Supplementary Information (SI) for Organic & Biomolecular Chemistry.
This journal is © The Royal Society of Chemistry 2024

Electronics Supporting Information

A Thiol-Selective and Acidicity-Stable Protein Modification Strategy using Electron-Deficient Yne Reagents

Zhi-Liang Chen,[a,b] *Wen Chen,*[a] *Feng-ling Wang,**[a] *Jian-hui Jiang*[a], and *Wan-Rong Dong**[a]

State Key Laboratory of Chemo/Biosensing & Chemometrics, College of Chemistry &Chemical Engineering, Hunan University, Changsha 410082, China

b.School of Pharmacy, Shaoyang University, Shaoyang, 422000, P. R China.

*E-mail: fengliw@hnu.edu.cn; wanrongdong@hnu.edu.cn

Table of Contents

1. Materials and reagents..S3

2. Adduct characterization..S3

3. Solid phase tetrapeptide synthesis..S4

4. Calculation of the ε of adducts..S5

5. Calculation of reaction rate constants...S5

6. Synthesis of IB-Y..S6

7. Protein modification procedure..S6

8. SDS-PAGE measurements..S7

9. Immunofluorescence imaging..S8

10. Addition Figures (Figures S1-S25)...S9-S22

11. References..S23

1. Materials and reagents

Ethanol (100%), acetonitrile (100%), iodomethane (> 95%), N-Methyl-N-(2-hydroxyethyl) - 4-aminobenzaldehyde (95%), piperidine (>98%), 1-ethyl-3-(3-dimethylamino-propyl) - carbodiimide (EDC), methyl propiolate (>98%), ethyl acetate (EA, >98%), 2-aminoethanethiol (>98%), mercaptoethanol (>98%), 2,3,3-trimethyl-3H-indole (>98%), propiolic acid (>98%), and DL-dithiothreitol (DTT, >98%) were purchased from Aldrich and Heowns. Anti-mouse second antibody was purchased from Proteintech. Other chemicals including sodium chloride (NaCl), Silica gel powder, N,N-dimethylformamide (DMF), dichloromethane, N,N-Diisopropylethylamine (DIPEA), Trifluoroacetic acid (TFA), and sodium hydroxide (NaOH) were purchased from China National Pharmaceutical Group Corporation. Ultrapure water (18 MΩ·cm) was prepared with a Millipore Milli-Q system (Billerica). MCF-7 and HepG-2 cells were purchased from Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Paraformaldehyde 4% solution and 1×PBS were supplied from Sangon Biotech (Shanghai, China). PBS buffer with different pH was prepared by adding NaOH (1 mol/L) or HCl (1 mol/L). Fetal bovine serum (FBS) was purchased from Biological Industries (BioInd). Methanol (chromatographic grade) was purchased from Sigma-Aldrich (MO, USA). The following amino acids and resins were purchased from GL BiochemShanghai used in the solid phase peptide synthesis: Fmoc-Ala-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ser(tBu)-OH, c-Cys(Trt)-OH.

2. Adduct characterization

The stability measurements of all samples were performed using an Aglient 1260 ChemStation equipment with a C-18 column. Chromatographic grade methanol (methanol : water = 6 : 4, v/v) was utilized as the mobile phase at a flow rate of 0.5 mL/min. The UV-Vis detector was set to detect at a wavelength of 280 nm. A typical injection volume of 100 μL was used, and all measurements were performed at a temperature of 30 ℃ . An FS5 fluorescence spectrometer (Edinburgh, England) was employed for the acquisition of fluorescence spectra of IB-Y. UV-vis spectra of all samples were obtainted using a UV-Vis spectrophotometer (Cary 8454, Agilent). MS spectra of all samples were acquired by an LCQ advantage ion trap mass spectrometry

(Thermo Fisher Scientific, Bremen, Germany). ^{1}H NMR spectra were obtained by a Bruker Avance-III 400 instrument (Bruker) with tetramethylsilane (TMS) serving as an internal standard.

3. Solid phase tetrapeptide synthesis

The tetrapeptide (Cys-Ser-Tyr-Ala) was prepared based on a previously reported method with minimal modifications.[1]

(1) Preloading of 2-chlorotrityl chloride resin: The 2-chlorotrityl chloride resin (0.4 mmol) was initially allowed to swell in dry DCM for 30 min, followed by subsequent washes with dry DCM (3 × 3 mL) and dry DMF (3 × 3 mL). The resin was then treated with a solution of Fmoc-Ala-OH (1.2 mmol) and DIPEA (2.4 mmol) in dry DMF (5 mL), followed by stirring the resulting suspension at room temperature overnight. The resin was then washed with dry DMF (3×3 mL), dry DCM (3× 3 mL) and dry DMF (3×3 mL).

(2) Deprotection: The resin was treated with 20% piperidine/DMF (3 × 5 min) and subsequently washed with DMF (3 × 3 mL), DCM (3 × 3 mL) and DMF again (3 × 3 mL). The residual solvents were removed by vacuum pump. A small amount of resin particles were then immersed in an ethanol solution of indene ketone (5 g/100 mL) and subjected to heating. If the color of the resin particles turned purple-black hue, it indicated the completion of Fmoc deprotection.

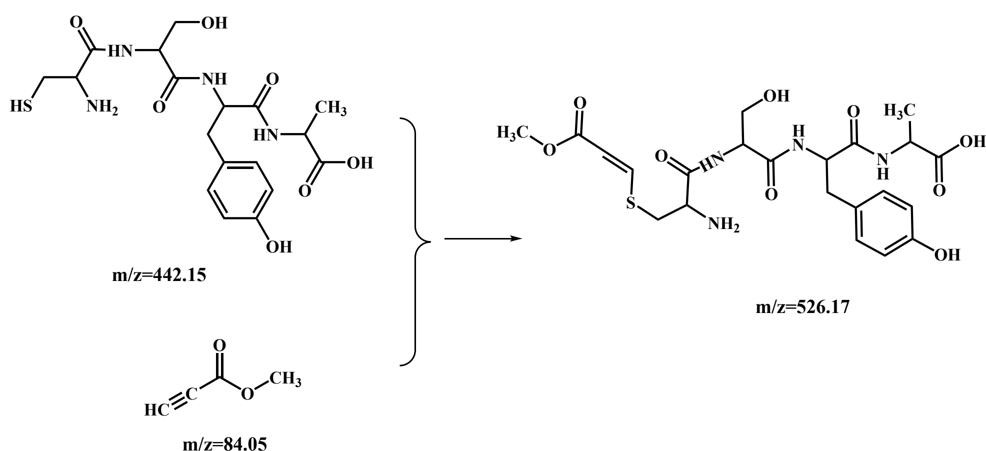
(3) Fmoc-Tyr(tBu)-OH loading: a solution of Fmoc-Tyr(tBu)-OH (1.2 mmol), PyBop (1.2 mmol), DIPEA (2.4 mmol) dispersed in 6 mL DMF was added to the resin. After 1.5 h, the resin was washed with DMF (3 × 3 mL), DCM (3 × 3 mL), and DMF (3 × 3 mL). A small portion of resin particles were then immersed in an ethanol solution containing indene ketone (5 g/100 mL) and subjected to heating. If the color of the resin particles turned transparent or light yellow, it indicated successful conjugation.

(4) Reiterate the aforementioned steps and consecutively couple Ser and Cys utilizing Fmoc-Ser(tBu)-OH and Boc-Cys(Trt)-OH, respectively.

(5) A mixture of TFA/DCM (1:100, v/v) were added to the resin (3 × 5 min), then the resin was washed with DCM.

(6) The combined solution was removed under vaccum and the residue was precipitated with Et_2O. The resulting white solid was then dispersed in water containing 0.1% TFA, purified by

semi-preparative HPLC and analyzed by MS spectrometry.



Scheme S1. Reaction route of tetrapeptide (Cys-Ser-Tyr-Ala) and methyl propiolate

4. Calculation of *ε* for the adduct.

Methyl propiolate (2 mM, 20 mL) and β-mercaptoethanol (2 mM, 20 mL) were shaken at 35 °C for 12 h. The mixture was purified by silica gel chromatography using H_2Cl_2-MeOH (20:1, v/v) as an eluent to afford a colorless sticky adduct. Absorption spectrum for the adduct was collected and the molar extinction coefficient (*ε*) in water at 286 nm was calculated by Eq. S1. The molar extinction coefficient (*ε*) of adducts at 286 nm in PBS buffer solution (20 mM, pH 7.38) was estimated to be 1.08×10^4.

$$A=\varepsilon cl \quad \text{(Eq. S1)}$$

Here, *A* is absorbance, *ε* is the molar extinction coefficient, *c* is concentration, and *l* is thickness of sample cell.

4. Calculation of reaction rate constants.

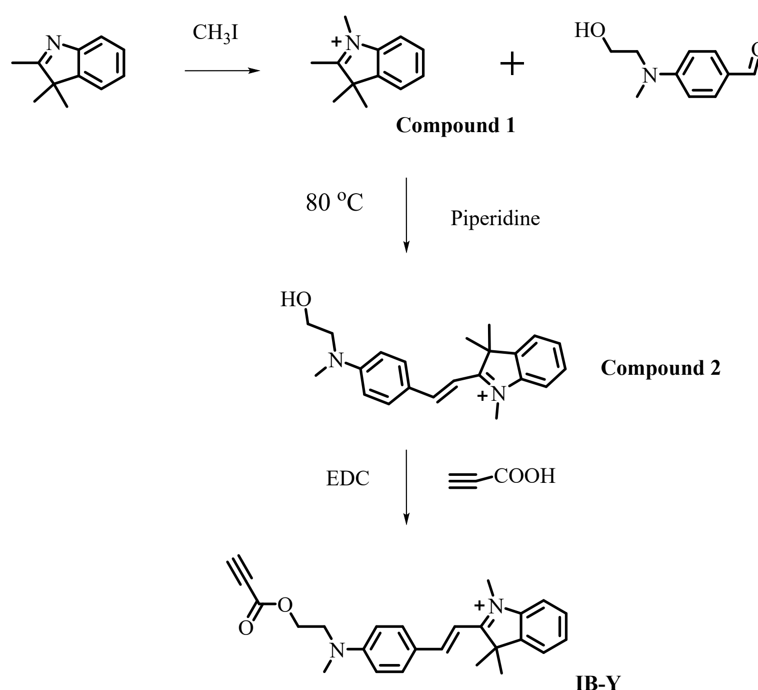
Methyl propiolate (200 μM, 1 mL) were mixed in mercaptoethanol (200 μM, 1 mL) in different pH conditions at 35 °C. The reaction dynamics was monitored by measuring the absorbance at 286 nm using a UV-Vis spectrometer. Reaction rate constants of the adducts were calculated by Eq. S2[2]

$$\frac{1}{a-c}-\frac{1}{a}=kt \quad \text{(Eq. S2)}$$

Where *a* is initial reaction concentration for methyl propiolate or mercaptoethanol, *c* is concentrations of the adduct, *k* is reaction rate constants, and *t* is time.

5. Synthesis of IB-Y

The synthetic procedures of IB-Y were conducted following a previously reported method with slight modifications (Scheme S1).[3,4] Approximately 500 mg (3.15 mmol) of 2,3,3-trimethyl-3H-indole was dispersed in ethanol (15 mL) containing iodomethane (CH_3I, 640 mg, 4.5 mmol). The resulting mixture was stirred at 50 °C for 12 hours under a nitrogen atmosphere. Subsequently, the grayish mixture was cooled to room temperature and the solvent along with residual CH_3I were removed using a rotary evaporator. Finally, the mixture was dried at 50 °C under vacuum conditions. Compound 1 was obtained as a brown product and used directly without any further purification.



Scheme S2. Synthetic routes for IB-Y.

Compound 1 (200 mg) was dispersed in ethanol (10 mL) containing N-methyl-N-(2-hydroxyethyl) -4-aminobenzaldehyde (190 mg), followed by the addition of 2 drops of piperidine. The resulting mixture was stirred at 80 °C for 8 h under a nitrogen atmosphere. Subsequently, the mixture was cooled to room temperature and the solvent was removed by a rotary evaporator. The obtained product, compound 2, which appeared as a red solid, was directly used without undergoing further purification..

Compound 2 (100 mg) was dispersed in DMF (15 mL) containing EDC (500 mg) and propiolic acid, and the resulting mixture was stirred at 40 °C for 48 h under nitrogen atmosphere. Subsequently, DMF was removed under vacuum to obtain a red sticky precipitate which was purified by silica gel chromatography using MeOH-EtOAc (1:20, v/v) as the eluent. The fluorophore IB-Y was obtained as a red product.

6. Protein modification

Conjugation of IgG with IB-Y was performed according to previous reports with slight modification.[5] Briefly, antibody conjugation was carried out by incubating 100 μg of IgG (1.0 mg/mL) with 2 μL of DTT (1 M) in 1×PBS (100 μL, pH 7.4) at 25 °C for 40 min. Excess DTT was removed using a desalting column (Thermo Fisher Scientific, USA) and washed with a solution of 1×PBS (pH 8.5). IB-Y dissolved in 1×PBS (pH 8.3) was quickly added to the IgG solution and the mixture was shaken at 25 ℃ for one hour. Excess IB-Y was removed using a size exclusion column packed with Superdex™200 prep grade medium (GE Corporation). The resulting IgG-IB conjugate was obtained and stored at a temperature of 5 ℃.

7. SDS-PAGE assays

10 μL of Samples were loaded into the wells of a precast PAGE gel (10%). Electrophoresis was carried out at 115 V for 1.5 h with a current of 15 mA in a running buffer (0.019 M Tris, 0.19 M glycine, 3.5 mM SDS). Subsequently, the gel was transferred into a polyvinylidene fluoride (PVDF) membrane in a transferring buffer (0.016 M Tris, 0.16 M glycine, 200 mL methanol, 1 L water) for 2 h at 100 V for 3.0 h. Finally, the gel was imaged by a Tanon 4200SF gel imaging system (Tanon Science & Technology, China).

8. Immunofluorescence imaging

The MCF-7 and HepG-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 g/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The cells were washed 3 times with 1×PBS and then incubated with 200 μL of paraformaldehyde solution (4%) for 30 min at room temperature. Subsequently, the cells were washed again 3 times with 1×PBS and incubated with primary antibody (mouse anti-PD-L1

antibody, proteintech, 1:1000 dilution with TBST) for 2 h at 37 ℃. Excess primary antibody was removed by washing 3 times with 1×PBS, followed by incubation of the cells with IgG-IB conjugates at 37 ℃ for 0.5 h. The cells were washed 3 times with 1×PBS,,and immunofluorescent images were collected using a Nikon TI-E+A1 SI confocal laser scanning microscope (Japan) under the following parameters, Ex: 561 nm, collection channel: 600 - 630 nm.

9. **Addition Figures (Figures S1-S25)**

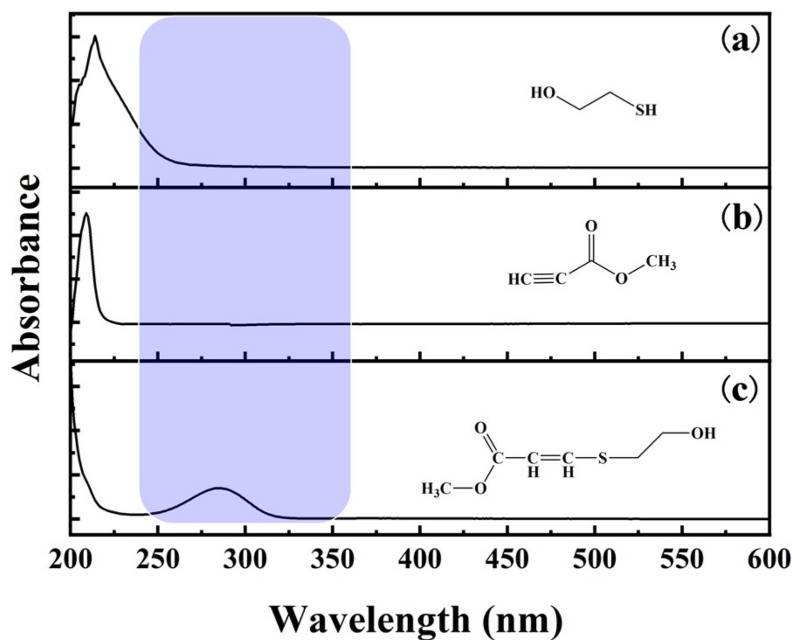


Fig. S1. UV-Vis spectra of β-mercaptoethanol (a) pre- (b) and post-incubation (c) with methyl propargynate

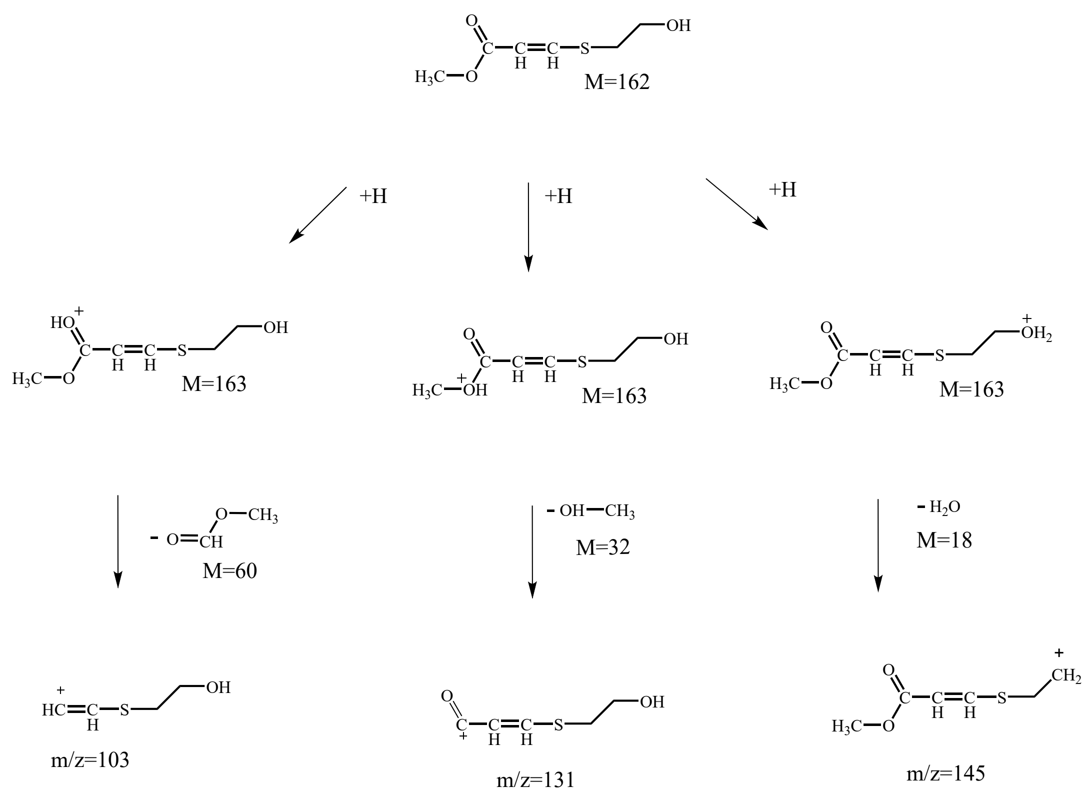


Fig. S2. Proposed fragmentation pathways analysis for methyl propiolate-β-mercaptoethanol

adduct.

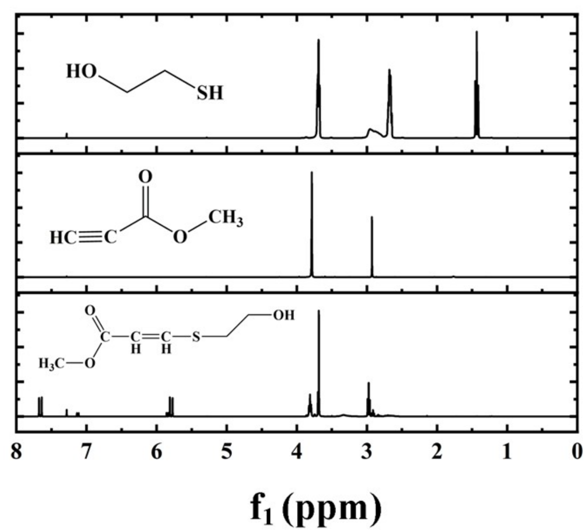


Fig. S3. ^{1}H NMR spectra of propargynate before and after incubtion of β-mercaptoethanol.

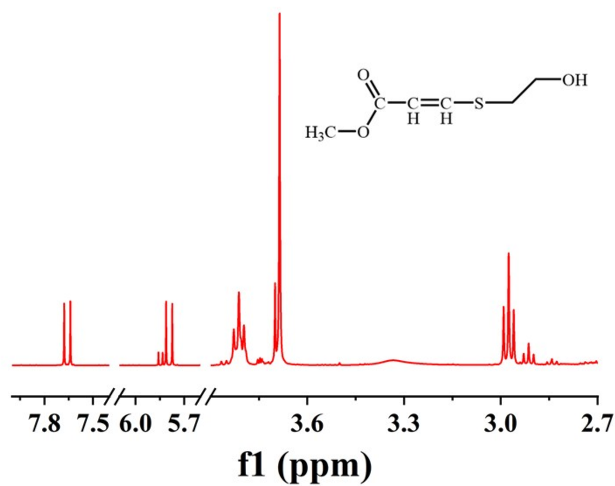


Fig. S4. Detailed ^{1}H NMR spectrum for the adduct.

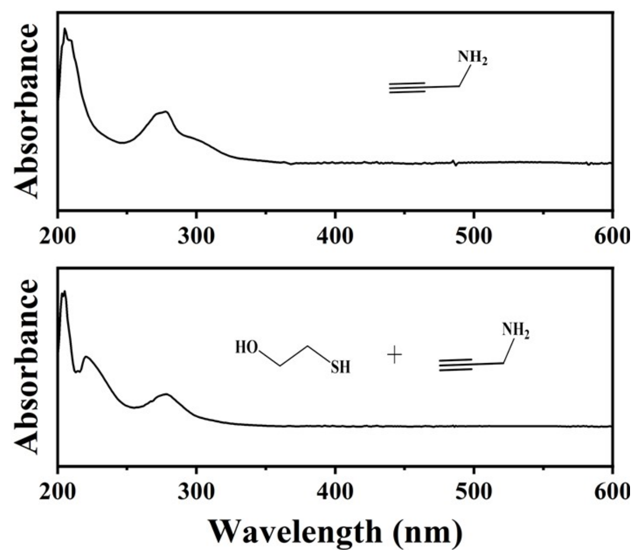


Fig. S5. UV-Vis spectra for methyl propargylamine after incubation with β-mercaptoethanol

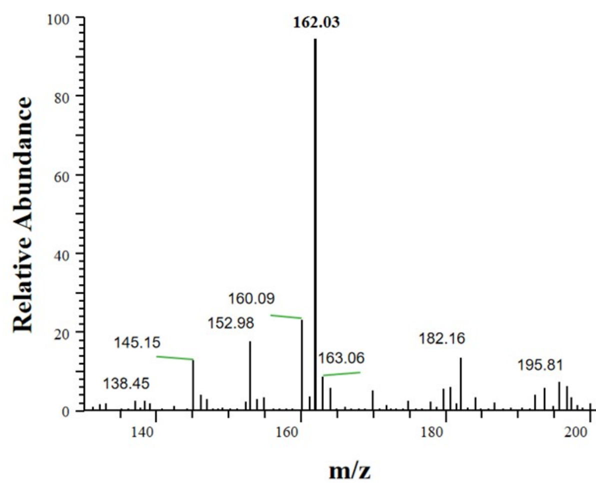


Fig. S6. MS spectrum for the adduct of methyl propiolate and 2-aminoethanethiol.

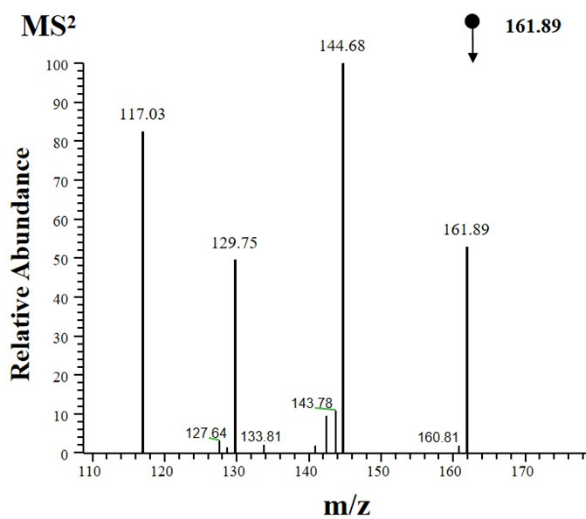


Fig. S7. MS-MS spectrum of the adduct peak at m/z 161.89.

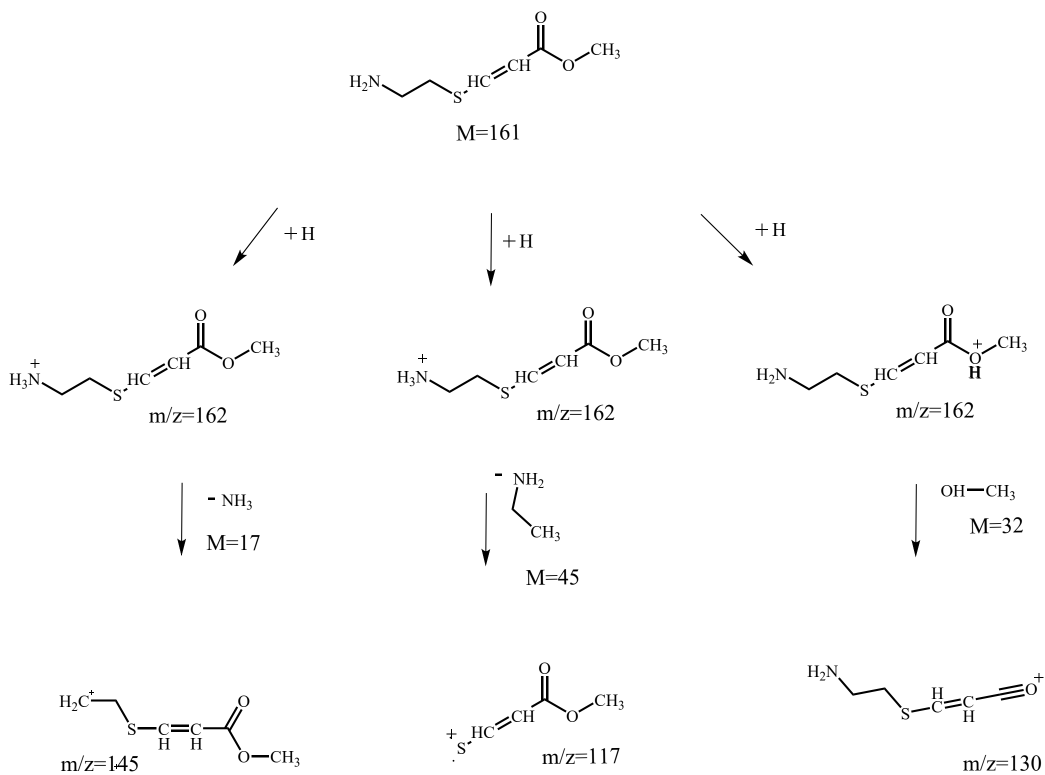


Fig. S8. Proposed fragmentation pathways analysis of the corresponding adduct.

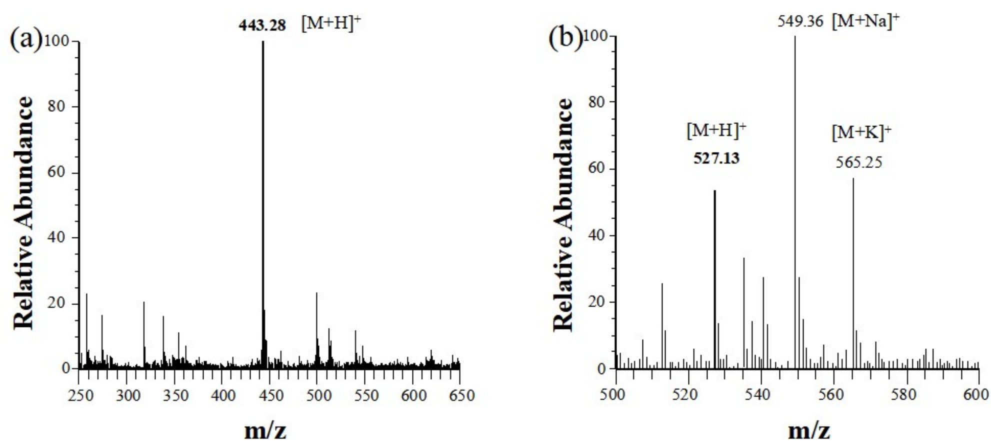


Fig. S9. MS pectrum of tetrapeptide pre- and post-incubation with methyl propargynate

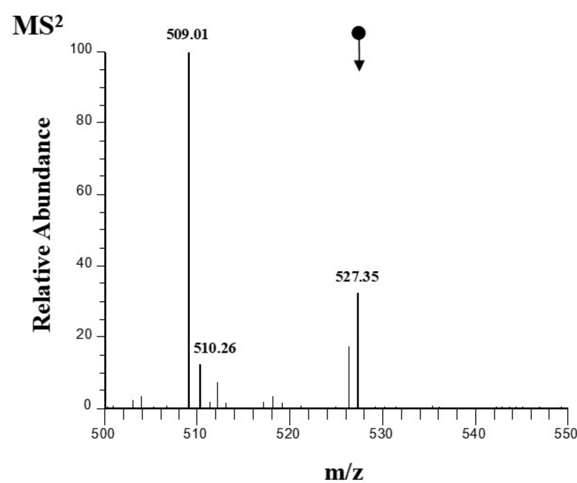


Fig. S10. MS-MS spectrum of the adduct peak at m/z 527. The peaks at m/z 509 and 510, indicating the detachment of the hydroxyl group (-OH) and amino group (-NH_2) from the adduct, providing evidence for the preferential reaction between the alkynyl group and thiol group of the tetrapeptide. The proposed fragmentation pathways analysis of the adduct (m/z=527) are presented in Fig. S11.

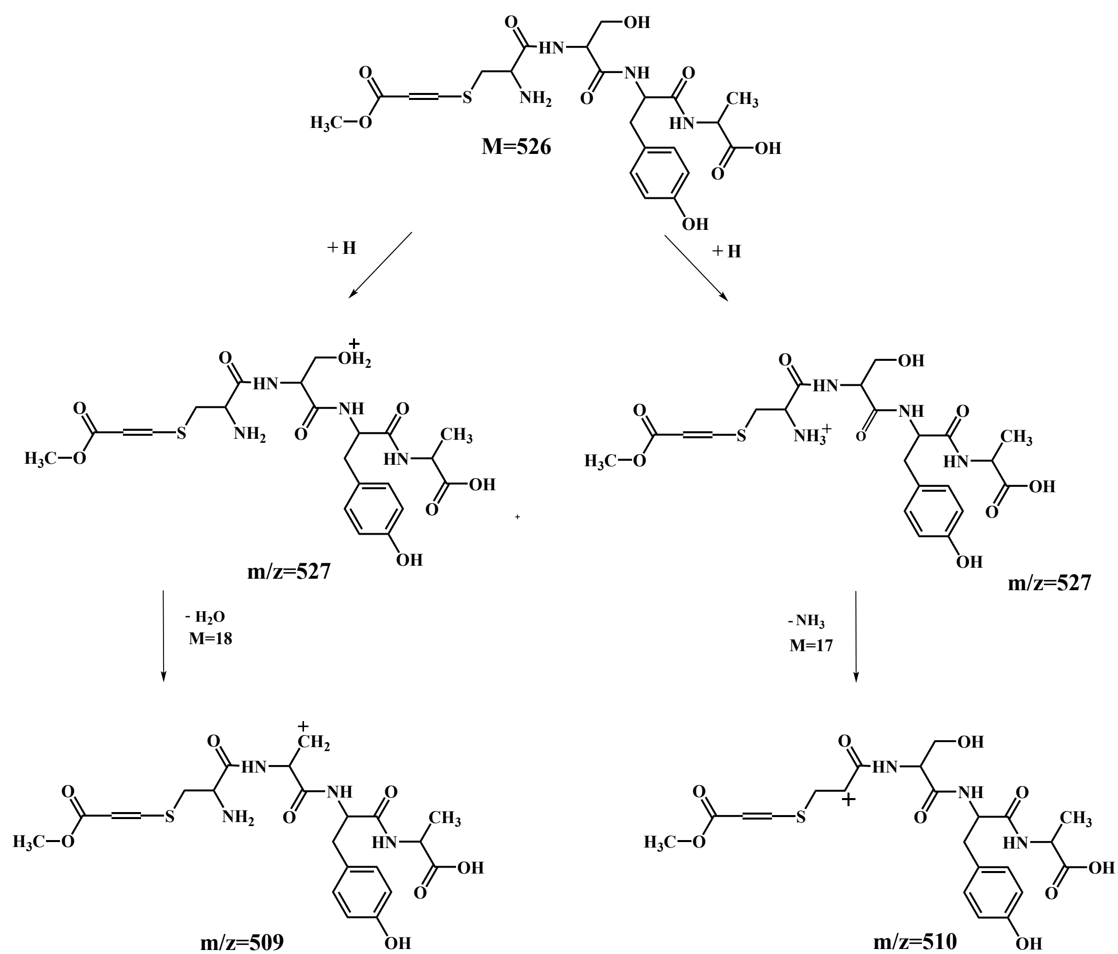


Fig. S11. Proposed fragmentation pathways analysis of the corresponding adduct.

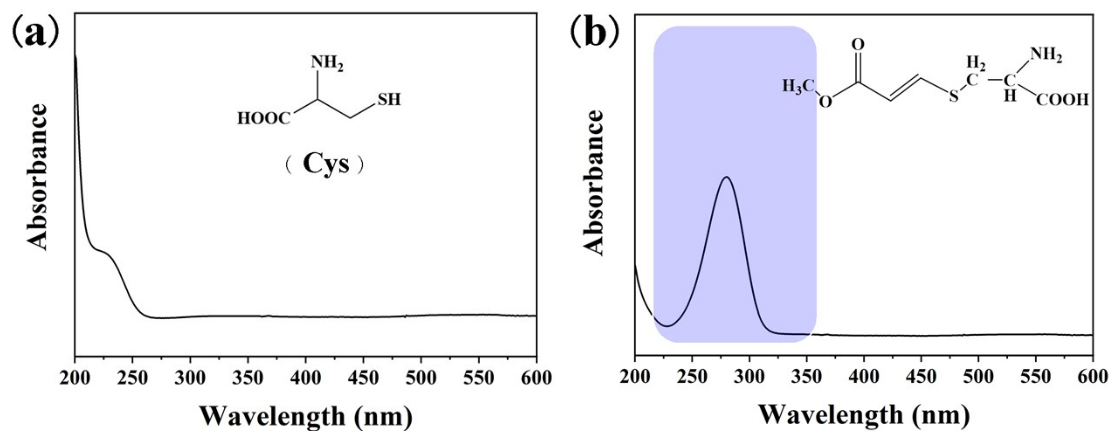


Fig. S12. UV -Vis spectrum of Cys before (a) and after (b) incubation with methyl propiolate.

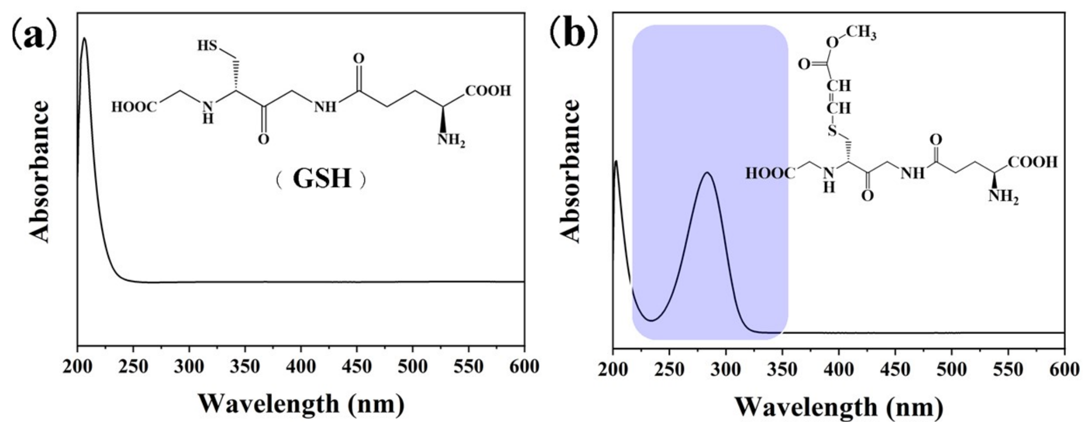


Fig. S13. UV -Vis spectrum of GSH before (a) and after (b) incubation with methyl propiolate.

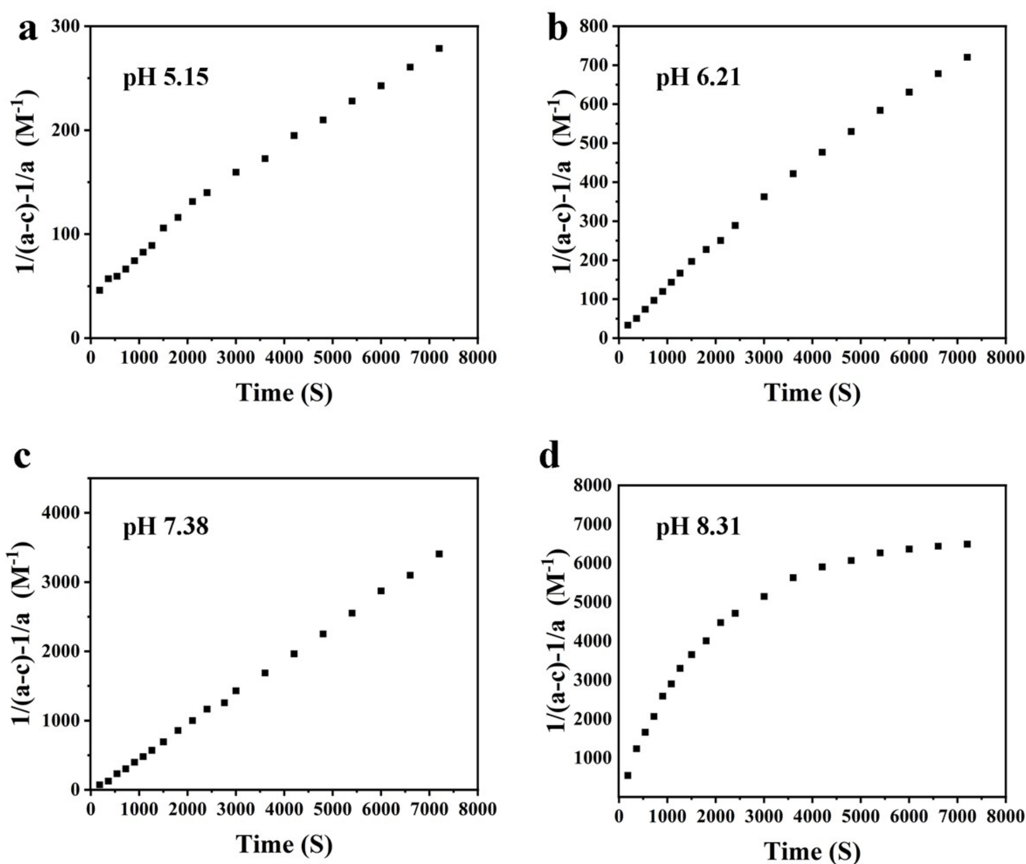


Fig. S14. Fitting curves for the second-order reaction constants at different pH.

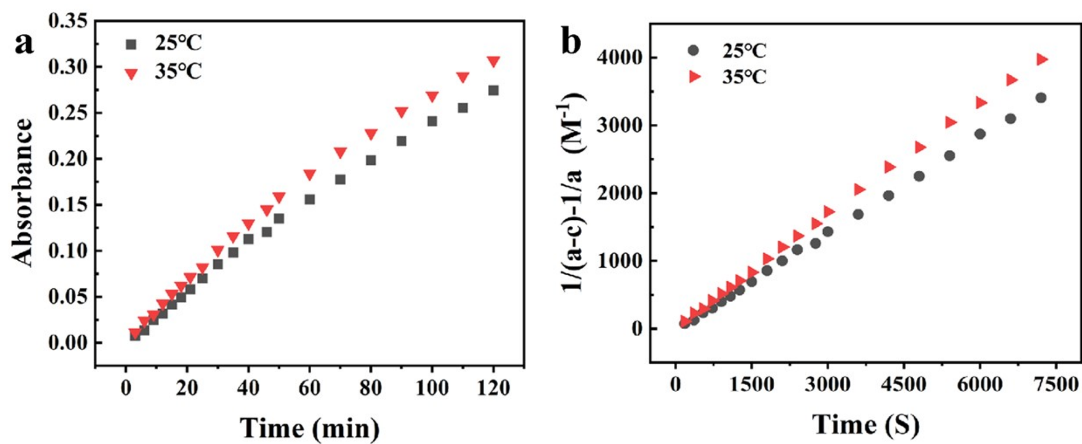


Fig. S15. (a) Dynamics of the reaction between methyl propiolate and β-mercaptoethanol at different temperature. (b) Fitting curve for the second order reaction constant. The reaction rate constants are 0.47 $M^{-1}S^{-1}$ at 25 °C, and 0.55 $M^{-1}S^{-1}$ at 35 °C.

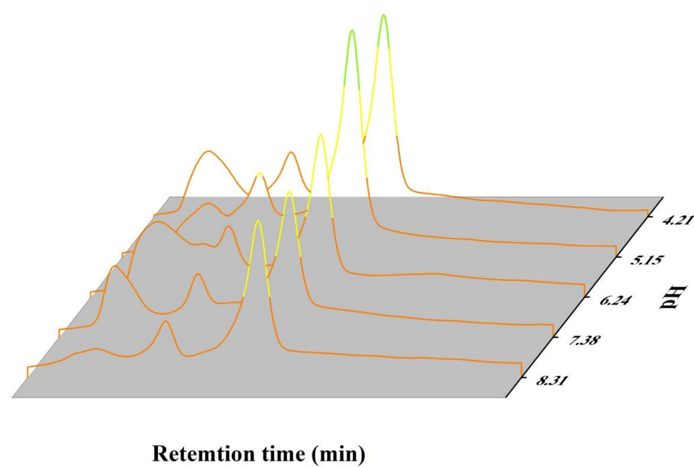


Fig. S16. Representative chromatograms of methyl propiolate-β-mercaptoethanol adduct at different pH.

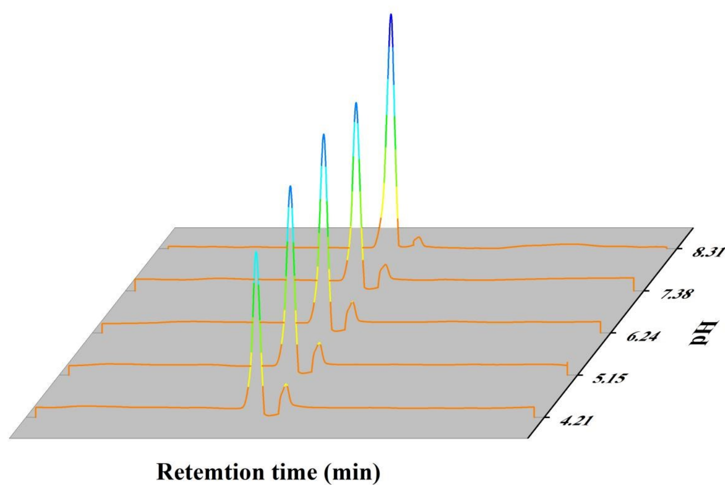


Fig. S17. Representative chromatograms of maleimide-β-mercaptoethanol adduct at different pH.

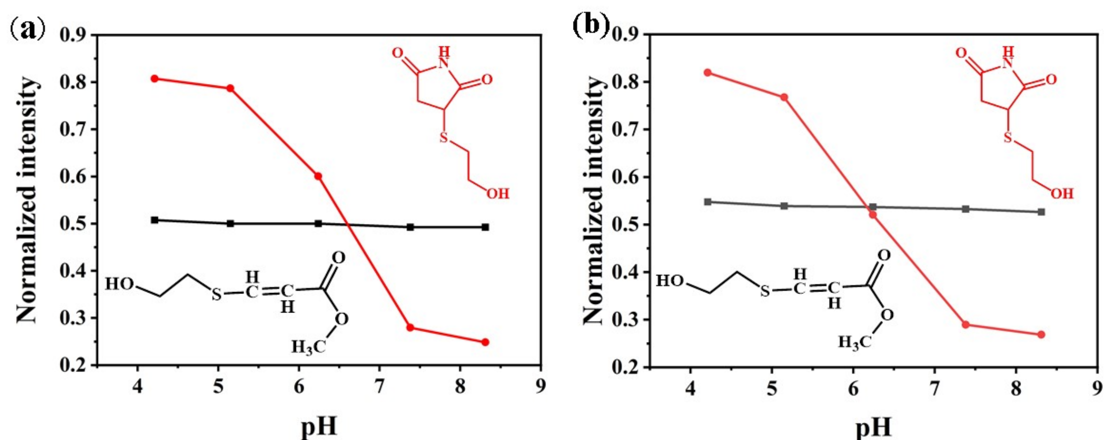


Fig. S18. Stability measurements for the adducts in the presence of 1.5 mM Cys (a) and 1.5mM GSH (b) at different pH over a period of 3 days. Black line:methyl propargynate- β-mercaptoethanol adduct, red line: maleimide-β-mercaptoethanol

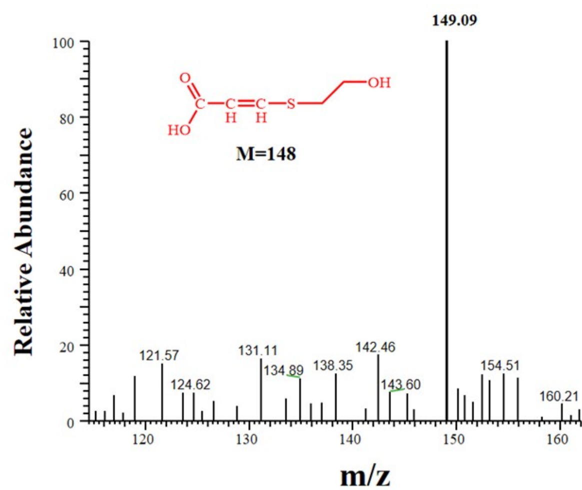


Fig. S19. MS spectrum of the adduct under alkaline environment in the presence of 1 mM Cys.

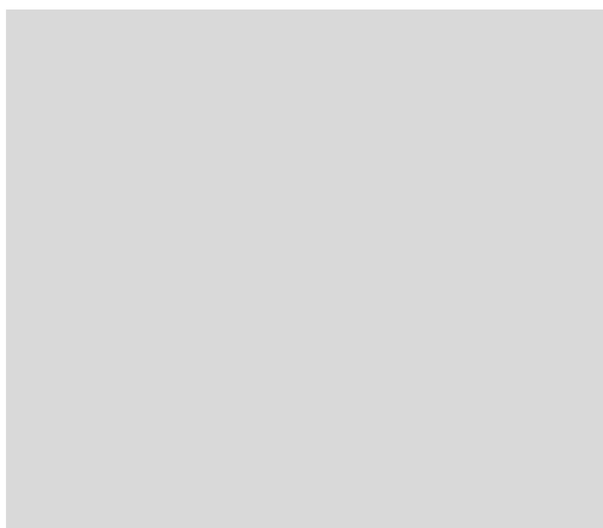


Fig. S20. MS-MS spectrum of the adduct under alkaline environment in the presence of 1.5 mM Cys.

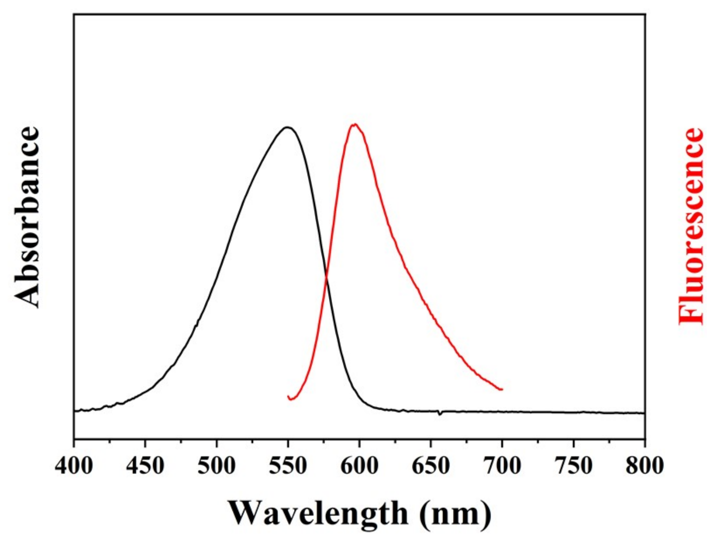


Fig. S21. Absorption and fluorescence spectra for IB-Y.

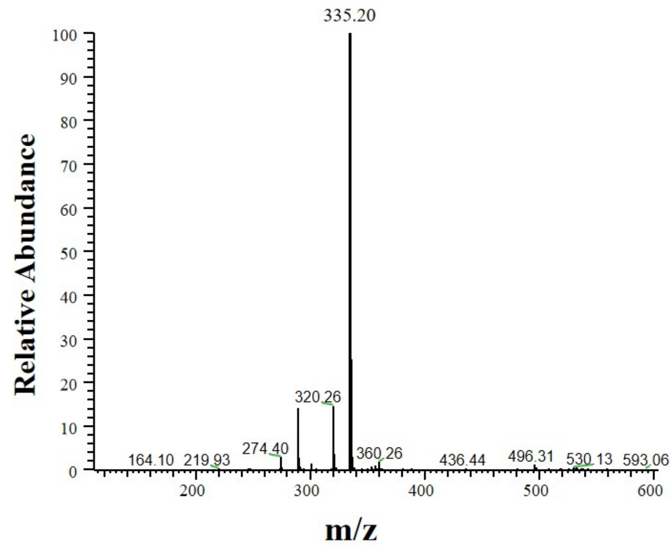


Fig. S22. MS spectrum for Compound 2.

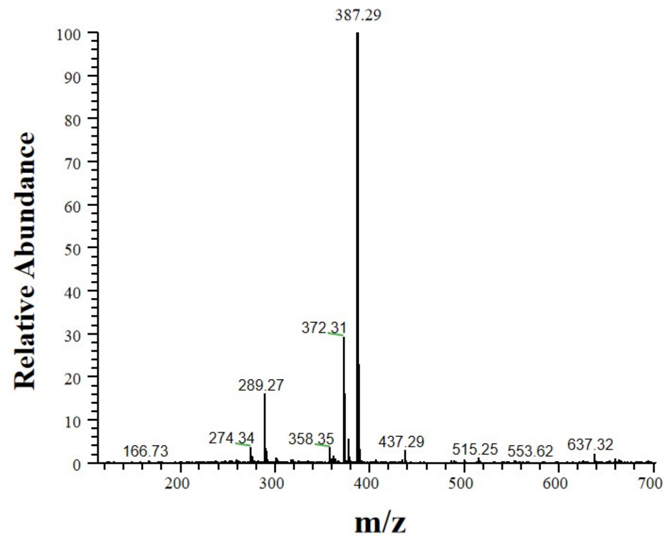


Fig. S23. MS spectrum for IB-Y.

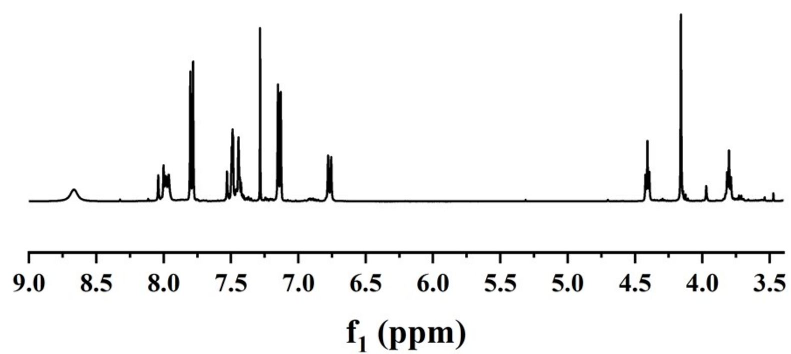


Fig. S24. ^{1}H NMR spectrum for IB-Y.

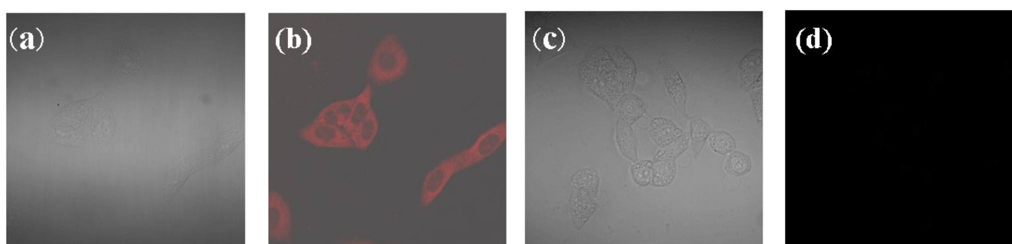


Fig. S25. Immunofluorescence images for fixed MCF-7 cells treated with (a, b) or without (c, d) PD-L1 primary antibody and stained with IgG-IB conjugates. Bright field (a, c) and fluorescence images (b, d).

10. References

1. J.-J. Du, L.-M. Xin, Z. Lei, S.-Y. Zou, W.-B. Xu, C.-W. Wang, L. Zhang, X.-F. Gao, J. Guo, *Chin. Chem. Lett*., 2018, *29*, 1127−1130.
2. Z. Y. Hong, C. Lv, A. A. Liu, S.-L. Liu, E.-Z. Sun, Z.-L. Zhang, A.-W. Lei, D.-W. Pang, *ACS Nano,* 2015, **9**, 11750−11760.
3. W. Liu, S.-J. Liu, Y.-Q. Kuang, F.-Y. Luo, J.-H. Jiang, *Anal. Chem*., 2016, **88**, 7867−7872.
4. X. Liu, Q.Y. Zhang, F. Wang, J.-H. Jiang, *Analyst,* 2019, **144**, 5980−5985.
5. M. Kasper, M. Glanz, A. Stengl, M. Penkert, S. Klenk, T. Sauer, D. Schumacher, J. Helma, E. Krause, M. C. Cardoso, H. Leonhardt, C. P. R. Hackenberger, *Angew. Chem. Int. Ed*., 2019, **58**, 11625−11630.