Electronic Supplementary Information

Optimized Molecular Design of PET Probe for Visualization of γ-Glutamyltranspeptidase Activity in Tumors

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Experimental section

Materials

All the initial chemicals and solvents purchased from commercial suppliers are available for use without further purification. 2-Cyano-6-aminobenzothiazole (NH_2-CBT) was purchased from Subo Chemical Technology Company (Shanghai, China). The amino acids including Boc-Gly-OH, Boc-D-Propargylglycine, N-Boc-S-Trityl-L-cysteine, and Boc-Glu-OtBu were obtained from Energy Chemical (Shanghai, China). 2-Azidoethyl-N,N-dimethylammoniomethyl-trifluoroborate ($AmBF_3$) was house-made according to the method in the literature.[1] GGT extracted from equine kidney was purchased from Sigma-Aldrich (Shanghai, China). The enzyme inhibitor 3-[(3-amino-3-carboxypropyl)methoxyphosphinyl]-oxy] benzeneacetic acid (GGsTop) was afforded from Toronto Research Chemicals (TRC, Canada). The primary antibody (anti-GGT1) for western blotting was purchased from Santa (Shanghai, China).

Apparatus

Electrospray mass spectrometry (ESI-MS) was employed to confirm chemical identity on a Waters Platform ZMD4000 LC/MS (USA). Analytical HPLC equipped with a waters 1525 pump, a waters 2487 radioactive detector (Radiomatic 610TR; Perkin Elmer; MA; USA), a UV/visible detector and a reverse C18 column (10 μm, 250 × 4.6 mm, Elite) was used for purity analysis. Semi-preparative HPLC equipped with a Waters 2489 UV/visible detector, a waters 1525 pump and a reverse C18 column (5 μm, 250 × 10 mm, Elite) was used for separation of compound. ^{1}H-NMR and ^{13}C-NMR spectra were analyzed by a Bruker 400 MHz nuclear magnetic resonance spectrometer (Bruker, Germany). The size measurement of the nanoparticles was performed on the Zeta Sizer Nano Series (Malvern Instruments; American) and transmission electron microscope (Talos F200C TEM, FEI, USA). PET imaging was recorded on an Inveon Dedicated micro-PET animal scanner (Siemens, Germany). The radioactivity was determined by using a γ counter (wizard 1470, Perkin Elmer, USA).

Synthesis procedure

Synthesis of compound JM-1

Isobutylchloroformate (IBCF) (195 μL, 1.5 mmol), and *N*-methylmorpholine (NMM) (330 μL, 3.0 mmol) were separately added to a solution of Boc-Gly-OH (315 mg, 1.8 mmol) in dry tetrahydrofuran (THF 10.0 mL), and the reaction mixture was stirred at 0 ℃ under N_2 for 2 h. Then 2-cyano-6-aminobenzothiazole (NH_2-CBT) (175 mg, 1.0 mmol) dissolved in THF (5 mL) was added to the reaction mixture at 0 ºC and dark environment and further stirred for 0.5 h. After further incubation at room temperature overnight, the reaction was quenched by adding hydrochloric acid (2 mL, 1 mol/L). Then, the organic solvent was removed by evaporating under reduced pressure. The residue was dissolved in ethyl acetate (EtOAc) and washed with aqueous $NaHCO_3$ (50 mL) respectively. The organic phase was collected and dried with Na_2SO_4 and then evaporated under reduced pressure. The crude product was purified by silica gel chromatography with the eluent of Hexane/EtOAc = 1/1 to get the compound **JM-1** as a yellow oily liquid (281 mg, yield: 85%). ESI-MS: *m*/*z* 355.24 $[M+Na]^+$, *m*/*z* 687.22 $[2M+Na]^+$. The retention time on HPLC chromatogram (λ = 320 nm) was 22.0 min.

Synthesis of compound JM-2

Trifluoroacetate (TFA, 2 mL) and dichloromethane (DCM, 2 mL) were added to a solution of compound **JM-2** and stirred for 0.5 h at room temperature. Then the reaction mixture was evaporated under reduced pressure and washed with cold diethyl ether. The crude product was purified by centrifugation to obtain the compound **JM-2** as a white solid (159 mg, yield: 99%). ESI-MS: *m*/*z* 233.07 $[M+H]^+$. The retention time on HPLC chromatogram (λ = 320 nm) was 9.5 min.

Synthesis of compound JM-3

The compound **JM-2** (159 mg, 0.842 mmol) and Boc-D-propargylglycine (200 mg, 0.939 mmol) were dissolved in dry THF (5 mL), and then HBTU (369 mg, 0.947 mmol) and DIPEA (368 μL, 2.106 mmol) were added. The reaction mixture was stirred for 3 h at room temperature under N_2, and then the solvent was removed by

evaporating under reduced pressure. The crude product was purified by silica gel chromatography (Hexane/EtOAc = 1/1) to yield the compound **JM-3** as a yellow oily liquid (310 mg, yield: 86%). ESI-MS: *m*/*z* 450.18 $[M+Na]^+$. The retention time on HPLC chromatogram (λ = 320 nm) was 22.3 min.

Synthesis of compound JM-4

Compound **JM-3** was dissolved in a mixed solvent of TFA (2 mL) and DCM (2 mL). The reaction solution was stirred at room temperature for 0.5 h. Then the solvent was removed by evaporating under reduced pressure and washed with cold diethyl ether. The crude product was purified by centrifugation to get the compound **JM-4** as a white solid (236 mg, yield: 99%). ESI-MS: *m*/*z* 328.23 $[M+H]^+$, 655.23 $[2M+H]^+$. The retention time on HPLC chromatogram (λ = 320 nm) was 11.0 min.

Synthesis of compound JM-5

The compound **JM-4** (236 mg, 0.721 mmol) was dissolved in dry THF (10 mL), and then N-Boc-S-Trityl-L-cysteine (341.8 mg, 0.793 mmol), HBTU (314.2 mg, 0.829 mmol), and DIPEA (314 μL, 0.803 mmol) were added at the same time. The reaction mixture was stirred for 3 h at room temperature under N_2 and then evaporated under reduced pressure. The crude product was purified by silica gel chromatography (DCM/MeOH = 15/1) to yield the compound **JM-5** as a yellow oily liquid (475 mg, yield: 85%). ESI-MS: *m*/*z* 795.41 $[M+Na]^+$. The retention time on HPLC chromatogram (λ = 320 nm) was 33.5 min.

Synthesis of compound JM-6

Compound **JM-5** was dissolved in a mixed solvent of TFA (2 mL) and DCM (2 mL). The reaction solution was stirred at room temperature for 0.5 h. Then the solvent was removed by evaporating under reduced pressure and washed with cold diethyl ether. The crude product was purified by centrifugation to get the compound **JM-6** as a white solid, which could be used directly in the following reaction without drying. ESI-MS: *m*/*z* 431.21 $[M+H]^+$, *m*/*z* 861.18 $[2M+H]^+$. The retention time on HPLC chromatogram (λ = 320 nm) was 12.3 min.

Synthesis of compound JM-7

The compound **JM-6** was dissolved in dry MeOH (6 mL), then Tips (120 μL) and 2-(ethyldisulfanyl) pyridine (102 μL, 0.675 mmol) were added. The resulting solution was stirred for 1 h at room temperature under N_2 protection and then evaporated under reduced pressure. The precipitation resulting from the solution was washed with cold diethyl ether. The crude product was purified by centrifugation to get the compound **JM-7** as a yellow solid (270 mg, yield: 90%). ESI-MS: *m*/*z* 491.21 $[M+H]^+$, *m*/*z* 513.19 $[M+Na]^+$, *m*/*z* 981.19 $[2M+H]^+$, *m*/*z* 1003.17 $[2M+Na]^+$. The retention time on HPLC chromatogram (λ = 320 nm) was 15.4 min.

Synthesis of compound JM-8

The compound **JM-7** (110 mg, 0.314 mmol) was dissolved in dry THF (10 mL), and then Boc-Glu-OtBu (75 mg, 0.248 mmol), HBTU (98 mg, 0.259 mmol) and DIPEA (97 μL, 0.563 mmol) were added at the same time. The reaction mixture was stirred for 4 h at room temperature under N_2 and then evaporated under reduced pressure. The crude product was purified by silica gel chromatography (DCM/ MeOH = 15/1) to yield the compound **JM-8** as a yellow oily liquid (148 mg, yield: 85%). ESI-MS: *m*/*z* 776.27 $[M+H]^+$, *m*/*z* 798.29 $[M+Na]^+$. The retention time on HPLC chromatogram (λ = 320 nm) was 29.5 min.

Synthesis of compound JM-9

The compound **JM-8** was dissolved in DCM (4 mL), and then TFA (4 mL) was added simultaneously. The solution was stirred for 1 h at room temperature and then evaporated under reduced pressure. The product was precipitated from cold diethyl ether, and then **JM-9** was obtained by centrifugation as a white solid (117 mg, yield: 99%). ESI-MS: *m*/*z* 620.20 $[M+H]^+$. The retention time on HPLC chromatogram (λ = 320 nm) was 15.6 min.

Synthesis of compound JM-10

The compound **JM-9** (31 mg, 0.05 mmol) was dissolved in DMF (2 mL) and H_2O (1 mL), and then $AmBF_3$ (294 μL, 015 mmol, 100 mg/mL), ligand (509 μL, 0.0068 mmol, 4 mg/mL) and tetrakis(acetonitrile)copper(I)hexa-fluorophosph (Cu (I), 18.6 mg, 0.05 mmol) were added. The resulting solution was stirred for 1 h at 45℃

under N_2 protection, and then evaporated under reduced pressure. The precipitate from the solution was washed with cold diethyl ether. The crude product was purified by semi-preparative HPLC to get the compound **JM-10** as a yellow solid (28 mg, yield: 68%, Mp: 125.5-126.5 ℃). ESI-MS: *m*/*z* 816.2 $[M+H]^+$. The retention time on HPLC chromatogram (λ = 320 nm) was 14.4 min. HR-MS *m*/*z*: $[M+H]^+$ calculated for $C_{30}H_{42}BF_3N_{11}O_6S_3^+$, 816.2449; measured, 816.2532. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.41 (s, 1H), 8.71 (s, 1H), 8.45 (d, *J* = 7.3 Hz, 2H), 8.35 (d, *J* = 7.2 Hz, 1H), 8.22 (s, 2H), 8.17 (d, *J* = 9.2 Hz, 1H), 7.90 (s, 1H), 7.79 – 7.71 (m, 1H), 4.80 (t, *J* = 5.7 Hz, 2H), 4.56 – 4.43 (m, 2H), 3.99 – 3.83 (m, 4H), 3.71 (s, 2H), 3.11 (d, *J* = 9.4 Hz, 2H), 2.94 (s, 6H), 2.80 – 2.69 (m, 1H), 2.65 (q, *J* = 6.9 Hz, 2H), 2.31 (dd, *J* = 31.2, 6.8 Hz, 4H), 1.96 (s, 2H), 1.18 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.77, 171.42, 171.24, 170.45, 168.66, 148.15, 143.68, 139.70, 137.18, 135.59, 125.33, 124.26, 121.24, 114.04, 111.82, 63.89, 53.58, 53.23, 52.59, 52.11, 44.06, 43.37, 41.14, 31.95, 31.09, 28.42, 26.40, 14.65.

Synthesis of compound JM-C

The compound **JM-7** (10 mg, 0.02 mmol) was dissolved in DMF (2 mL) and H_2O (1 mL), and then $AmBF_3$ (120 μL, 0.06 mmol, 100 mg/mL), ligand (207 μL, 0.002 mmol, 4 mg/mL) and tetrakis(acetonitrile)copper(I)hexa-fluorophosph (Cu (I), 7.5 mg, 0.02 mmol) were added. The resulting solution was stirred for 1 h at 45℃ under N_2 protection, and then evaporated under reduced pressure. The precipitate from the solution was washed with cold diethyl ether. The crude product was purified by semi-preparative HPLC to get the compound **JM-C**. (8.5 mg, yield: 62%). ESI-MS: *m*/*z* 687.33 $[M+H]^+$, *m*/*z* 709.34 $[M+Na]^+$. The retention time on HPLC chromatogram (λ = 320 nm) was 11.1 min.

Cell culture

Human colon cancer cell line HCT116, human glioblastoma cell line U87 and human non-small cell lung cancer cell line NCI-H1299 were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM or RPMI 1640 medium contained fetal bovine serum (FBS, 10% v/v), 37 °C and 5%

CO_2.

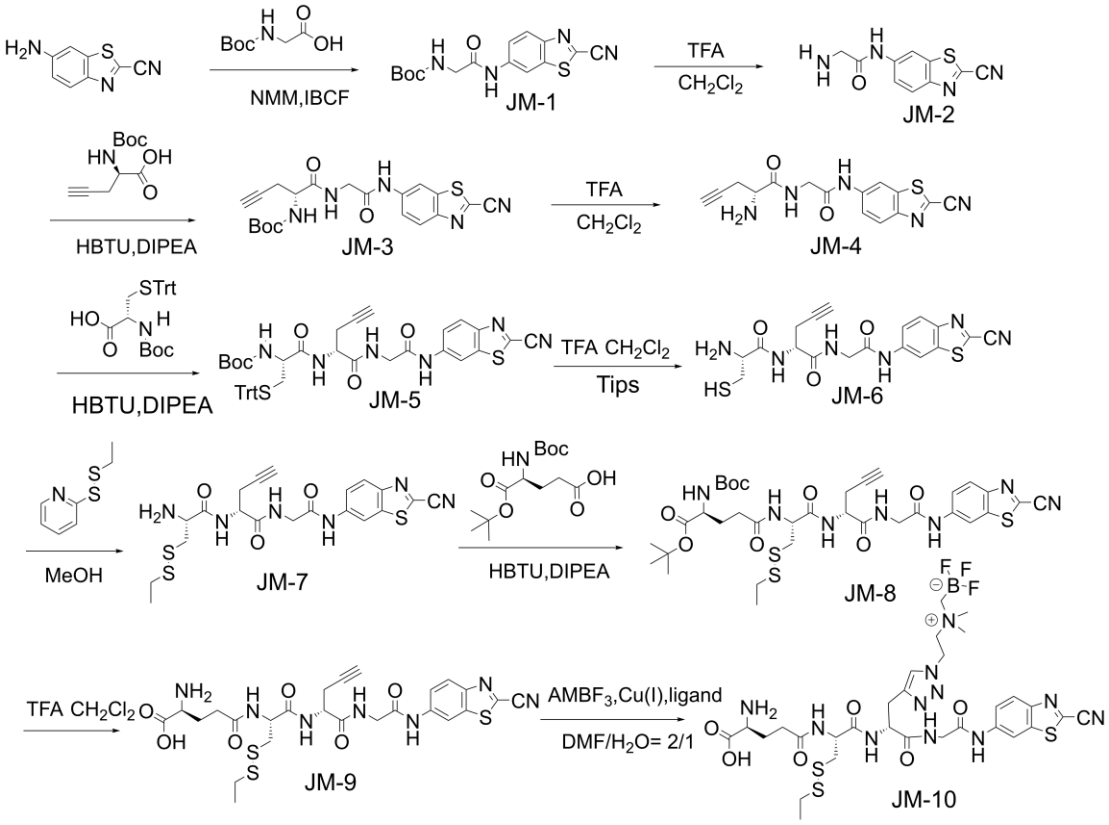
Western blotting analysis of GGT expression

To screen GGT-positive and negative cells, western blotting (WB) analysis was used to evaluate the expression level of GGT in different cancer cell lines. The cells were cultured in a 60 × 60 mm petri dish. When the density of cells reached 90%, the cells were lysed with 200 μL of RIPA lysate (containing 2 μL of protease inhibitor PMSF). After extracting the protein and determining the protein concentration, 20 μg protein was added to the same volume of 0.9% NaCl solution, and the mixed solution was added to a 10% sodium dodecyl sulfate-polyacrylamide gel prepared in advance for electrophoretic separation. The initial voltage was set to 80 V, and adjusted to 120 V after entering the separation gel. After the protein bands were utterly separated, they were transferred to a nitrocellulose membrane, and then blocked with 5% skim milk for 1 h. The primary rat anti-glutamyl transpeptidase monoclonal antibody (Anti-GGT1) and β-actin were diluted with a primary antibody solution at a ratio of 1: 500, and the membrane was immersed in the primary antibody at 4 °C overnight. Subsequently, the primary antibody was recovered, and the membrane was washed three times with 1 × TBST solution. Then the goat anti-mouse or rabbit IgG-HRP secondary antibody was added, and incubated on a shaker at room temperature for 1 h. Finally, the blotted protein was detected by Chemi DOC XRS and gel imaging system.

In vitro cytotoxicity test

The cytotoxicity of nonradioactive probe **JM-10** against U87 and NCI-H1299 cell lines was evaluated by standard 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, respectively. The cells were seeded in a 96-well plate (8×10^3 cells and 100 μL DMEM medium containing 10% FBS per well) and cultured at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. Then, **JM-10** diluted with DMEM or RPMI 1640 culture medium to the indicated concentration of 0, 6.25, 12.5, 25.0, 50.0, and 100.0 μM was added into each well, followed by incubating for 24 h and 48 h,

respectively. Then MTT (5 mg/kg, 20 μL) was employed to bind living cells for 4 h, specifically. Cellular viability was figured out based on the absorbance of each well at 490 nm measured by an ELISA reader. Cell viability % = (mean OD value of experimental group/mean OD value of control group) × 100%. All tests were performed for at least three independent experiments.



Scheme S1. Synthetic route of nonradioactive compound **JM-10**.

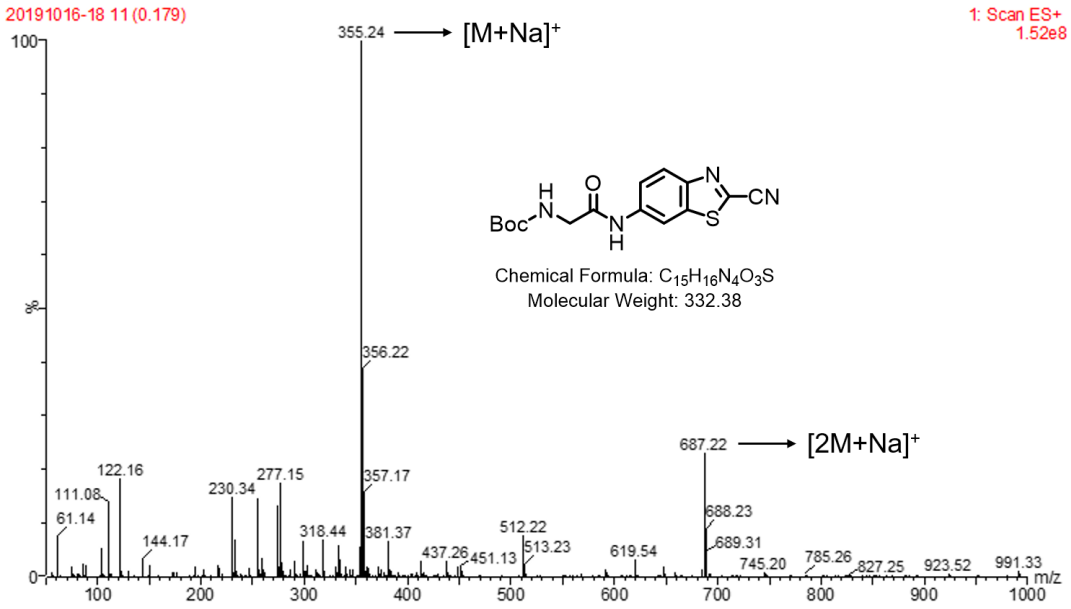


Fig. S1. ESI-MS analysis of compound **JM-1**.

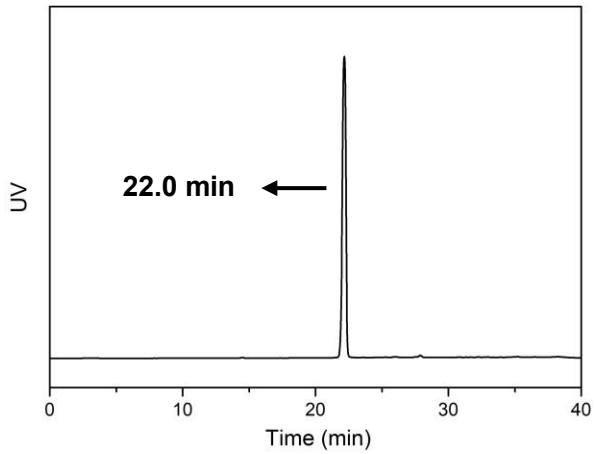


Fig. S2. HPLC trace of compound **JM-1**.

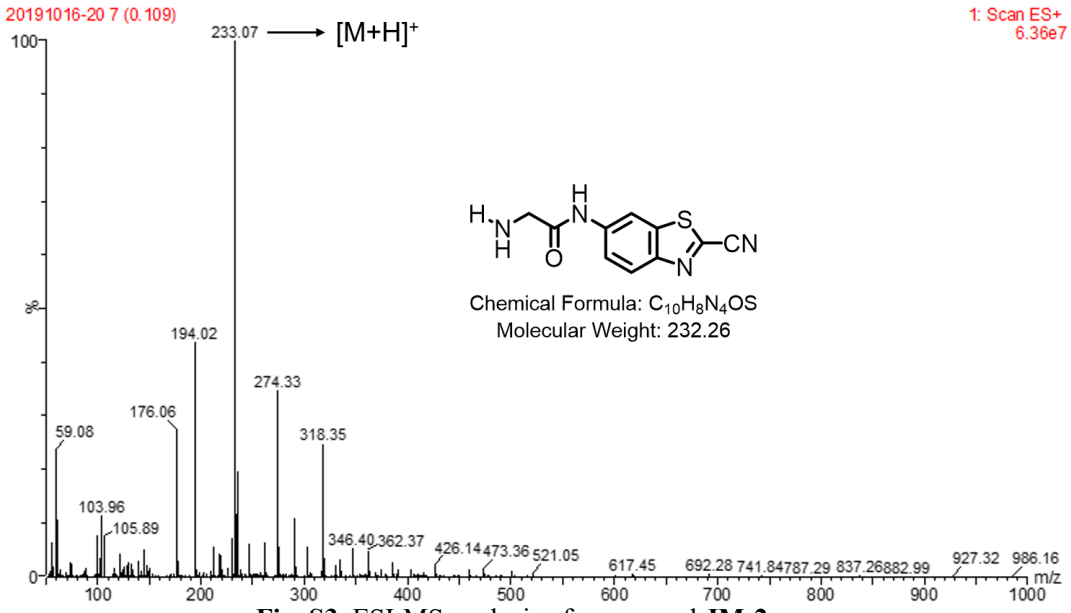


Fig. S3. ESI-MS analysis of compound **JM-2**.

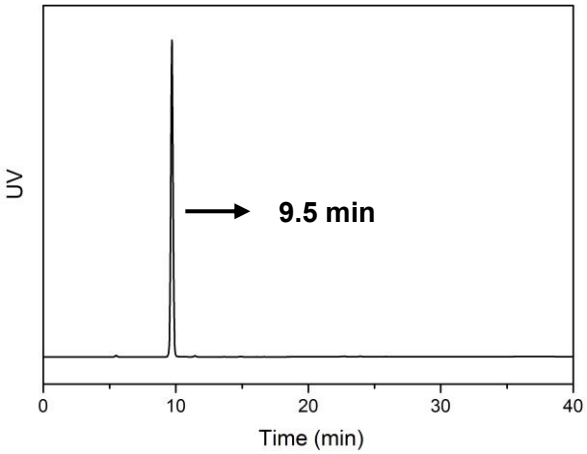


Fig. S4. HPLC trace of compound **JM-2**.

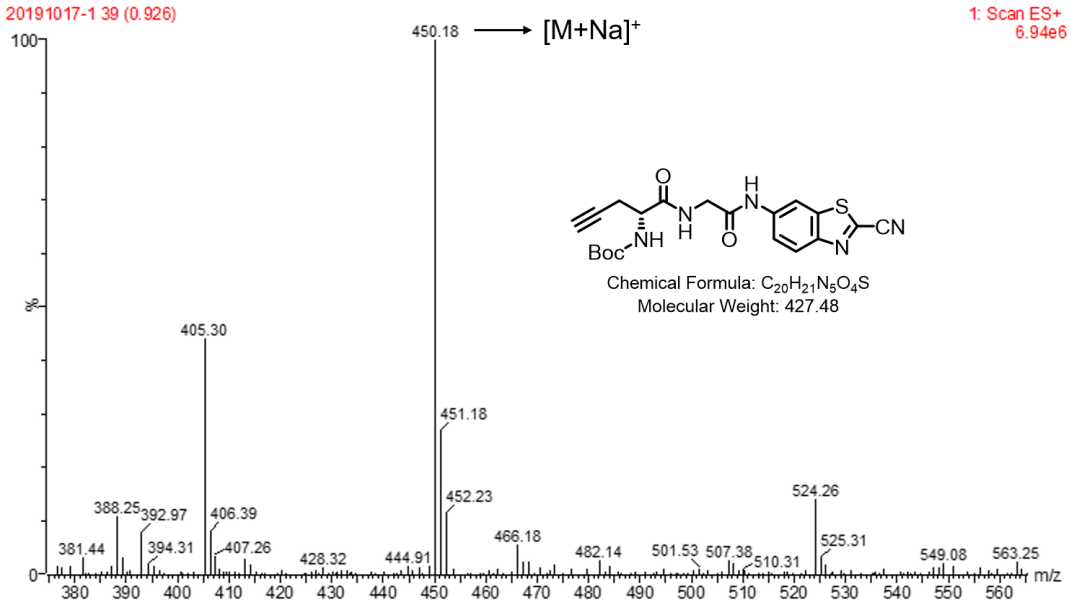


Fig. S5. ESI-MS analysis of compound **JM-3**.

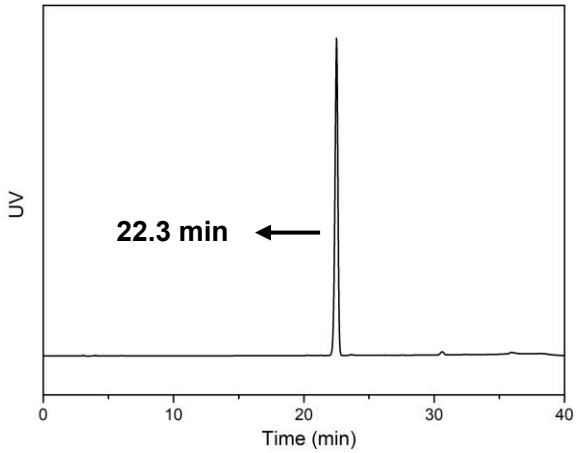


Fig. S6. HPLC trace of compound **JM-3**.

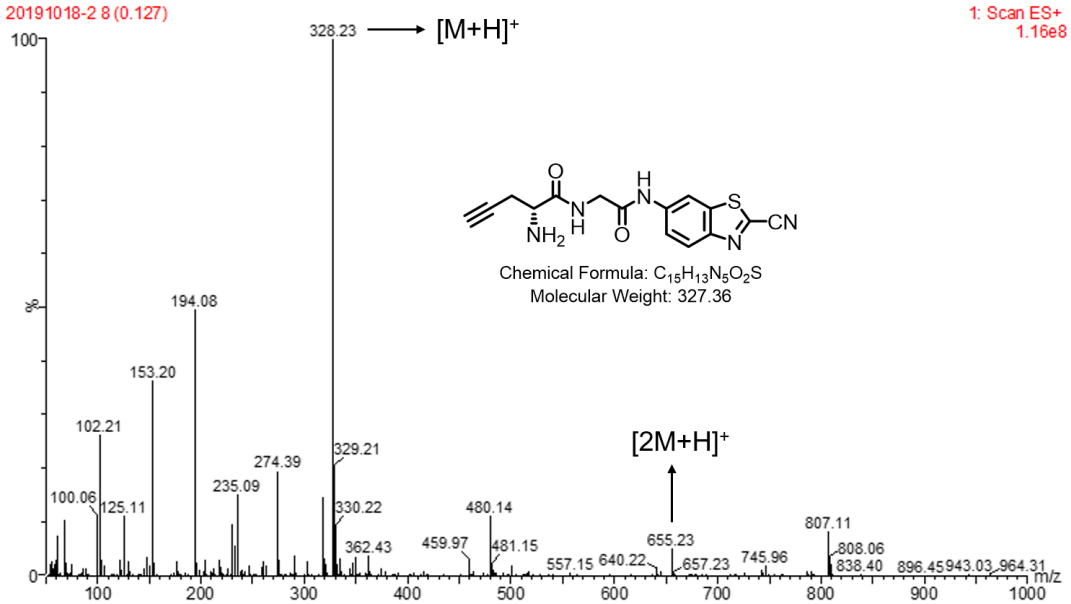


Fig. S7. ESI-MS analysis of compound **JM-4**.

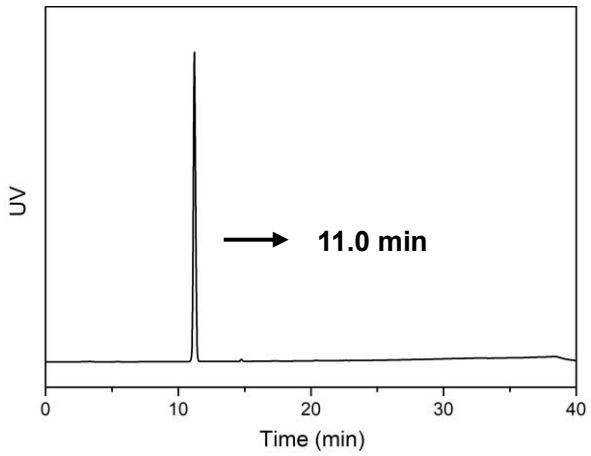


Fig. S8. HPLC trace of compound **JM-4**.

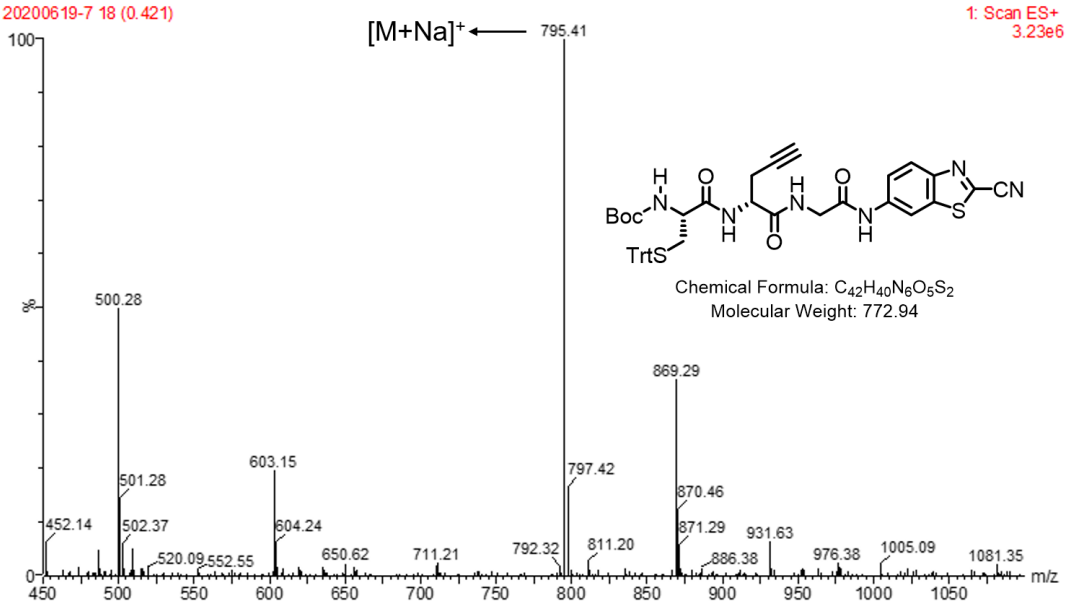


Fig. S9. ESI-MS analysis of compound **JM-5**.

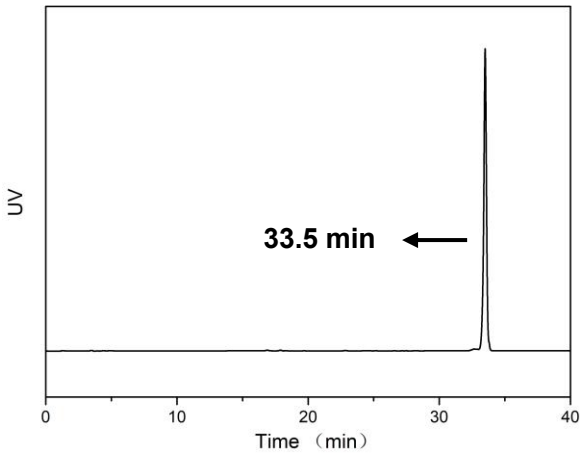


Fig. S10. HPLC trace of compound **JM-5**.

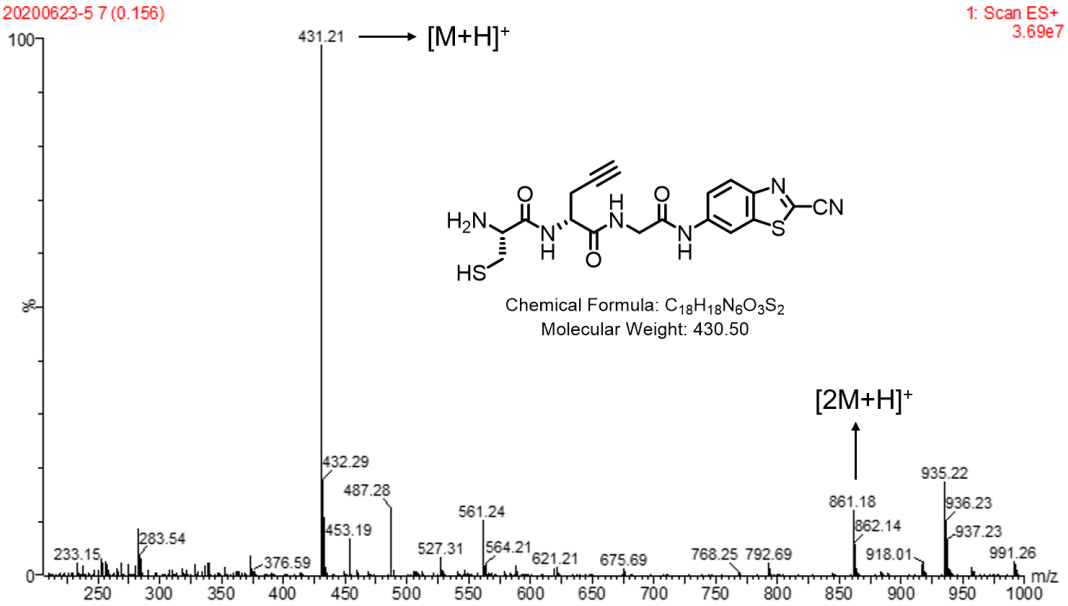


Fig. S11. ESI-MS analysis of compound **JM-6**.

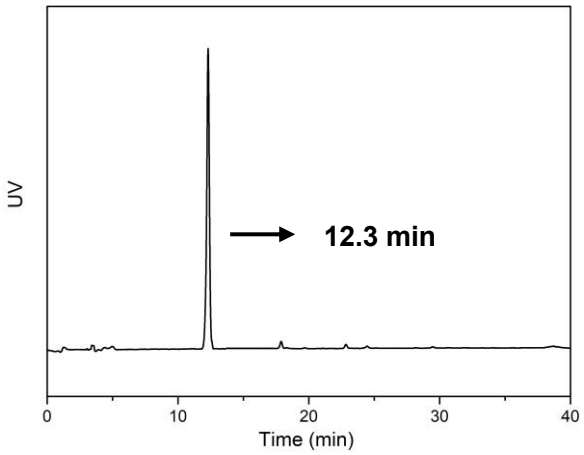


Fig. S12. HPLC trace of compound **JM-6**.

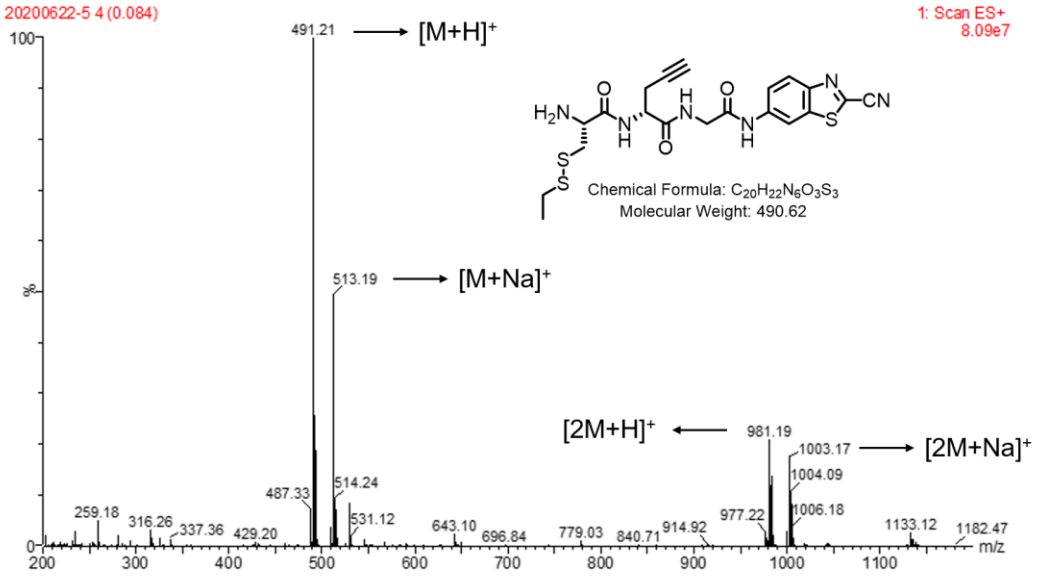


Fig. S13. ESI-MS analysis of compound **JM-7**.

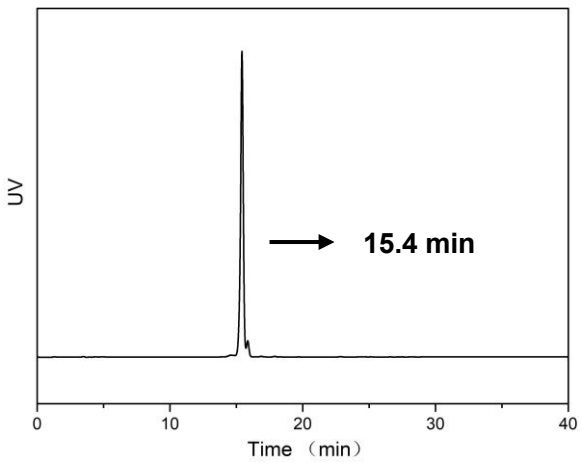


Fig. S14. HPLC trace of compound **JM-7**.

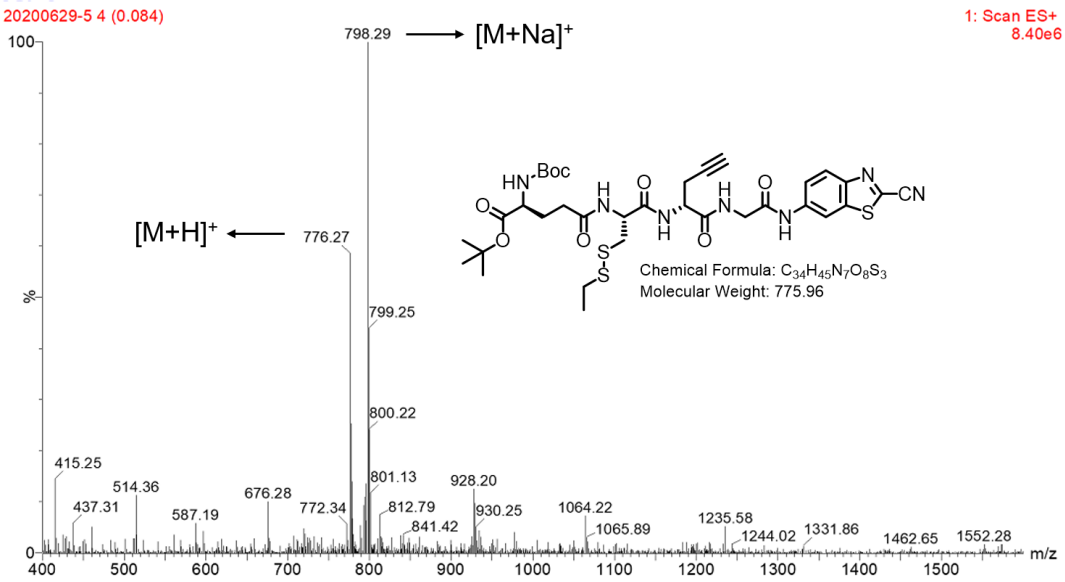


Fig. S15. ESI-MS analysis of compound **JM-8**.

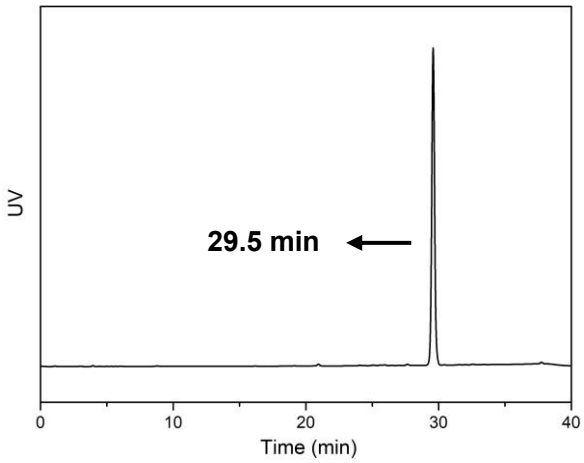


Fig. S16. HPLC trace of compound **JM-8**.

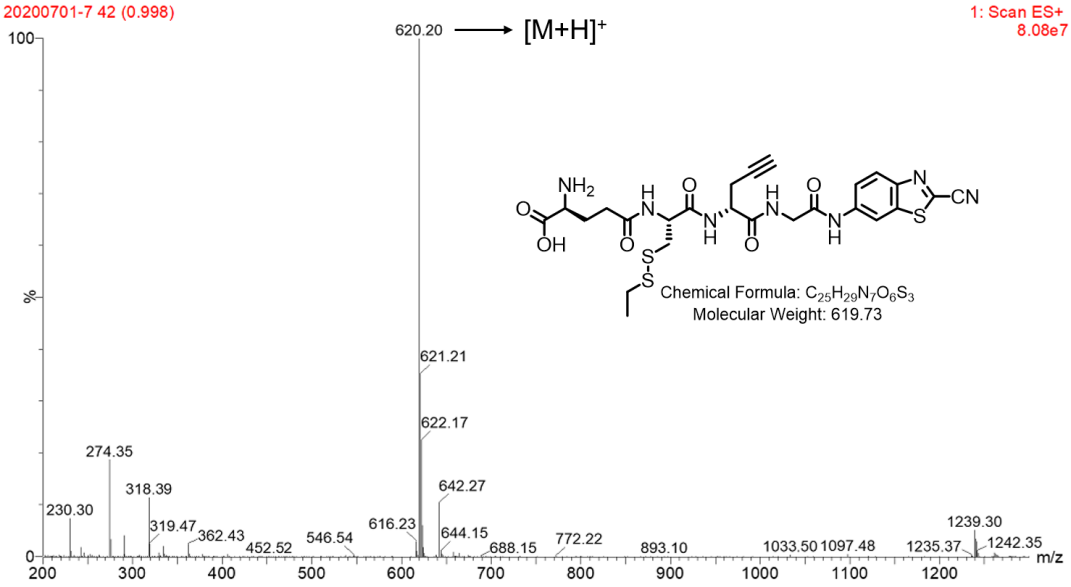


Fig. S17. ESI-MS analysis of compound **JM-9**.

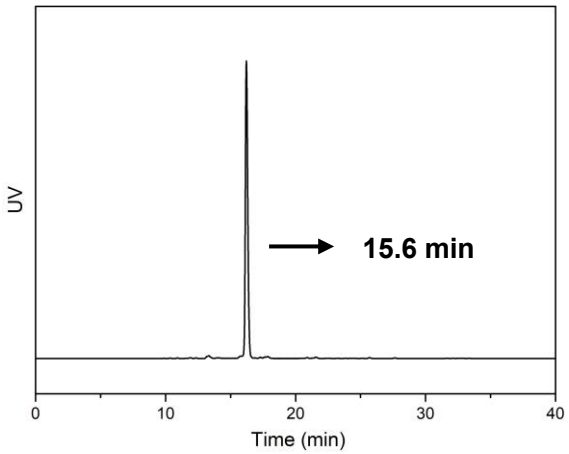


Fig. S18. HPLC trace of compound **JM-9**.

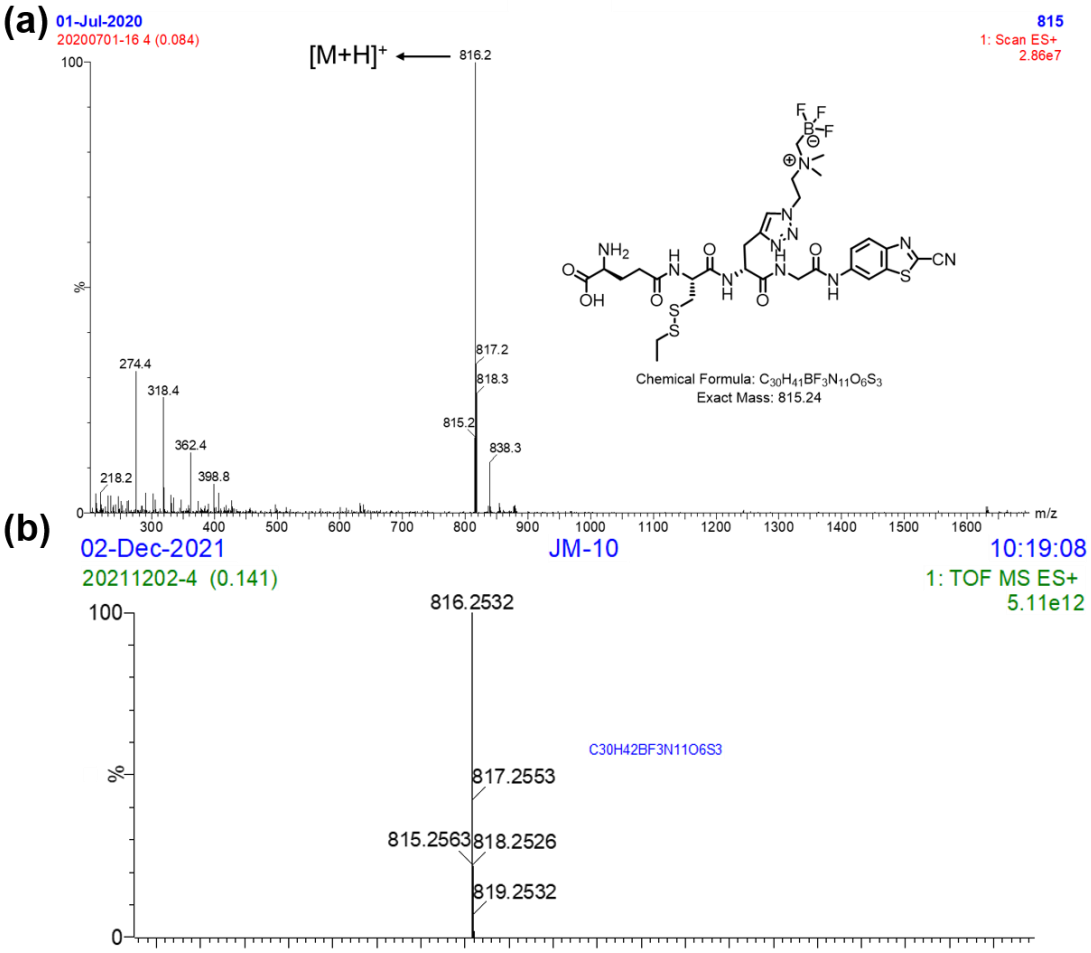


Fig. S19. (a) ESI-MS and (b) MALDI-TOF analysis spectrograms of compound **JM-10**.

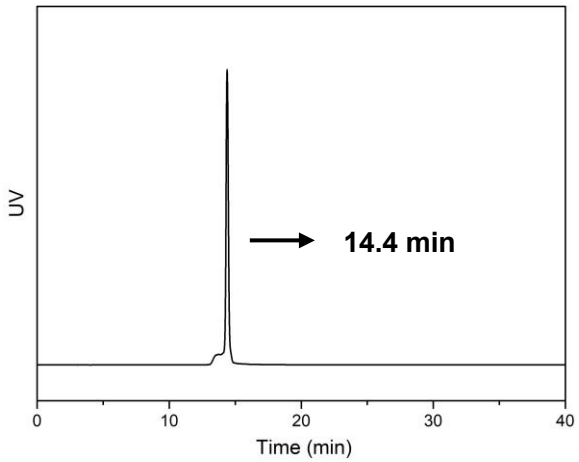


Fig. S20. HPLC trace of compound **JM-10**.

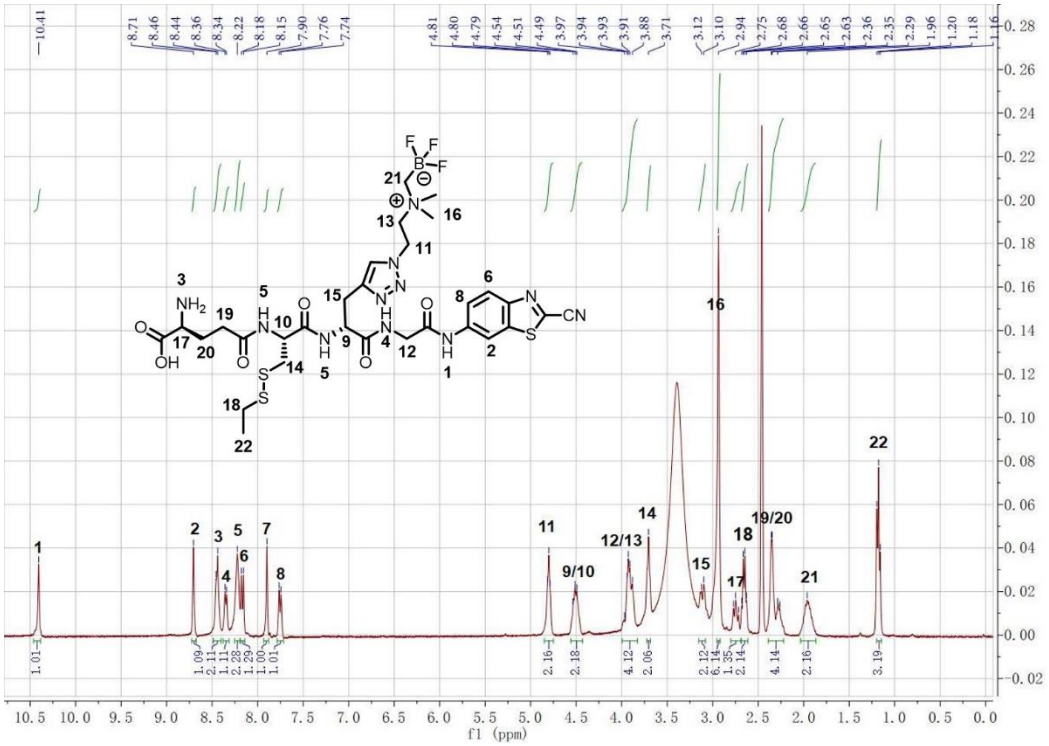


Fig. S21. ^{1}H NMR spectrum of compound **JM-10**.

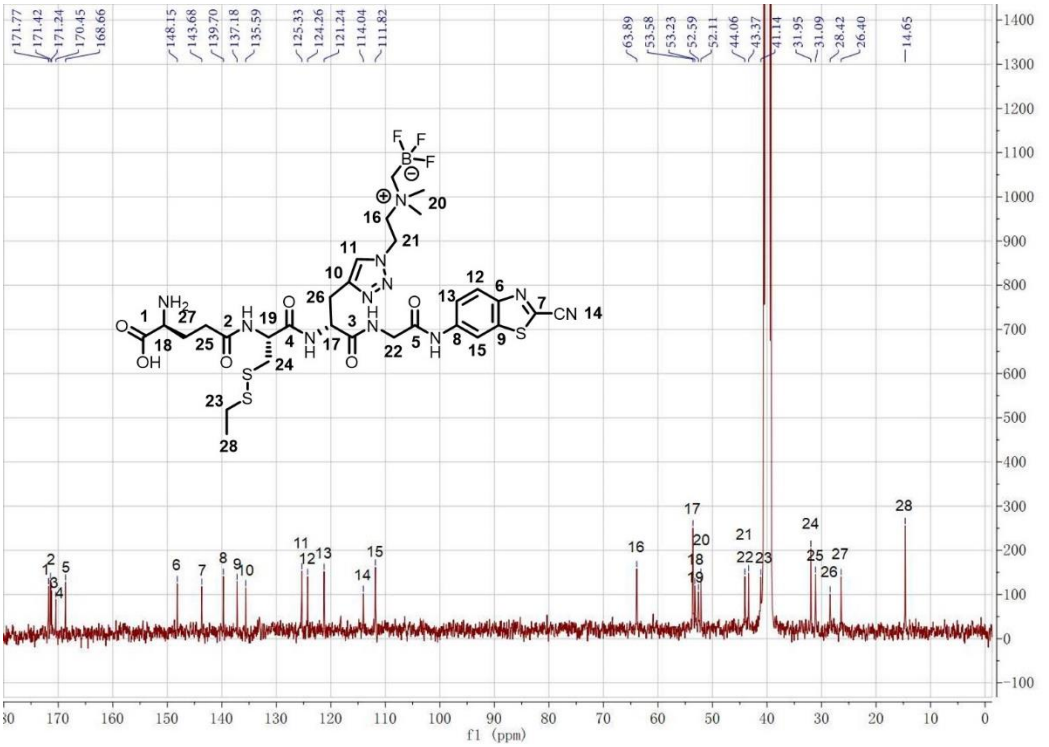


Fig. S22. ^{13}C NMR spectrum of compound **JM-10**.

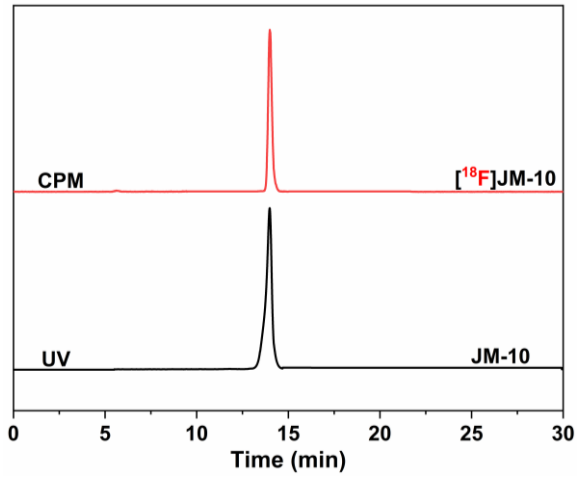


Fig. S23. Stability of **JM-10** and **[^{18}F]JM-10** in pyridazine hydrochloride (pH = 2.5) at 80 °C for 1 h.

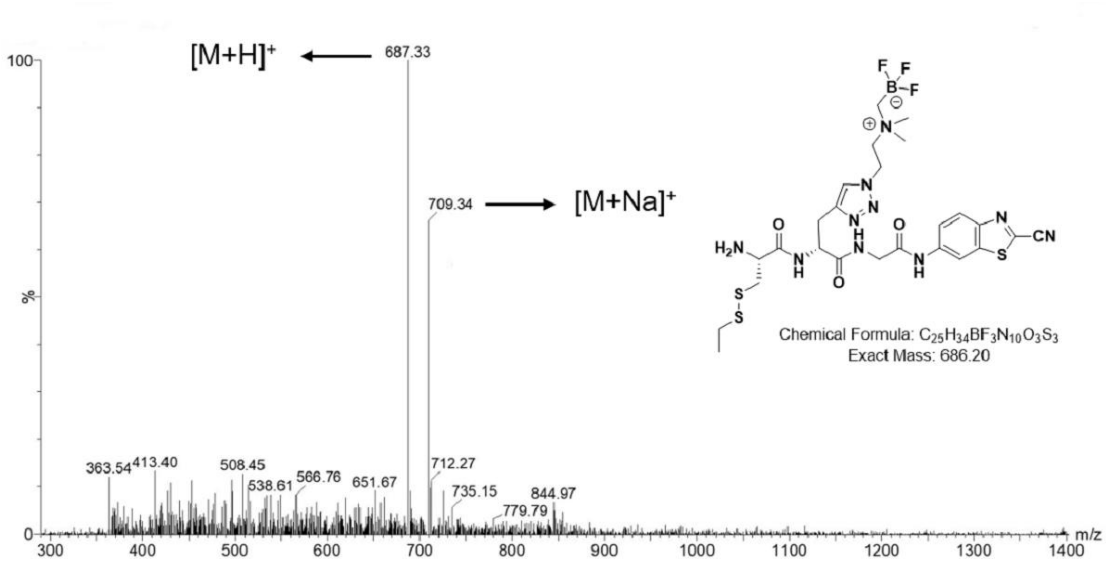


Fig. S24. ESI-MS analysis of compound **JM-C**.

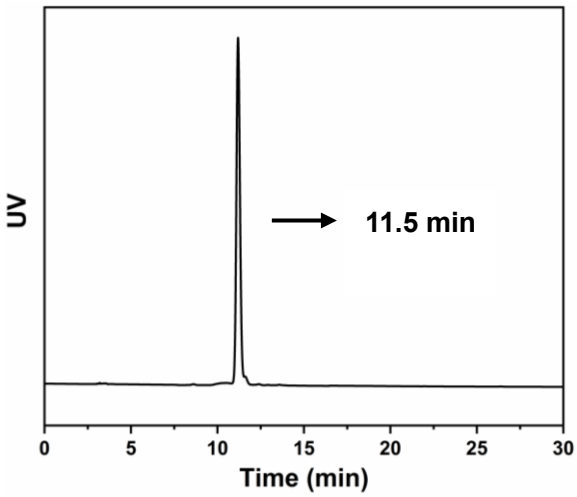


Fig. S25. HPLC trace of compound **JM-C.**

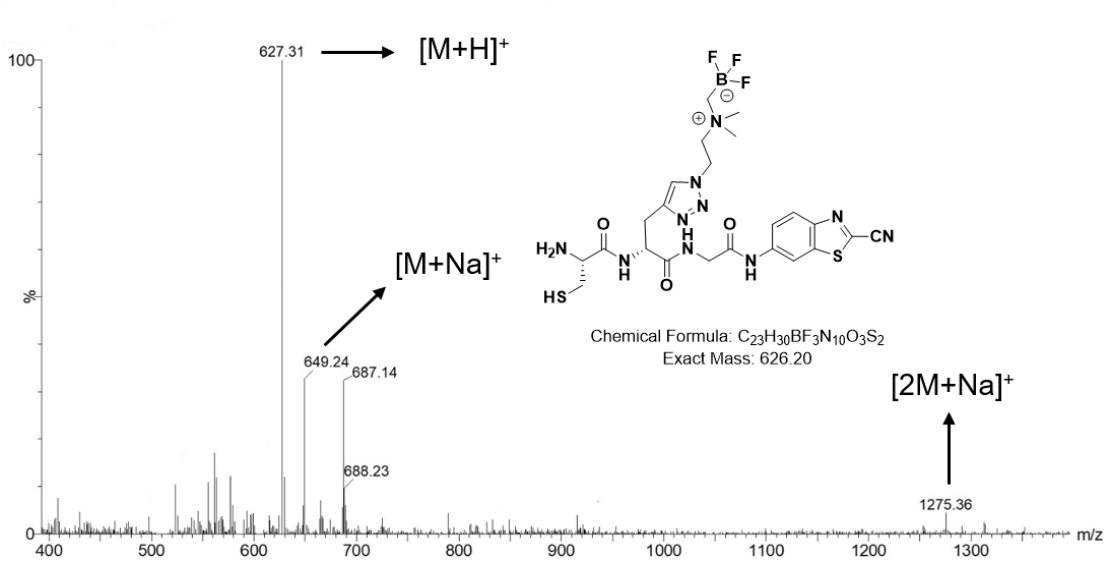


Fig. S26. ESI-MS analysis of compound **JM-C-R**.

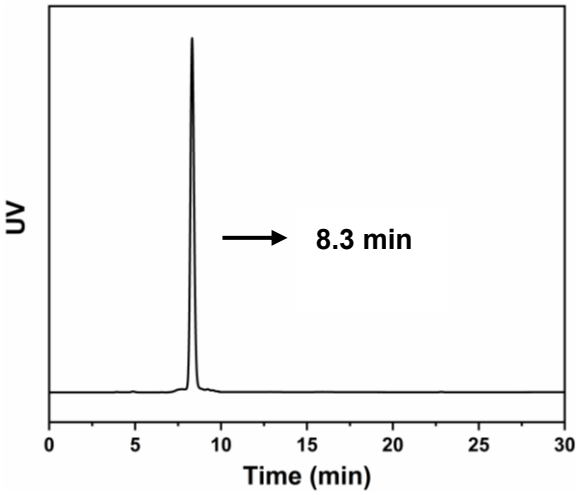


Fig. S27. HPLC trace of compound **JM-C-R.**

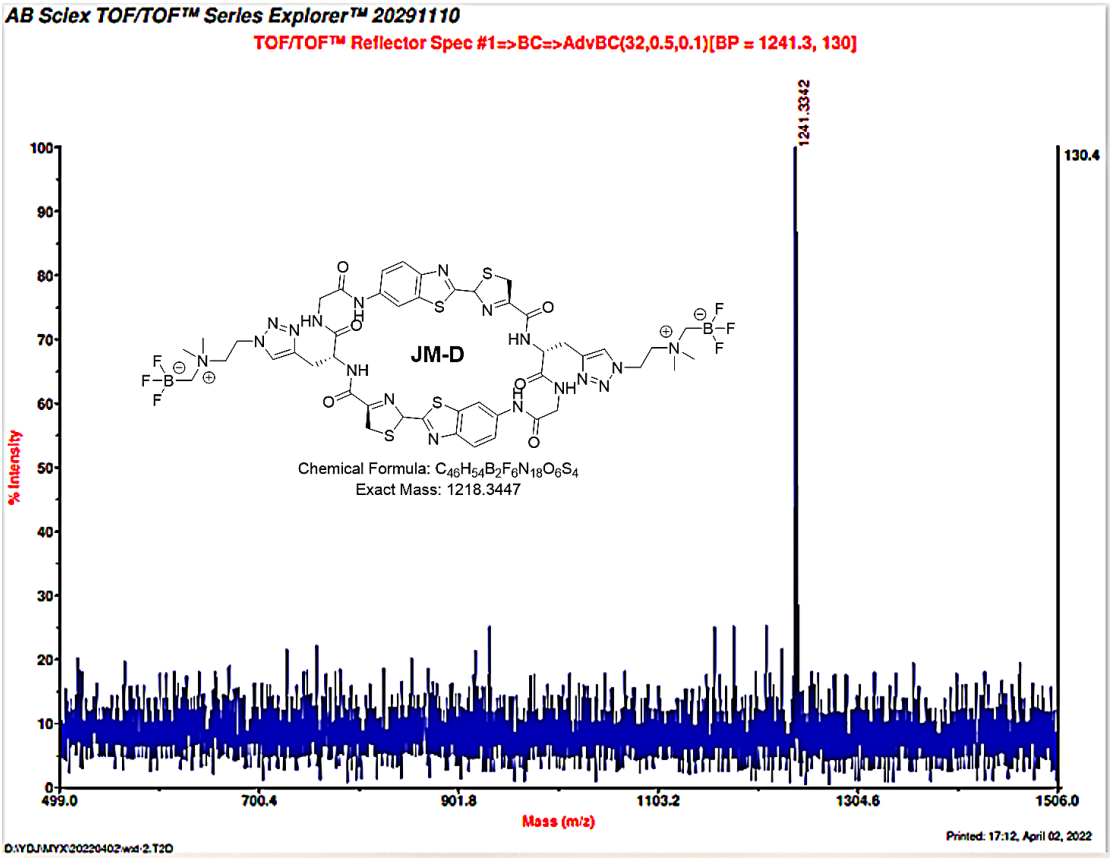


Fig. S28. MALDI-TOF of compound **JM-D.**

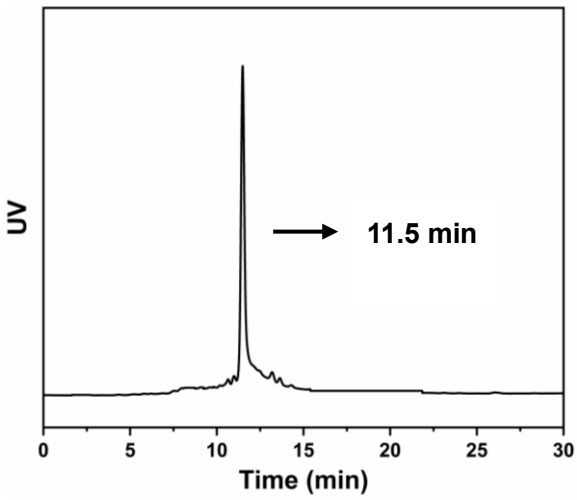


Fig. S29. HPLC trace of compound **JM-D.**

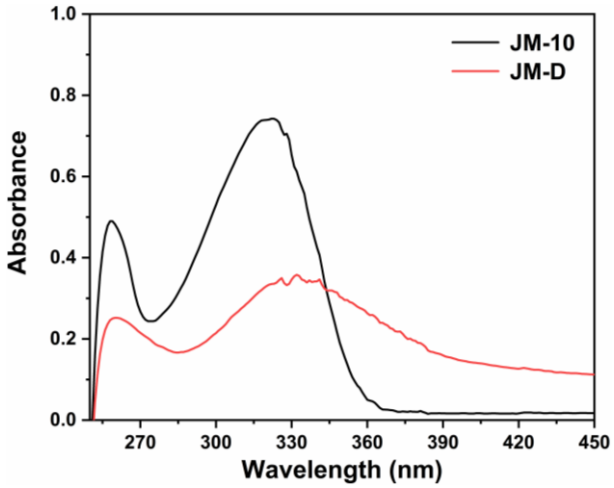


Fig. S30. UV-Vis spectrum of compound **JM-10** and compound **JM-D.**

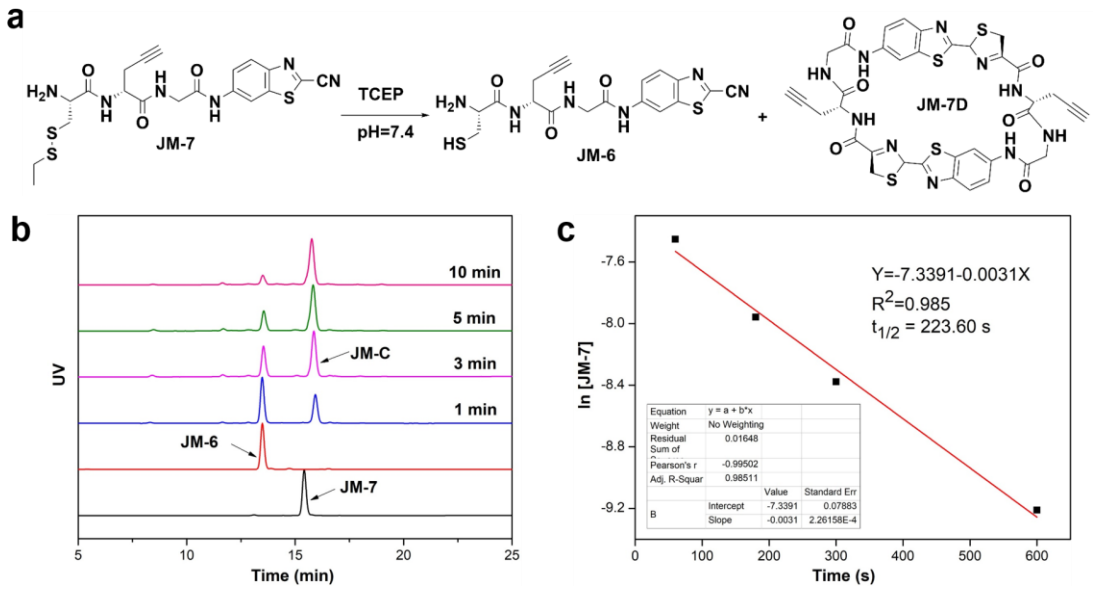


Fig. S31 (a) Possible action mechanism of compound **JM-7** under reductive conditions (GSH or TCEP). (b) HPLC trace of **JM-7** (250 μM) incubated in the buffer containing TCEP (5 mM) at 37 °C for different time. (c) Linear fitting of HPLC data to determine the first-order reaction rate k1 for the cyclization reaction of JM-7.

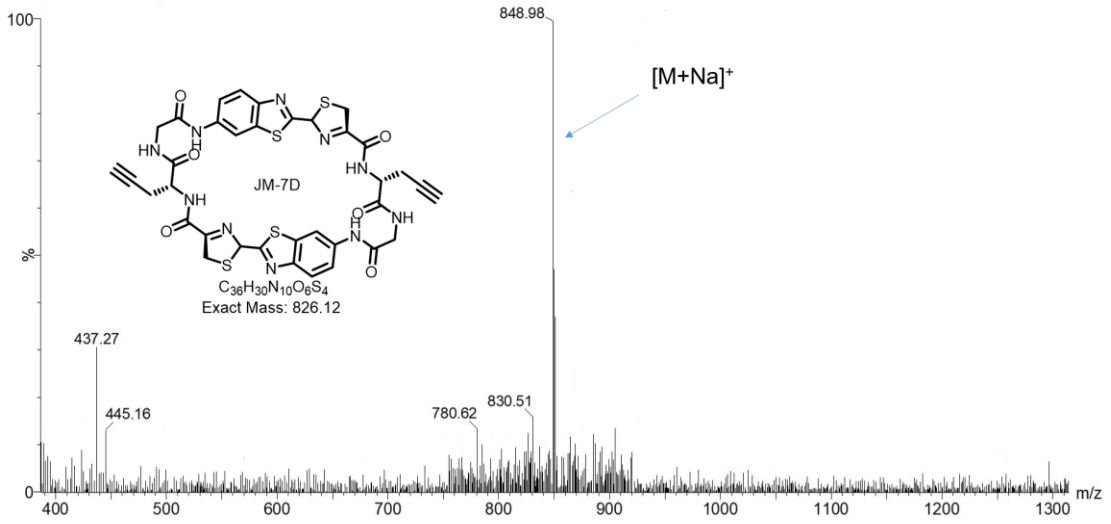


Fig. S32. ESI-MS analysis of condensation product **JM-7D**.

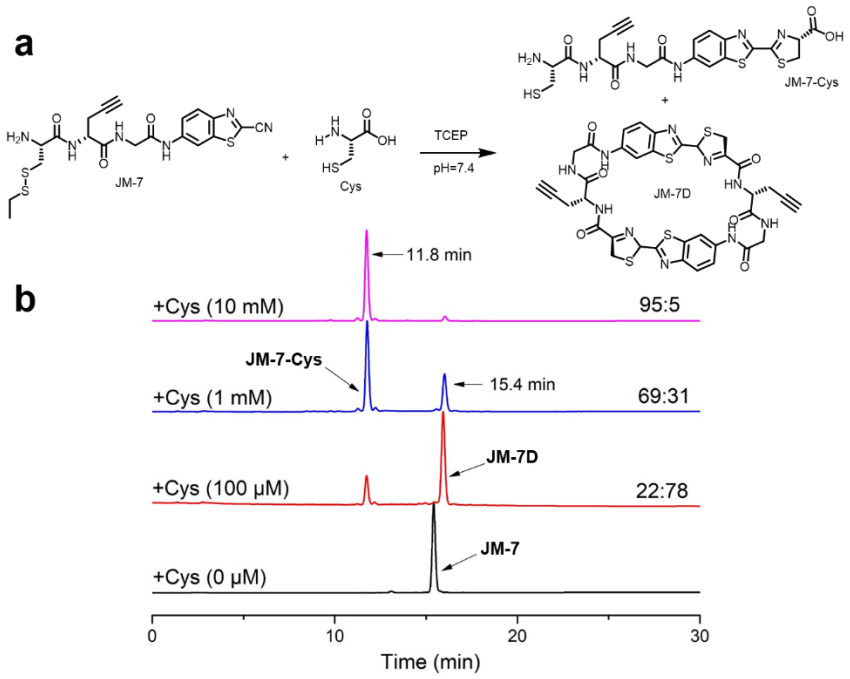


Fig. S33. (a) Possible reaction mechanism of compound **JM-7** with L-cysteine. (b) HPLC trace of **JM-7** (100 μM) incubated with varying concentration of L-cysteine in a buffer (pH = 7.4, 40% DMF) containing TCEP (2 mM) at 37 °C for 30 min.

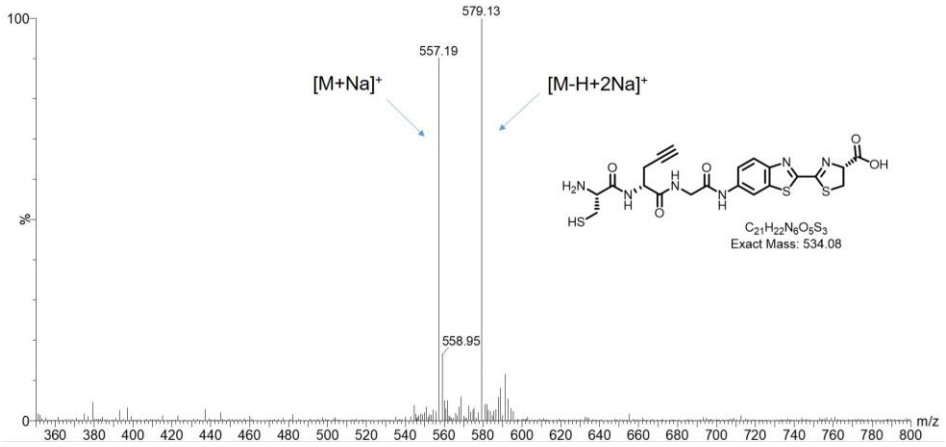


Fig. S34. ESI-MS analysis of **JM-7-Cys**.

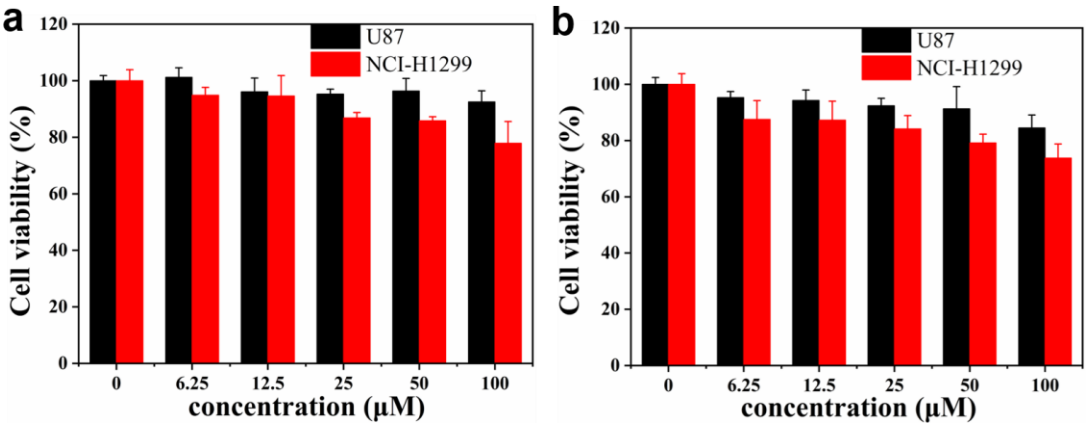


Fig. S35. Cytotoxicity assay of nonradioactive probe **JM-10** against U87 and NCI-H1299 cells for 24 h (a) and 48 h (b).

Table S1. Semi-preparative HPLC conditions for purification of nonradioactive probe **JM-10**.

Time/min	Flow (mL/min)	H_2O % (0.1%TFA)	MeCN% (0.1%TFA)
Initial	3	80	20
3	3	80	20
5	3	75	25
25	3	65	35
30	3	10	90
35	3	80	20

Table S2. Analytical HPLC conditions for all compounds.

Time/min	Flow (mL/min)	H_2O % (0.1%TFA)	MeCN% (0.1%TFA)
Initial	1	80	20
3	1	80	20
35	1	10	90
40	1	80	20

Reference

1. Z. Liu, M. Pourghiasian, M. A. Radtke, J. Lau, J. Pan, G. M. Dias, D. Yapp, K. S. Lin, F. Bénard and D. M. Perrin, An organotrifluoroborate for broadly applicable one-step ^{18}F-labeling, *Angewandte Chemie*, 2014, **53**, 11876-11880.