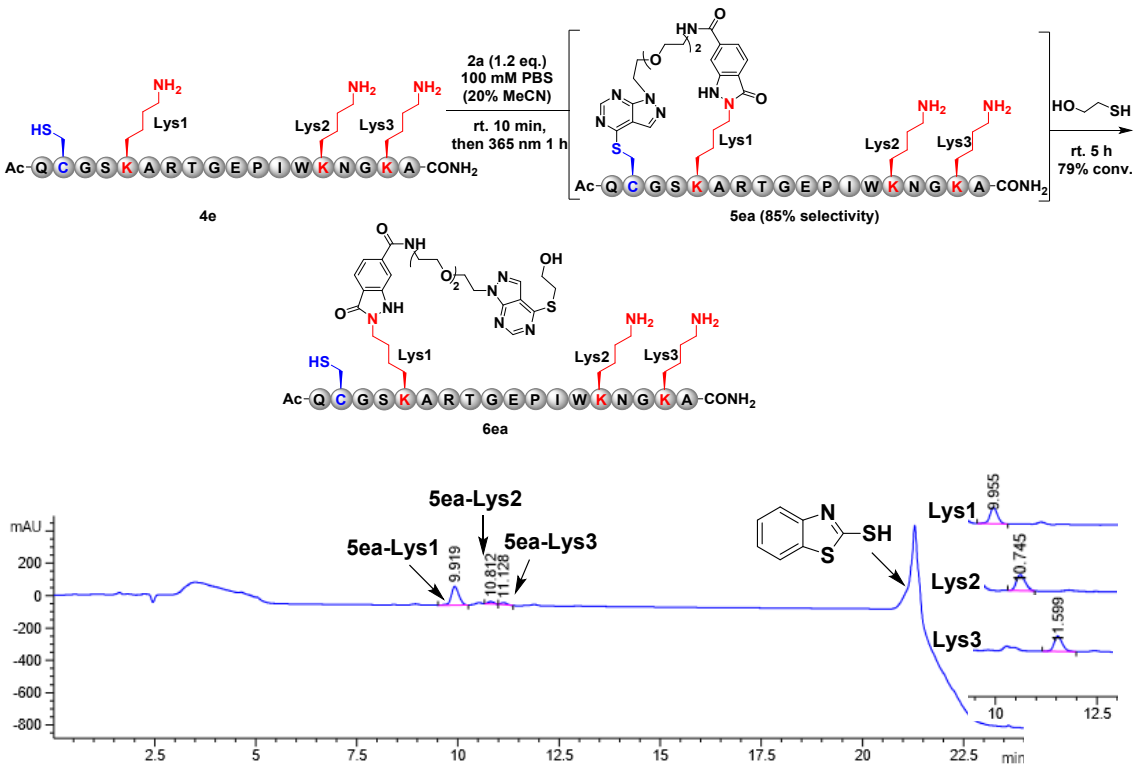


Figure S104. LC-MS UV spectrum of the first step on 220 nm. Selectivity to Lys1: 85%. Gradient used：Method E.

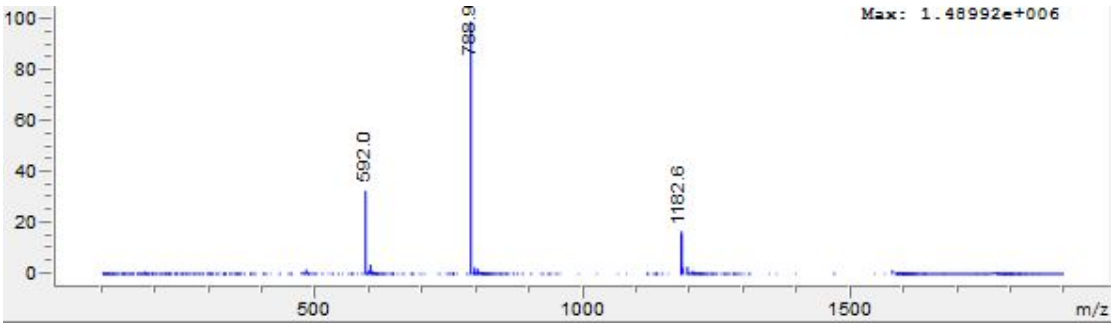


Figure S105. ESI Mass spectrum of the product **5ea**. Calculated Mass $[M+2H]^{2+}$: 1182.6, $[M+3H]^{3+}$: 788.7, $[M+4H]^{4+}$: 591.8; Mass Found (ESI+) $[M+2H]^{2+}$: 1182.6, $[M+3H]^{3+}$: 788.9, $[M+4H]^{4+}$: 592.0.

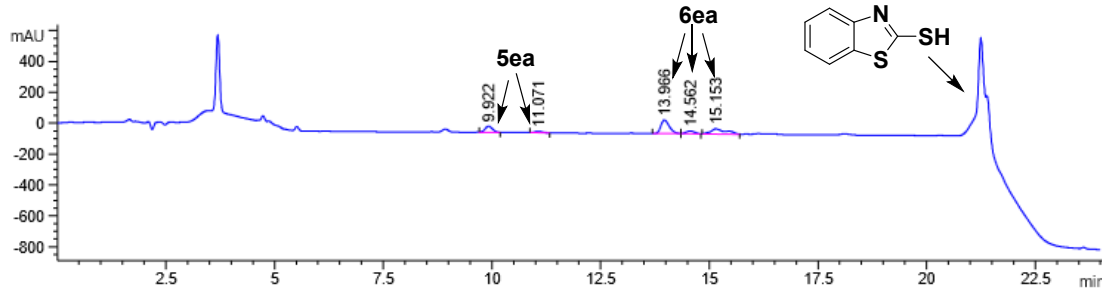


Figure S106. LC-MS UV spectrum of the whole process for **6ea** on 220 nm. Gradient used：Method E.

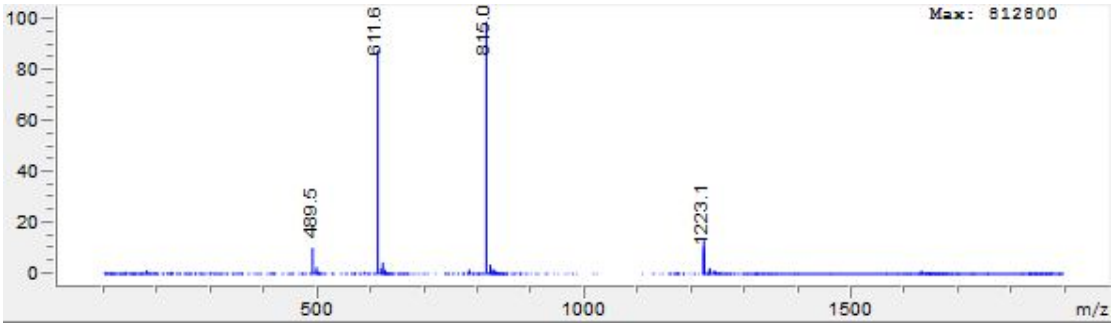


Figure S107. ESI Mass spectrum of the product **6ea**. Calculated Mass $[M+3H]^{3+}$: 814.7, $[M+4H]^{4+}$: 611.3; Mass Found (ESI+) $[M+3H]^{3+}$: 815.0, $[M+4H]^{4+}$: 611.6.

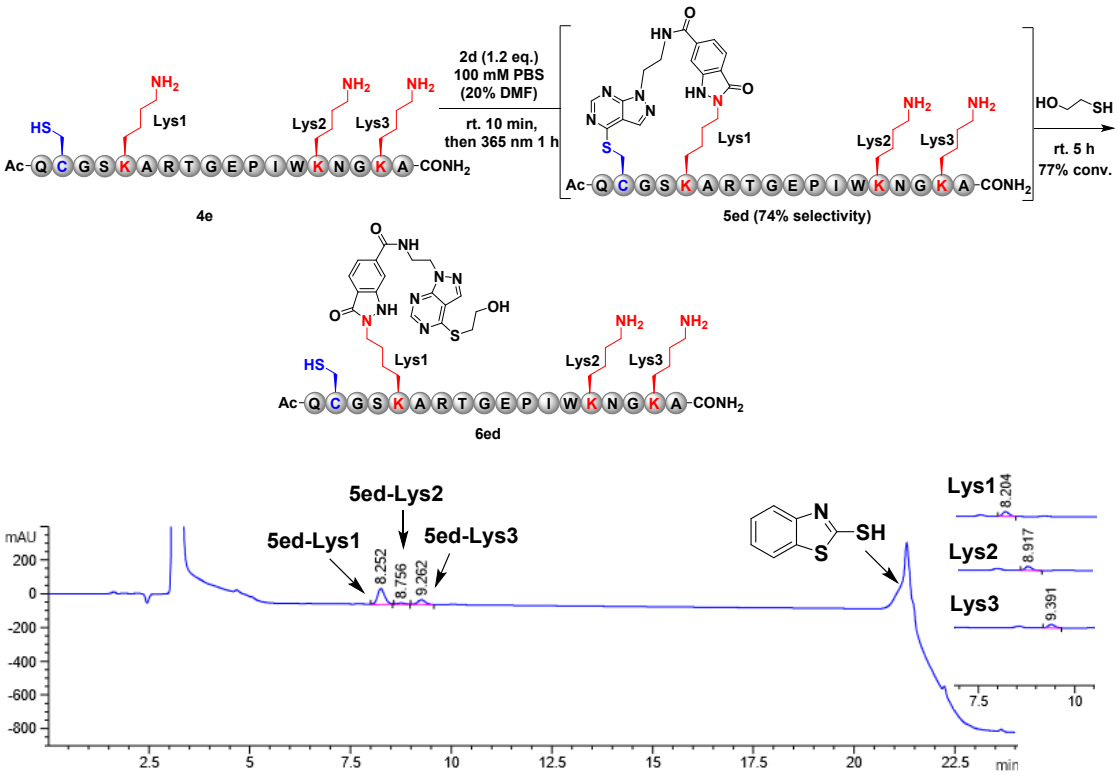


Figure S108. LC-MS UV spectrum of the first step on 220 nm. Selectivity to Lys1: 74%. Gradient used：Method E.

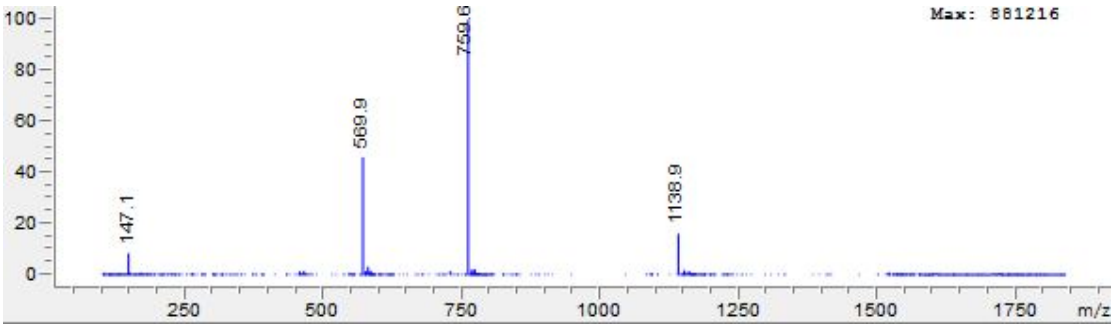


Figure S109. ESI Mass spectrum of the product **5ed**. Calculated Mass $[M+2H]^{2+}$: 1138.5, $[M+3H]^{3+}$: 759.4, $[M+4H]^{4+}$: 569.8; Mass Found (ESI+) $[M+2H]^{2+}$: 1138.9, $[M+3H]^{3+}$: 759.6, $[M+4H]^{4+}$: 569.9.

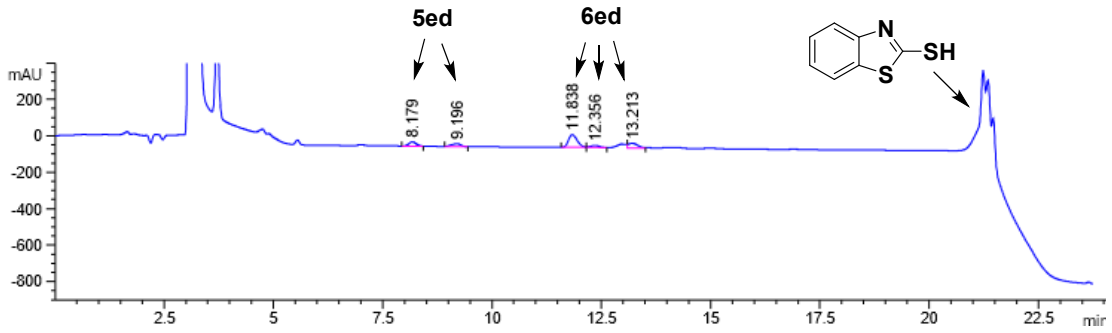


Figure S110. LC-MS UV spectrum of the whole process for **6ed** on 220 nm. Gradient used：Method E

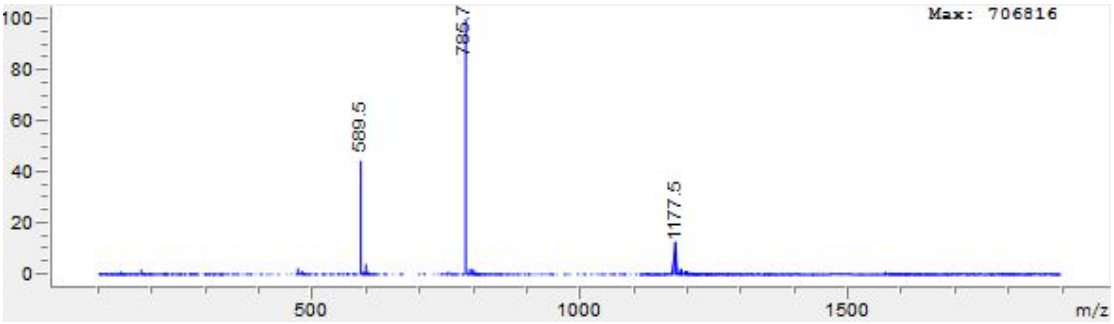


Figure S111. ESI Mass spectrum of the product **6ed**. Calculated Mass $[M+2H]^{2+}$: 1177.6, $[M+3H]^{3+}$: 785.4, $[M+4H]^{4+}$: 589.3; Mass Found (ESI+) $[M+2H]^{2+}$: 1177.5, $[M+3H]^{3+}$: 785.7, $[M+4H]^{4+}$: 589.5.

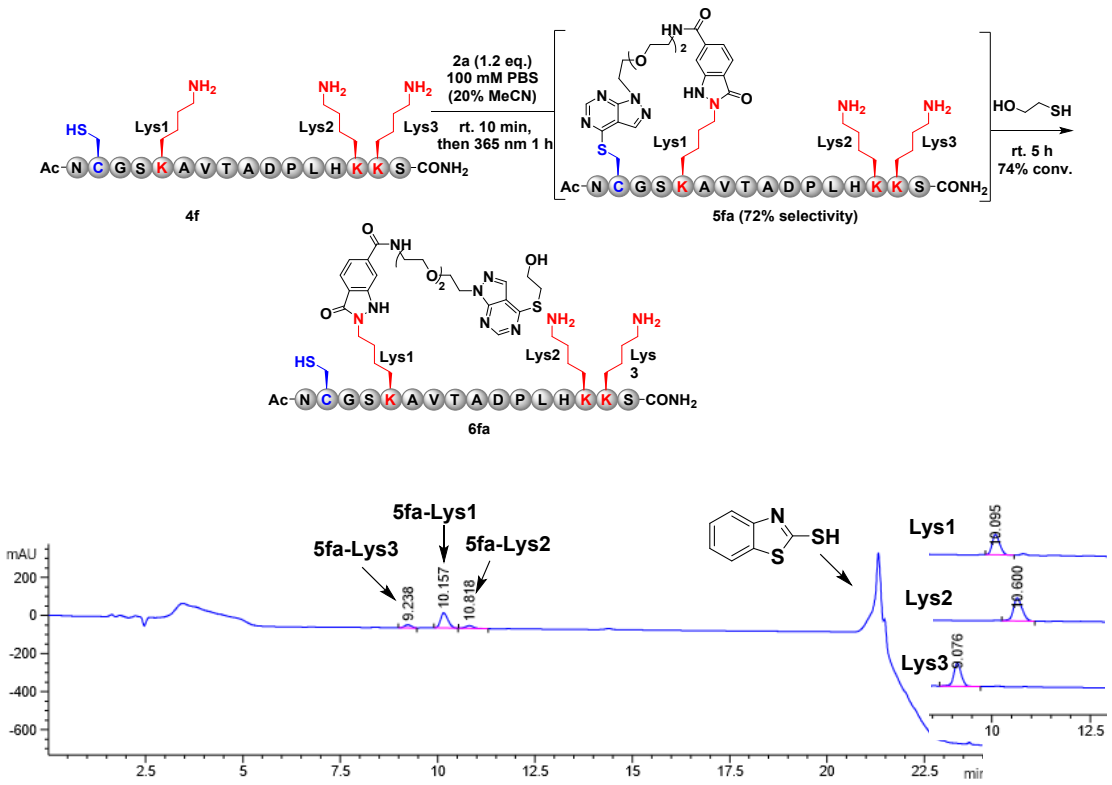


Figure S112. LC-MS UV spectrum of the first step on 220 nm. Selectivity to Lys1: 72%. Gradient used：Method E.

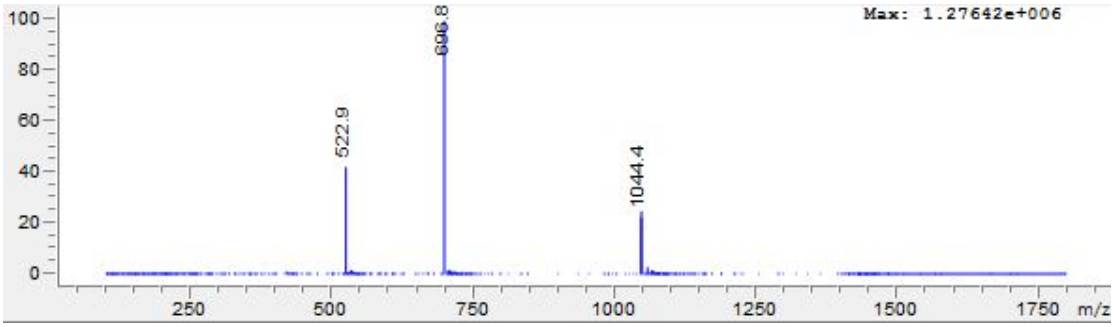


Figure S113. ESI Mass spectrum of the product **5fa**. Calculated Mass $[M+2H]^{2+}$: 1045.0, $[M+3H]^{3+}$: 697.0, $[M+4H]^{4+}$: 523.0; Mass Found (ESI+) $[M+2H]^{2+}$: 1044.4, $[M+3H]^{3+}$: 696.8, $[M+4H]^{4+}$: 522.9.

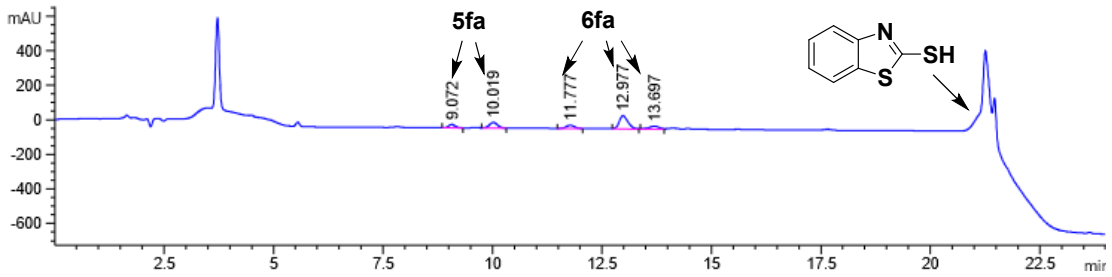


Figure S114. LC-MS UV spectrum of the whole process for **6fa** on 220 nm. Gradient used：Method E.

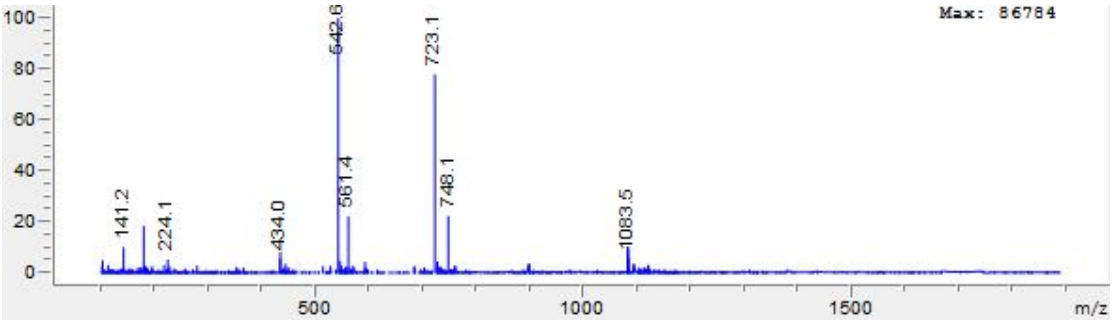
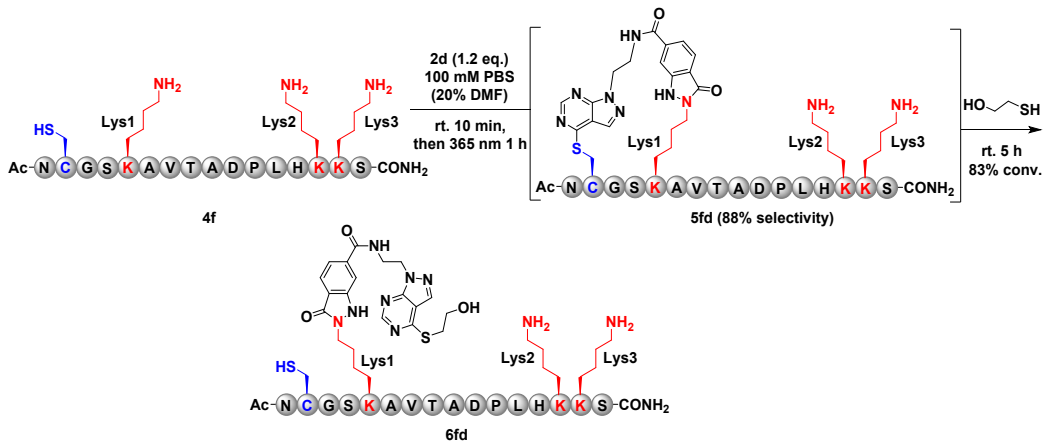


Figure S115. ESI Mass spectrum of the product **6fa**. Calculated Mass $[M+2H]^{2+}$: 1084.0, $[M+3H]^{3+}$: 723.0, $[M+4H]^{4+}$: 542.5; Mass Found (ESI+) $[M+2H]^{2+}$: 1083.5, $[M+3H]^{3+}$: 723.1, $[M+4H]^{4+}$: 542.6.



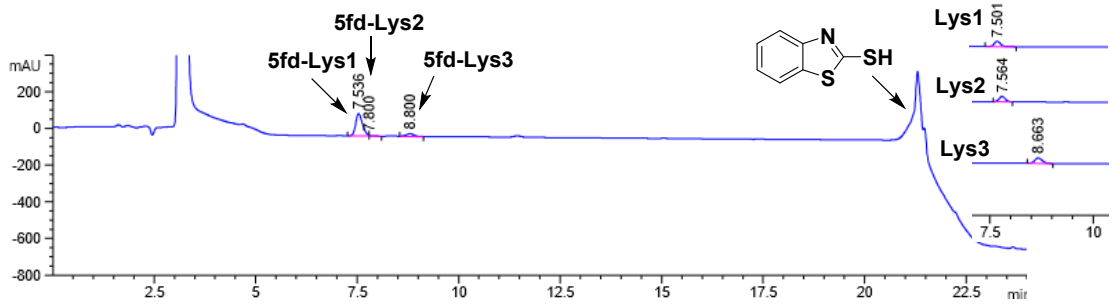


Figure S116. LC-MS UV spectrum of the first step on 220 nm. Selectivity to Lys1: 88%. Gradient used：Method E.

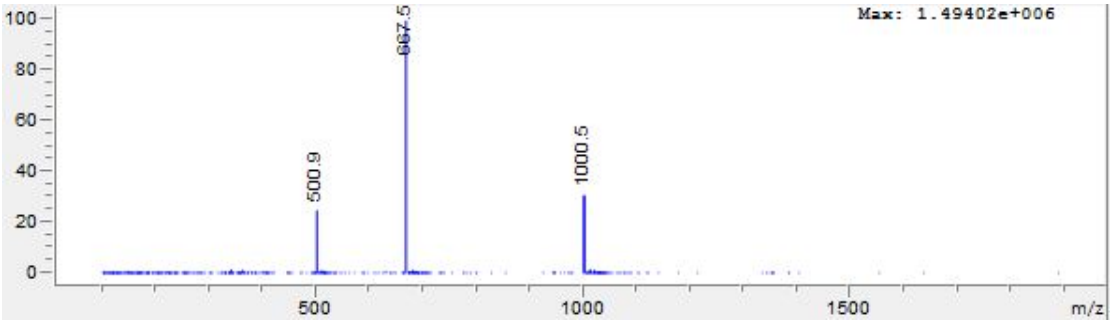


Figure S117. ESI Mass spectrum of the product **5fd**. Calculated Mass $[M+2H]^{2+}$: 1001.0, $[M+3H]^{3+}$: 667.7, $[M+4H]^{4+}$: 501.0; Mass Found (ESI+) $[M+2H]^{2+}$: 1000.5, $[M+3H]^{3+}$: 667.5, $[M+4H]^{4+}$: 500.9.

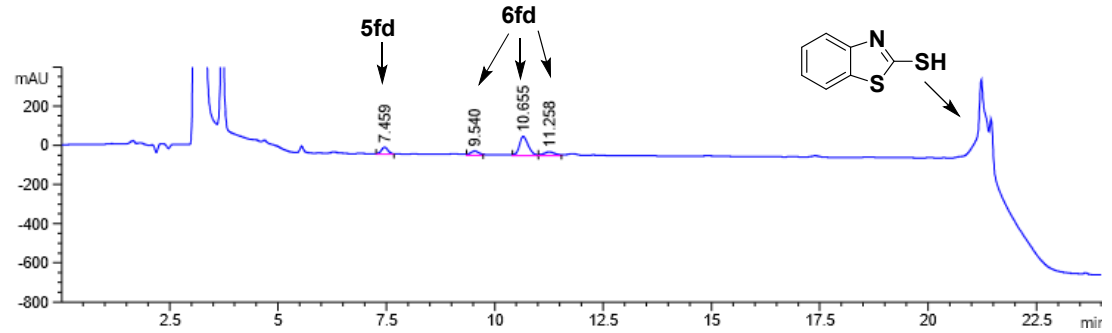


Figure S118. LC-MS UV spectrum of the whole process for **6fd** on 220 nm. Gradient used：Method E.

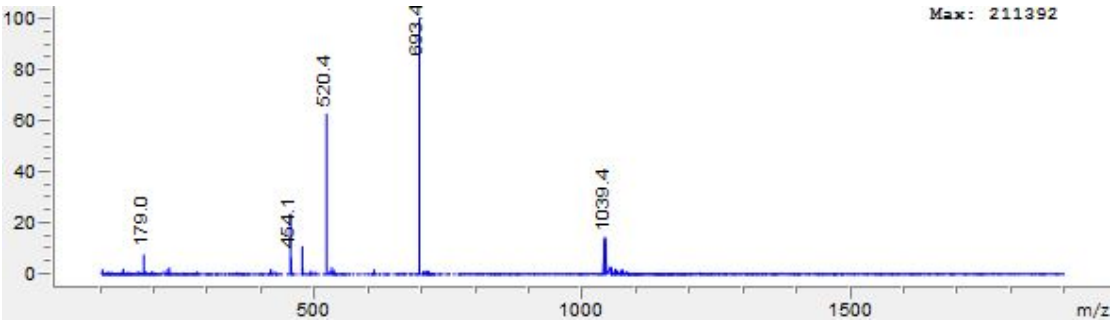


Figure S119. ESI Mass spectrum of the product **6fd**. Calculated Mass $[M+2H]^{2+}$: 1040.0, $[M+3H]^{3+}$: 693.7, $[M+4H]^{4+}$: 520.5; Mass Found (ESI+) $[M+2H]^{2+}$: 1039.4, $[M+3H]^{3+}$: 693.4, $[M+4H]^{4+}$: 520.4.

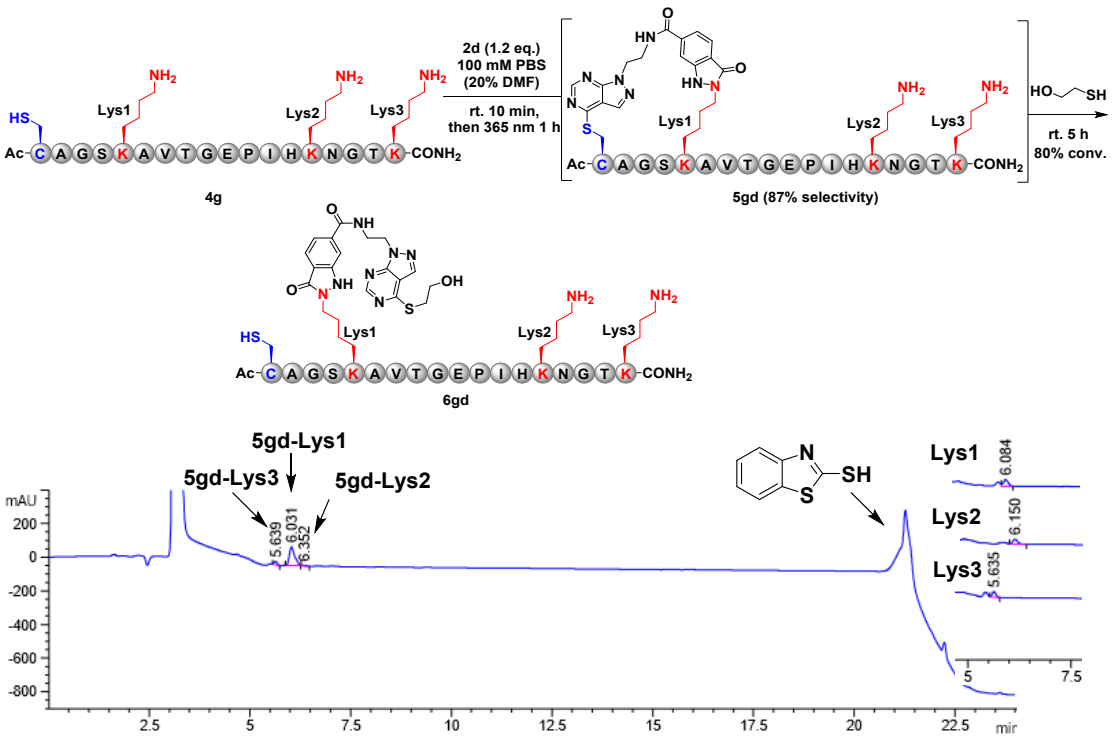


Figure S120. LC-MS UV spectrum of the first step on 220 nm. Selectivity to Lys1: 87%. Gradient used：Method E.

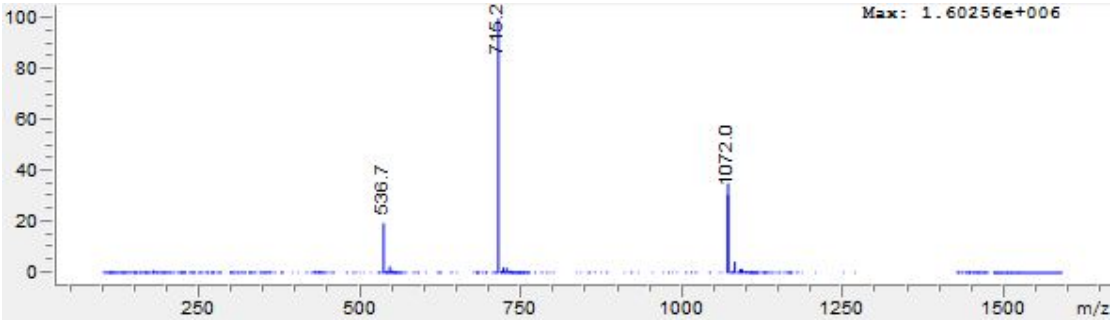


Figure S121. ESI Mass spectrum of the product **5gd**. Calculated Mass $[M+2H]^{2+}$: 1072.0, $[M+3H]^{3+}$: 715.0, $[M+4H]^{4+}$: 536.5; Mass Found (ESI+) $[M+2H]^{2+}$: 1072.0, $[M+3H]^{3+}$: 715.2, $[M+4H]^{4+}$: 536.7.

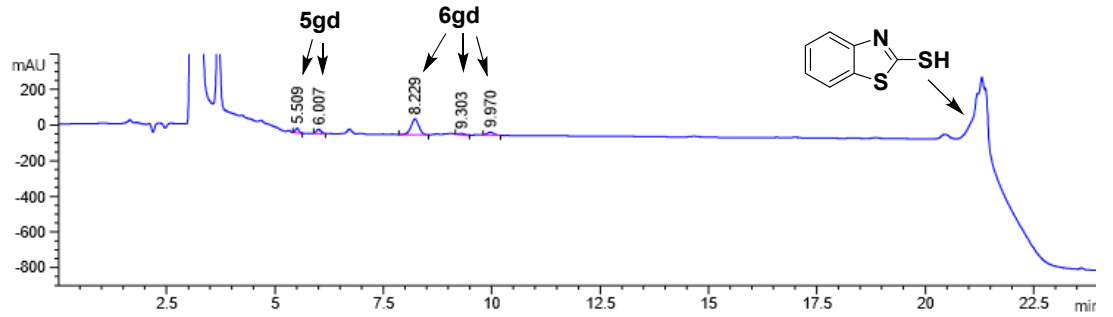


Figure S122. LC-MS UV spectrum of the whole process for **6gd** on 220 nm. Gradient used：Method E.

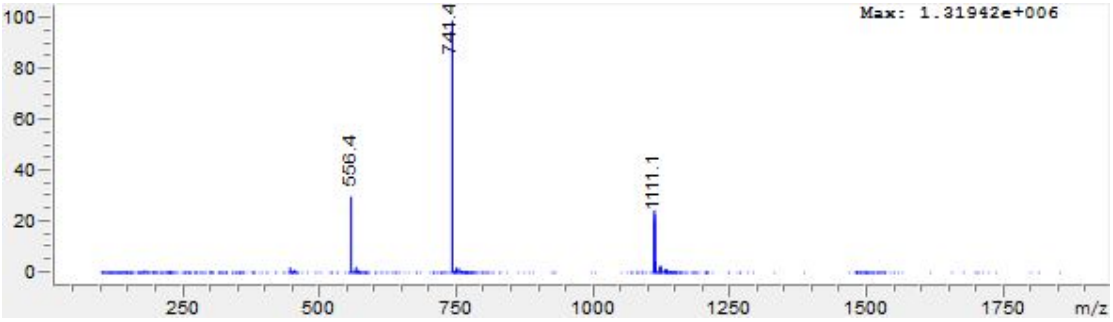


Figure S123. ESI Mass spectrum of the product **6gd**. Calculated Mass $[M+2H]^{2+}$: 1111.0, $[M+3H]^{3+}$: 741.0, $[M+4H]^{4+}$: 556.0; Mass Found (ESI+) $[M+2H]^{2+}$: 1111.1, $[M+3H]^{3+}$: 741.4, $[M+4H]^{4+}$: 556.4.

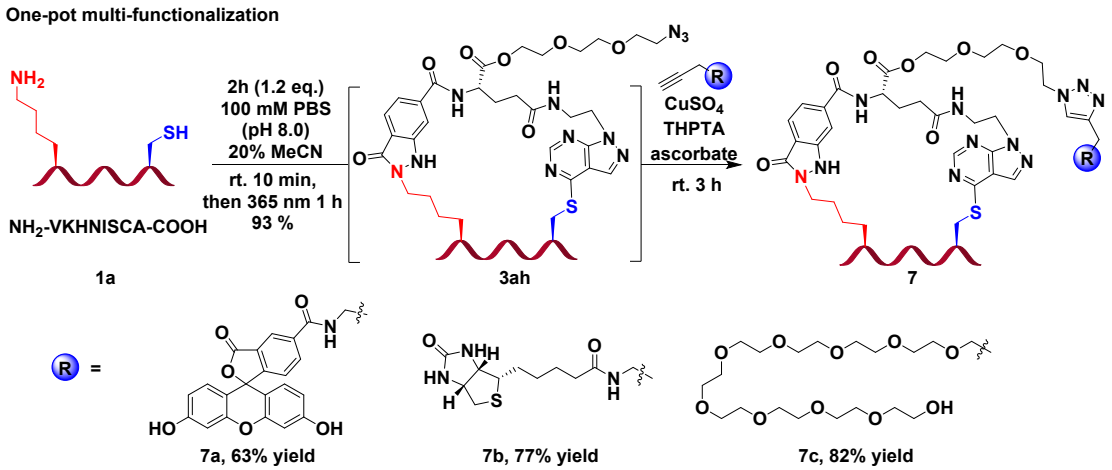
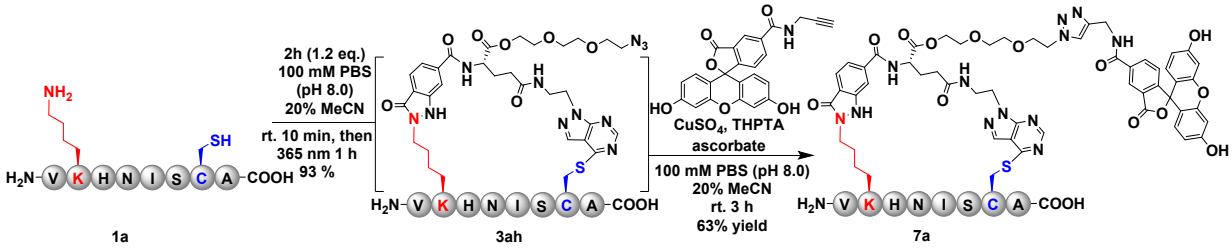


Figure S124. One-pot multi-functionalization via CuAAC.

General procedure for the following one-pot multi-functionalization via click reaction: 0.5 μmol peptide (**1a**) was dissolved in 400 μL non-degassed PBS buffer (100 mM, pH 8.0). Then, corresponding stapling reagent (**2h**, 0.6 μmol) in 100 μL MeCN was added. The resulting solution was stirred at room temperature for 10 min, followed by irradiating at 365 nm for another 1 h to afford the **3ah**. Then, corresponding alkyne (5.0 eq.) was dissolved in reaction mixture, $CuSO_4$ (20 mol%) and THPTA (100 mol%) in water (10 μL) was added, followed by sodium ascorbate (200 mol%) in water (10 μL) was added to the reaction mixture. The mixture was stirred at room temperature for 3 h. After that, the reaction was analyzed by HPLC-MS.



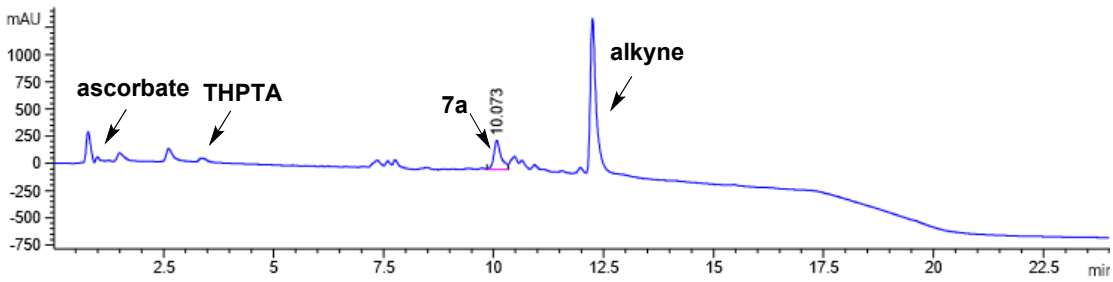


Figure S125. LC-MS UV spectrum of the whole process for **7a** on 220 nm. Gradient used：Method A

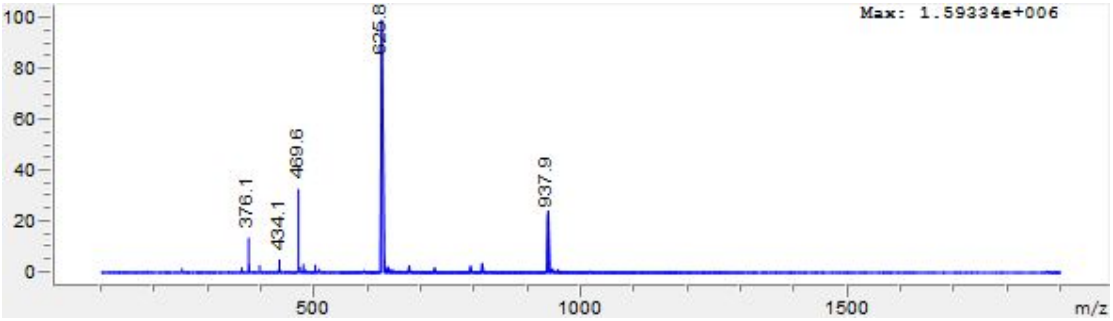


Figure S126. ESI Mass spectrum of the product **7a**. Calculated Mass $[M+2H]^{2+}$: 937.9, $[M+3H]^{3+}$: 625.6, $[M+4H]^{4+}$: 469.4; Mass Found (ESI+) $[M+2H]^{2+}$: 937.9, $[M+3H]^{3+}$: 625.8, $[M+4H]^{4+}$: 469.6.

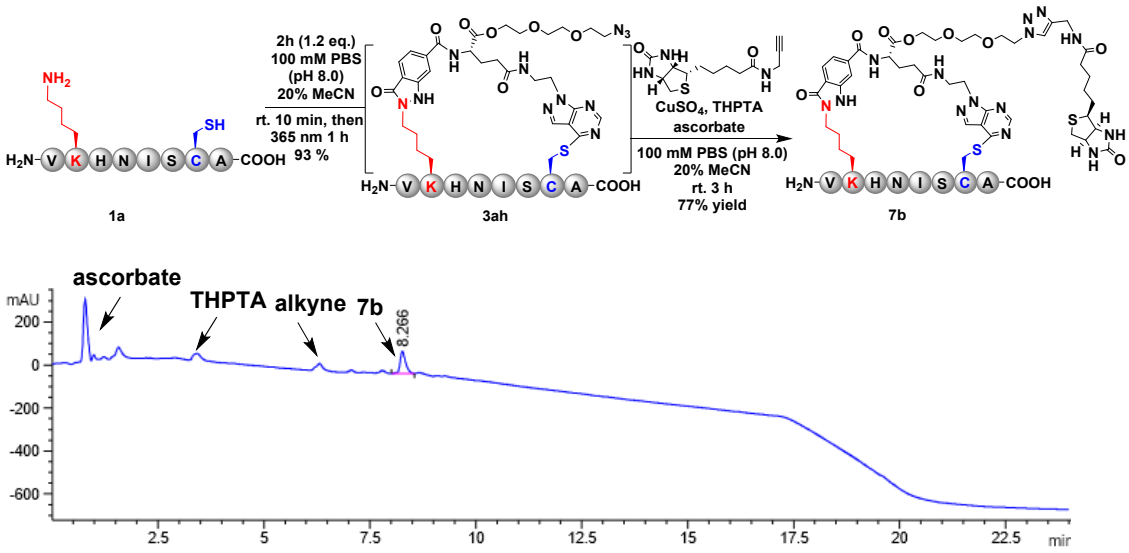


Figure S127. LC-MS UV spectrum of the whole process for **7b** on 220 nm. Gradient used：Method A

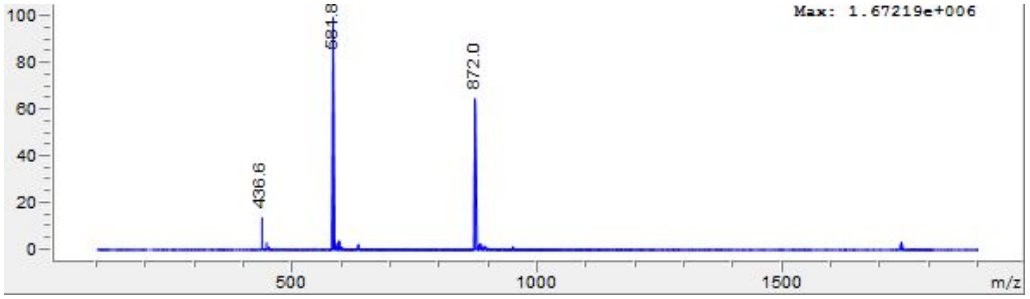


Figure S128. ESI Mass spectrum of the product **7b**. Calculated Mass $[M+2H]^{2+}$: 871.9, $[M+3H]^{3+}$: 581.6; Mass Found (ESI+) $[M+2H]^{2+}$: 872.0, $[M+3H]^{3+}$: 581.8.

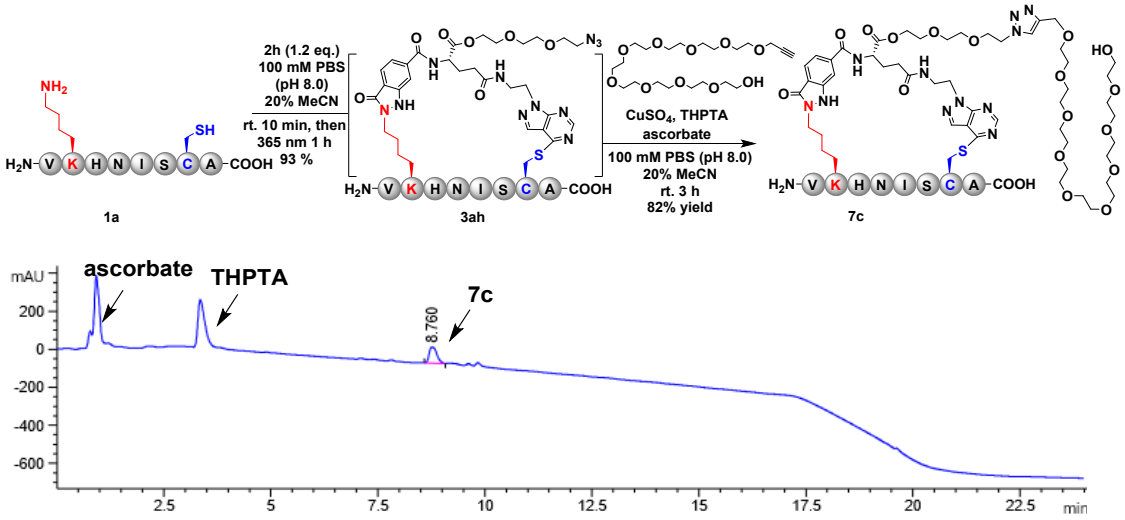


Figure S129. LC-MS UV spectrum of the whole process for **7c** on 220 nm. Gradient used：Method A

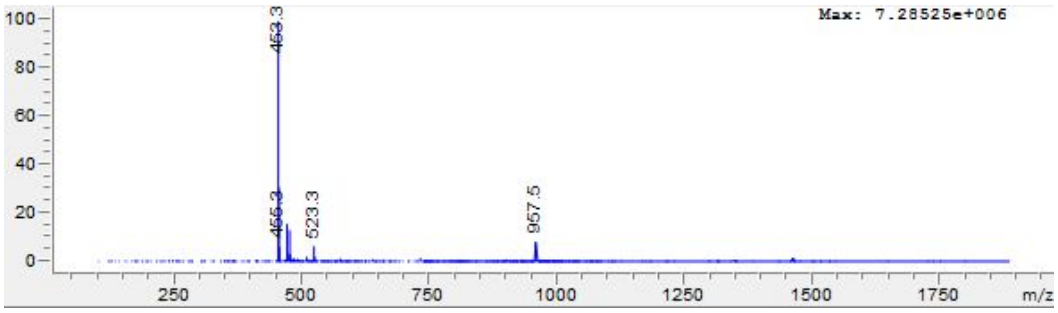
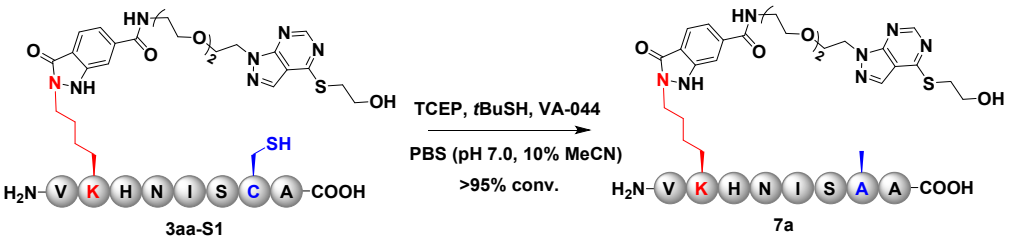


Figure S130. ESI Mass spectrum of the product **7c**. Calculated Mass $[M+2H]^{2+}$: 957.5; Mass Found (ESI+) $[M+2H]^{2+}$: 957.5.

9. Desulfurization of the directed Cys to Ala

General procedure for desulfurization: 0.25 μmmol peptide (**3aa-S1** or **6ca**) was dissolved in 300 μL PBS and 50 μL MeCN in a 2 mL Eppendorf reaction tube. 125 μL 0.4 M TCEP, 10 μL *t*BuSH and 10 μL 0.25 M VA-044 were added to the tube. The solution was stirred at 37 °C for 2 h and analyzed by LC-MS.



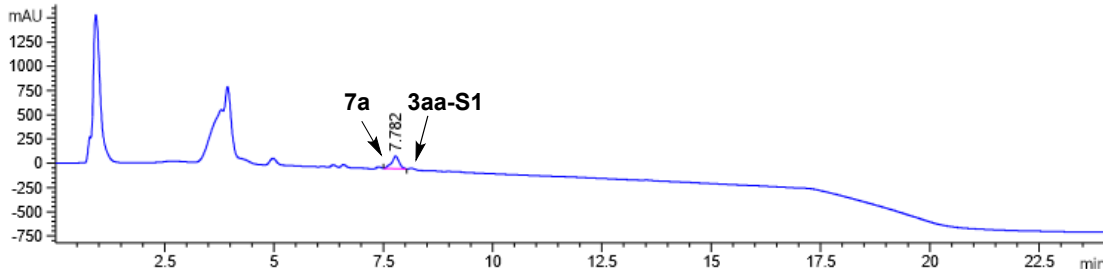


Figure S131. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

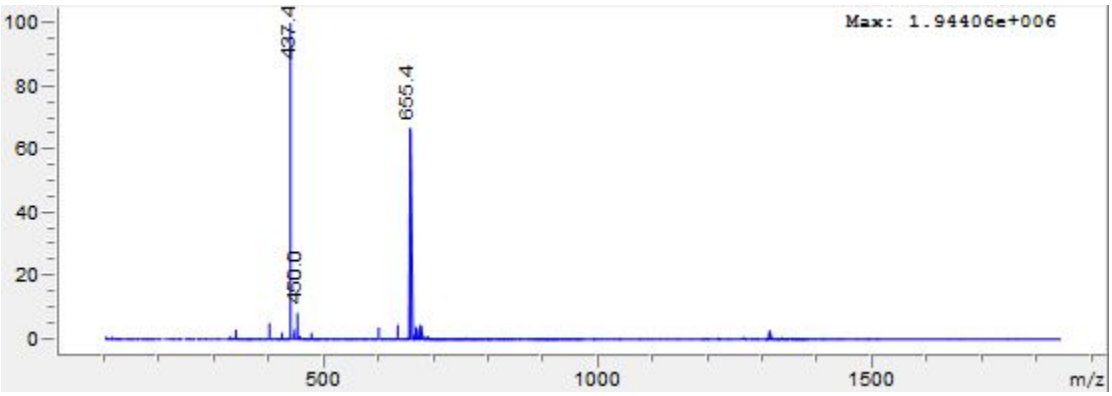
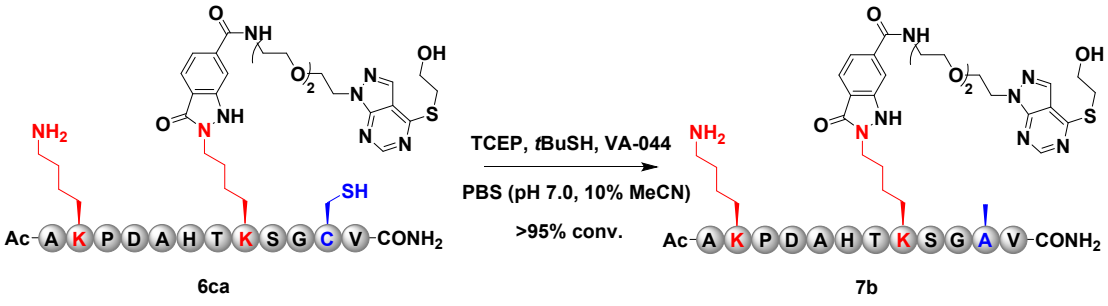


Figure S132. ESI Mass spectrum of the product **7a**. Calculated Mass $[M+2H]^{2+}$: 655.3, $[M+3H]^{3+}$: 437.2; Mass Found (ESI+) $[M+2H]^{2+}$: 655.4, $[M+3H]^{3+}$: 437.4.



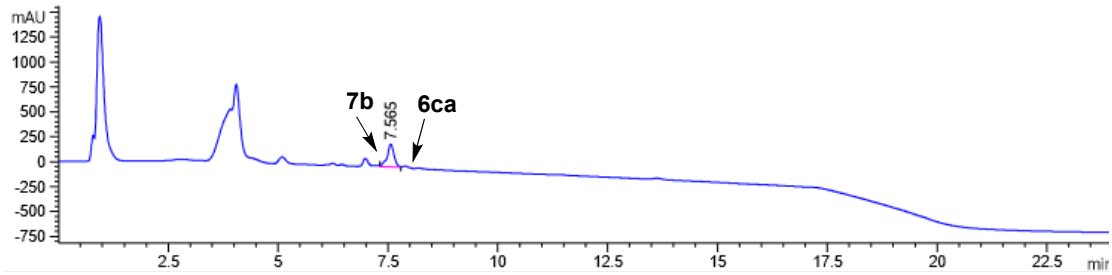


Figure S133. LC-MS UV spectrum of reaction mixture on 220 nm. Gradient used：Method A

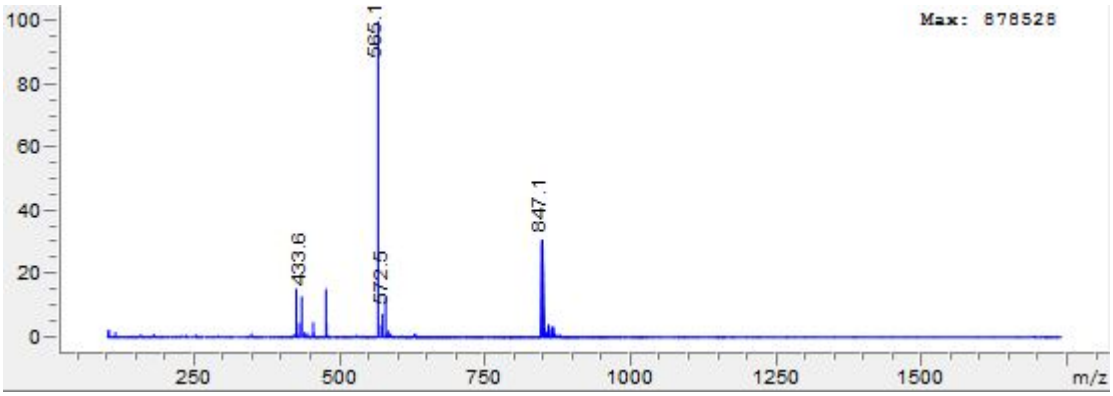


Figure S134. ESI Mass spectrum of the product **7b**. Calculated Mass $[M+2H]^{2+}$: 846.9, $[M+3H]^{3+}$: 564.9; Mass Found (ESI+) $[M+2H]^{2+}$: 847.1, $[M+3H]^{3+}$: 565.1.

10. Cys directed Lys stapling on proteins

Bovine serum albumin (BSA) was purchased from Sigma and used without further purification. BSA is composed of 583 amino acids and contains one free cysteine Cys34 (35 Cys residues in total, and 34 cysteine residues are in the form of disulfide bonds) and 59 lysines. Calculated M.W. (average): 66430 Da

Sequence:

DTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHVKLVNELTEFAKTCVAD
ESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQEPERNECFLSHKDDSPD
LPKLKPDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVFQEC
CQAEDKGACLLPKIETMREKVLTSSARQRLRCASIQKFGERALKAWSVARLSQKFP
KAEFVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDK
PLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRH
PEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQF
EKLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTED
YLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTF
HADICTLPDTEKQIKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEA
CFAVEGPKLVV STQTALA

Histone H3.3 human was purchased from Sigma and used after removal of excess reagents by buffer exchange. H3.3 is composed of 136 amino acids and contains one

cysteine and 13 lysines. Calculated M.W. (average): 15197 Da

Sequence:

ARTKQTARKSTGGKAPRKQLATKAARKSAPSTGGVKKPHRYRPGTVALREIRRYQ KSTELLIRKLPFQRLVREIAQDFKTGLRFQSAAIGALQEASEAYLVGLFEDTNLCAIHA KRVTIMPKDIQLARRIRGERA

General procedure for BSA stapling: In a 1.5 mL eppendorf tube, bovine serum albumin (BSA) solution in 100 mM PBS buffer (pH = 8.0) (10 nmmol, 0.9 mL), **2a** (1.2 eq. in MeCN) or **2h** (1.2 eq. in DMSO) was added and shook at room temperature for 5 h. Then, removing excess reagents by repeated ultracentrifugation into 100 mM PBS buffer (pH = 8.0) using an Amicon centrifugal filter (30k MWCO, Merck Millipore, Darmstadt, Germany) and the solution was irradiated at 365nm for 1 h. After that, the protein samples was buffer-exchanged with 20 mM ammonium acetate (AA) at a concentration of 10 μM using Amicon centrifugal filters (Merck Millipore, Darmstadt, Germany) with a molecular weight cutoff of 30 k. For denatured nano ESI-MS analysis, the desalted protein samples were further diluted with 49.5/49.5/1 (v/v/v) methanol/water/formic acid mixture solution to a concentration of 3 μM. BSA protein aliquots (3 μL) were analyzed using a quadrupole ion mobility time-of-flight mass spectrometer (Synapt G2-Si HDMS, Waters, UK) in the positive ion mode according to the previously described method.[3] Briefly, BSA protein ions were generated by nano-ESI from a homemade borosilicate capillary emitter (1.0 mm o.d./0.58 mm i.d., Sutter Instruments, Novato, CA, USA) with a tip i.d. of ~1 μm pulled using a P-97 puller (Sutter Instruments, Novato, CA, USA). The detailed instrumental parameters were as follows: capillary voltage 0.7 kV, sampling cone 100 V, extraction cone 80 V, and source temperature 30 °C.

General procedure for histone H3.3 stapling: The procedure for H3.3 stapling was similar as BSA but **2a** or **2d** (10 eq. in MeCN) and Amicon centrifugal filter (3k MWCO, Merck Millipore, Darmstadt, Germany) were used. The mixture was reacted at rt. for 3 h and irradiated at 365nm for another 1 h.

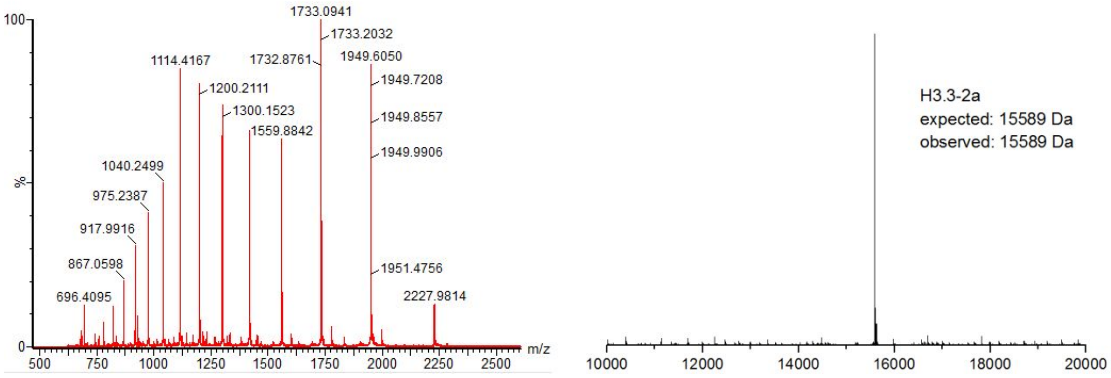


Figure S135. Non-deconvoluted (left) and deconvoluted (right) mass spectrum of **H3.3-2a**.

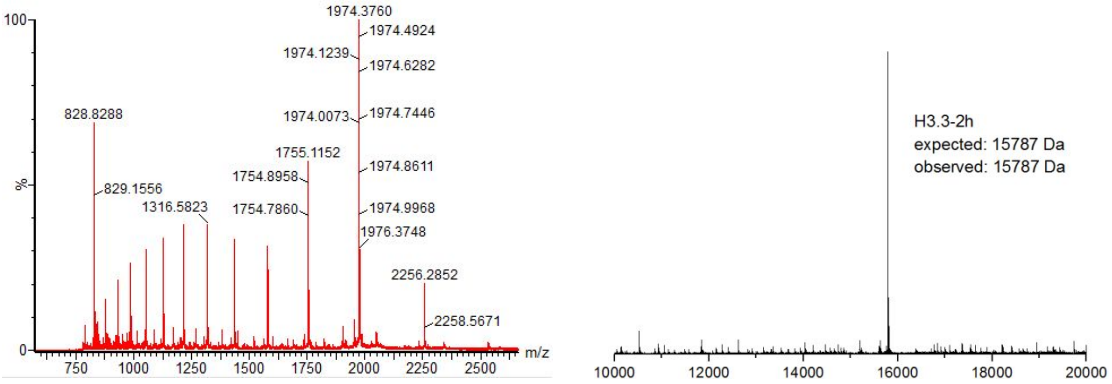


Figure S136. Non-deconvoluted (left) and deconvoluted (right) mass spectrum of **H3.3-2h**.

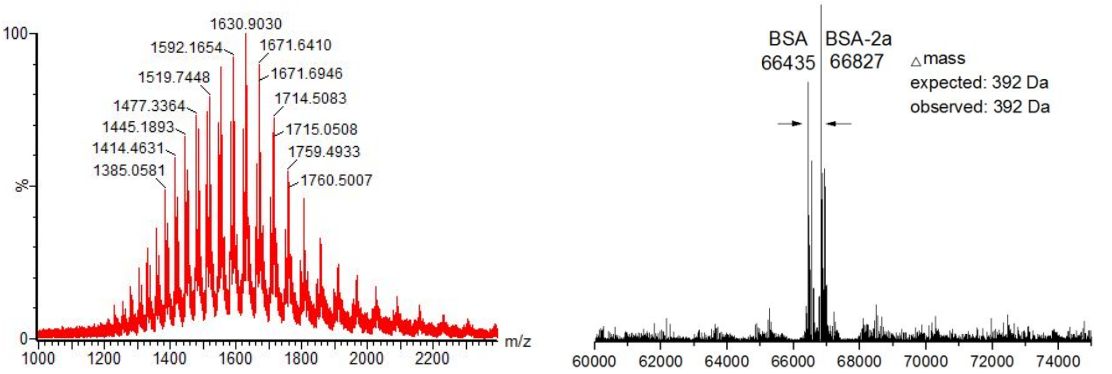


Figure S137. Non-deconvoluted (left) and deconvoluted (right) mass spectrum of **BSA-2a**.

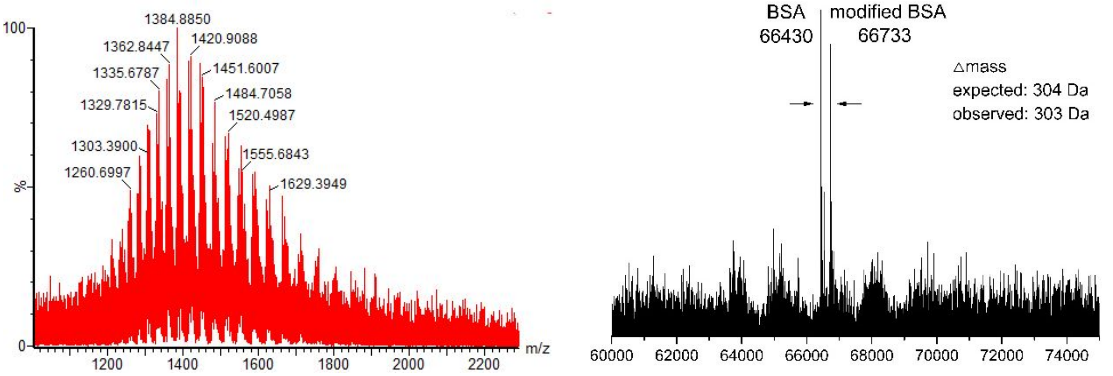
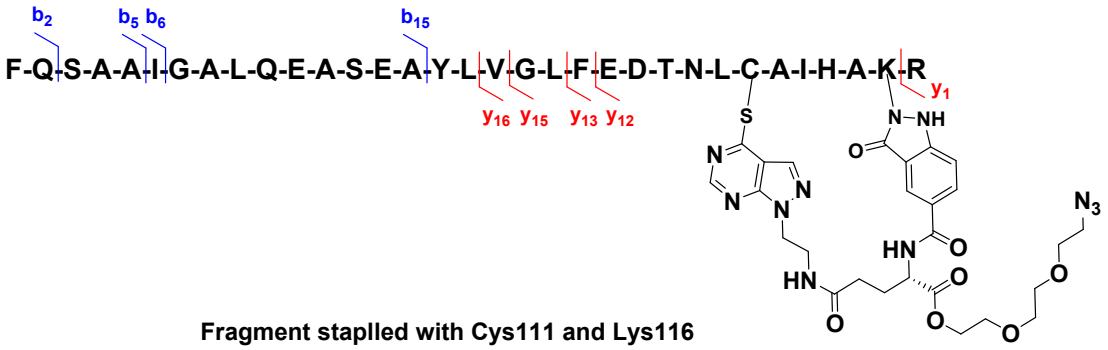


Figure S138. Non-deconvoluted (left) and deconvoluted (right) mass spectrum of **BSA-2d**.

Determination of the Lys site in **H3.3-2h** stapling reaction by trypsin digestion: Reaction mixture of histone H3.3 with **2h** (1 μg protein) was mixed with 50 mM NH_4HCO_3 buffer (50 μL) and trypsin stock solution (0.5 μL 0.1 μg/μL). The reaction was heated at 37 °C for 12 h. Then the mixture was centrifuged, and analyzed by LC-MS/MS. According to reported methods,[4,5] a mass of the next fragment was identified, indicating the crosslink of fragments containing Cys111 and Lys116.



Fragment staplled with Cys111 and Lys116

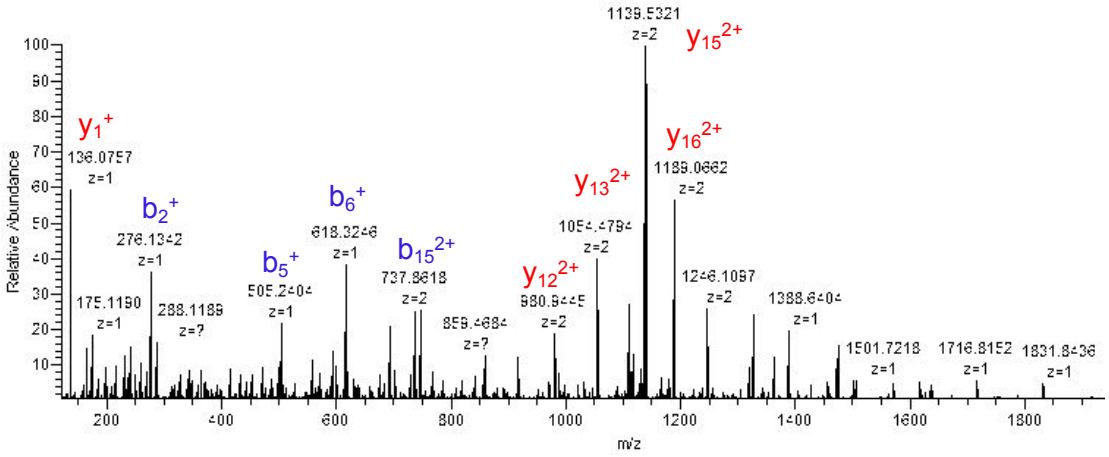
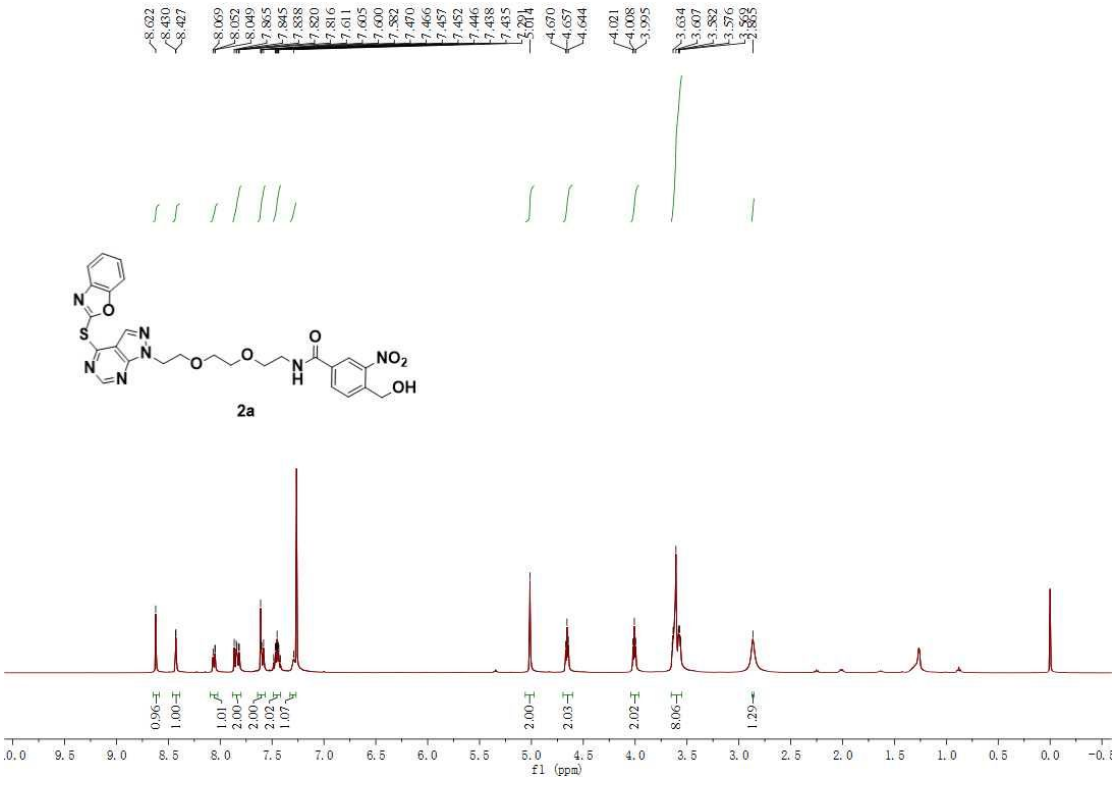
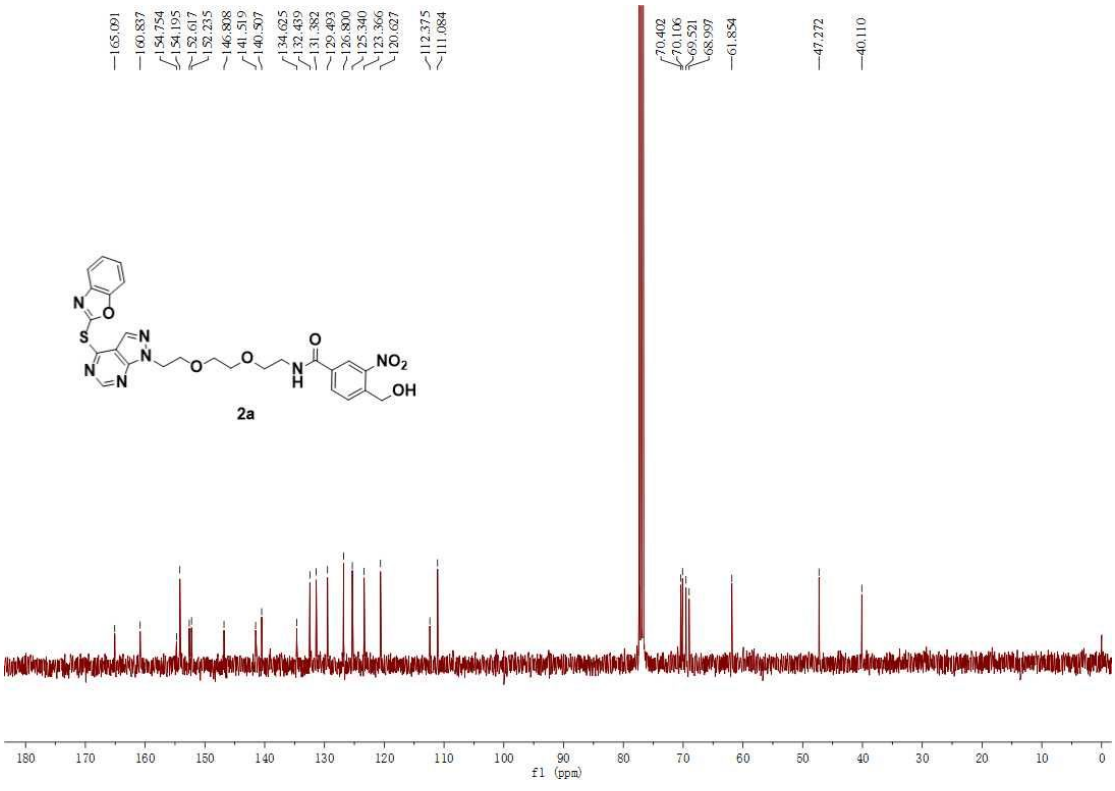
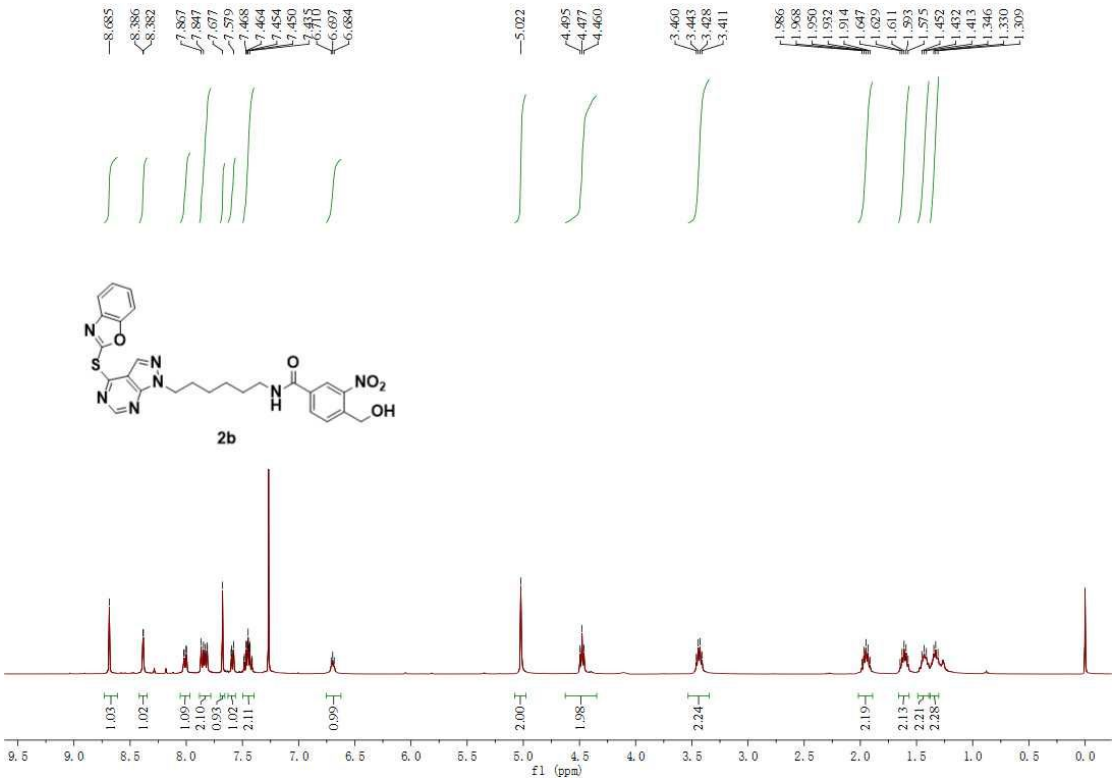


Figure S139. MS/MS fragmentation of the trypsin digested stapled peptides derived from the **H3.3-2h** stapling reaction. b_n indicates the b ion from *N*-terminus of the stapled peptide to residue n. y_n indicates the y ion from the residue n to the *C*-terminus of the stapled peptide.

11. Spectra of new compounds



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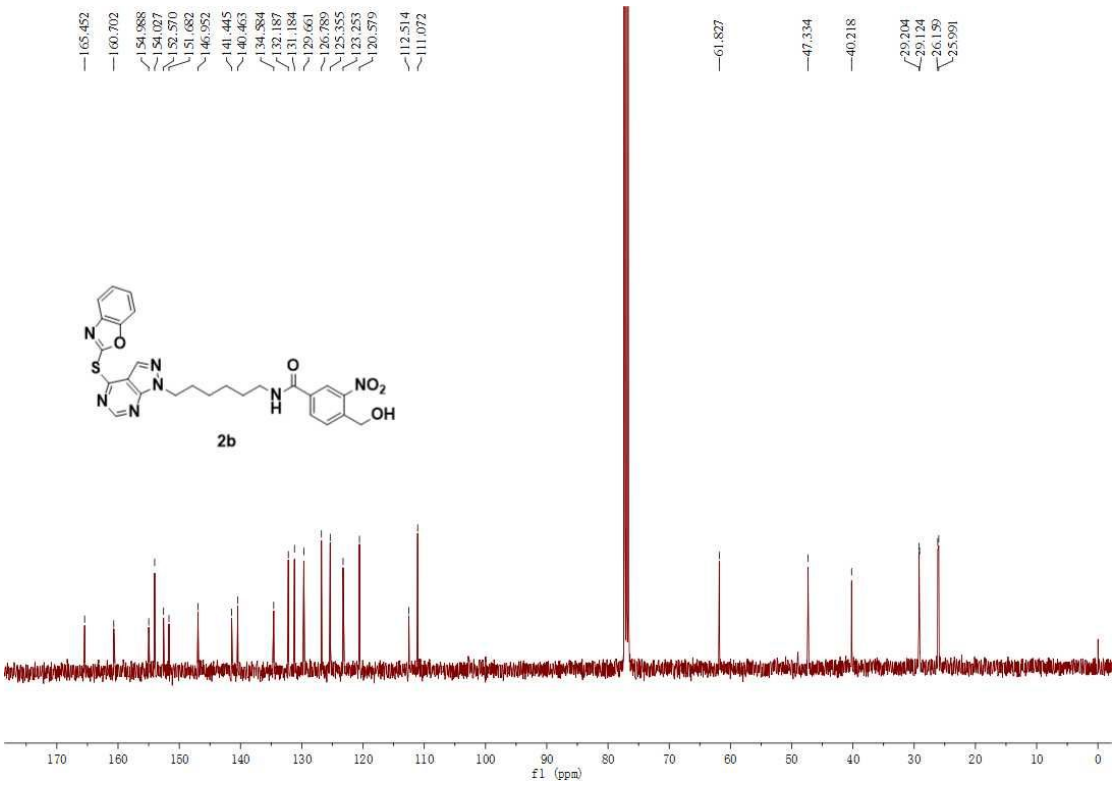
8.386
8.382
7.867
7.847
7.677
7.579
7.468
7.464
7.454
7.450
6.710
6.697
6.684
5.022
4.495
4.477
4.460
3.460
3.443
3.428
3.411
1.986
1.968
1.950
1.932
1.914
1.647
1.629
1.611
1.593
1.575
1.452
1.432
1.413
1.346
1.330
1.309
2b
1.03
1.02
1.09
2.10
0.93
1.02
2.11
0.99
2.00
1.98
2.24
2.19
2.13
2.21
2.28
f1 (ppm)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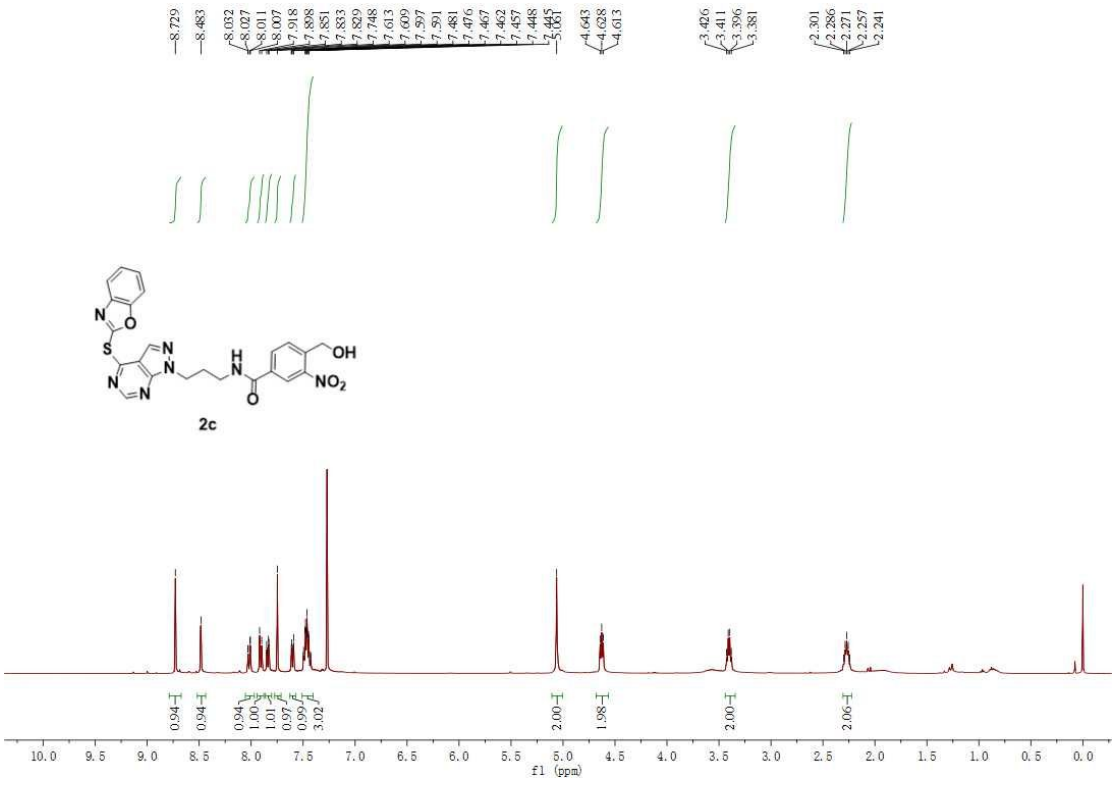
160.702
154.988
154.027
152.570
151.682
146.952
141.445
140.463
134.584
132.187
131.184
129.661
126.789
125.355
123.253
120.579
112.514
111.072
61.827
47.334
40.218
29.204
29.124
26.159
25.991
2b
f1 (ppm)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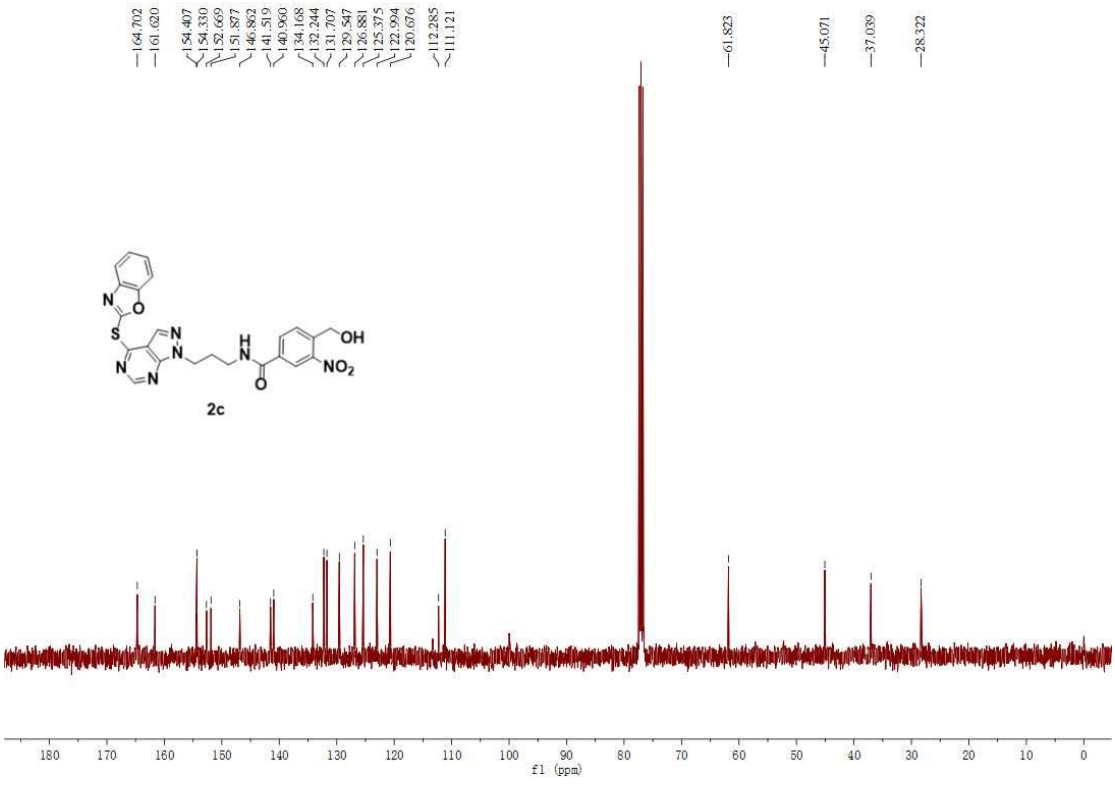
8.483
8.032
8.027
8.011
8.007
7.918
7.898
7.851
7.833
7.829
7.748
7.613
7.609
7.597
7.591
7.481
7.476
7.467
7.462
7.457
7.448
7.445
5.061
4.643
4.628
4.613
3.426
3.411
3.396
3.381
2.301
2.286
2.271
2.257
2.241
OH
NO2
2c
0.94
0.94
0.94
1.00
1.01
0.97
0.99
3.02
2.00
1.98
2.00
2.06
f1 (ppm)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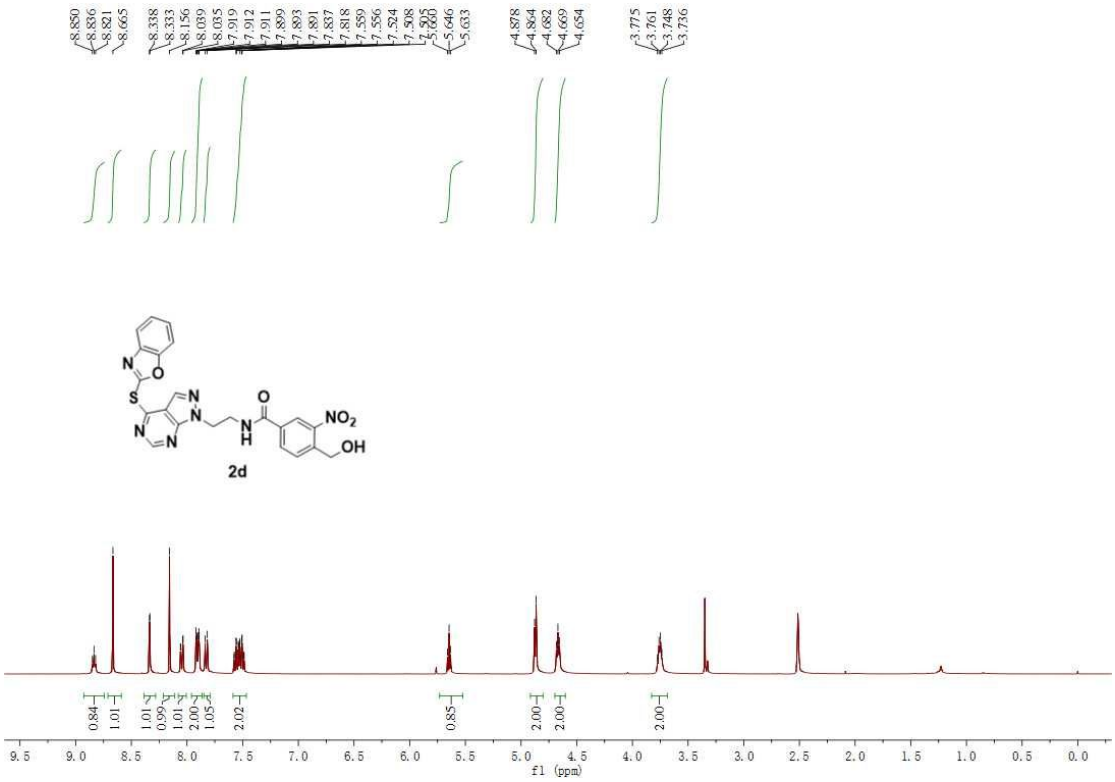
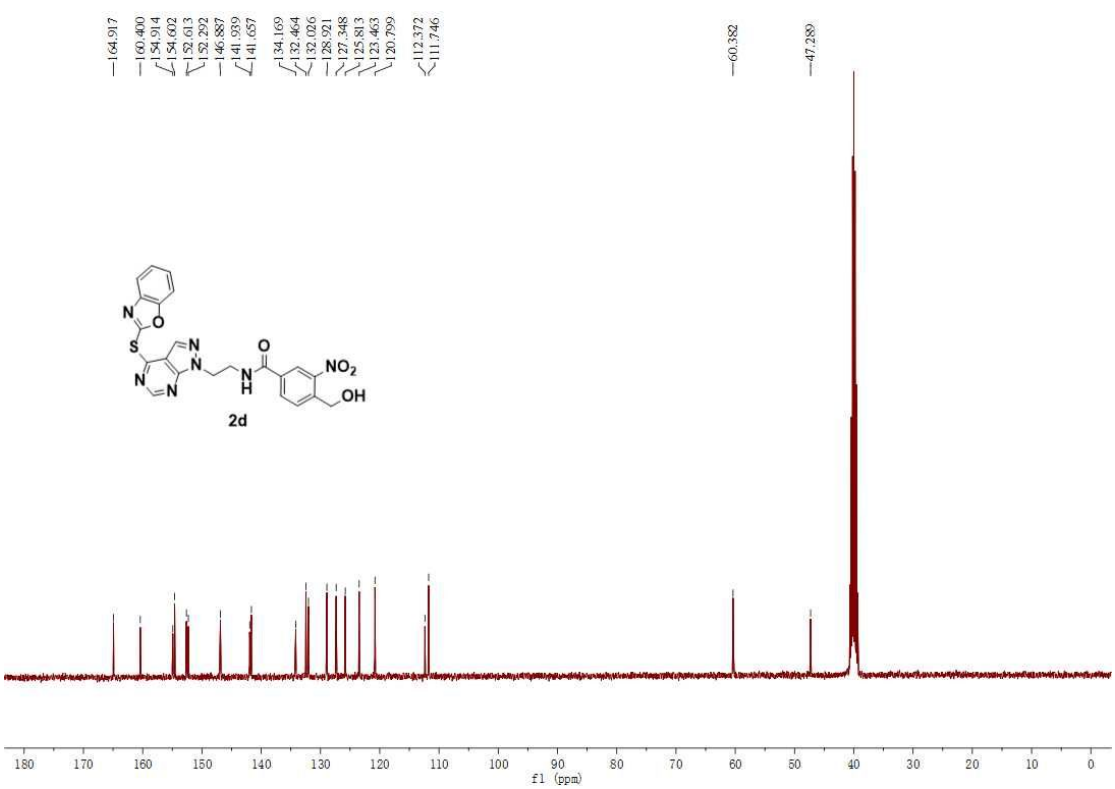
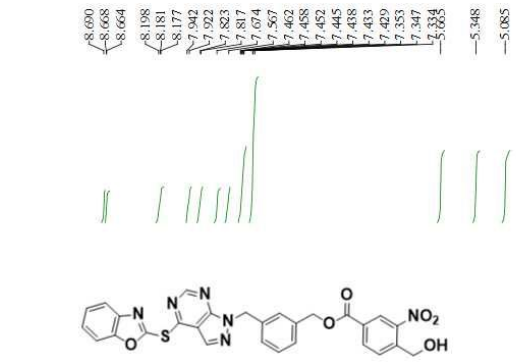
161.620
154.407
154.330
152.669
151.877
146.862
141.519
140.960
134.168
132.244
131.707
129.547
126.881
125.375
122.994
120.676
112.285
111.121
61.823
45.071
37.039
28.322
OH
NO2
2c
f1 (ppm)


8.850
8.836
8.821
8.665
8.338
8.333
8.156
8.039
8.035
7.919
7.912
7.911
7.899
7.893
7.891
7.837
7.818
7.559
7.556
7.524
7.508
5.646
5.633
4.878
4.864
4.682
4.669
4.654
3.775
3.761
3.748
3.736
2d
0.84
1.01
1.01
0.99
1.01
2.00
1.05
2.02
0.85
2.00
2.00
2.00
f1 (ppm)


164.917
160.400
154.914
154.602
152.613
152.292
146.887
141.939
141.657
134.169
132.464
132.026
128.921
127.348
125.813
123.463
120.799
112.372
111.746
60.382
47.289
2d
f1 (ppm)


8.690
8.668
8.664
8.198
8.181
8.177
7.942
7.922
7.823
7.817
7.674
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7.462
7.458
7.452
7.445
7.438
7.433
7.429
7.353
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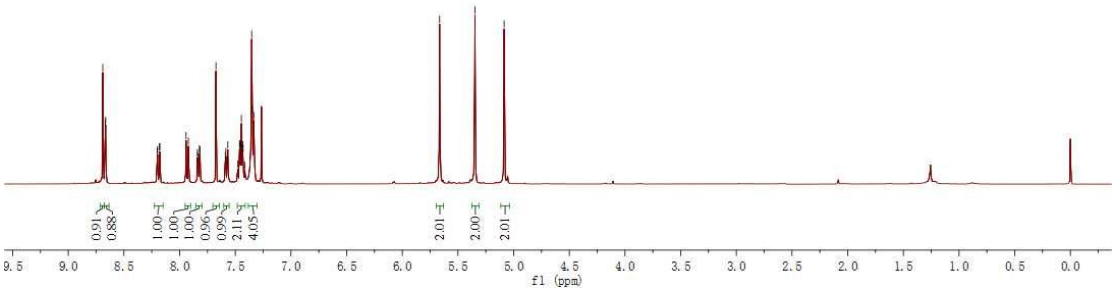
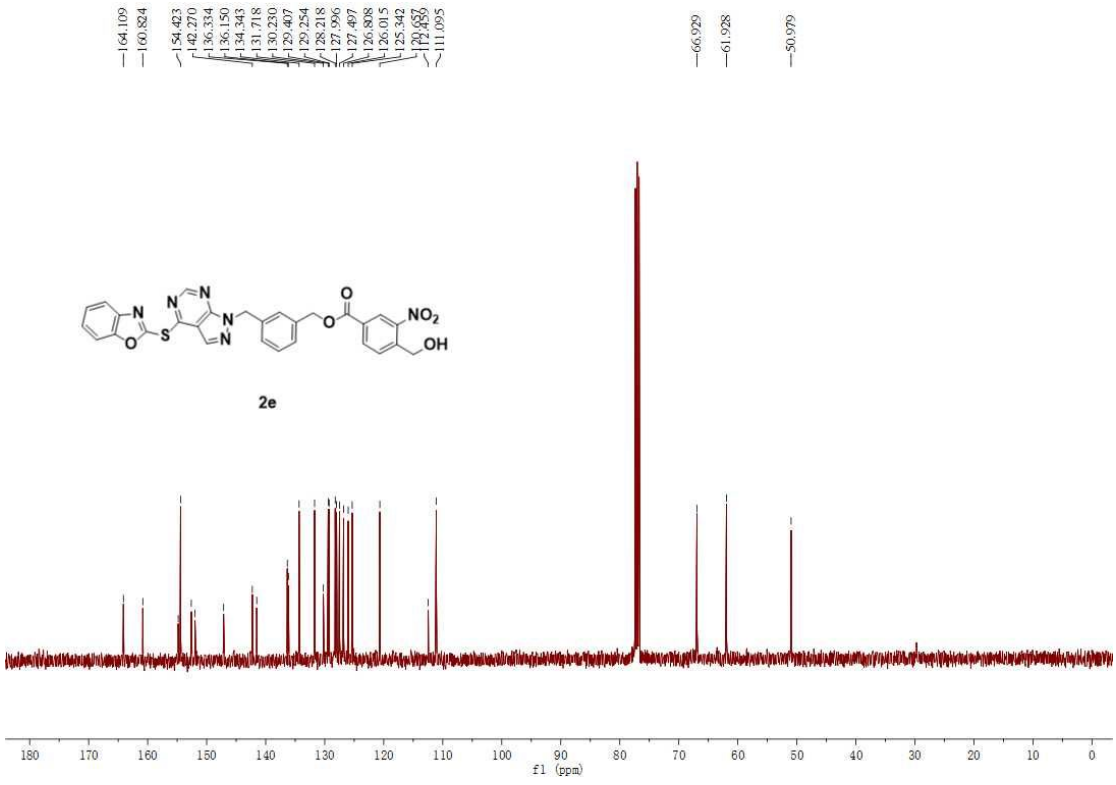
N
N
S
O
O
O
NO2
OH

2e


0.91
0.88
1.00
1.00
1.00
0.96
0.99
2.11
4.05
2.01
2.00
2.01
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)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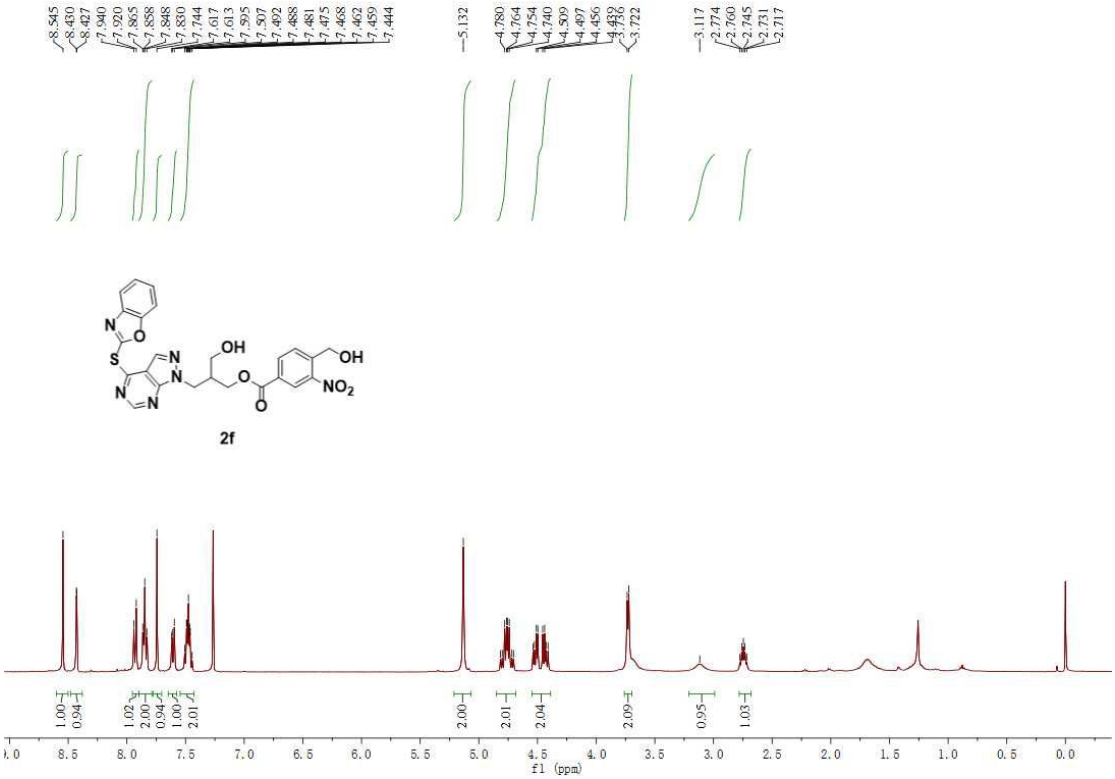
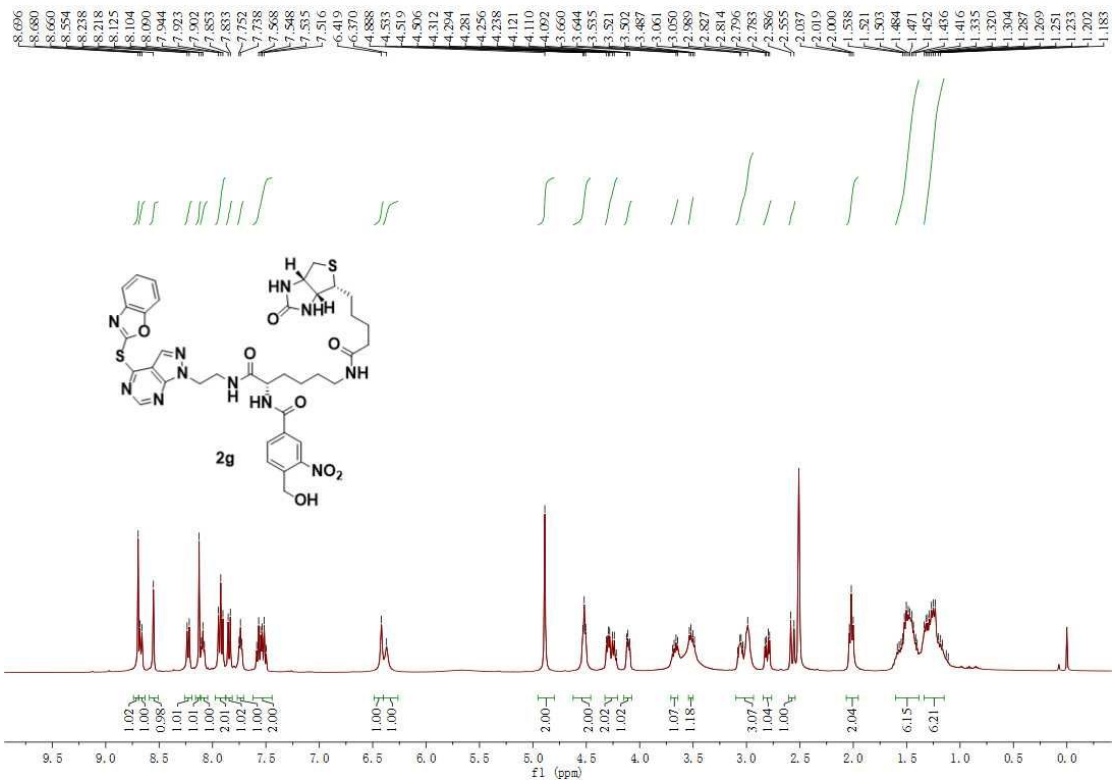
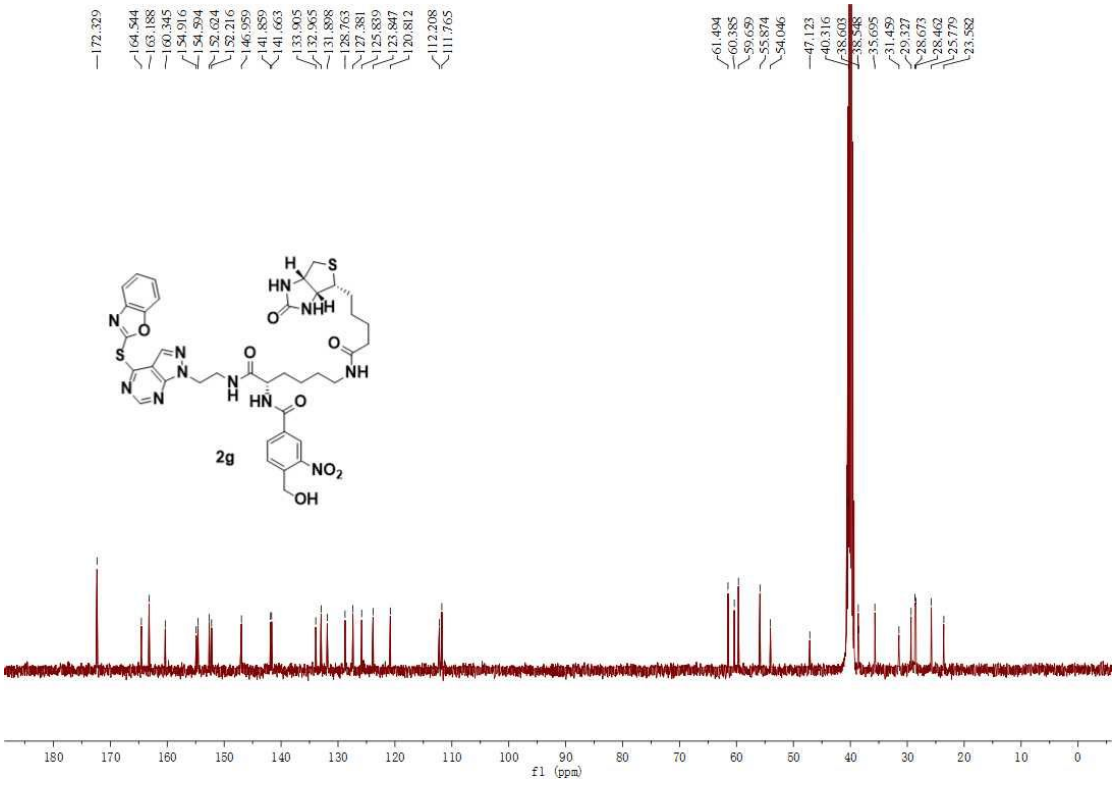
160.824
154.423
142.270
136.334
136.150
134.343
131.718
130.230
129.407
129.254
128.218
127.996
127.497
126.808
126.015
125.342
120.687
112.459
111.095
66.929
61.928
50.979
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

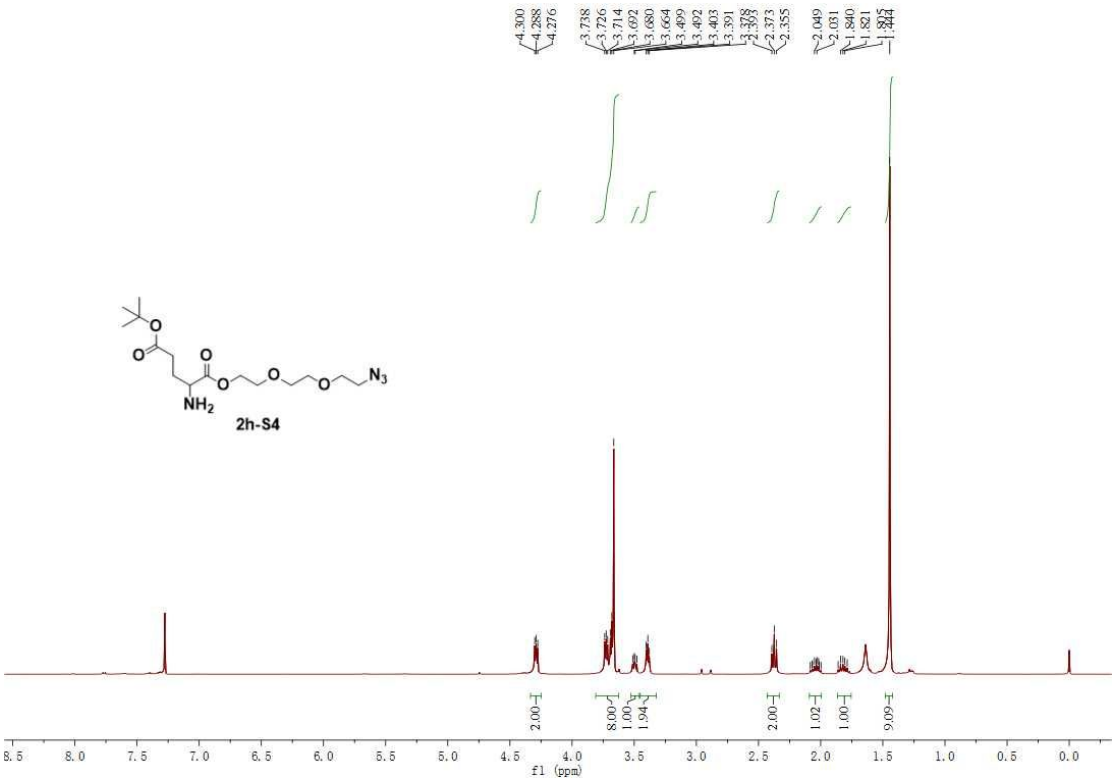
N
N
N
N
S
O
O
O
NO2
OH

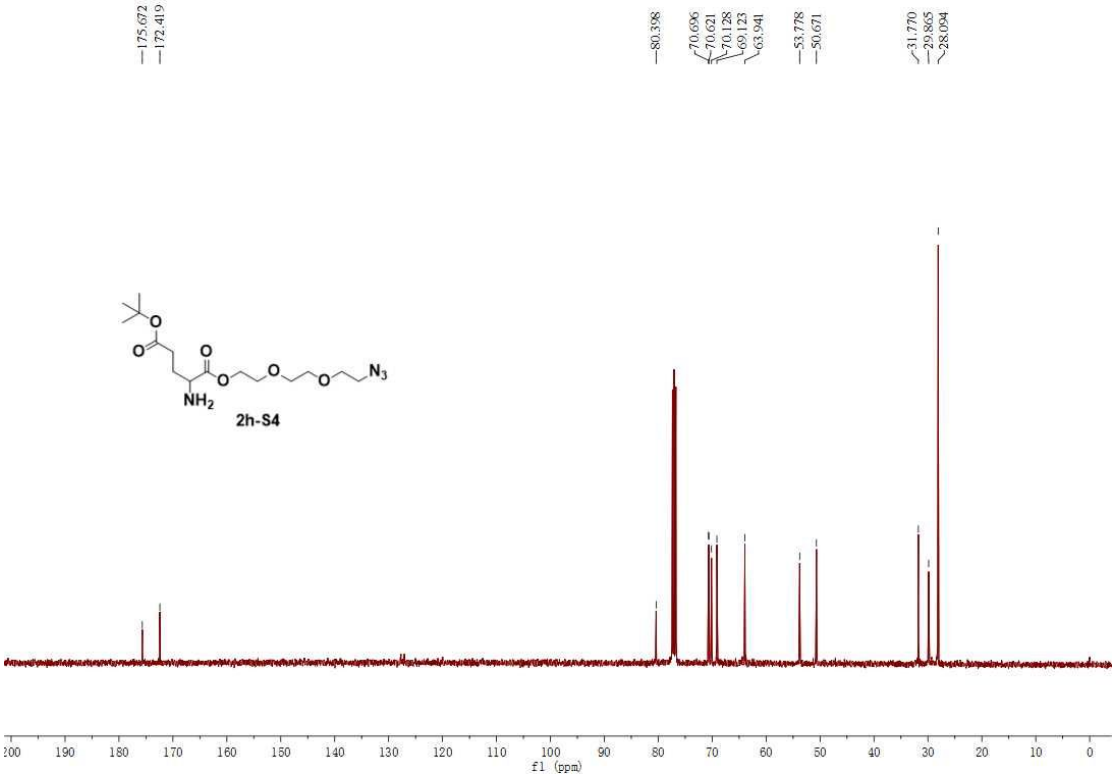
2e

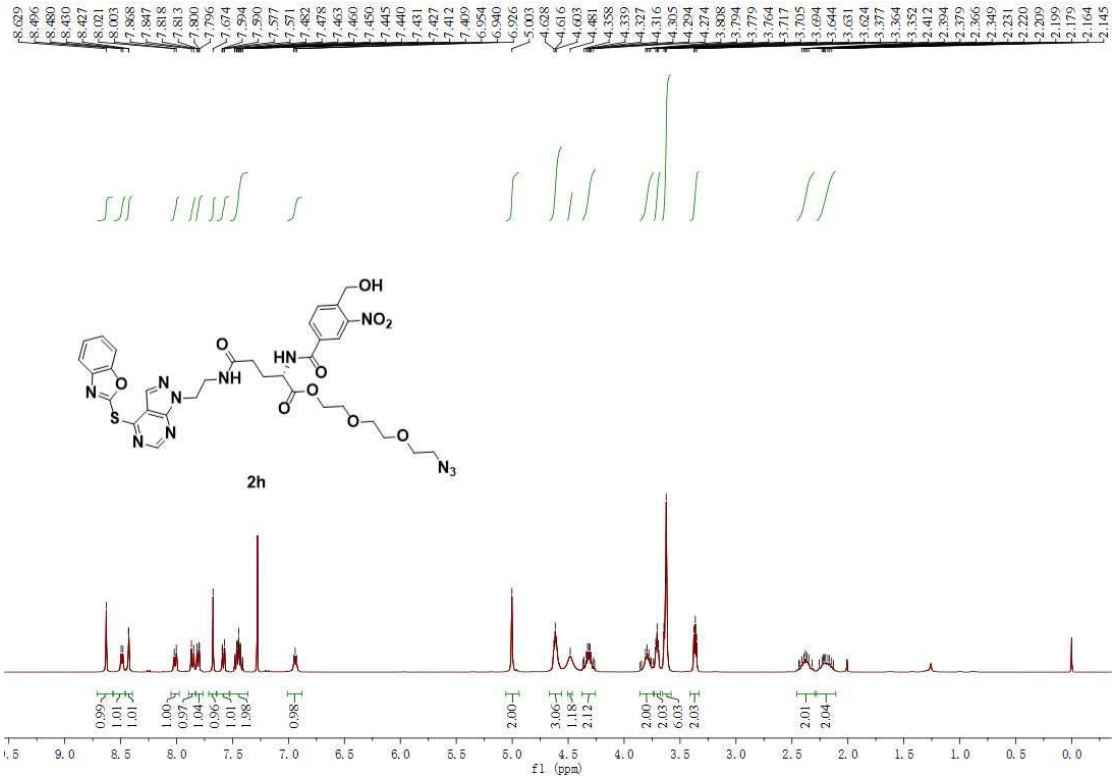

8.545
8.430
8.427
7.940
7.920
7.865
7.858
7.848
7.830
7.744
7.617
7.613
7.595
7.507
7.492
7.488
7.481
7.475
7.468
7.462
7.459
7.444
5.132
4.780
4.764
4.754
4.740
4.509
4.497
4.456
4.439
4.436
3.722
3.117
2.774
2.760
2.745
2.731
2.717
OH
OH
NO2
2f
1.00
0.94
1.02
2.00
0.94
1.00
2.01
2.00
2.01
2.04
2.09
0.95
1.03
f1 (ppm)
163.951
161.288
154.241
154.154
152.739
152.097
146.933
142.883
141.331
133.743
131.426
129.603
129.172
127.010
125.613
125.456
120.623
111.996
111.190
64.942
61.845
60.346
46.592
40.917
OH
OH
NO2
2f
f1 (ppm)

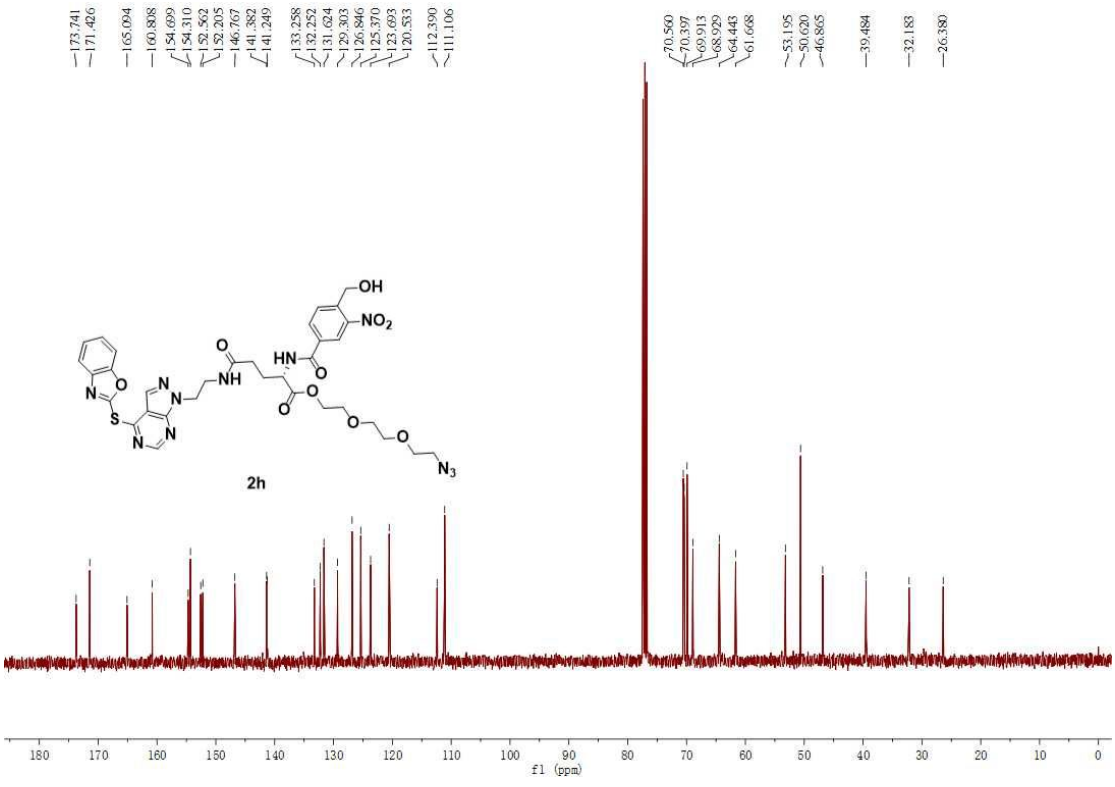

2g
f1 (ppm)

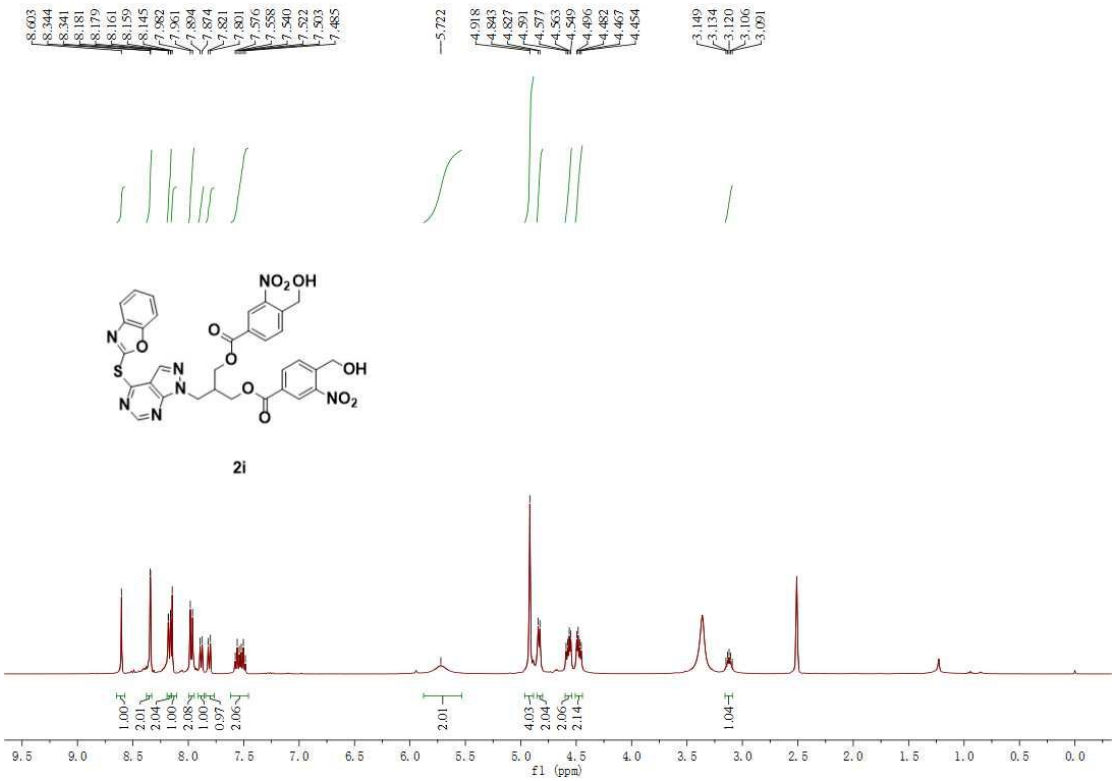

2g
f1 (ppm)

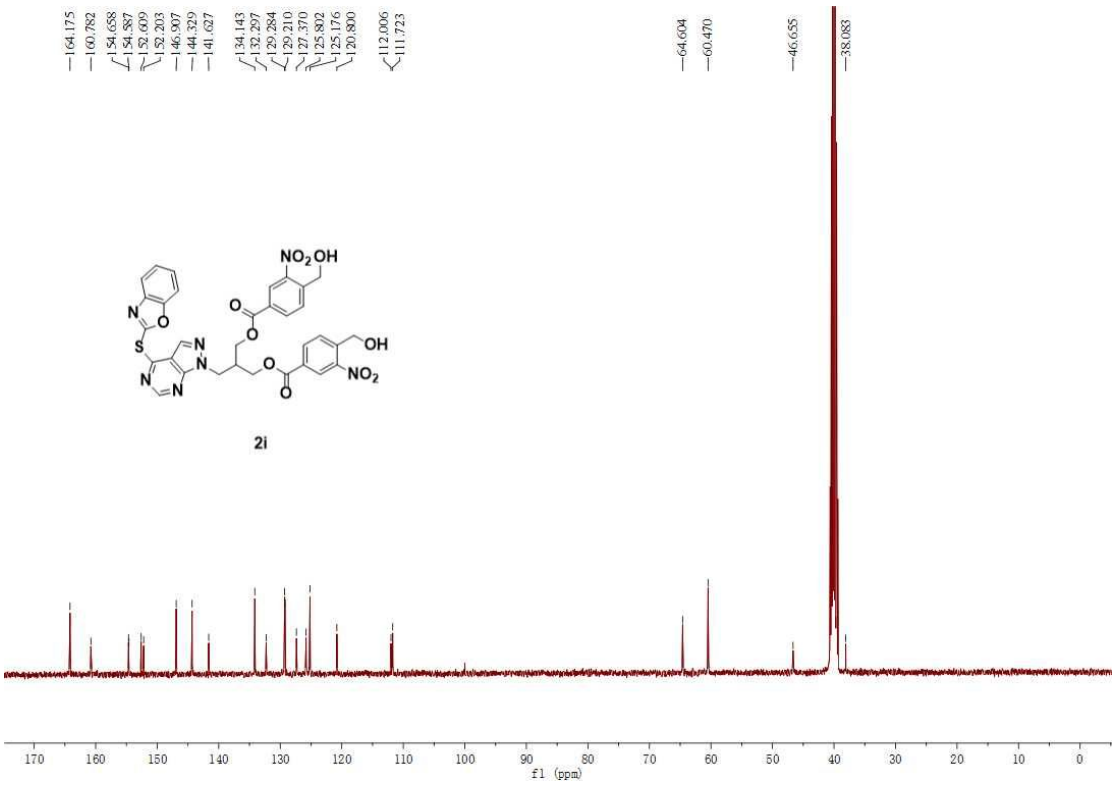

4.300
4.288
4.276
3.738
3.726
3.714
3.692
3.680
3.664
3.499
3.492
3.403
3.391
2.373
2.355
2.049
2.031
1.840
1.821
1.805
1.444
2.00
8.00
1.00
1.94
2.00
1.02
1.00
9.09
f1 (ppm)
2h-S4

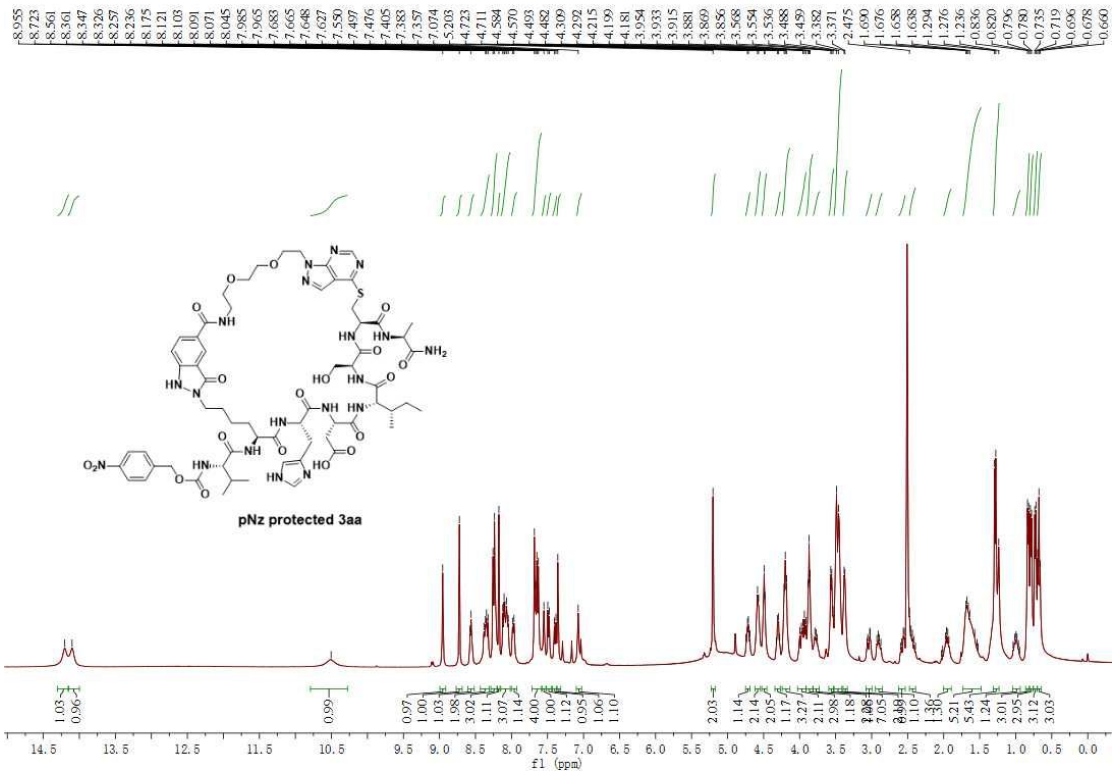

175.672
172.419
80.398
70.696
70.621
70.128
69.123
63.941
53.778
50.671
31.770
29.865
28.094
f1 (ppm)
2h-S4

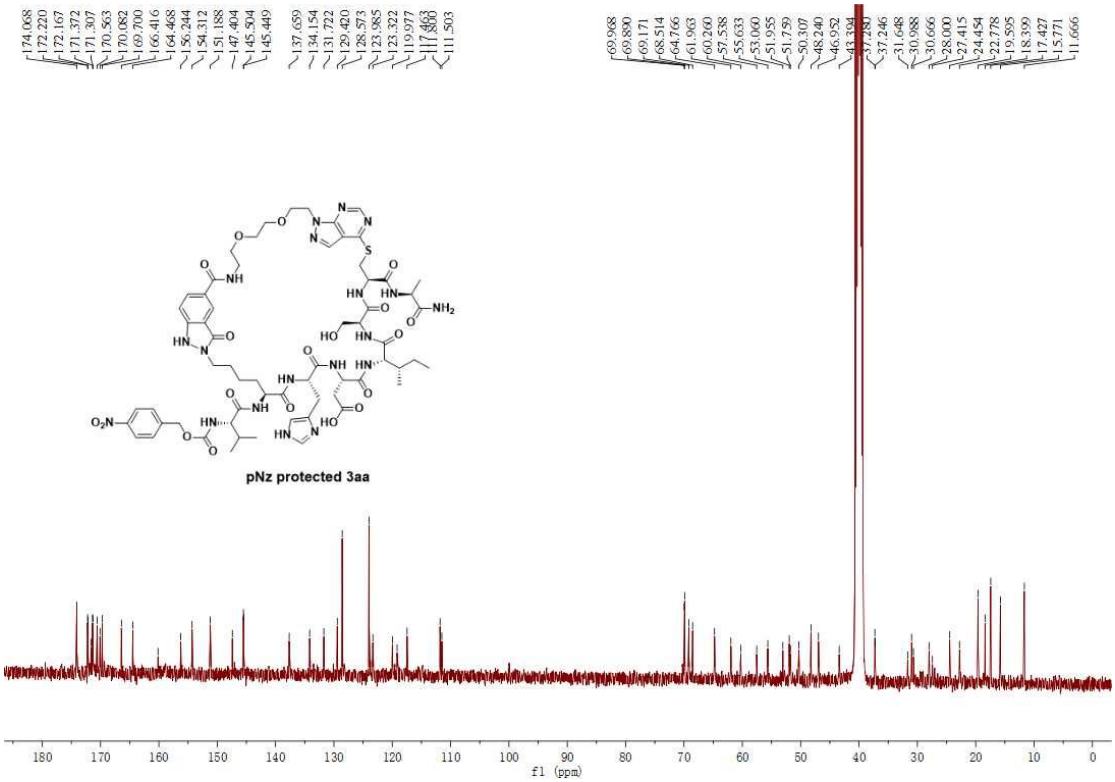

8.629
8.496
8.480
8.430
8.427
8.021
8.003
7.868
7.847
7.818
7.813
7.800
7.796
7.674
7.594
7.590
7.577
7.571
7.482
7.478
7.463
7.460
7.450
7.445
7.440
7.431
7.427
7.412
7.409
6.954
6.940
6.926
5.003
4.628
4.616
4.603
4.481
4.358
4.339
4.327
4.316
4.305
4.294
4.274
3.808
3.794
3.779
3.764
3.717
3.705
3.694
3.644
3.631
3.624
3.377
3.364
3.352
2.412
2.394
2.379
2.366
2.349
2.231
2.220
2.209
2.199
2.179
2.164
2.145
OH
NO2
HN
NH
O
N
S
N3
2h
0.99
1.01
1.01
1.00
0.97
1.04
0.96
1.01
1.98
0.98
2.00
3.06
1.18
2.12
2.00
2.03
6.03
2.03
2.01
2.04
f1 (ppm)

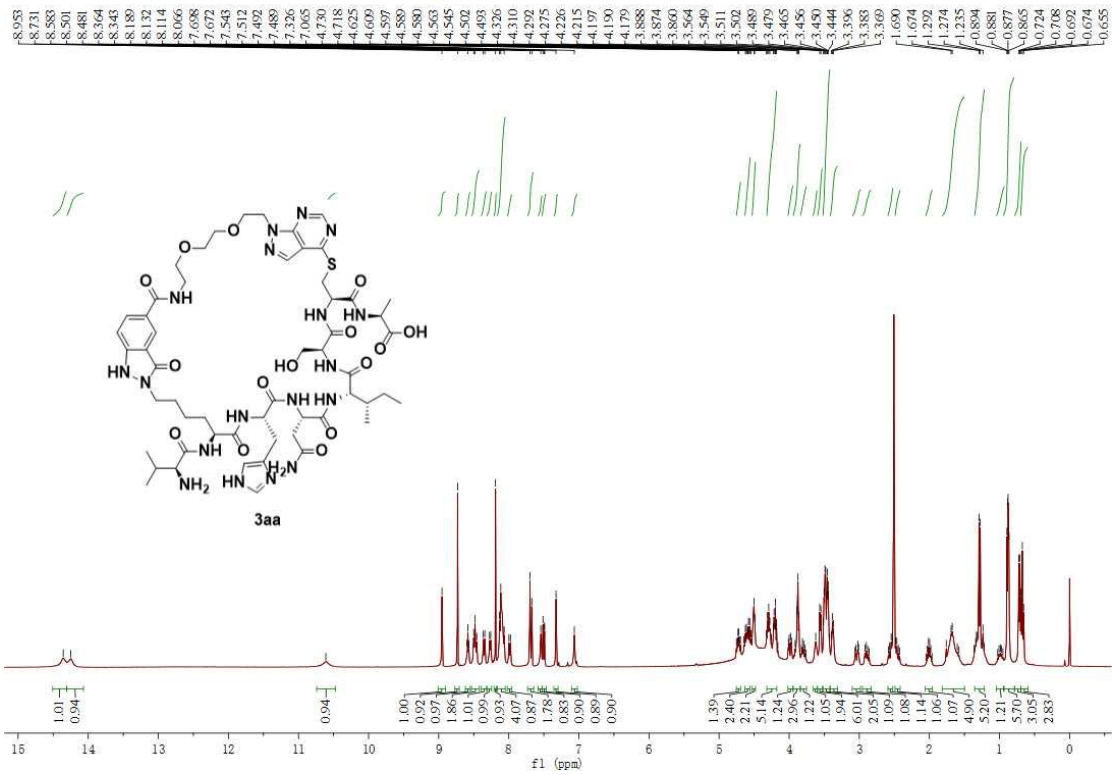

173.741
171.426
165.094
160.808
154.699
154.310
152.562
152.205
146.767
141.382
141.249
133.258
132.252
131.624
129.303
126.846
125.370
123.693
120.533
112.390
111.106
70.560
70.397
69.913
68.929
64.443
61.668
53.195
50.620
46.865
39.484
32.183
26.380
OH
NO2
HN
NH
O
N
S
N3
2h
f1 (ppm)

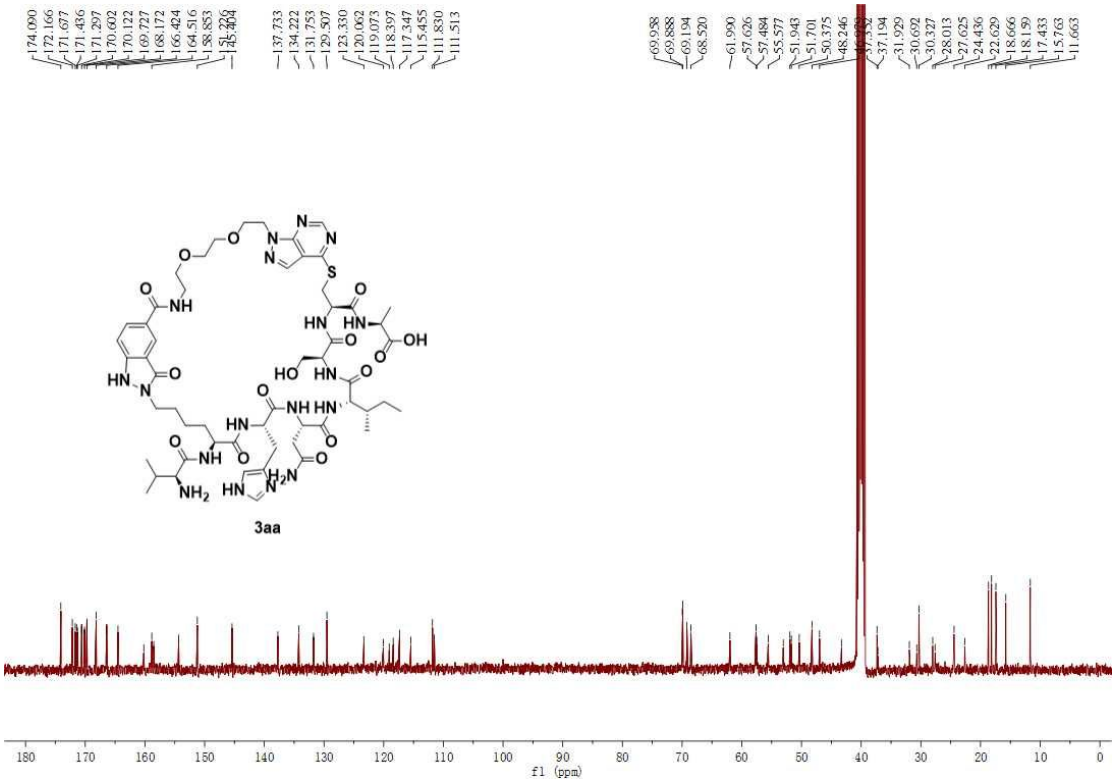

8.603
8.344
8.341
8.181
8.179
8.161
8.159
8.145
7.582
7.561
7.894
7.874
7.821
7.801
7.576
7.558
7.540
7.522
7.503
7.485
5.722
4.918
4.843
4.827
4.591
4.577
4.563
4.549
4.496
4.482
4.467
4.454
3.149
3.134
3.120
3.106
3.091
NO2OH
OH
NO2
2i
1.00
2.01
2.04
1.00
2.08
1.00
0.97
2.06
2.01
4.03
2.04
2.06
2.14
1.04
f1 (ppm)


164.175
160.782
154.658
154.387
152.609
152.203
146.907
144.329
141.627
134.143
132.297
129.284
129.210
127.370
125.802
125.176
120.800
112.006
111.723
64.604
60.470
46.655
38.083
NO2OH
OH
NO2
2i
f1 (ppm)


pNz protected 3aa
f1 (ppm)


pNz protected 3aa
f1 (ppm)


3aa
f1 (ppm)


3aa
f1 (ppm)

12. Reference

[1] Hu W, Yuan Y, Wang CH, Tian HT, Guo A-D, Nie HJ, Hu H, Tan M, Tang Z, Chen XH. *Chem*, 2019, 5: 2955-2968
[2] Li J, Deng JJ, Yin Z, Hu QL, Ge Y, Song Z, Zhang Y, Chan ASC, Li H, Xiong XF. *Chem Sci*, 2021, 12: 5209-5215
[3] Yin Z, Huang J, Miao H, Hu O, Li H. *Anal Chem*, 2020, 92: 12312-12321
[4] Kubota K, Dai P, Pentelute BL, Buchwald SL, *J Am Chem Soc*, 2018, 140: 3128-3133
[5] Silva M, Faustino H, Coelho JAS, Pinto MV, Fernandes A, Companon I, Corzana F, Gasser G, Gois PMP. *Angew Chem Int Ed*, 2021, 60: 10850-10857