Supporting Information

Copper-Catalyzed Click Reaction inside Living Cells

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1. General information, cell lines and culture conditions

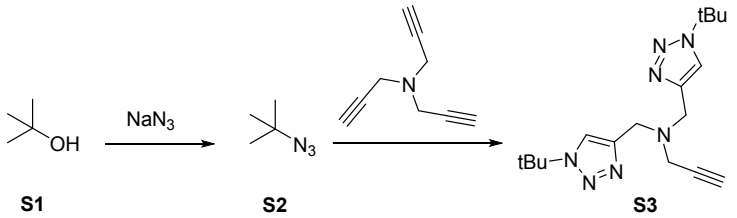
All reagents obtained from commercial sources were used without further purification. NMR spectra were recorded on JEOL 400 or 500 MHz spectrometer. Electrospray ionization mass spectrometry (ESI-MS) was performed at a Thermo LCQ Deca XP Plus with a Surveyor HPLC system. MALDI-TOF spectrums were acquired with a Voyager Biospectrometry Workstation (Applied Biosystem Inc.). HR-MS was performed at an Agilent 6530 Accurate-Mass Q-Tof LC/MS.

The endothelial cell growth medium (EGM^{TM}-2, Lonza, Allendale, NJ, USA), fetal bovine serum (FBS, Atlanta biologicals, Lawrenceville, GA), methionine-free DMEM High Glucose (DMEM, Invitrogen, Grand Island, NY), trypsin (Hyclone laboratories Inc. Logan, Utah) and Phosphate buffered saline solution (10 × PBS, Sigma-Aldrich, St Louis, MO) were used for cell culture. Click-iT^{R} HPG (*L*-homopropargylglycine) was purchased from Invitrogen (Grand Island, NY). The MTS assay kit (Promega, Madison, WI) and avidin-FITC from egg white (Sigma-Aldrich, St Louis, MO) was used for characterizing the cells.

The HUVECs were kindly provided by Dr. Changyi Chen (Molecular Surgeon Research Center, Baylor College of Medicine, Houston, TX, USA). OVCAR5 were provided by Dr. Rathindra Bose (Department of Biology and Biochemistry, University of Houston, Houston, TX, USA). The HUVEC cells were cultured in EGM^{TM}-2 medium with 10% FBS at 37 ºC with 5% CO_2. HUVEC cells of passage 5 to 9 were used for the experiments. OVCAR5 were cultured using RPMI-1640 medium with 10% FBS in 37 ºC with 5% CO_2. For MTS assay, cells at a density of 5×10^3 cells/well were seeded into 96-well plates. For microscopic imaging, cells at a density of 1×10^5 cells/slide were seeded onto a glass cover slip (1×1 cm^2) in 30 mm culture dish. For LC-MS/MS analysis, cells at a density of 1×10^6 cells/well were seeded onto 12-well plates, or at a density of 1×10^7 cells on a 100 mm culture dish. For the ICP-MS analysis, cells at a density of 1×10^6 cells/well were seeded into 12-well plates. For western blotting analysis, cells were seeded into a 100 mm culture dish with the amount of 1×10^7 cells. After seeded, cells were cultured for another 24 h before the experiment.

2. Synthesis Procedure

1.1. Compound **S3**

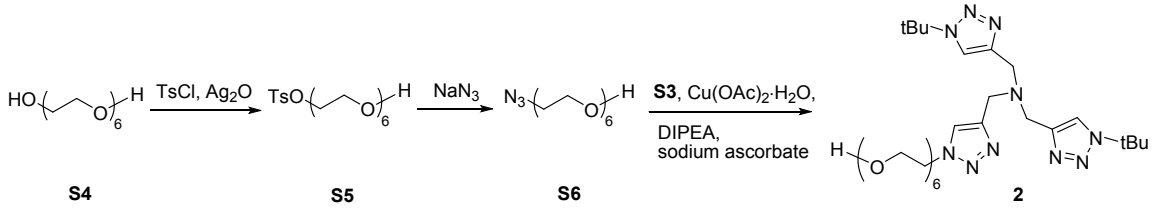


Compound **S2** was prepared from **S1** according to the reported procedures.[1]

*Caution: sodium azide and tButyl azide **S2** are explosive! They should be handled only in a small quantity strictly following the safety guidelines for these hazardous chemicals.*

Under nitrogen atmosphere, 4 mL MeCN was added to the mixture of compound **S2** (0.8 g, 8.1 mmol), tripropargylamine (0.54 g, 4.1mmol), 2,6-lutidine (0.44 g, 4.1 mmol) and $Cu(OAc)_2 \cdot H_2O$ (41 mg, 0.2 mmol). The mixture was stirred for 19 h at room temperature, followed by the evaporation of solvent under reduced pressure. The crude product was purified by flash chromatography using 5% MeOH/ethyl acetate as eluent to obtain the desired product **S3**[2] (509 mg, 38% yield).

1.2. Ligand **2**



Compound **S6** was prepared in two steps from **S4** and **S5** according to the reported procedures.[3]

Under nitrogen atmosphere, 1 mL THF was added to the mixture of **S6** (33 mg, 0.1 mmol), **S3** (36 mg, 0.1 mmol), *N*,*N*-diisopropylethylamine (14 mg, 0.16 mmol), $Cu(OAc)_2 \cdot H_2O$ (2 mg, 0.01 mmol) and sodium ascorbate (4 mg, 0.02 mmol). The reaction mixture was stirred at 60 ºC for 14 h. CupriSorb™ was added and the mixture was stirred for 2 h under ambient conditions to remove copper. After filtration and solvent evaporation, the residue was purified by silica gel column chromatography (ethyl acetate/methanol = 9/1) to afford product **2** (38 mg, 55%) as a yellow wax.

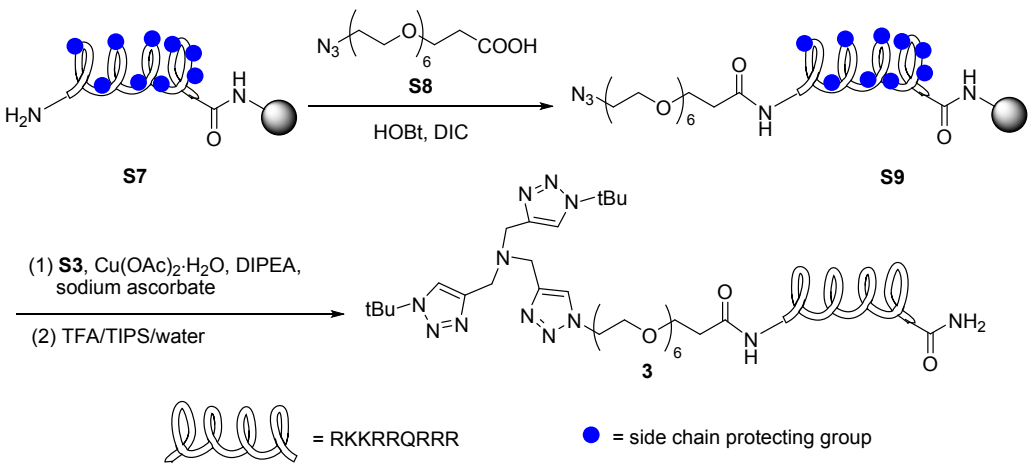
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.94 (s, 1H), 7.86 (s, 2H), 4.53 (t, *J* = 5.3 Hz, 2H), 3.87 (t, *J* = 5.3 Hz, 2H), 3.77 (s, 2H), 3.74 (s, 4H), 3.69 (t, *J* = 5.0 Hz, 2H), 3.64-3.56 (m, 18H), 1.66 (s, 18H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 125.15, 121.28, 72.74, 70.63, 70.56, 70.29, 69.58, 61.66, 59.34, 50.27, 47.40, 47.00, 30.11. HRMS (ESI-TOF) m/z: $[M + Na]^+$ Calcd for $C_{29}H_{52}N_{10}O_6Na$ = 659.3964; Found 659.3951.

1.3. Solid-phase synthesis of the Tat peptide RKKRRQRRR on resin (**S7**)

Rink Amide ChemMatrix® resin (0.48 mmol g^{-1}, 208 mg, 0.1 mmol) was swelled with DMF/CH_2Cl_2 (1/1, vol/vol) for 30 min in a solid-phase reactor. For Fmoc deprotection, 20% piperidine in DMF was added to resin and allowed to shake (550 rpm) for 2.5 min at 80 ºC. After reaction, the reagents were drained and the resin was washed with 5 cycles of DMF, 2 cycles of CH_2Cl_2 and 3 cycles of NMP. The deprotection step was repeated one more time. To attach the Fmoc-protected amino acids to the resin, under nitrogen, Fmoc-amino acid (0.5 mmol), HOBt (68 mg, 0.5 mmol) and DIC (77μL, 0.5 mmol) were dissolved in 2 mL NMP and the solution was added to the resin. The mixture was shaken at 550 rpm for 20 min at 80 ºC. The reagents were drained and the resin was washed with 2 cycles of NMP and 2 cycles of DMF. The above deprotection and coupling steps were repeated and finally the resin was washed with CH_2Cl_2 and dried to provide the side-chain protected Tat peptide (RKKRRQRRR) on the resin (**S7**, 310 mg).

1.4. Azido-EG_6-Tat bound resin **S9**

Under nitrogen, the above resin bound Tat peptide (**S7**, 280 mg, 0.08 mmol) was swelled in DMF/CH_2Cl_2 (1/1, vol/vol) for 30 min in a solid-phase reactor. The azido-PEG-acid (**S8**, 91 mg, 0.14 mmol, Quanta BioDesign), HOBt (33 mg, 0.24 mmol) and DIC (40 μL, 0.24 mmol) were dissolved in 2 mL anhydrous THF, and added to the swelled resin **S7**. The mixture was allowed to shake for 2 h at 50 ºC. The reagent was drained. The resin was washed with THF, and the above procedure was repeated one more time. The resin was washed with CH_2Cl_2 and dried to provide the azido-EG_6-Tat bound resin **S9** (360 mg).

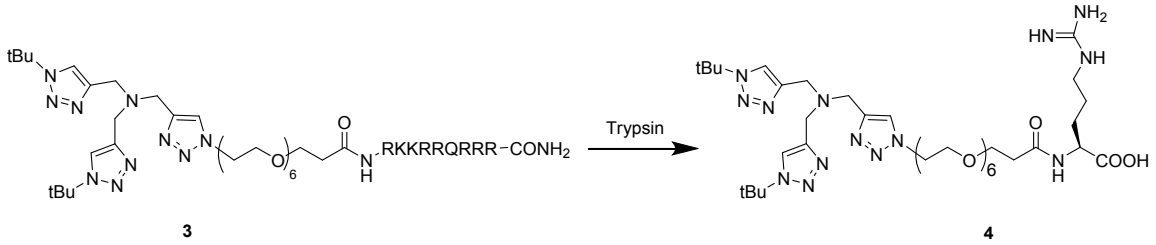


1.5. Tat-ligand **3**:

The resin **S9** (360 mg, 0.08 mmol) was swelled in DMF for 30 min in a solid-phase reactor. Under nitrogen, $Cu(OAc)_2 \cdot H_2O$ (32 mg, 0.16 mmol) and sodium ascorbate (63 mg, 0.32 mmol) were dissolved in degassed water to prepare the Cu(I) stock solution that was used immediately. Compound **S3** (79 mg, 0.24 mmol) and DIPEA (67 μL, 0.38 mmol) was dissolved in 2.7 mL DMF, followed by the addition of 0.3 mL of Cu(I) stock solution. The solution was transferred to the resin **S9**. The mixture was shaken for 2 days at room temperature in nitrogen atmosphere. The solution was drained, and the resin was washed with 3 cycles of 5 mg/mL EDTA (a.q.), 3 cycles of water, and 2 cycles of THF, and 2 cycles of CH_2Cl_2 and dried. To cleave the side-chain protecting groups and release the Tat-ligand **3** from the resin, the resin was treated with a cleavage solution containing TFA/TIPS/water (95/2.5/2.5, vol/vol/vol) and allowed to react at room temperature for 2 h with gentle shaking. The solution was then filtered and TFA was removed by vacuum. Cold diethyl ether was added into the mixture to precipitate the peptide product. After standing for 30 min in ice bath, the peptide precipitate was isolated by centrifugation (2200 rpm, 5 min). The precipitate was washed by diethyl ether for three times and freeze-dried to yield the crude peptide. HPLC purification was performed with Shimadzu Prominence HPLC system with a UV detector at 215 nm and a Waters XTerra C18 column (50 × 19 mm, 5 μm) with gradient of 5-50% acetonitrile. The Tat-ligand **3** was obtained in 70.8 mg (44% yield).

MALDI-TOF-MS: $[M+H]^+$ calcd for $C_{85}H_{162}N_{41}O_{17}$ = 2030.4, Found 2030.4, $[M+2H]^{2+}$ calcd for $C_{85}H_{163}N_{41}O_{17}/2$ = 1015.7, Found 1016.1.

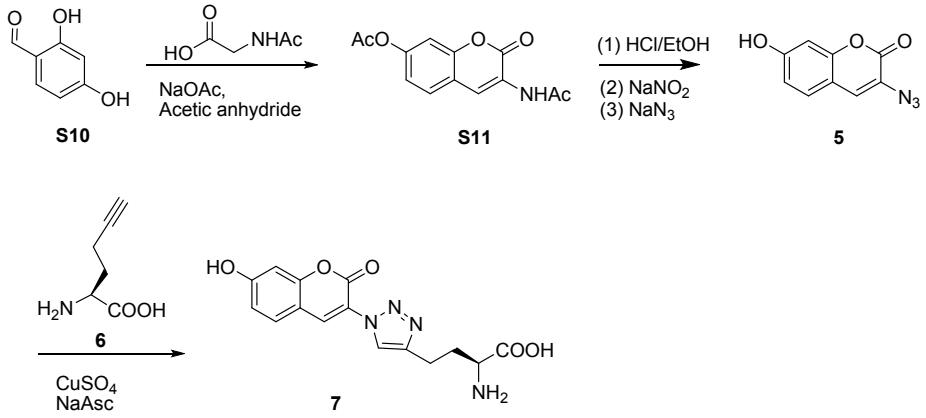
1.6. Enzymatic hydrolysis of the Tat-ligand **3** to **4**



A mixture of Tat-ligand **3** (16.4 mg, 8 μmol) and trypsin (from bovine pancreas, Sigma, 16 mg) in 200 μL ammonium acetate (50 mM) aqueous solution was incubated at 37 ºC for one day. The progress of hydrolysis was monitored by LC-ESI MS. The crude product was purified by HPLC to yield **4** (1.7 mg, 24%) as a white solid.

HRMS (ESI-TOF) m/z: $[M + Na]^+$ Calcd for $C_{38}H_{68}N_{14}O_9Na$ = 887.5186; Found 887.5170.

1.7. Compound **7**



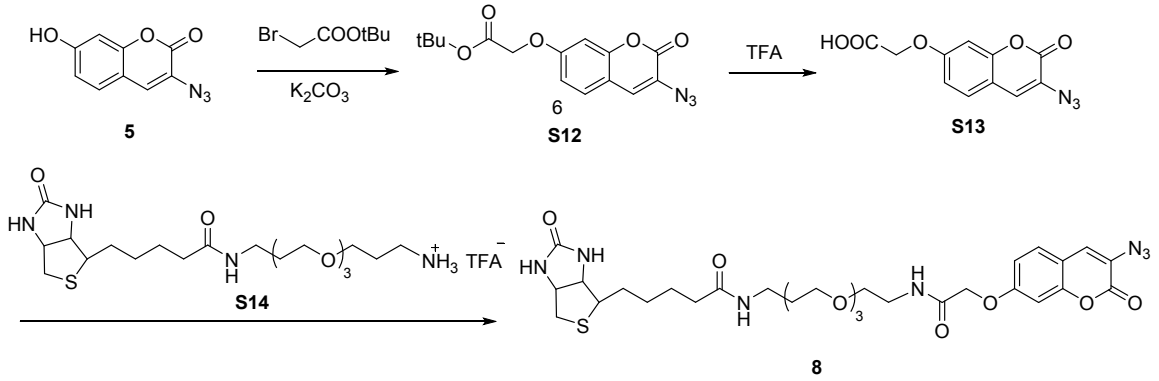
Compound **5** was prepared in two steps from **S10** according to the reported procedures.[4] Compound **7** was prepared in one step from **5** and **6** according to the reported procedures.[5]

1.8. Compound **8**

Compound **S13** was prepared in two steps from **5** according to the reported procedures.[4]

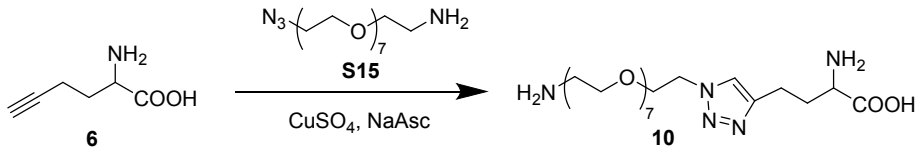
A mixture of **S13** (20.6 mg, 80 μmol), HBTU (33 mg, 87 μmol) and DIPEA (50 μL, 290 μmol) in 0.5 mL DMF was prepared. After 20 min, a solution of **S14** (40.2 mg, 72 μmol, Quanta Biodesign) in 2 mL CH_2Cl_2 was added to the mixture under nitrogen. The reaction mixture was stirred at room temperature for 3 h, and

diluted with CH_2Cl_2 (60 mL), and washed with 40 mL 10% citric acid (aq.). The organic layer was evaporated under vacuum. The crude product was purified by flash chromatography (MeOH/CH_2Cl_2 = 11:89, R_f = 0.28) to give **8** (19.7 mg, 40% yield) as a yellow wax.



^{1}H NMR (500 MHz, $CDCl_3$): δ 7.36 (d, *J* = 8.4 Hz, 1H), 7.24 (t, *J* = 5.5 Hz, 1H), 7.17 (s, 1H), 6.92 (d, *J* = 2.4 Hz, 1H), 6.90 (q, *J* = 2.5 Hz, 1H), 6.64 (t, *J* = 5.3 Hz, 1H), 6.34 (s, 1H), 5.57 (s, 1H), 4.53 (s, 1H), 4.50 – 4.46 (m, 1H), 4.33 – 4.26 (m, 1H), 3.65 – 3.50 (m, 12H), 3.44 (dd, *J* = 12.4, 6.3 Hz, 2H), 3.31 (dd, *J* = 12.2, 6.2 Hz, 2H), 3.12 (dd, *J* = 11.9, 7.3 Hz, 1H), 2.88 (dd, *J* = 12.8, 4.9 Hz, 1H), 2.72 (d, *J* = 12.8 Hz, 1H), 2.17 (t, *J* = 7.5 Hz, 2H), 1.89 – 1.53 (m, 8H), 1.46 – 1.34 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d6) δ 172.44, 170.18, 163.25, 129.37, 128.36, 127.92, 127.69, 125.07, 119.69, 113.95, 110.15, 70.28, 70.07, 61.57, 60.30, 59.72, 55.97, 55.46, 54.08, 49.13, 42.34, 36.23, 35.73, 29.93, 28.76, 25.84, 18.59, 17.25, 12.99. HRMS (ESI-TOF) m/z: $[M + Na]^+$ Calcd for $C_{31}H_{43}N_7O_9SNa$ = 712.2735; Found 712.2719.

1.9. Compound **10**

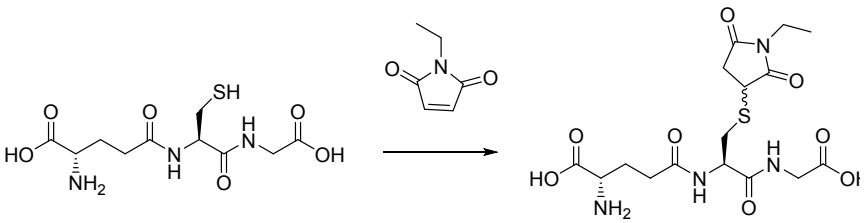


Under nitrogen, sodium ascorbate (8 mg, 0.04 mmol) was added to a stirred mixture of **6** (25.8 mg, 0.2 mmol), **S15 (**80 mg, 0.2 mmol), $CuSO_4 \cdot 5H_2O$ (3 mg, 0.02 mol) and DIPEA (57 uL, 0.32 mmol) in methanol (2 mL). The reaction mixture was stirred at 60 ℃ for 2 days. CupriSorb resin was added and mixture was stirred for 4 h to remove copper. The mixture was filtered and the solvent was removed by vacuum to obtain an

yellowish crude product which was purified using Shimadzu Prominence HPLC system and a C18 column with 2% – 40% acetonitrile/water gradient to yield the product **10** (41 mg, 40%) as a white powder.

^{1}H NMR (500 MHz, D_2O): δ 7.76 (s, 1H), 4.45 (t, *J* = 5.0 Hz, 2H), 3.81 (t, *J* = 5.0 Hz, 2H), 3.61 (t, *J* = 5.0 Hz, 2H), 3.58 – 3.43 (m, 25H), 3.05 (t, *J* = 5.0 Hz, 2H), 2.80 – 2.69 (m, 2H), 2.21 – 2.02 (m, 2H). ^{13}C NMR (126 MHz, D_2O): δ 172.82, 123.95, 122.50, 69.56, 68.75, 66.37, 53.07, 49.98, 39.10, 29.78, 20.62. HRMS (ESI-TOF) m/z: $[M + Na]^+$ Calcd for $C_{22}H_{43}N_5O_9Na$ = 544.2953; Found 544.2949.

1.10. Compound **S17**[6]



15.3 mg GSH and 26 mg *N*-ethylmalemide was mixed in reaction vessel. Under nitrogen protection, 1 mL methanol was added to the mixture. The suspension was stirred overnight at room temperature. Solvent was removed by vacuum to obtain the crude. The crude was purified by reversed-phase HPLC to yield product **S17** (18.2 mg, 84%) as white powder.

MS (ESI): $[M+H]^+$ calcd for $C_{16}H_{25}N_4O_8S$ = 433.1, Found 433.1.

3. MTS cytotoxicity assay on solvents and ascorbate concentrations during CuAAC reaction

A MTS working solution (100 μL, prepared by mixing phenazine methosulfate (PMS, Promega), MTS (Promega), and culture medium (EGM™-2 for HUVECs, RPMI-1640 for OVCAR5s) in 1/20/105 volume ratio) was added to HUVECs after subjecting to various CuAAC reaction conditions in a 96-well plate. Cells were kept in dark and incubated at 37 ºC in 5% CO_2 for 2 hours. The solutions were then transferred to a new 96-well plate for measurement of absorbance at 492 nm using Bioassay reader (HTS 7000, Perkin Elmer). The MTS solution incubated under the same condition without cells was used as blank. Blank signal was subtracted in all results.

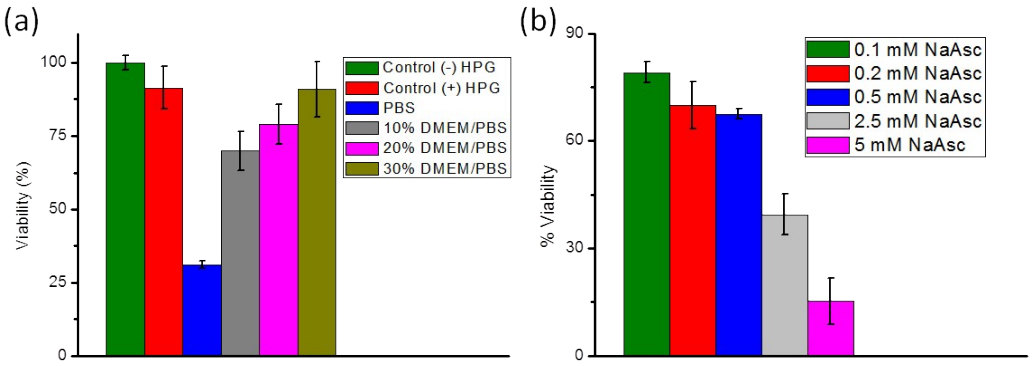


Fig. S1 HUVEC viability after subjected to the following CuAAC reaction conditions. (a) CuAAC reaction conditions: 100 μM **8**, 100 μM $CuSO_4$, 200 μM ligand **1**, 500 μM sodium ascorbate in various media for 10 minutes in air at room temperature. (b) CuAAC reaction conditions: 100 μM **8**, 100 μM $CuSO_4$, 200 μM ligand **1**, various concentration of sodium ascorbate in 10/90 DMEM/PBS. The error bars represent the standard deviation (N = 3).

4. Fluorogenic CuAAC reaction assay on ligands, media and ascorbate concentrations

To perform the fluorogenic CuAAC reaction assay (Figure 2), the stock solutions of the reagents dissolved in PBS at the concentration listed in **Table S1** were prepared. The stock solutions were mixed in a 96-well plate to provide reaction mixtures of 100 μL/well with the final concentration of the reagents listed in **Table S1**; sodium ascorbate stock solution was prepared freshly prior to use, and was added at last with a multi-channel pipette to start the reaction. Fluorescence was measured using a Bioassay reader (HTS 7000, Perkin Elmer) with excitation at 340 nm and emission at 465 nm.

Table S1. Reaction mixture composition for the fluorogenic CuAAC reaction assay for Figure 2.

	Stock solution conc.	Final conc.
Methionine-free DMEM		Varied
L-homopropargylglycine (**6**)	50 mM	50 μM
$CuSO_4$	0.5 mM	0.1 mM
5	1 mM	0.1 mM
Ligand	4 mM	0.2 mM
Sodium ascorbate	25 mM	Varied

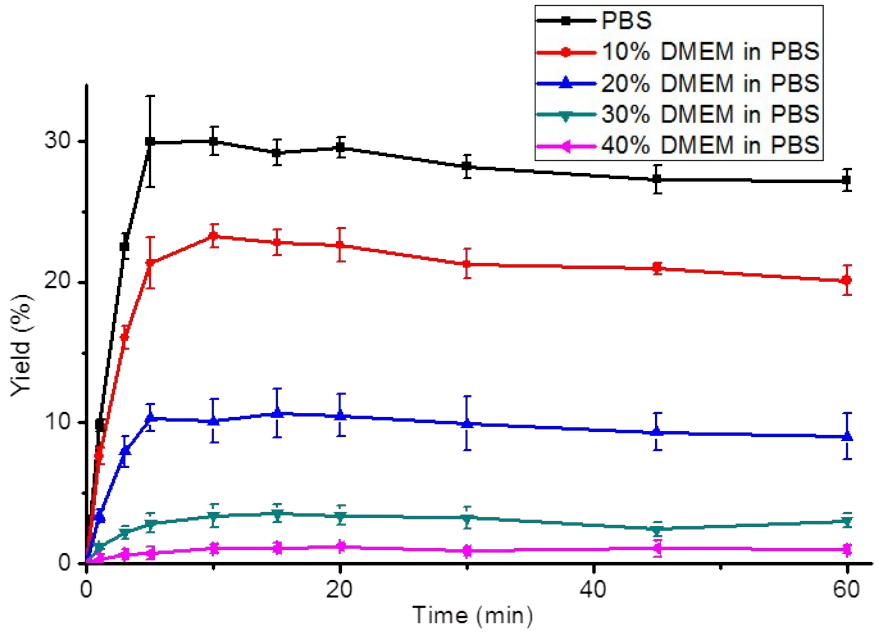


Fig. S2 Conversion-time profiles of ligand **1** assisted CuAAC reaction between **5** and **6** in different solvents. Reaction conditions: 100 μM **5**, 50 μM **6**, 100 μM $CuSO_4$, 200 μM ligand **1**, 500 μM sodium ascorbate. The error bars represent the standard deviation of data from three samples.

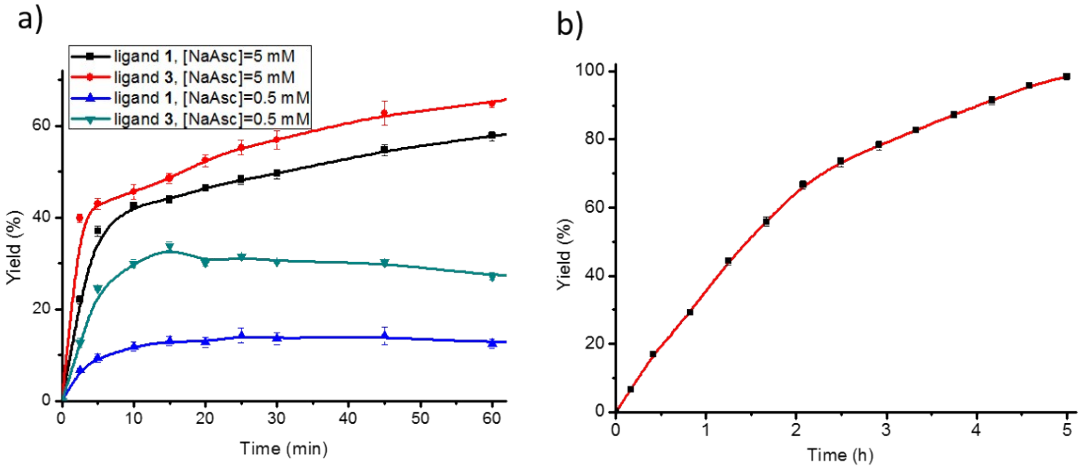


Fig. S3 (a) Conversion-time profiles of ligand **1** and ligand **3** assisted CuAAC reaction of **5** and **6** with different concentration of sodium ascorbate in 10/90 (v/v) methionine-free DMEM/PBS. (b) Conversion-time profiles of ligand **1** assisted CuAAC reaction of **5** and **6** in 0.2% SDS/PBS. The error bars represent the standard deviation of data from three samples.

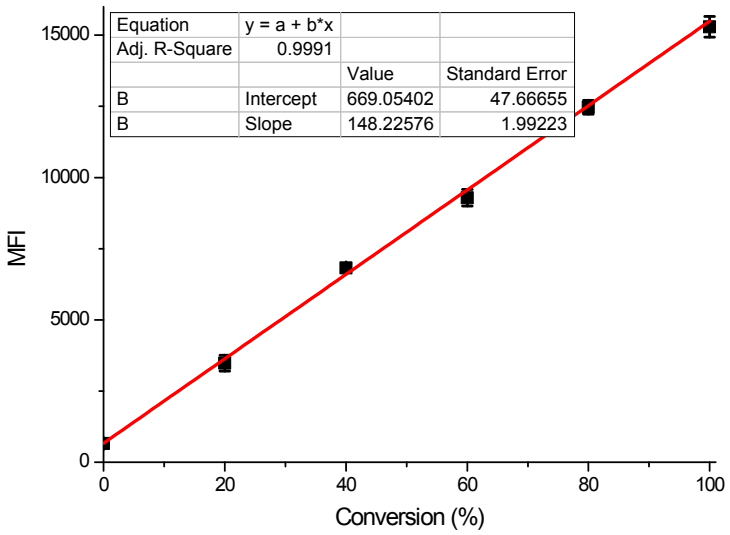


Fig. S4 Standard calibration curve for the mean fluorescence intensity (MFI) vs the amounts of compound **7** which is converted to the % conversion of the reaction. The calibration standards were prepared by mixing **7** (0, 10, 20, 30, 40, 50 μM) with **5** (100, 90, 80, 70, 60, 50 μM), respectively, in 100 μL PBS. The error bar represents standard deviation of data from three samples.

Due to the concern of the variation in fluorescence intensity in different reaction mixture, the fluorogenic reaction mixture was also analyzed by LC-ESI MS. The molecular ion of product **7** (m/z 331.0) was used to generate an extracted ion chromatogram. The integrated peak area was used for the determination of the yield (%) of the fluorogenic CuAAC reaction.

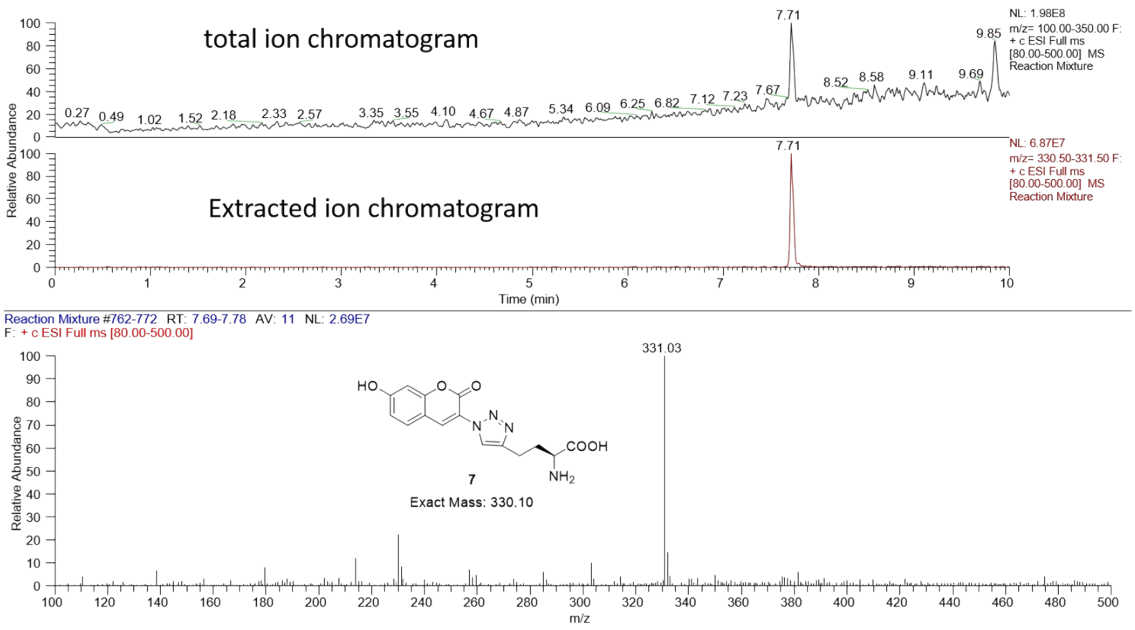


Fig. S5 Total ion chromatogram, extracted ion chromatogram and mass spectrum of the fluorogenic reaction mixture.

Table S2. Yield (%) of the fluorogenic CuAAC reaction between **5** and **6** in various reaction conditions as determined by LC-ESI MS method. The error bar is the standard deviation of at least three samples. Reaction conditions: 100 μM **5**, 50 μM **6**, 100 μM $CuSO_4$, 200 μM ligand and 500 μM sodium ascorbate in water.

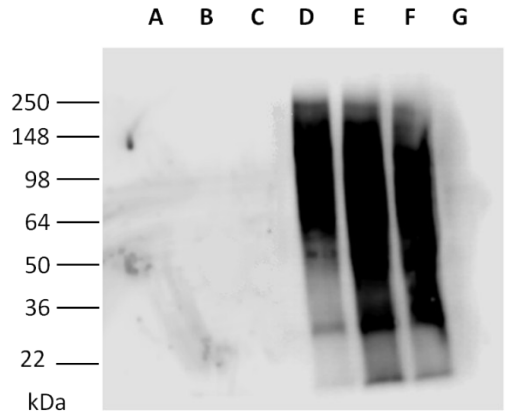
	Reaction Time (min)	Yield (%)
Ligand **1**	10	18.2 ± 1.5
Ligand **2**	10	20.1 ± 1.5
Ligand **3**	10	30.4 ± 2.1
(-) HPG	10	0

5. Optimization of metabolic labeling with HPG

The 80% confluence HUVEC and OVCAR5 cells were incubated in HBS (HEPES-buffered saline) for 30 min to exhaust methionine. Then cells were incubated in methionine-free DMEM supplemented with 4 mM HPG (**6**, Life Technologies) for 2 to 4 hours. The incorporation of HPG was confirmed by western blotting after conjugation with the biotin-azide (**9**) through CuAAC (see below). We also found that using lower concentration (50 μM) of **6** as recommend by the vendor resulted in a low incorporation to the proteins as shown by the following assay.

Thus, adhered HUVECs were treated with methionine-free DMEM supplemented with various concentrations of **6** for 2 hours. After removal of the media, the adhered cells were washed with PBS for three times, then lysed with 1% SDS in PBS. The suspension was collected in an Eppendorf tube and centrifuged at 16,000 × g for 20 minutes. The supernatant with the extracted proteins was collected, and mixed with a solution of the biotin-azide **9** (100 μM), $CuSO_4$ (100 μM), ligand **1** (200 μM), and sodium ascorbate (1 mM). The mixture was incubated for 1 hour in nitrogen (with O_2 < 1 ppm). The proteins were separated by SDS-PAGE (Biorad), and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in TBST (Tris-buffered saline and Tween 20) for 1 hour at room temperature, followed by incubation with NeutrAvidin-HRP (Thermo Pierce, 1:4000 – 1:3000) in the above blocking buffer for 1 hour. The membrane was washed with TBST for 6 times. After adding Amersham ECL select western blotting detection reagent, the chemiluminescence signal was detected with a LI-COR C-DiGit blot scanner. The result is showed in **Fig. S6**.

The above procedure with 4 mM HPG (**6**) was then used for incorporating HPG to the newly synthesized proteins in both HUVEC and OVCAR5 cell lines.



	HPG concentration
A	50 μM
B	100 μM
C	400 μM
D	2 mM
E	4 mM
F	8 mM
G	0 mM

Fig. S6 Western blot analysis of proteins extracted from HUVECs after incubation with various concentrations of HPG (**6**) for 2 hours. The result shows that 2 mM of HPG is needed for efficient incorporation of HPG into newly synthesized proteins.

6. Comparison of fluorescence response for coumarin 5 and 8

100 μL of solution containing100 μM of **5** or **8** were mixed with HPG (6, 100 μM), 100 μM $CuSO_4$, 200 μM **1** and 5 mM sodium ascorbate were prepared in a 96-well plate. The fluorescence intensity was monitored with excitation at 340 nm and emission at 465 nm.

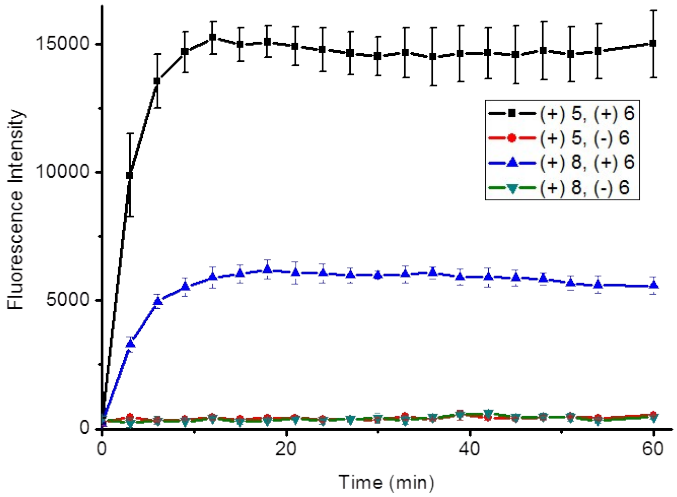


Fig. S7 Comparison of fluorescence intensity of **5** and **8** in CuAAC reaction with HPG (**6**). The error bars represent the standard deviation of data from three samples.

7. Fluorescence microscopic assay

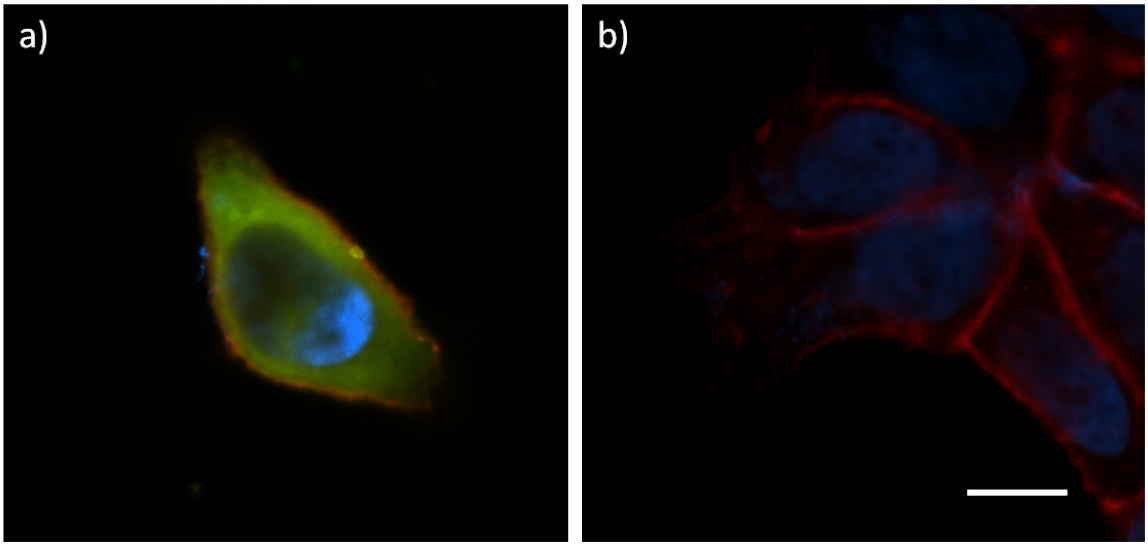


Fig. S8 Widefield fluorescent images of HPG-incorporated OVCAR5 cells after treatment with a mixture of **8** (100 μM), $CuSO_4$ (100 μM), **3** (200 μM) and sodium ascorbate (500 μM) in 10/90 (v/v) methionine-free

DMEM/PBS for 10 minutes (a), a mixture of **8** (100 μM) and sodium ascorbate (500 μM) in 10/90 (v/v) methionine-free DMEM/PBS for 10 minutes (b). Green: FITC fluorescence of avidin-FITC. Red: Stain for plasma membrane with wheat germ agglutinin Alexa Fluor 555 conjugate. Blue: Stain for cell nucleus with 4',6-diamidino-2-phenylindole (DAPI). Scale bar: 10 μm.

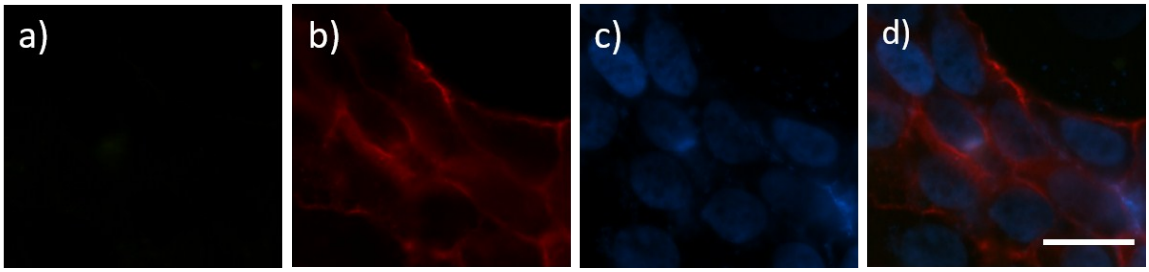


Fig. S9 Confocal microscopy images of blank control samples. HPG-incorporated OVCAR5 cells were treated with a mixture of **8** (100 μM), $CuSO_4$ (100 μM), **3** (200 μM) and sodium ascorbate (500 μM) in 10/90 (v/v) methionine-free DMEM/PBS for 10 minutes. The cells were fixed and stained with wheat germ agglutinin Alexa Fluor 555 conjugate and DAPI. Scale bar is 20 μm. The fluorescence images of FITC (a), Alexa fluor 555 (b), DAPI (c) and overlay of three fluorescence channels (d).

8. Quantification of the removal of extracellular ligands after CuAAC reaction

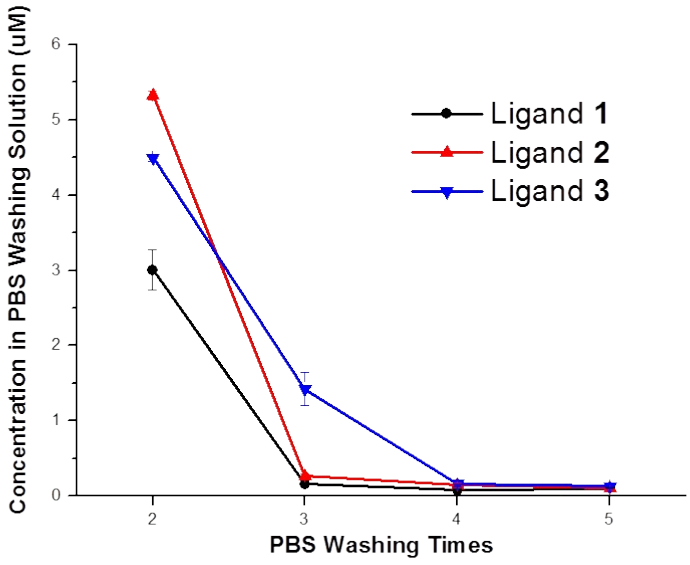


Fig. S10 The concentration of ligands **1**–**3** in PBS washing solution measured by LC-ESI-MS/MS. Four times washing is sufficient to remove the weakly bounded ligands. The error bars represent the standard deviation of data from three samples.

After the HUVEC and OVCAR5 cells in a 12-well plate were subjected to the above CuAAC reaction conditions, the adhered cells were washed with 500 μL PBS four times. The efficiency of PBS washing for removal of the ligands outside the cells was analyzed by LC-ESI-MS/MS as described below. To the collected PBS washing solution, 50 μM trypsin is added. The solution is incubated for 24 hours and enriched with C18 ziptip to a 100 μL solution. The solution was analyzed by the LC-ESI-MS/MS method described below. The result (**Fig. S10**) shows that four times washing with PBS removed the ligands outside the cells to less than 0.2 μM.

9. Measurement of intracellular ligand concentrations by LC-ESI-MS/MS

LC-ESI-MS/MS was performed on a Thermo LCQ Deca XP Plus mass spectrometer with a Surveyor HPLC system. Liquid chromatography was performed on a Kinetex XB C18 column (Phenomenex, 50 × 2.1 mm, 5 μm) eluted with a linear gradient from 8% – 47% of mobile phase B (acetonitrile containing 0.1% formic acid) in mobile phase A (water containing 0.1% formic acid) for 7 minutes at a flow rate of 200 μL/min. The mass spectrometer was set at positive electrospray ionization with the following conditions: 300 ºC capillary temperature, 45 units Sheath gas, 10 units aux gas and 5.0 kV spray voltage. All other conditions were optimized with the automatic tuning function for each individual compound.

For the preparation of standard calibration curve, a stock solution containing 1 ng/μL of ligand **1**, **2**, and **4** was diluted with cell lysate to 9 concentration levels of 1, 2, 5, 10, 20, 50, 100, 200, 500 pg/μL, respectively. All samples were spiked with internal standard (ISTD) (**Fig. S11**) at a final concentration of 100 pg/μL. The standard samples were analyzed with five replicate injections at each concentration and a blank (water) injection between two concentrations. The chromatogram was generated by summing the most intense product ions observed in the full-scan MS/MS spectra (**Fig. S11**) acquired for compound **1** (m/z 375.0), compound **2** (m/z 581.2 + 609.3), compound **4** (m/z 405.4) and compound ISTD (m/z 361.1). The extracted ion chromatograms of all analytes were showed in **Fig. S12**. The calibration curve for compound **1**, **2** and **4**

over the entire range of the assay is showed in **Fig. S13**. For compound **1**, the correlation coefficient was 0.9981 over the dynamic range from 5 pg to 2.5 ng on-column. For compound **2**, the correlation coefficient was 0.9976 over the dynamic range from 5 pg to 2.5 ng on-column. For compound **4**, the correlation coefficient was 0.9978 with the linearity range from 5 pg to 2.5 ng on-column.

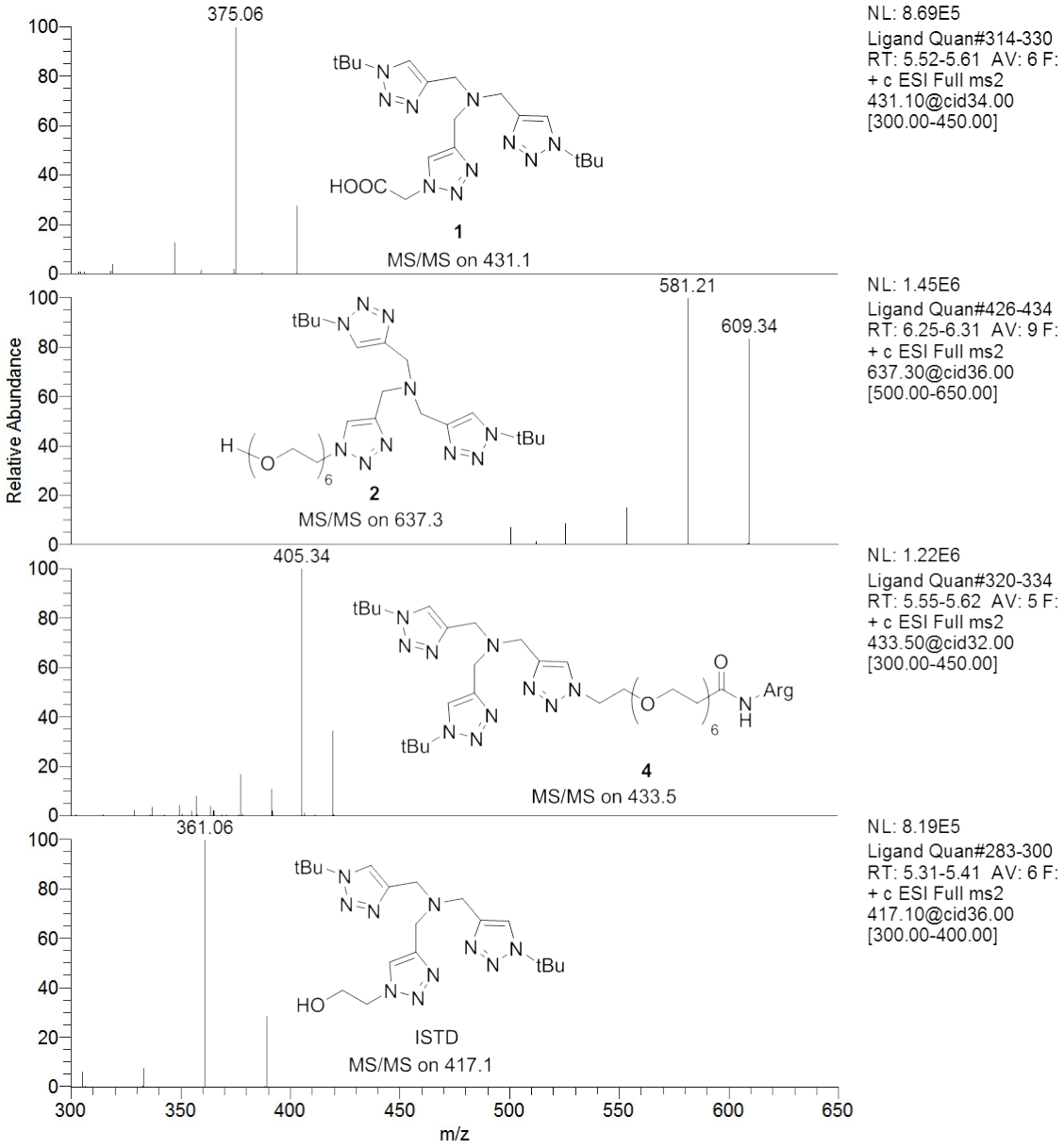


Fig. S11. MS/MS spectra of compound **1**, **2**, **4** and ISTD.

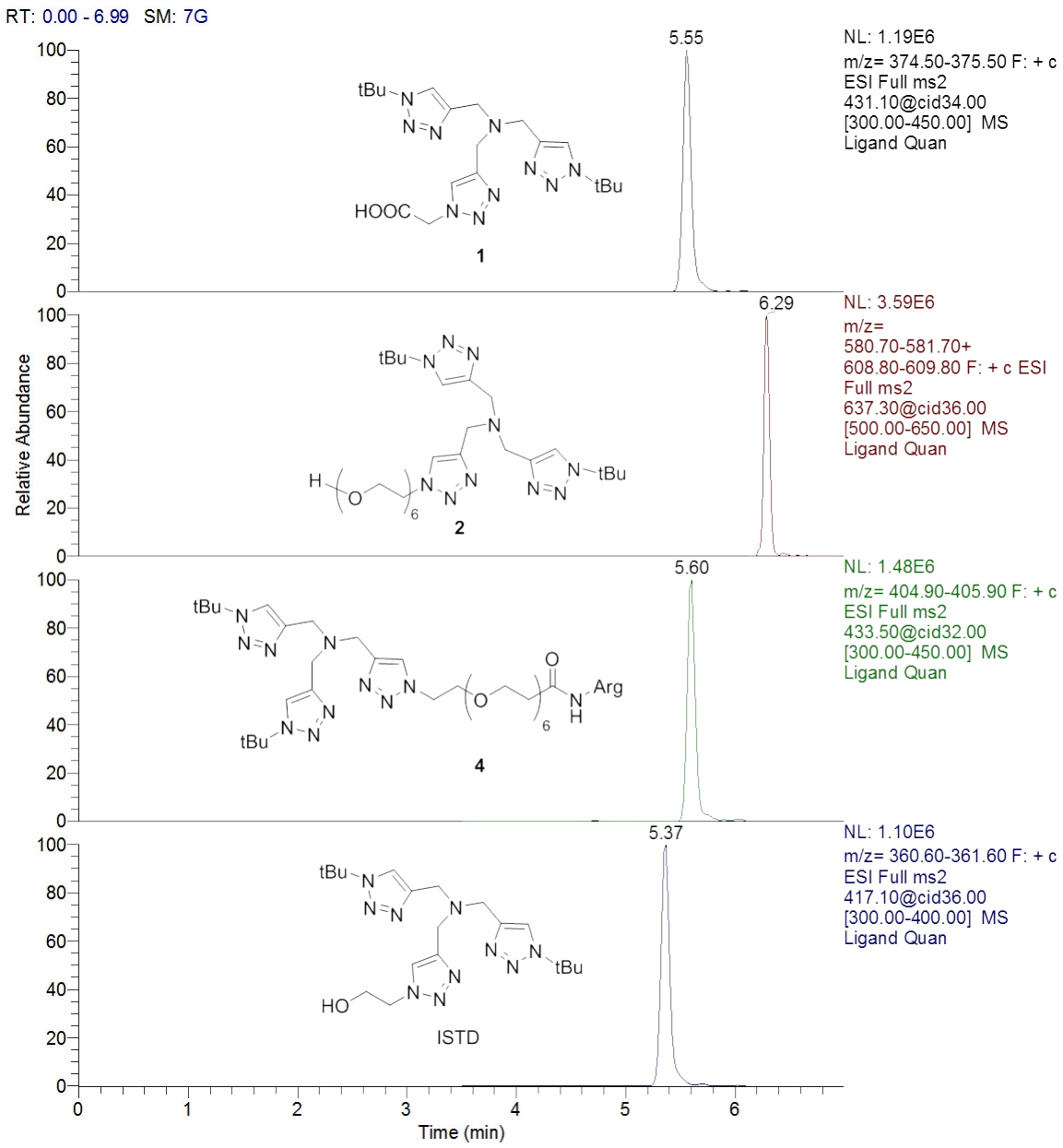


Fig. S12. Extracted ion-chromatograms of compound **1**, **2**, **4** and ISTD.

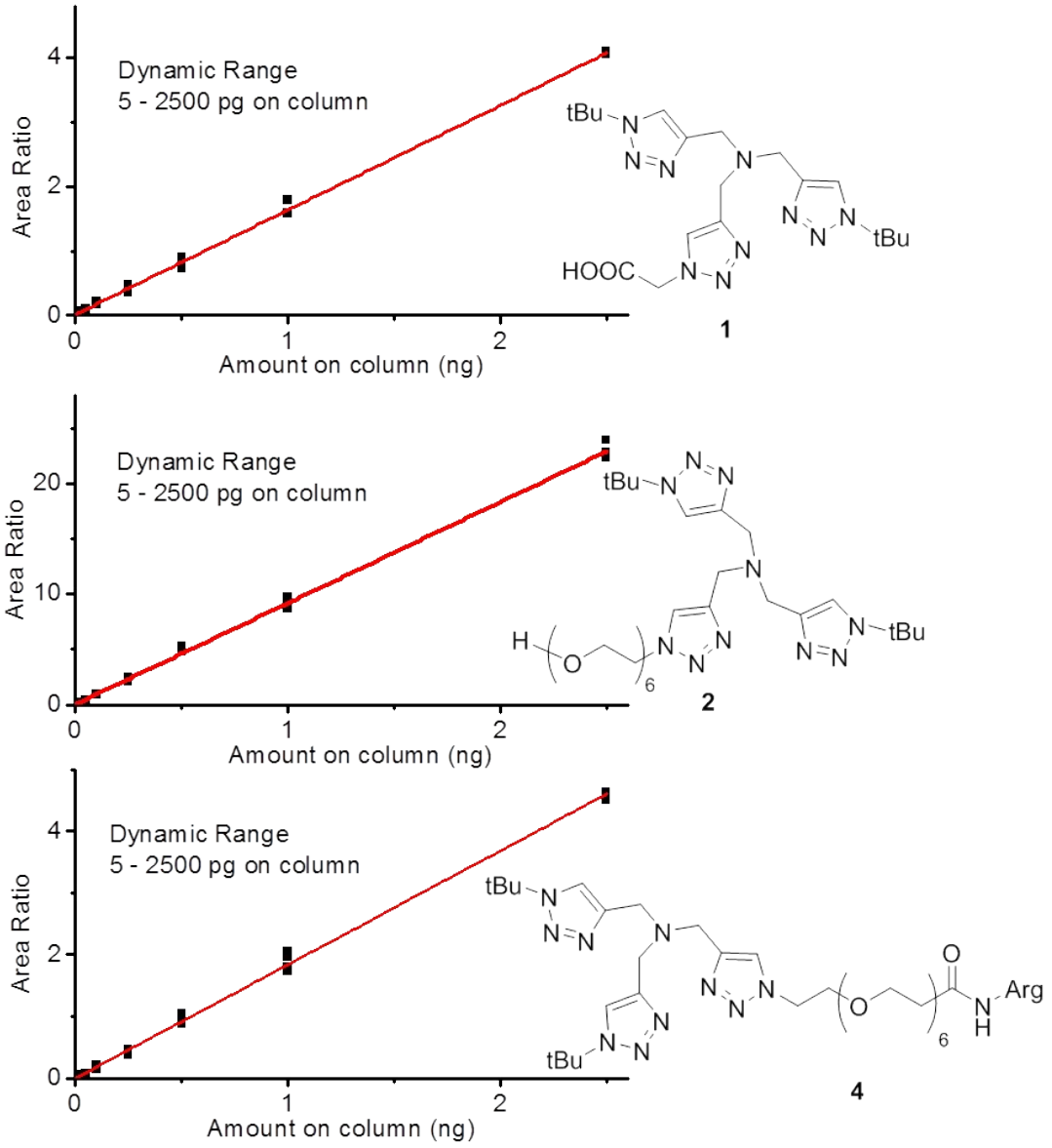


Fig. S13. Calibration curve obtained with internal standard for quantification of **1**, **2** and **4**.

After the HUVEC and OVCAR5 cells in a 12-well plate were subjected to the above CuAAC reaction and washing with PBS for four times, 300 μL trypsin-EDTA solution was added to each well to detach the adhered cells. Hemacytometer (Bright-Line 3100, Hausser Scientific) was used for the estimation of cell number in trypsin solution. The cell suspension was collected in an Eppendorf tube and lysed by repeated freezing/thawing and centrifuge to disrupt the cell membrane. The solution was then incubated at 37 ºC for 1 day to hydrolyze the Tat peptide. The solution was centrifuged (16,000 × g, 20 min), with the supernatant

purified by C18 ziptip and eluted with 40% acetonitrile. Internal standard (final concentration 100 pg/μL) was added to the sample solution and the mixture was subjected to the LC-ESI-MS/MS analysis as described above. The above measured total amount of ligand inside the cells divided by the measured number of cells and the cell volume assuming to be 1.7 picoliter[7] then gives the intracellular ligand concentrations presented in Table 1.

10. Measurement of the biotin-azide 9 concentration by LC-MS/MS

Liquid chromatography was run in a linear gradient from 9 – 60% B, 200 μL/min flow rate in 7.5 minutes using Kinetex XB C18 column (Phenomenex, 50 × 2.1 mm, 5 μm). Mobile phase A: water containing 0.1% formic acid. Mobile phase B: acetonitrile containing 0.1% formic acid. Mass spectrometer was set at positive electrospray ionization with the following conditions: 250 ºC capillary temperature, 45 units Sheath gas, 10 units aux gas, 4.5 kV spray voltage.

For the preparation of standard calibration curve, a stock solution containing 1 ng/μL of compound **9** was diluted with cell lysate to 8 concentration levels of 0.5, 1, 2, 5, 10, 20, 50 and 100 pg/μL respectively. All samples were spiked with internal standard (**Fig. S14**) to obtain the final concentration of 20 pg/μL. The standard samples were analyzed with five replicate injections at each concentration level and a blank (water) injection between each level. The chromatogram was generated by summing the most intense product ions observed in the full-scan MS/MS spectra (**Fig. S14**) acquired for compound **9** (m/z 270.1) and ISTD (m/z 182.1). The extracted ion chromatograms of all analytes are showed in **Fig. S15**. The calibration curve for compound **10** over the entire range of the assay is showed in **Fig. S16**. For compound **9**, the correlation coefficient was 0.999 over the dynamic range from 2.5 pg to 500 pg on-column.

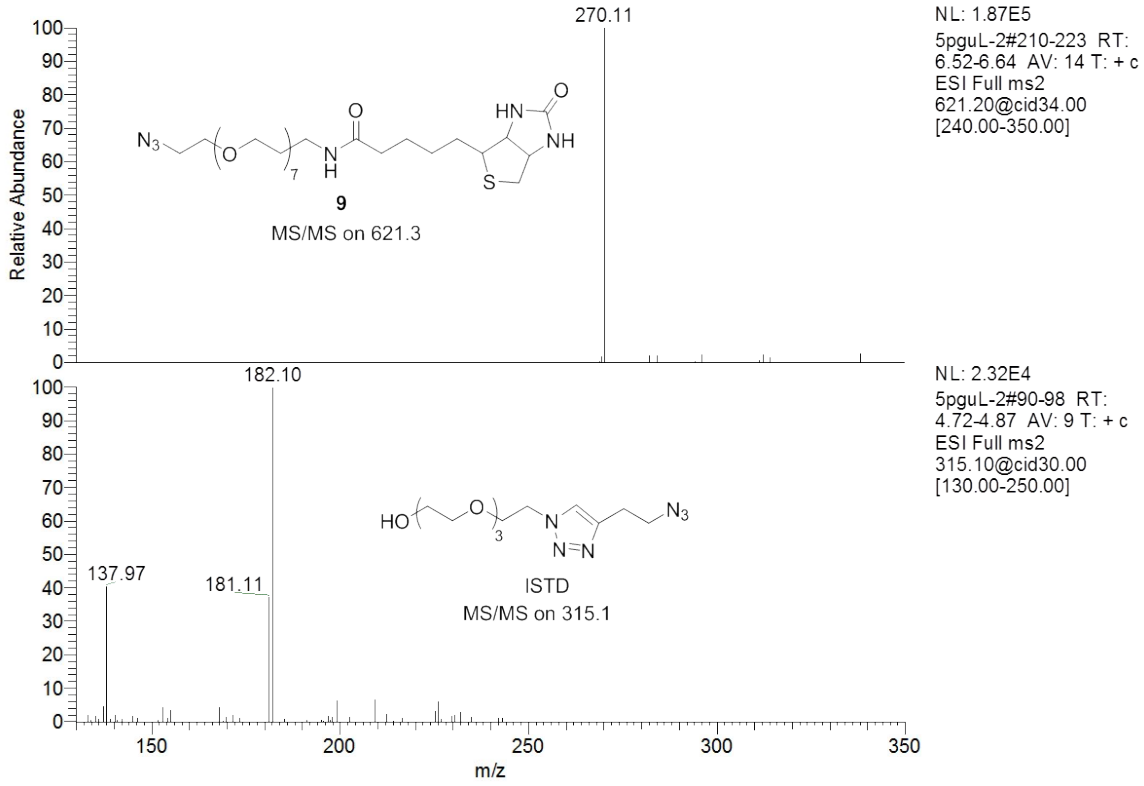


Fig. S14. MS/MS spectra of compound **9** and ISTD.

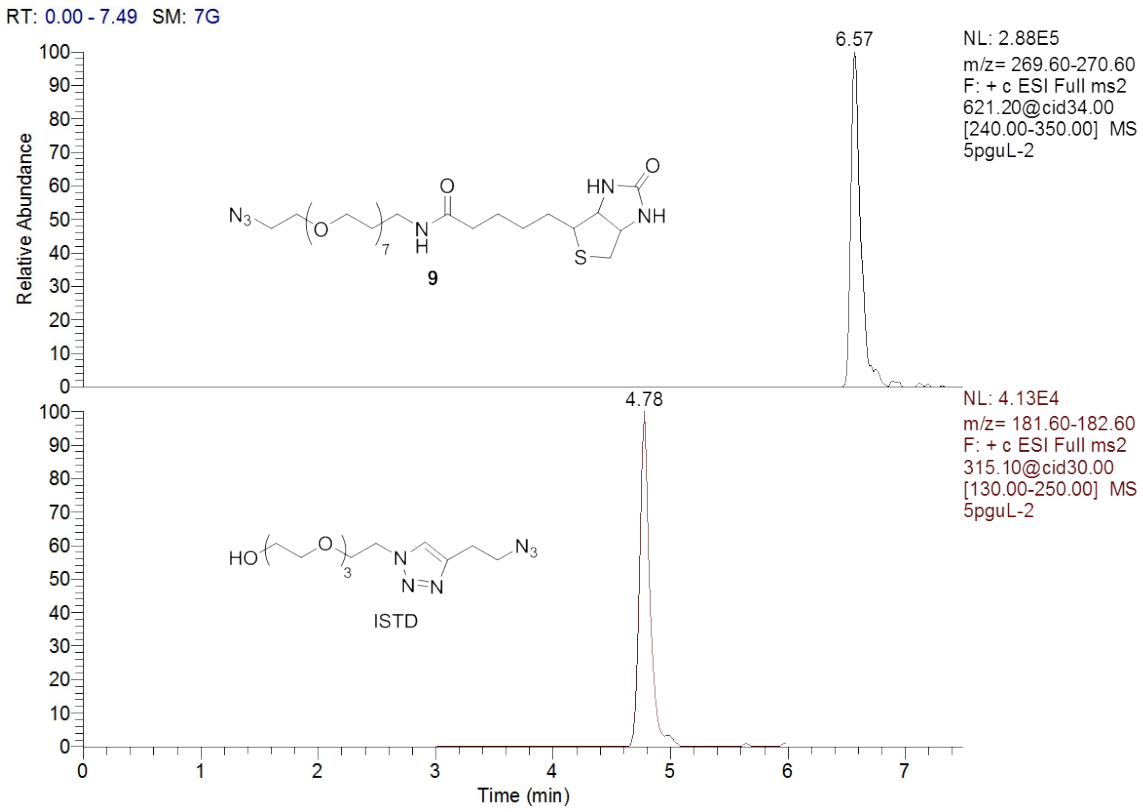


Fig. S15. Extracted ion-chromatograms of compound **9** and ISTD.

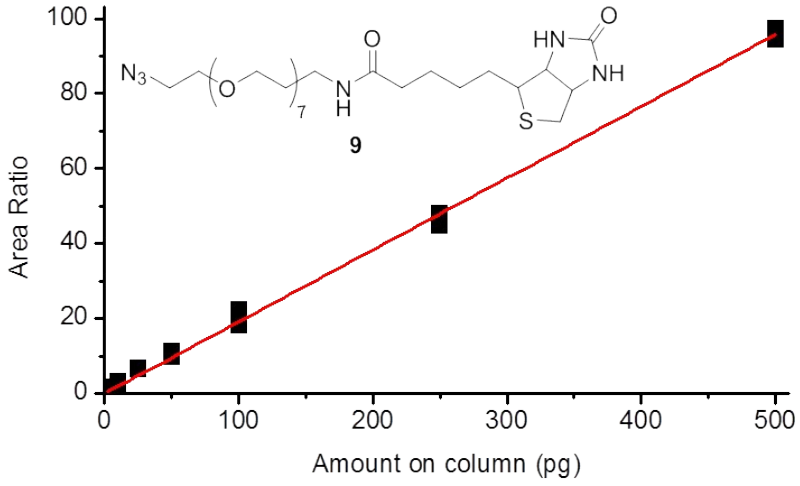


Fig. S16. Calibration curve obtained with internal standard for quantification of **9**.

OVCAR5 cells in a 6-well plate were incubated with 2 mL HBS supplemented with 100 µM biotin-azide **9** for 10 min. The solution was discarded. The cells were washed with 3 mL PBS five times and transferred to

an Eppendorf tube. Trichloroacetic acid (60% w/v) was added to the solution to lyse the cells and precipitate the proteins. After centrifugation at 10,000 × g for 10 minutes, the supernatant was collected and mixed with the internal standard (final concentration 20 pg/μL). The mixture was subjected to the LC-ESI-MS/MS analysis as described above.

11. Determination of the contamination of the cytosolic proteins by the membrane proteins

We used a Mem-PER™ plus membrane protein extraction kit to separate the membrane proteins and native cytosolic proteins of the cells. According to the manufacturer, this kit generates typically less than 10% cross-contamination between the cytosolic protein and membrane protein fractions. The low cross-contamination was also indicated by the absence of a specific membrane protein marker (Na-K ATPase) in the cytosolic protein fraction.[8] To measure the cross-contamination of the cytosolic proteins by the membrane proteins during the process, we fluorescently labeled the isolated membrane proteins, mixed it with the cytosolic proteins, re-isolated them and detected any fluorescence from the membrane proteins in the cytosolic protein fraction as described below.

About 2 × 10^7 native OVCAR5 cells and HPG-incorporated OVCAR5 cells were collected in Eppendorf tubes, respectively. The cells were lysed with Mem-PER™ plus membrane protein extraction kit (Life Technologies) following the manufacturer-recommended procedure to obtain the membrane protein fraction and cytosolic protein fraction. Briefly, 200 μL of membrane permeabilization buffer was added to the cell pellet. The suspension was centrifuged (15,000 × g, 15 minutes), and the supernatant (200 μL) containing the cytosolic proteins was collected. The pellet was then treated with 200 μL of the cell solubilization buffer. The suspension was centrifuged (15,000 × g, 15 minutes), and the supernatant containing the membrane proteins was collected. The HPG-incorporated membrane proteins were precipitated using the chloroform/methanol precipitation method. Briefly, 800 μL methanol and 200 μL chloroform were added to the protein solution, followed by the addition of 600 μL water. The suspension was centrifuged (15,000 × g, 2 minutes) for phase separation. With the protein staying at the interface, the top aqueous layer was discarded. Methanol (800 μL) was added to precipitate the proteins from organic phase, followed by centrifuged (15,000 × g, 2 minutes). The membrane protein pellet was dried in air and reconstituted with 0.2% SDS/PBS (200 μL) containing 50 μM **5**, 100 μM $CuSO_4$, 200 μM ligand **1** and 500 μM sodium ascorbate. The reconstituted

membrane protein solution was incubated for 5 hours at room temperature in nitrogen atmosphere. The **5**-tagged membrane proteins were subsequently isolated using the above chloroform/methanol precipitation method, and reconstituted in cell solubilization buffer. The protein concentrations of all protein fractions were measured by Micro BCA™ protein assay (Life Technologies). The obtained protein solutions were typically between 2.0 – 3.5 μg/μL in a total volumes of 200 μL. The fluorescence of all protein fractions was measured by a Bioassay reader (Synergy HT, Biotek) with excitation at 360 nm and emission at 460 nm (**Figure S17**). A standard calibration curve for the mean fluorescence intensity (MFI) and the concentration of **5**-tagged membrane proteins are shown in **Figure S18**.

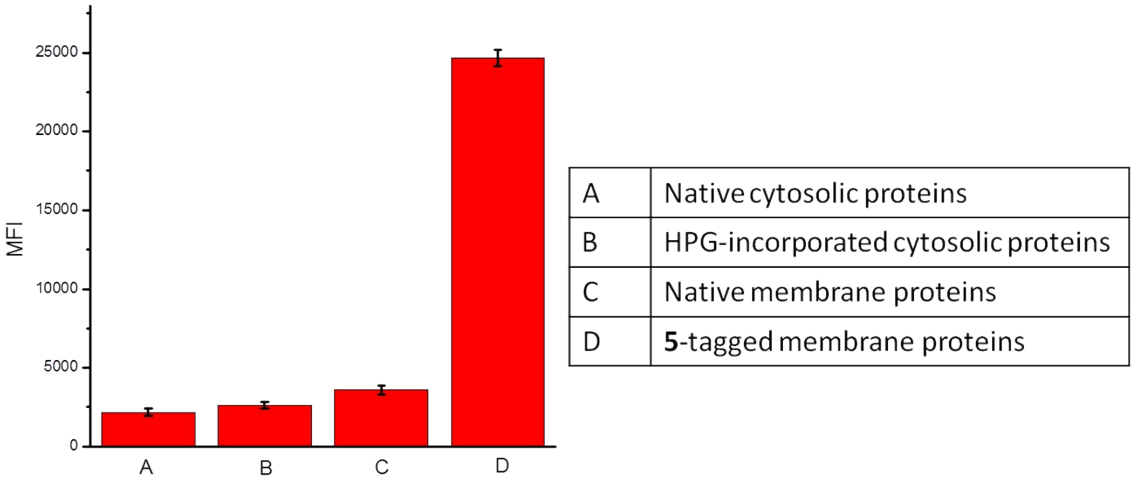


Fig. S17 Mean fluorescence intensity (MFI) of different protein fractions. All proteins were diluted to 1.4 μg/μL. The error bars represent the standard deviation of data from three samples.

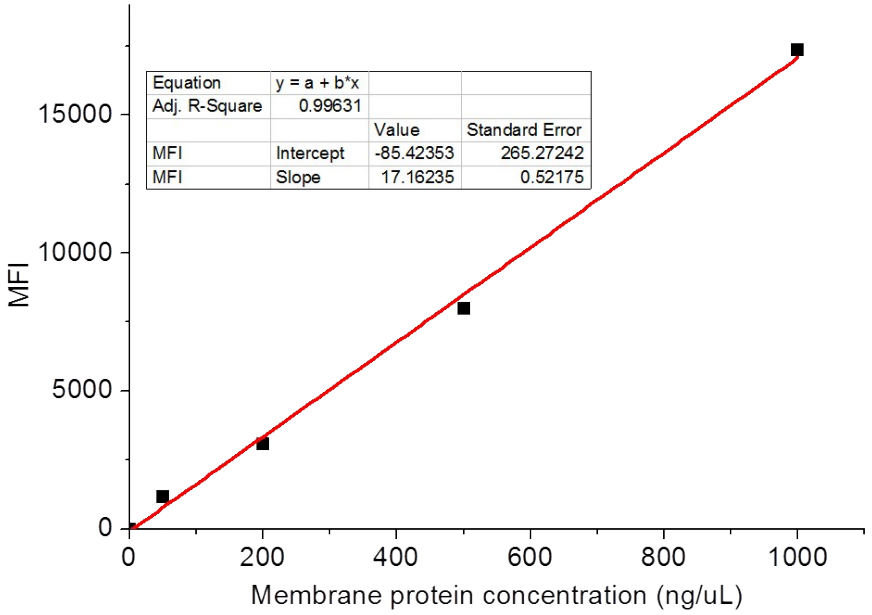


Fig. S18 Standard calibration curve for the mean fluorescence intensity (MFI) and the concentration of **5**-tagged membrane proteins. The calibration standards were prepared by mixing **5**-tagged membrane proteins (0, 50, 200, 500, 1000 ng/μL) with native cytosolic proteins (1000, 950, 800, 500, 0 ng/μL), respectively. The fluorescent intensity of the native cytosolic proteins was subtracted from all obtained data.

To examine the cross-contamination of membrane proteins into the cytosolic protein fraction, 100 μg of native cytosolic proteins were mixed with 100 μg of **5**-tagged membrane proteins, followed by chloroform/methanol protein precipitation to remove the detergent-containing buffer. The protein precipitate was reconstituted in 100 μL membrane permeabilization buffer followed by centrifugation (15,000 × g, 15 minutes) to pellet the insoluble proteins. The supernatant was transferred to a 96-well plate for fluorescence measurement. The obtained fluorescence intensity was correlated to the standard calibration curve to determine the amount of **5**-tagged membrane proteins in the solution. The result is presented in **Table S3**, showing the presence of less than 2% membrane proteins in the cytosolic proteins.

Table S3. Determination of the amount of fluorescent membrane proteins in the cytosolic proteins.

	Total protein (μg)	MFI (a.u.)[a]	**5**-tagged membrane protein (μg)
Cytosolic protein fraction	90	255	1.9 ± 0.6
		241	
		234	
Membrane protein fraction	92	16899	98.9 ± 1.4
		16917	
		16870	

[a] After subtraction of the fluorescence intensity of the native cytosolic proteins shown in Fig. S17. Experiment was run in triplicate to determine the average amount of **5**-tagged membrane protein.

12. Measurement of the amount of product 10 per μg proteins to determine the reaction yields

The CuAAC reaction yields for the membranes and cytosolic proteins of the live cells were determined by measuring the amount (pg) of the reaction product **10** per μg of the membrane and cytosolic proteins using LC-MS/MS, and normalized it to the amount (pg) of **10** per μg of total proteins on which the accessible HPG groups were fully converted to **10** by the *ex vivo* CuAAC reaction in cell lysate under optimum conditions (see below).

CuAAC reaction in suspended cells. OVCAR5 cells on 150 mm petri dish were cultured to ~80% confluence, and the medium was changed to HBS. After 30 min of incubation to exhaust methionine, the medium was then changed again to methionine-free DMEM supplemented with 4 mM HPG (**6**). After incubation for 4 h, the cells were washed with PBS (5 mL, three times). The HPG-incorporated OVCAR5 cells (~10^8 cells) were gently detached in ice-cold PBS using a cell scraper (Nunc™, ThermoFisher), followed by centrifugation at 1000 rpm in an Eppendorf tube. The pelleted cells were resuspended after addition of a freshly prepared solution (1 mL) of CuAAC reagents (100 μM **9**, 100 μM $CuSO_4$, 200 μM ligand, 500 μM sodium ascorbate in 10% methionine-free DMEM/PBS). The reaction mixture was incubated on an orbital shaker for 10 min at room temperature. The solution was removed by centrifugation at 1000 rpm for 5 minutes followed by discarding the supernatant. The cell pellet was resuspended in PBS followed by centrifugation (1000 rpm, 5 minutes) and removal of the supernatant. The washing step was repeated four times.

CuAAC reaction in adherent cells. OVCAR5 cells on a 150 mm petri dish were cultured to ~80% confluence, and the medium was changed to HBS. After 30 min of incubation to exhaust methionine, the medium was

then changed again to methionine-free DMEM supplemented with 4 mM HPG (**6**). For the Control 1 (Table 2), the DMEM medium was supplemented with 4 mM HPG and 0.2 mM methionine. After incubation for 4 h, the cells were washed with PBS (5 mL, three times), and treated with a solution of CuAAC reagents (100 μM **9**, 100 μM $CuSO_4$, 200 μM ligand) in 10% methionine-free DMEM/PBS; the sodium ascorbate stock solution was prepared freshly and added at the final concentration of 500 μM to initiate the CuAAC reaction. After incubation on an orbital shaker for 10 min at room temperature, the solution was carefully removed by suction with vacuum, and the cells were immediately washed with PBS (5 mL, four times).

CuAAC reaction in adhered cells after GSH depletion by BSO. OVCAR5 cells on 150 mm petri dish were cultured to ~80% confluence. The medium was changed to RPMI-1640 medium containing 20 μM of BSO (racemic mixture). After incubation for 24 hours, the solution was discarded, and the cells were incubated in HBS for 30 min to exhaust methionine. The medium was changed to methionine-free DMEM supplemented with 4 mM HPG (**6**). After incubation for 4 h, the cells were washed with PBS (5 mL, 3 times), and treated with a solution of CuAAC reagents (100 μM **9**, 100 μM $CuSO_4$, 200 μM ligand) in 10% methionine-free DMEM/PBS; the sodium ascorbate stock solution was prepared freshly and added at the final concentration of 500 μM to initiate the CuAAC reaction. After incubation on an orbital shaker for 10 min at room temperature, the solution was carefully removed by suction with vacuum, and the cells were immediately washed with PBS (5 mL, four times).

CuAAC reaction in adhered cells after biothiols depletion by NEM. OVCAR5 cells on 150 mm petri dish were cultured to ~80% confluence, and the medium was changed to HBS. After 30 min of incubation to exhaust methionine, the medium was then changed again to methionine-free DMEM supplemented with 4 mM HPG (**6**). After incubation for 4 h, the cells were washed with PBS (5 mL, three times), and treated with a solution of 100 μM *N*-ethylmalemide (NEM) in HBS for 10 minutes. The cells were immediately treated with a solution of CuAAC reagents (100 μM **9**, 100 μM $CuSO_4$, 200 μM ligand) in 10% methionine-free DMEM/PBS; the sodium ascorbate stock solution was prepared freshly and added at the final concentration of 500 μM to initiate the CuAAC reaction. After incubation on an orbital shaker for 10 min at room temperature, the solution was carefully removed by suction with vacuum, and the cells were immediately washed with PBS (5 mL, four times).

Separation of membrane and cytosolic proteins: OVCAR5 cells on 150 mm petri dish were cultured to ~80% confluence. The adhered cells were gently detached in ice-cold PBS using a cell scraper (Nunc™, ThermoFisher), followed by centrifugation at 1000 rpm in a centrifuge tube. The cells pellet in the Eppendorf tube was lysed with a Mem-PER™ plus Membrane protein extraction kit. Briefly, 200 µL of membrane permeabilization buffer was added to the cell pellet. The suspension was centrifuged (15,000 × g, 15 minutes), and the supernatant containing the cytosolic proteins was collected. The pellet was then treated with 200 µL of the cell solubilization buffer. The suspension was centrifuged (15,000 × g, 15 minutes), and the supernatant containing the membrane proteins was collected. The protein concentrations of the protein fractions were measured by Micro BCA™ protein assay (Life Technologies). The obtained protein solutions were typically between 2.0 – 3.5 µg/µL in a total volumes of 200 µL.

CuAAC reaction on extracted total cellular proteins under optimum conditions (Control 2, Table 2). OVCAR5 cells on 150 mm petri dish were cultured to ~80% confluence, and the medium was changed to HBS. After 30 min of incubation to exhaust methionine, the medium was then changed again to methionine-free DMEM supplemented with 4 mM HPG (**6**). After incubation for 4 h, the cells were washed with PBS (5 mL, three times). The HPG-incorporated OVCAR5 cells (~10^8 cells) were gently detached in ice-cold PBS using a cell scraper (Nunc™, ThermoFisher), followed by centrifugation at 1000 rpm in an Eppendorf tube. The cell pellet was lysed in 100 µL 1% SDS/PBS (containing EDTA-free Halt™ Protease Inhibitor, ThermoFisher). The suspension was centrifuged (15,000 × g, 15 min) and the supernatant was collected in a new centrifuge tube. Protein concentration was measured by Micro BCA™ assay (ThermoFisher Scientific). A freshly prepared solution of CuAAC reagents was mixed with the protein solution to a final concentration of 100 µM **9**, 100 µM $CuSO_4$, 200 µM ligand, 500 µM sodium ascorbate in 500 µL solution. The reaction was performed in nitrogen atmosphere for 5 hours. The proteins were precipitated by methanol/chloroform precipitation method. Hence, we assumed that all the accessible HPGs on proteins were tagged with **9** under such conditions, and the measured amount of the triazole **10**, 49.18 ± 0.95 pg per µg proteins, was resulted from a 100% conversion (Table 2 Control 2).

Determination of the protein concentrations: The protein concentrations in the above total cellular protein solution, and the membrane and cytosolic fractions were measured by Micro BCA™ assay (ThermoFisher Scientific) using the manufacturer-recommended procedure. Briefly, 1 mL of a working reagent prepared by

mixing Reagent MA, MB and MC (25/24/1, v/v/v) was added to BSA standards and the protein samples in a test tube, respectively. All samples were incubated at 60 ºC for 30 minutes. The solutions were transferred to a clear-bottom 96-well plate and their absorbance at 562 nm were measured using a Bioassay reader (HTS 7000, Perkin Elmer). The obtained protein solutions were typically between 1–4 μg/μL in total volumes of 100–200 μL and the ratio of the amounts of the cytosolic/membrane proteins was approximately 2 to 1.

*Hydrolysis of the proteins to give the triazole product **10** for LC-MS/MS analysis:* After measurement of the concentrations, the solutions of membrane, cytosolic and total proteins were freeze-dried and reconstituted in 400 μL of 6 M HCl for protein hydrolysis. The hydrolysis was performed at 110 ºC for 24 hours in a sealed glass reactor in nitrogen atmosphere, followed by removal of the solvent by vacuum. The sample was dissolved in 300 μL of 0.1% TFA (aq.), and adsorbed to a 100 μL C18 Ziptip, washed with water (2 × 100 μL) and eluted with 45% acetonitrile (100 μL). The eluted sample was spiked with the ISTD at final concentration of 20 pg/μL. The mixture was subjected to LC-MS/MS analysis as described below.

LC-MS/MS: Liquid chromatography was run in a linear gradient from 2% – 60% B, 200 μL/min flow rate in 6.5 minutes using Kinetex XB C18 column (Phenomenex, 50 × 2.1 mm, 5 μm). Mobile phase A: water containing 0.1% formic acid. Mobile phase B: acetonitrile containing 0.1% formic acid. The mass spectrometer was set at positive electrospray ionization with the following conditions: 250 ºC capillary temperature, 45 units Sheath gas, 10 units aux gas, 4.5 kV spray voltage.

For the preparation of standard calibration curve, a stock solution containing 1 ng/μL of compound **10** was diluted with cell lysate to 8 concentration levels of 0.5, 1, 2, 5, 10, 20, 50 and 100 pg/μL, respectively. All samples were spiked with the internal standard (ISTD, **Figure S19**) to obtain the final concentration of 20 pg/μL. The standard samples were analyzed with five replicate injections at each concentration level and a blank (water) injection between each level. The chromatogram was generated by summing the most intense product ions observed in the full-scan MS/MS spectra (**Figure S19**) acquired for compound **10** (m/z 238.6) and the ISTD (m/z 182.1). The extracted ion chromatograms of all analytes are showed in **Figure S20**. The calibration curve for compound **10** over the entire range of the measurement is showed in **Figure S21**. For compound **10**, the correlation coefficient was 0.996 over the dynamic range from 2.5 pg to 500 pg on-column.

Data and calculation of the yields:

Eq S1,

Amount of **10** (pg)/protein (μg) = $\frac{Test\ sample\ volume\ (\mu L) \times Concentration\ of\ 10\ (test\ sample,\ pg/\mu L) \times dilution\ factor}{Proten\ (\mu g)}$

Sample	Concentration of **10** (Test sample, pg)	Sample Volume (μL)	Protein (μg)	Amount of **10** (pg)/protein (μg)
Control 2	2.49	200	203	48.97
	3.63	200	289	50.22
	2.90	200	240	48.35
Membrane (CuAAC with **1**)	1.09	200	134	3.25
	1.21	200	156	3.09
	0.87	200	126	2.76
Membrane (CuAAC with **3**)	1.23	200	149	8.25
	1.51	200	178	8.46
	1.54	200	158	9.77
(-)-Cytosolic (CuAAC with **1**)	0.54	150	240	0.34
	0.66	150	275	0.36
	0.51	150	249	0.31
(-)-Cytosolic (CuAAC with **3**)	0.72	150	258	0.42
	0.96	150	277	0.52
	0.99	150	302	0.49
Scraping- Cytosolic (CuAAC with **1**)	2.13	150	278	1.15
	2.09	150	296	1.06
	2.29	150	321	1.07
Scraping- Cytosolic (CuAAC with **3**)	4.31	150	303	4.27
	3.29	150	246	4.01
	4.06	150	267	4.56

NEM-Cytosolic (CuAAC with **1**)	1.48	150	254	1.75
	1.68	150	227	2.22
	1.89	150	249	2.28
NEM-Cytosolic (CuAAC with **3**)	6.59	150	298	6.63
	5.96	150	257	6.96
	7.79	150	313	7.47
BSO-Cytosolic (CuAAC with **1**)	0.89	150	286	0.45
	0.87	150	279	0.47
	0.96	150	263	0.55
BSO-Cytosolic (CuAAC with **3**)	1.92	150	279	1.03
	2.14	150	292	1.10
	2.02	150	309	0.98

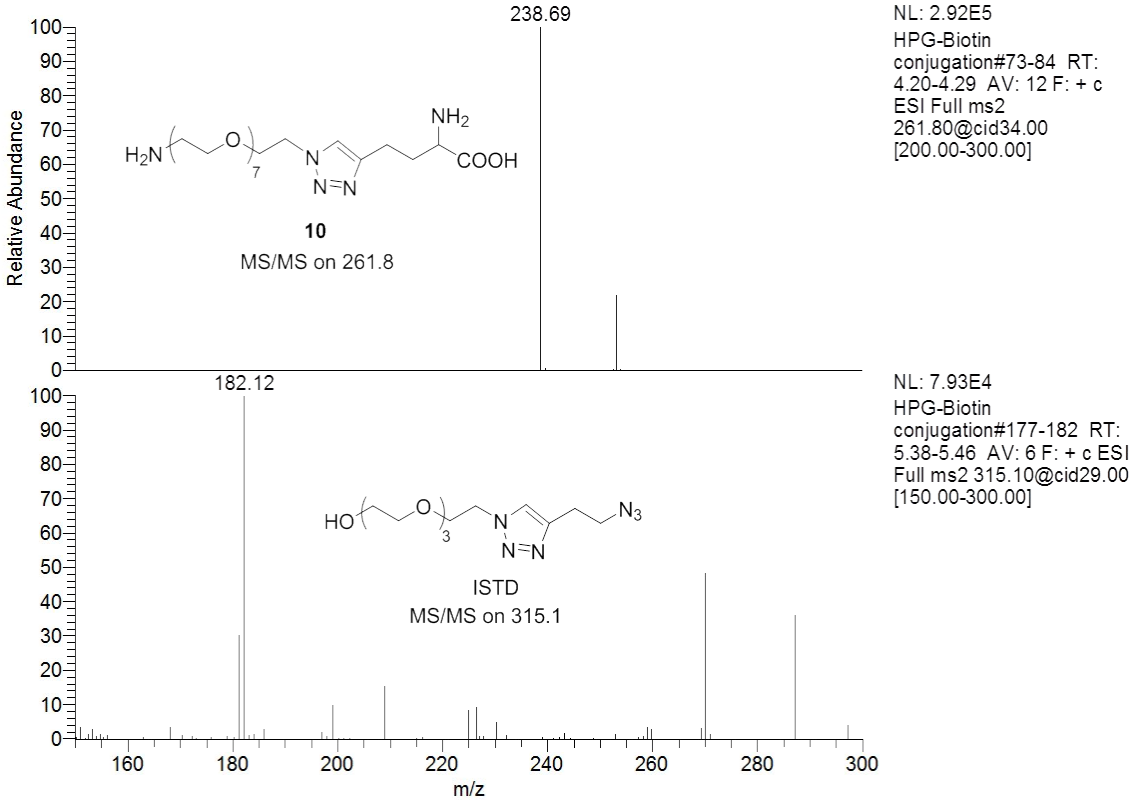


Fig. S19 MS/MS spectra of compound **10** and ISTD.

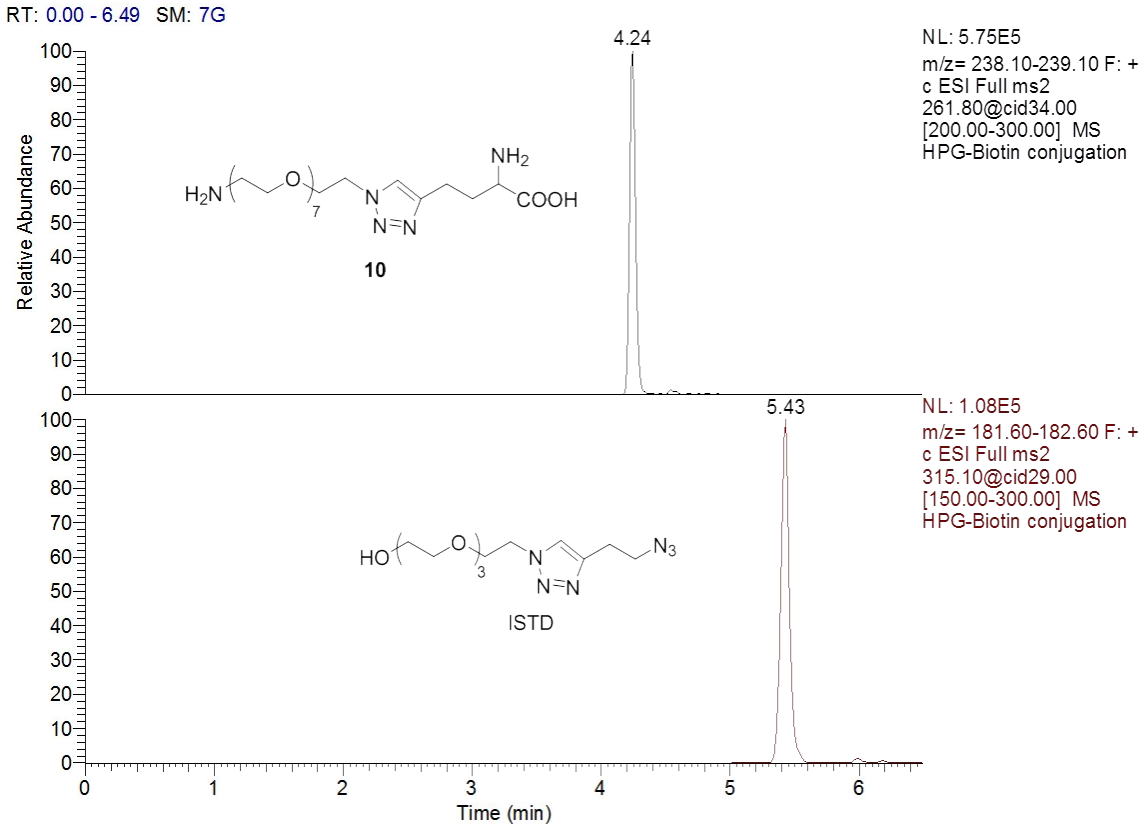


Fig. S20 Extracted ion-chromatograms of compound **10** and ISTD.

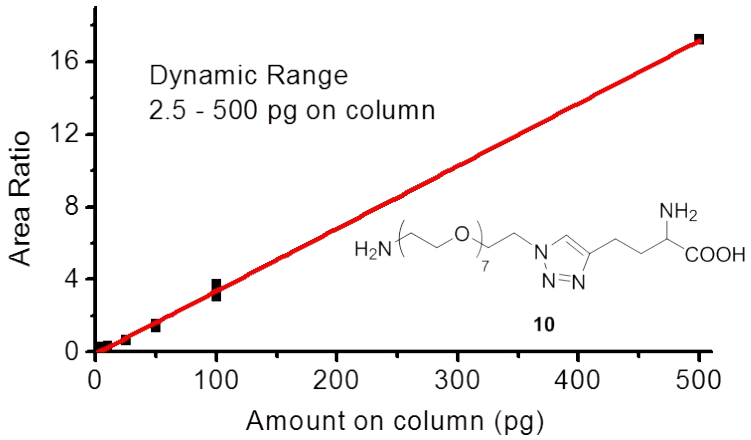
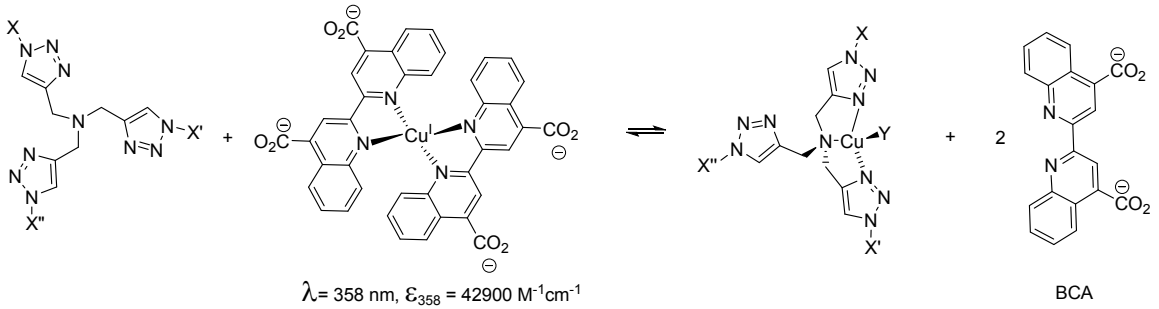


Fig. S21 Calibration curve obtained with internal standard for quantification of **10**.

13. Measurement of Cu^{I}-ligand dissociation constant

A competition assay with bicinchoninic acid (BCA) is used for the determination of Cu^{I}-ligand dissociation constant (K_D). Two molecules of BCA chelate with one Cu^{+} to form complex $[Cu^{I}(BCA)_2]^{3-}$ that has a strong absorbance at 358 nm.



By varying the ligand concentration in the mixture, $[Cu^{I}(BCA)_2]^{3-}$ concentration can be determined by absorbance at 358 nm, which can be further converted to $K_D(Cu^{I}$-ligand) by the following equation:

$$K_D = \frac{[Cu^I][L]}{[Cu^I L]} = \frac{[L]}{[Cu^I L]} \frac{[\{Cu^I(Bca)_2\}^{3-}]}{[Bca]^2} \frac{1}{\beta_2}$$

Where $\beta_2 = 10^{17.2}$ M^{-2} for $[Cu^{I}(BCA)_2]^{3-}$.

Sample preparation was conducted in an anaerobic chamber. Briefly, different amounts of ligand were added to a MES buffer solution containing $[Cu^{I}(bCA)_2]^{3-}$ prepared by mixing $[Cu^{I}(MeCN)_4]PF_6$ (16 μM final concentration) and Na_2Bca (40 μM final concentration). Sodium ascorbate (100 μM final concentration) was added to maintain the Cu^{I} oxidation state. All samples were transferred to UV cuvettes (1 cm path) and sealed with septum cap until UV analysis was finished. After 20 minutes incubation, the transfer of Cu^{I} from Bca to ligand was estimated by the change in absorbance at 358 nm using a Varian Cary 50 Bio UV/Visible Spectrophotometer. Solution prepared under the same condition without $[Cu^{I}(MeCN)_4]PF_6$ was used as blank. Numerical data are listed in **Table S4**.

Table S4. Estimation of Dissociation Constant K_D by competition for Cu^I between BCA and Cu^I-chelating ligand **1**, **2**, **3** and GSH in MES buffer.

Ligand	$[Bca]_{total}$ (μM)	$[Ligand]_{total}$ (μM)	A (358)	$[Cu^I(BCA)_2]^{3-}$ (μM)	$[Cu^I(Ligand)]$ (μM)	K_D (10^{-11} M)	K_D Ave ± SD (10^{-11} M)
	40	0	0.66	16	0		
	40	500	0.56	13.61	2.39	10.97	
1	40	1000	0.52	12.57	3.43	10.41	12.13±1.68
	40	2000	0.49	11.87	4.13	13.68	
	40	3000	0.46	11.09	4.91	13.47	
	40	0	0.46	16.00	0		
	40	500	0.42	14.71	1.29	32.00	
2	40	2000	0.36	12.57	3.43	20.94	25.87±4.94
	40	4000	0.33	11.56	4.45	22.96	
	40	8000	0.31	10.57	5.43	27.58	
	40	0	0.42	16.00	0		
	40	250	0.34	13.18	2.82	3.93	
3	40	500	0.30	11.56	4.44	2.85	3.52±0.52
	40	1000	0.28	10.84	5.16	3.92	
	40	4000	0.19	7.37	8.63	3.37	
	40	0	0.62	16.00	0		
	40	250	0.33	8.36	7.64	3.08	
GSH	40	1000	0.19	5.00	11.00	3.15	3.10±0.04
	40	4000	0.08	2.16	13.84	3.08	

14. Evaluation of the inhibition of CuAAC reaction by GSH

The fluorogenic CuAAC reaction assay in the presence of GSH at a series of concentrations was first performed. Thus, the stock solutions of the reagents dissolved in PBS were mixed into the final concentration of 69 μM **3**, 163 μM $CuSO_4$, 100 μM **5**, 50 μM **6** and various concentration of GSH. Sodium ascorbate (500

μM) was added to initiate the reaction. The fluorescence intensity was measured 10 minutes after the addition of sodium ascorbate and the result is presented in **Fig. S22**.

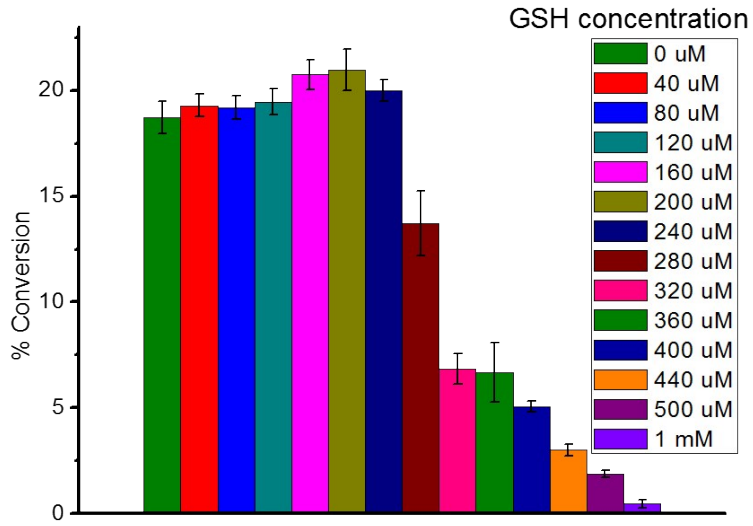


Fig. S22 Conversion of ligand **3**-assisted CuAAC reaction of **5** and **6** in the reaction mixture containing various concentrations of GSH. The error bar is the standard deviation in a triplicate experiment.

LC-MS/MS based CuAAC reaction assay was also performed for the evaluation of GSH inhibitory effect. The stock solutions of the reagents dissolved in PBS were mixed into the final concentration of 69 μM **3**, 163 μM $CuSO_4$, 1.3 μM **S15**, 20 μM **6** and various concentration of GSH. Sodium ascorbate (500 μM) was added to initiate the reaction. After 10 min reaction, 20 mM EDTA was added to the mixture to terminate the reaction. The formation of product **10** was determined by the above LC-MS/MS method. The results are presented in **Table S5**.

Table S5. Yield of ligand **3** assisted CuAAC reaction of **S15** and **6** in the reaction mixture containing various concentrations of GSH. The error bar is the standard deviation of at least three samples.

GSH (μM)	Yield (%)
0	24.78 ± 1.83
100	19.53 ± 1.03
200	16.89 ± 2.10
300	7.17 ± 1.14
500	2.17 ± 0.24

15. Measurement of cellular GSH concentration

GSH is prone to oxidation in extracellular environment. To accurately measure cellular GSH concentration, *N*-ethylmaleimide was used to alkylate the GSH thiol to form the stable conjugate **S17** before cell lysis.[6] The following modified LC-MS/MS based method was used in this study to determine the intracellular GSH concentration. Specifically, a derivatization solution of 50 mM *N*-ethylmalemide and 5 mM EDTA in 400 μL water was used to suspend the cell pellet. The suspension was incubated for 2 hours at room temperature. Trichloroacetic acid (50 μL, 60% w/v) was added to precipitate the proteins. The suspension was centrifuged (16,000 × g, 16 minutes) and the supernatant was collected for the following LC-MS/MS analysis.

Liquid chromatography was run in a linear gradient from 2% – 60% B, 200 μL/min flow rate in 6.7 minutes using a Kinetex XB C18 column (Phenomenex, 50 × 2.1 mm, 5 μm). Mobile phase A: water containing 0.1% formic acid. Mobile phase B: acetonitrile containing 0.1% formic acid. Mass spectrometer was set at positive electrospray ionization with the following conditions: 250 ºC capillary temperature, 45 units Sheath gas, 10 units aux gas, 4.5 kV spray voltage.

For the preparation of standard calibration curve, a stock solution containing 1 ng/μL of compound **S17** was diluted with cell lysate to 8 concentrations: 0.5, 1, 2, 5, 10, 20, 50 and 100 pg/μL. All samples were spiked with internal standard to obtain the final concentration of 20 pg/μL. The standard samples were analyzed with five replicate injections at each concentration level and a blank (water) injection between each concentration. The chromatogram was generated by summing the most intense product ions observed in the full-scan MS/MS spectra (**Fig. S23**) acquired for **S17** (m/z 304.0). The extracted ion chromatograms of **S17** as a mixture of 1:1 diasteromers is showed in **Fig. S23**. The calibration curve for compound **S17** over the entire range of the assay is showed in **Fig. S24.** For compound **S17**, the correlation coefficient was 0.996 over the dynamic range from 2.5 pg to 500 pg on-column.

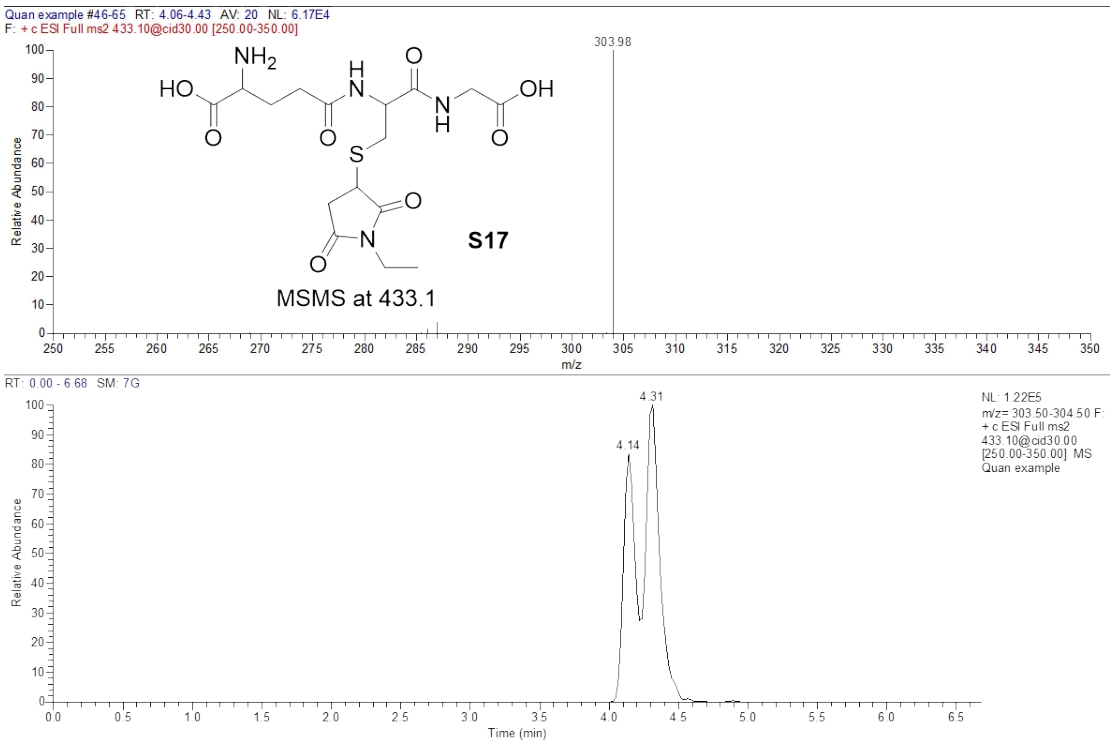


Fig. S23 MS/MS spectrum and extracted ion chromatogram of compound **S17**.

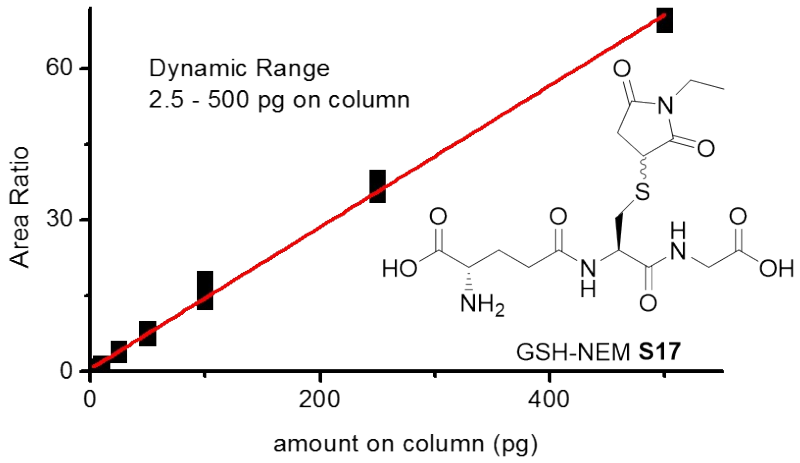


Fig. S24 Standard calibration curve for quantification of compound **S17**.

16. Depletion of cellular GSH

OVCAR5 cells were cultured to 80% confluence on a 100 mm petridish in RPMI-1640 media supplemented with various concentration (0, 20, 40, 100 and 200 μM) of buthionine sulfoximine (BSO) for 12 or 24 hours.

The solution was discarded, and the cells were washed three times with PBS. The adhered cells were detached by incubation with 0.25% trypsin-EDTA solution for 3 minutes at room temperature. Each cell suspension was collected in an Eppendorf tube. Cell number was estimated by a hemocytometer. Cells were pelleted by centrifugation (1000 rpm for 5 min) and the supernatant was discarded. The amount of the intracellular GSH in the cell pellet was measured as described in Section 15, and divided by the cell number and the average cell volume assuming to be 1.7 picoliter[7]. The data is summarized in **Table S6** and **Table 2**.

To determine the depletion of GSH after detachment of the cells using a cell scraper, OVCAR5 cells were cultured to 80% confluence on a 100 mm petri dish in RPMI-1640 media. The cells were gently detached and suspended in ice-cold PBS using a cell scraper (Nunc™, ThermoFisher). Cell number was estimated by a hemacytometer to be ~ 10^7. The cell suspension in PBS was centrifuged (1000 rpm, 5 min). The cell pellet was immediately subjected to the measurement of the intracellular GSH concentration as described in Section 15. The measured amount of GSH was divided by the cell number and the average cell volume assuming to be 1.7 picoliter[7] to give the intracellular GSH concentration. The average of the triplicate measurement is presented in **Table 2**.

Table S6. Cellular GSH concentration. The error bars represent the standard deviation of data from three samples.

Incubation time	BSO concentration (μM)[a]	Cellular GSH concentration (μM)	Remaining % GSH
12 h	0	1585.3 ± 28.6	100
	20	58.8 ± 2.3	3.9
	40	31.7 ± 3.3	2.1
	100	13.9 ± 0.5	0.9
	200	6.6 ± 0.1	0.4
24 h	20	4.9 ± 0.4	0.3

[a] A racemic mixture of BSO was used in this study.

17. MTS assay to evaluate cell death following GSH depletion and CuAAC reaction

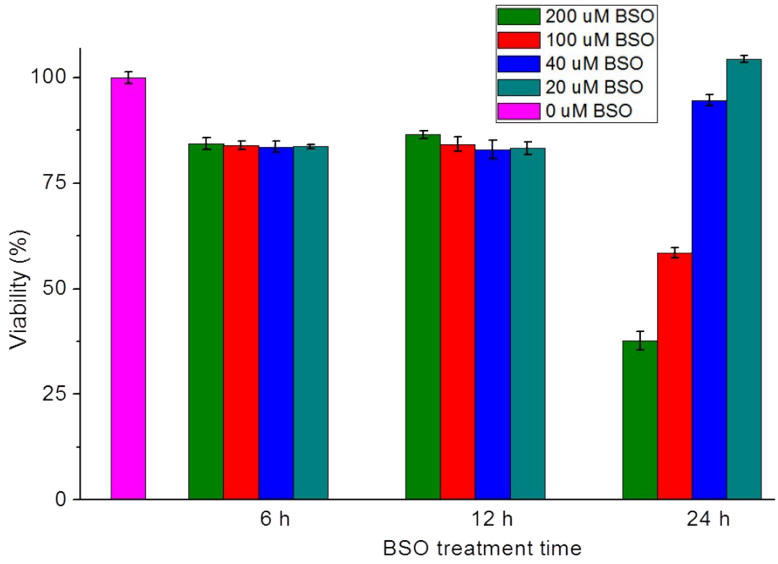


Fig. S25 Cell viability after the treatment with different concentration of BSO (racemix) for various time (6, 12, 24 h) to deplete the cellular GSH as described in Section 16. The MTS assay is described in Section 3. The error bars represent the standard deviation of data from at least three samples.

To evaluate the cell death following the slow GSH depletion by BSO, OVCAR5 cells in 96-well plate were incubated in RPMI-1640 medium containing 20 μM of BSO (racemic mixture) for 24 hours. The solution was then discarded, and the cells were incorporated with HPG, and subjected to CuAAC reaction as described in Section 12. After removal of the reaction solution, the adhered cells were washed with PBS three times and incubated in RPMI-1640 medium supplemented with 5 mM glutathione reduced ethyl ester (GSH-OEt) for 4 hours. The culture medium was then replaced with RPMI-1640 medium until the cell viability was determined by the MTS assay. The result is shown in **Fig. S26a**.

To evaluate the cell death following the rapid GSH depletion by NEM, OVCAR5 cells in 96-well plate were first incorporated with HPG, then treated with 100 μM *N*-ethylmalemide (NEM) in HBS, followed by CuAAC reaction as described in Section 12. After removal of the reaction solution, the cells were washed with PBS three times and incubated in RPMI-1640 medium supplemented with 5 mM GSH-OEt for 2 hours until the cell viability was determined by MTS assay. The result is shown in **Fig. S26b**.

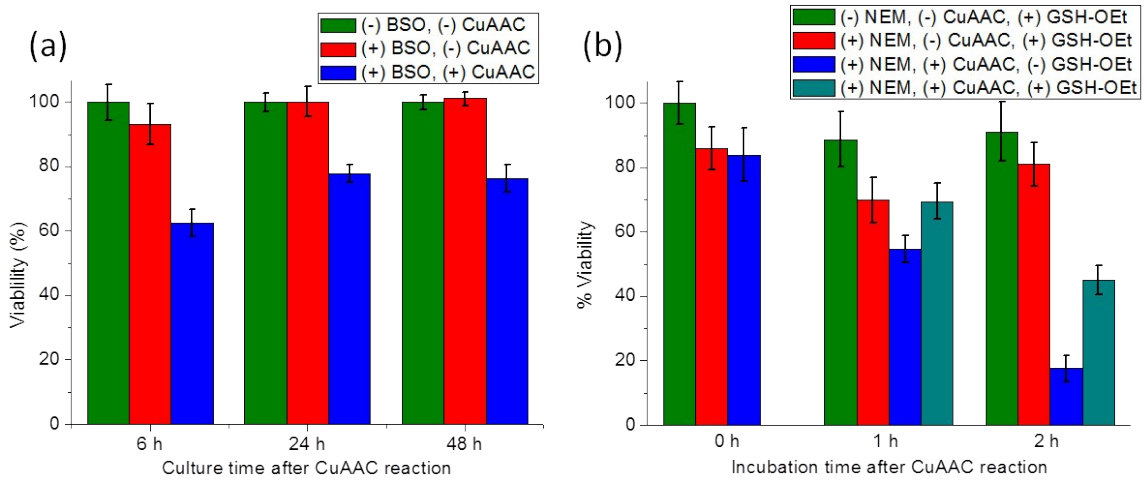


Fig. S26 Cell viability after treatment with BSO (a) or NEM (b) and CuAAC reaction. (a) OVCAR5 cells were pretreated with 20 μM BSO followed by treatment of CuAAC reagents for 10 minutes. (b) OVCAR5 cells were pretreated with 100 μM NEM in HBS followed by treatment of CuAAC reagents for 10 minutes. The error bars represent the standard deviation of data from at least three samples.

18. References

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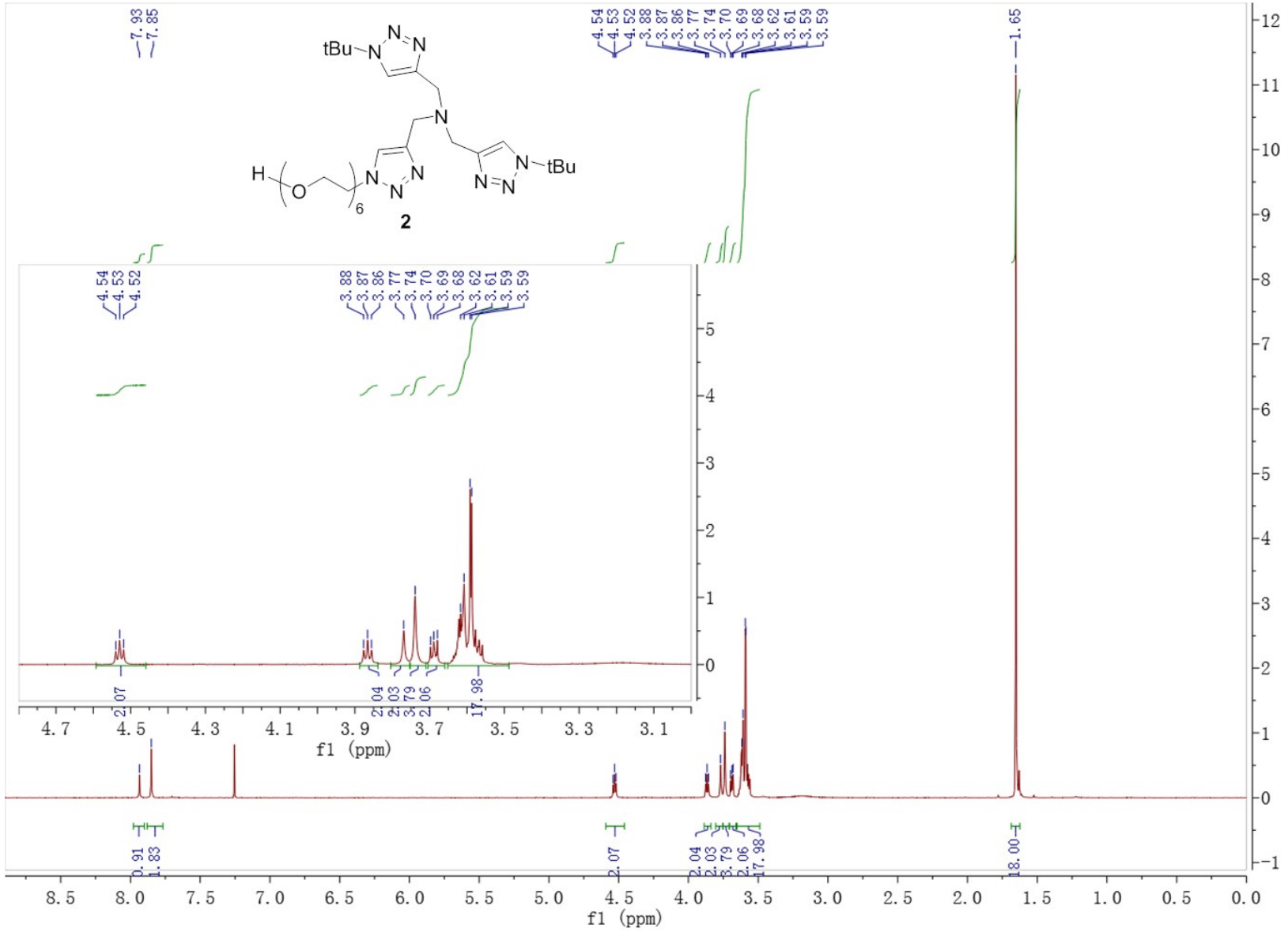
19. NMR and MS spectra, HPLC chromatogram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7.85
4.54
4.53
4.52
3.88
3.87
3.86
3.77
3.74
3.70
3.69
3.68
3.62
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3.59
3.59
1.65
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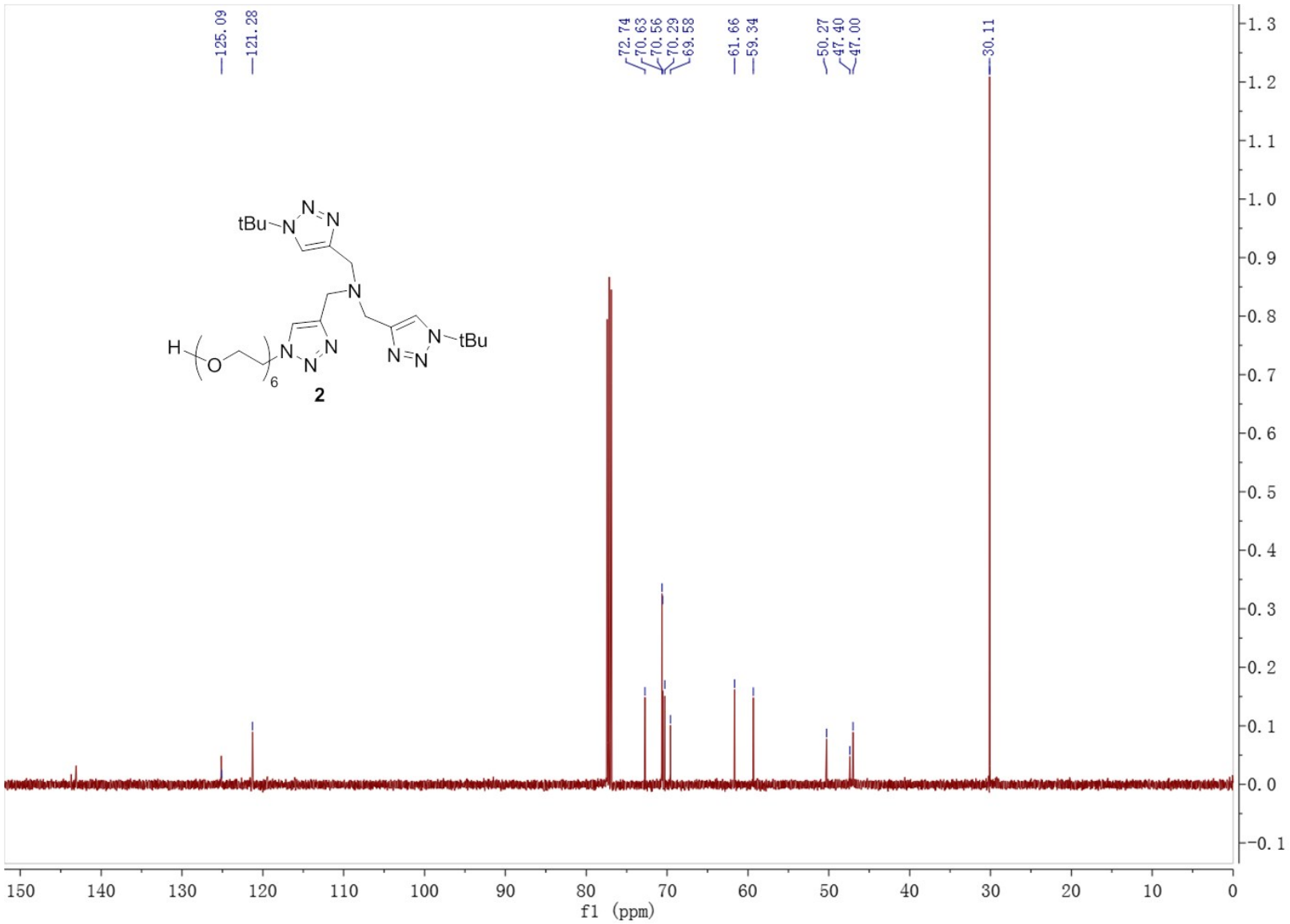
tBu
H
O
6
2
f1 (ppm)
f1 (ppm)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72.74
70.63
70.56
70.29
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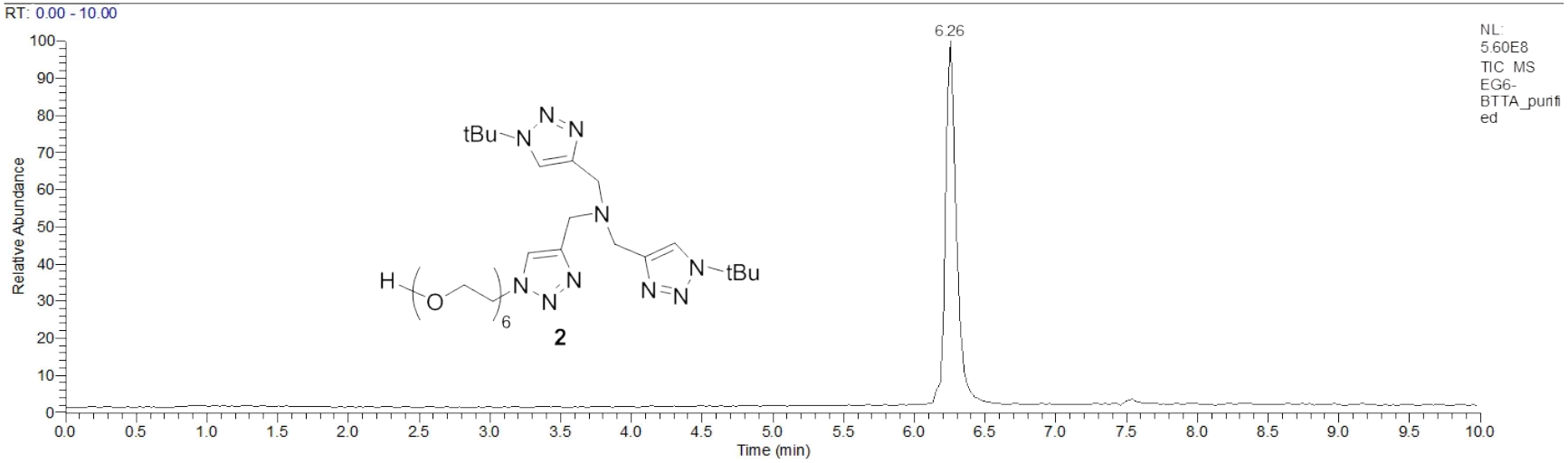
tBu
tBu
H
O
6
2
f1 (ppm)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NL:
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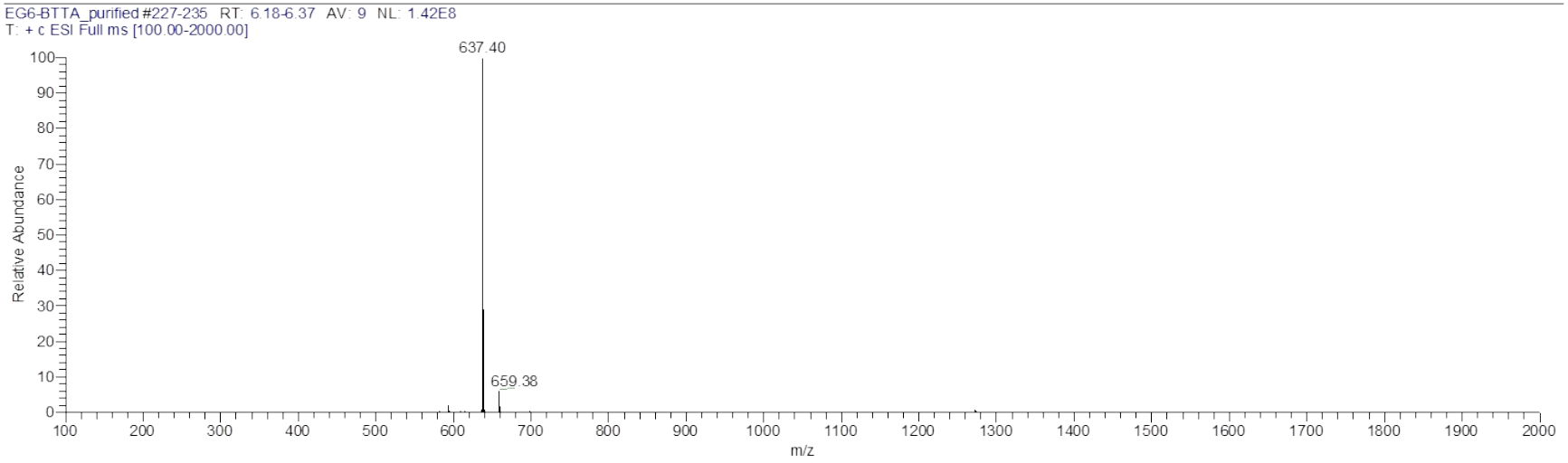
TIC MS
EG6-
BTTA_purifi
ed
Relative Abundance
Time (min)
tBu
tBu
H
O
6
2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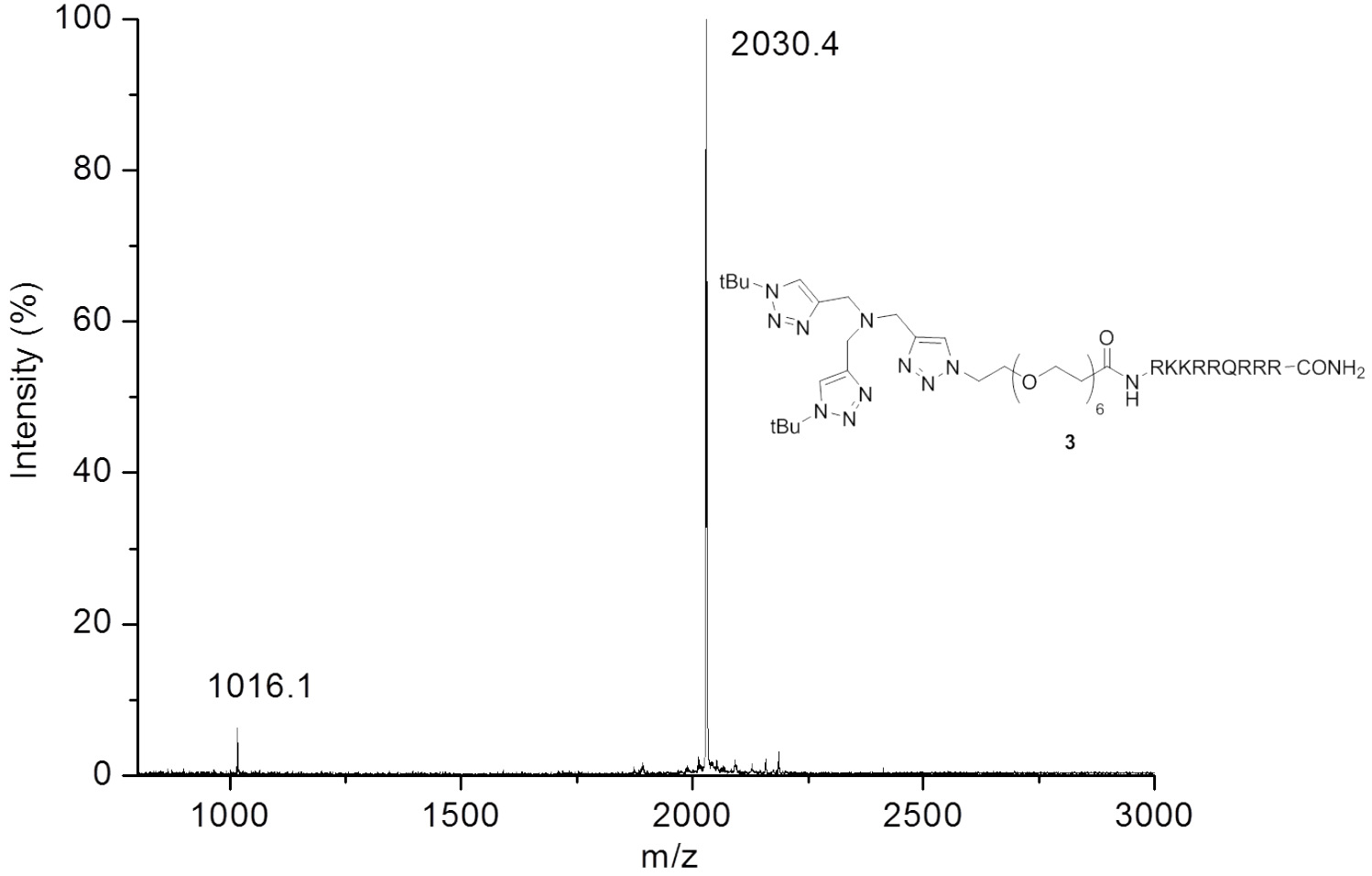
659.38
Relative Abundance
m/z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80
60
40
20
0
Intensity (%)
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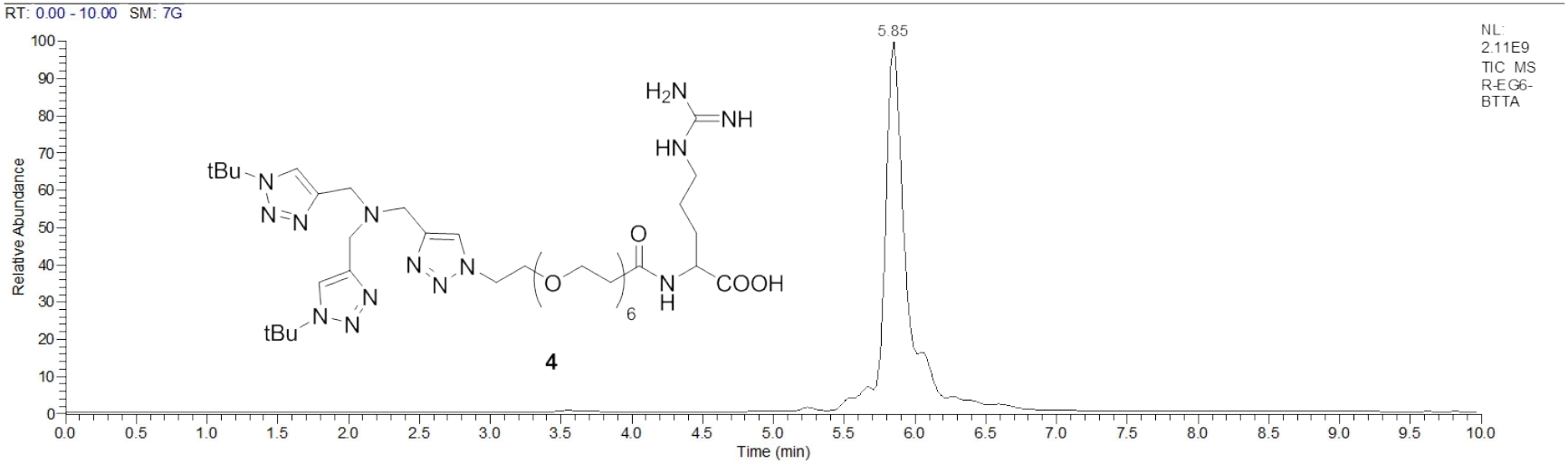
1016.1
1000
1500
2000
2500
3000
m/z
tBu
tBu
RKKRRQRRR–CONH2
3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NL:
2.11E9
TIC MS
R-EG6-
BTTA
Relative Abundance
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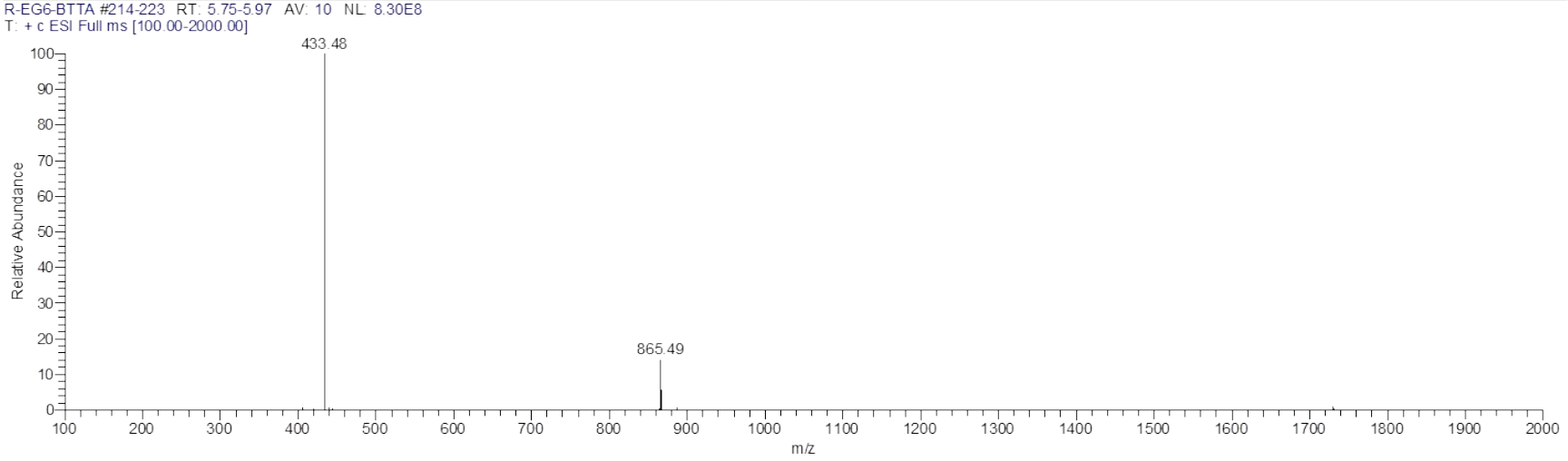
NH
HN
O
N
H
COOH
6
4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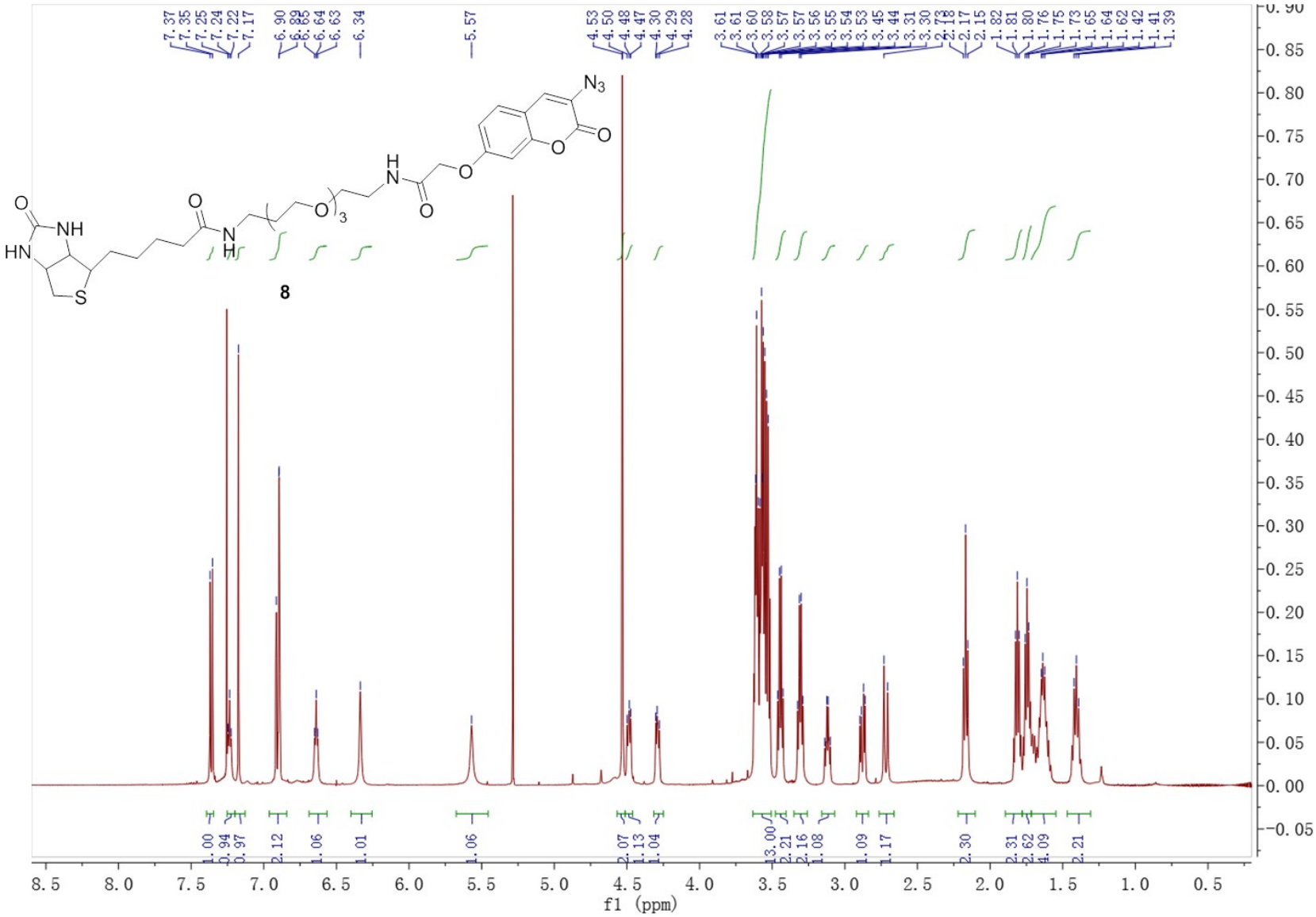
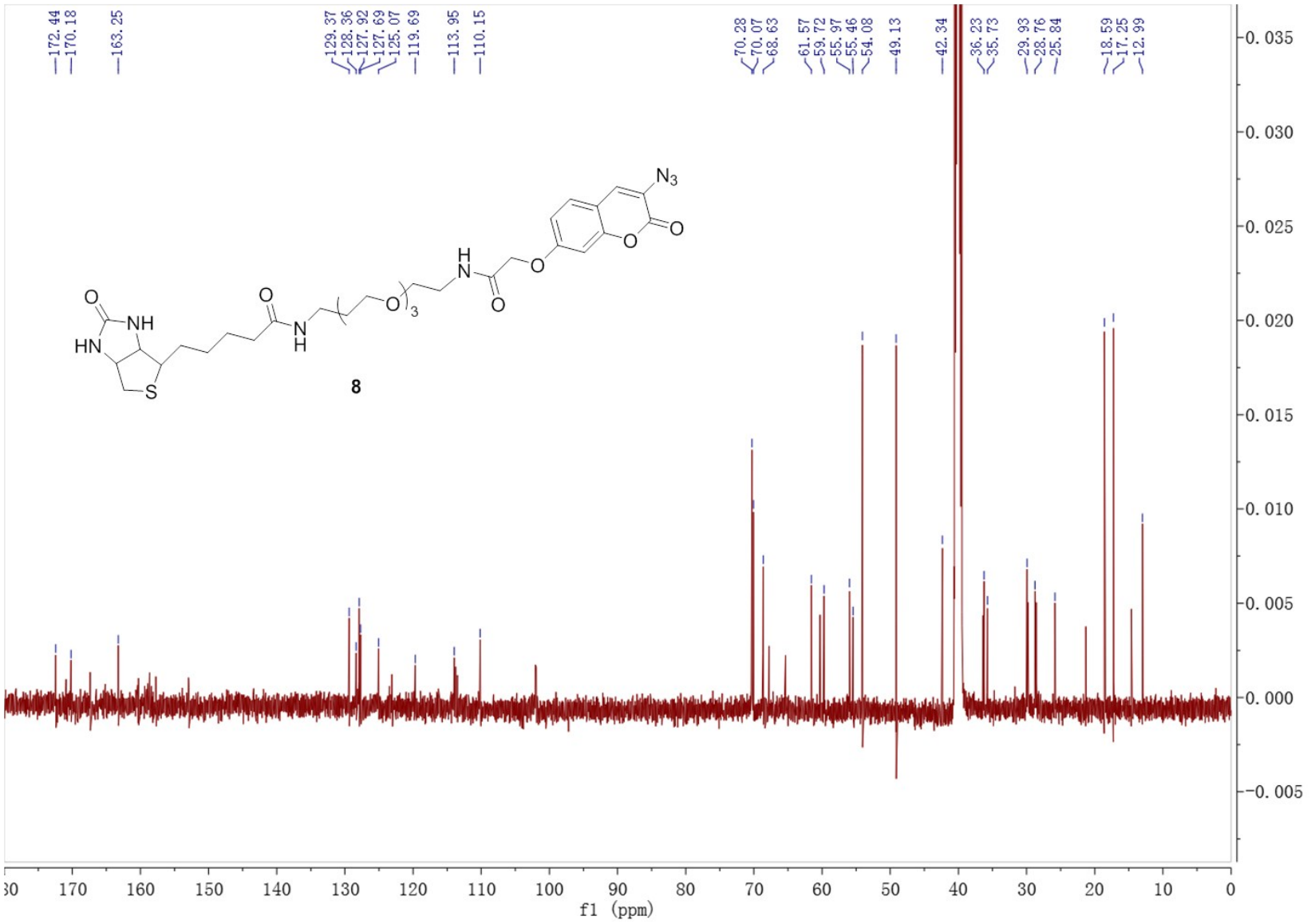
Relative Abundance
m/z


8
f1 (ppm)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110.15
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68.63
61.57
59.72
55.97
55.46
54.08
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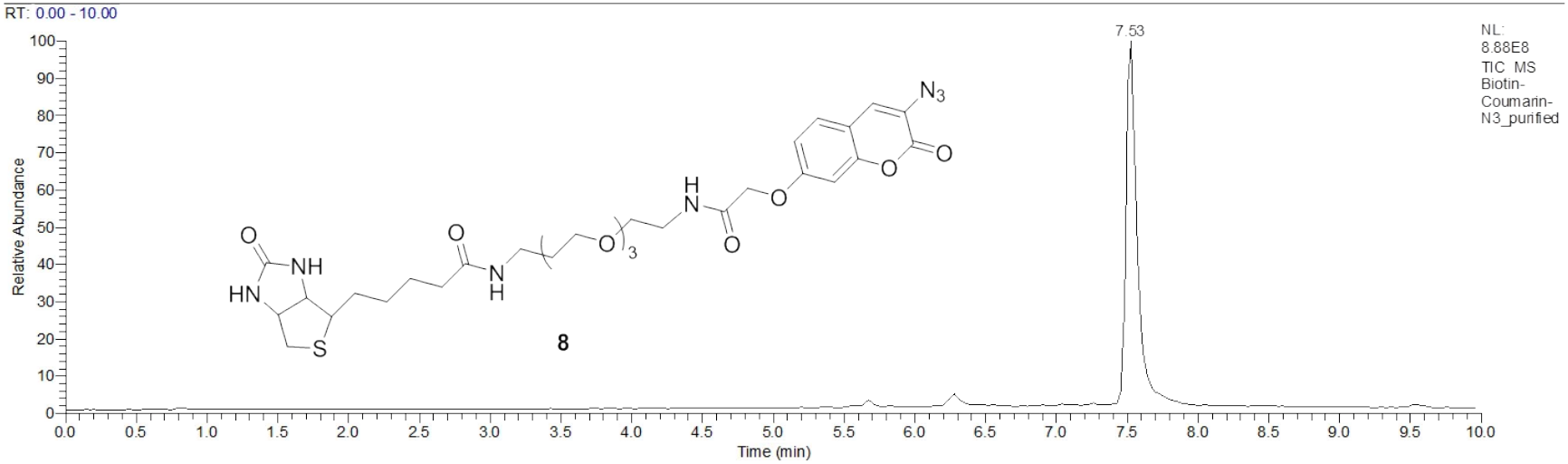
42.34
36.23
35.73
29.93
28.76
25.84
18.59
17.25
12.99
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
-0.005
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)
8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NL:
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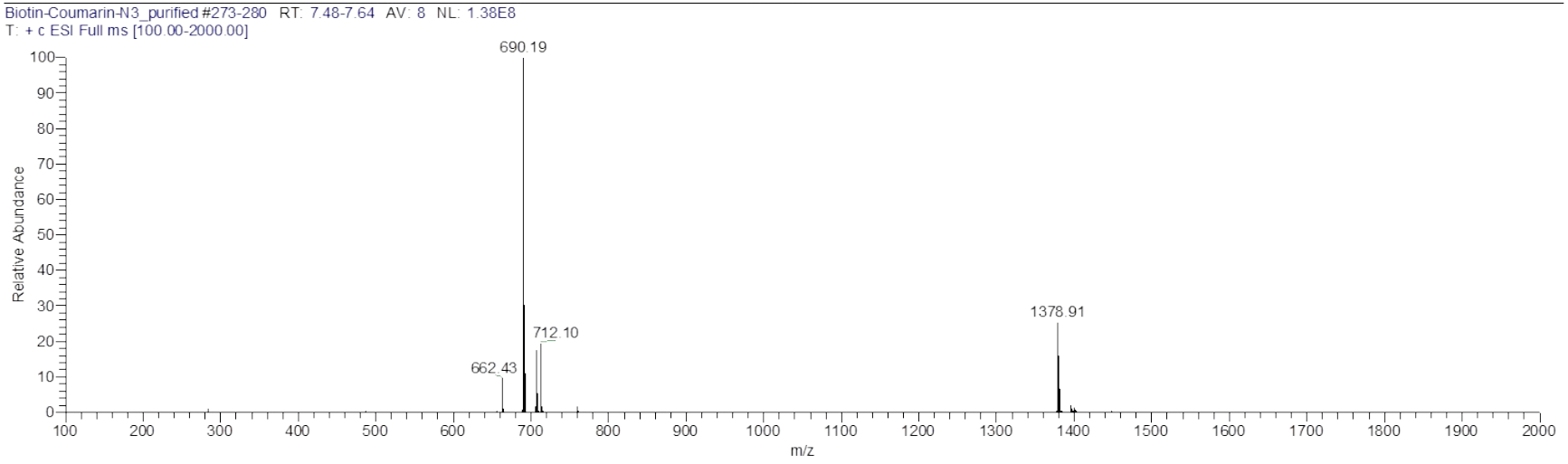
TIC MS
Biotin-
Coumarin-
N3_purified
Relative Abundance
Time (min)
8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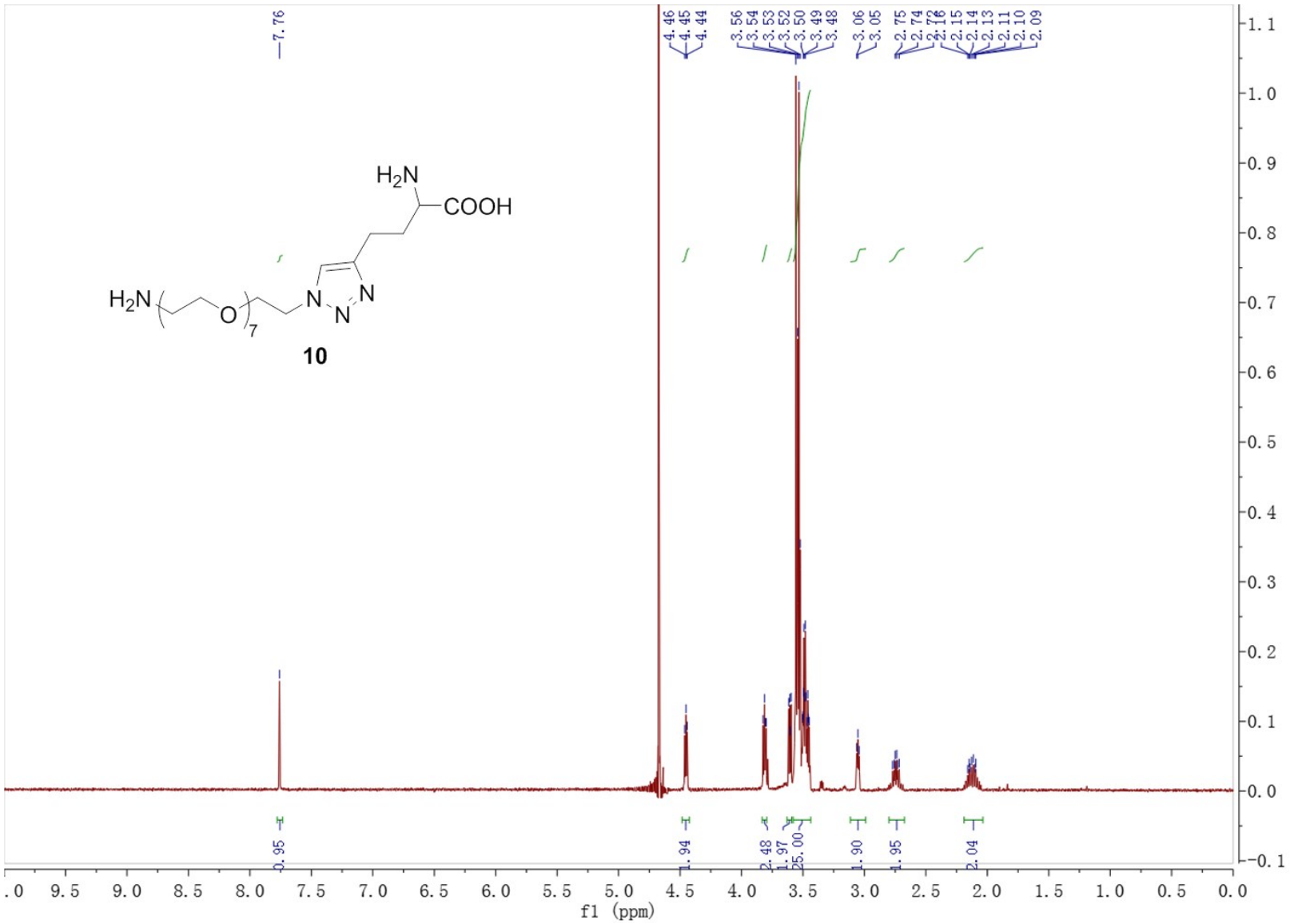
Relative Abundance
m/z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2.15
2.14
2.13
2.11
2.10
2.09
H2N
COOH
H2N
O
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N
N
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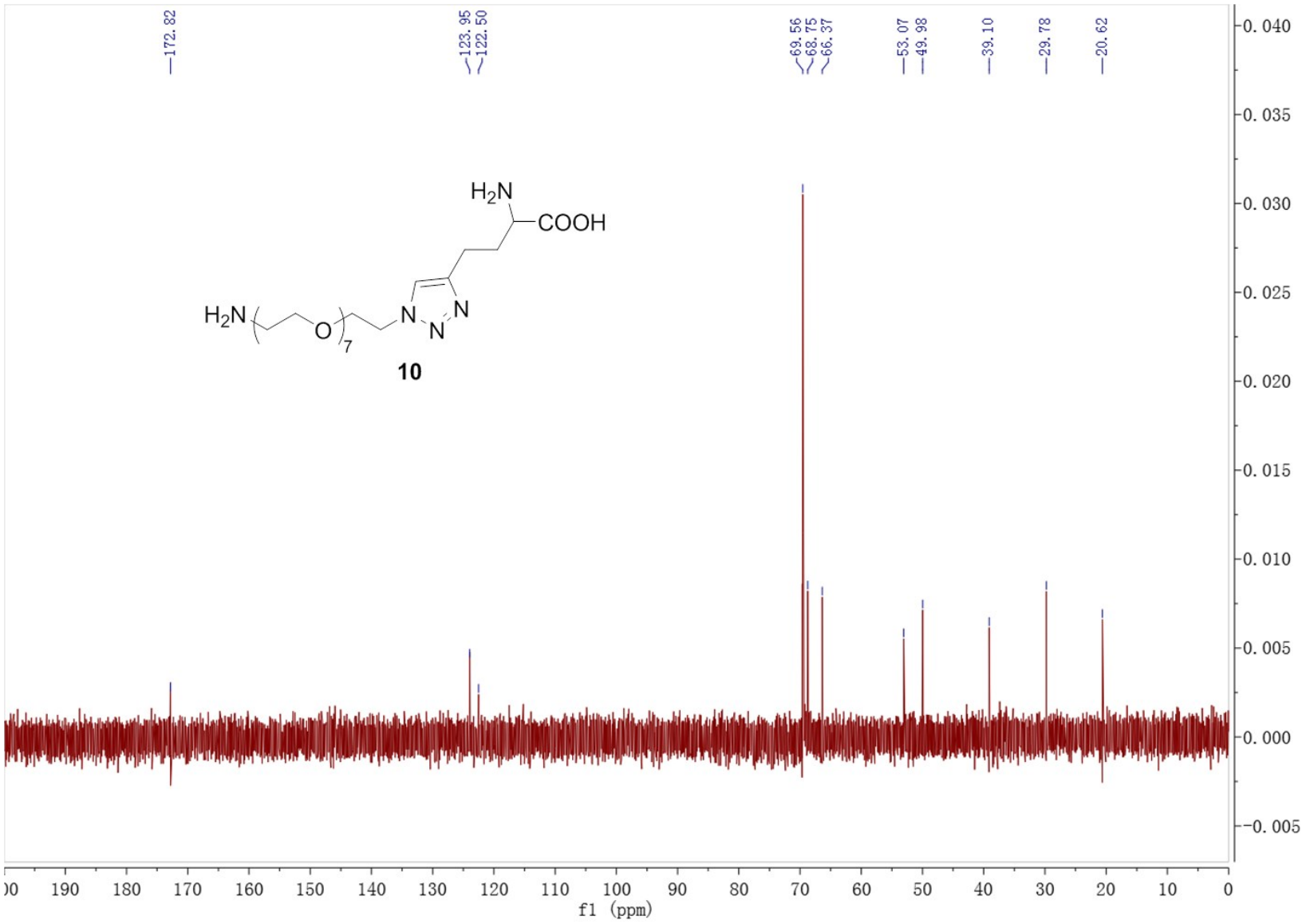
f1 (ppm)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COOH
H2N
O
7
N
N
N
10
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0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
-0.005
190
180
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160
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30
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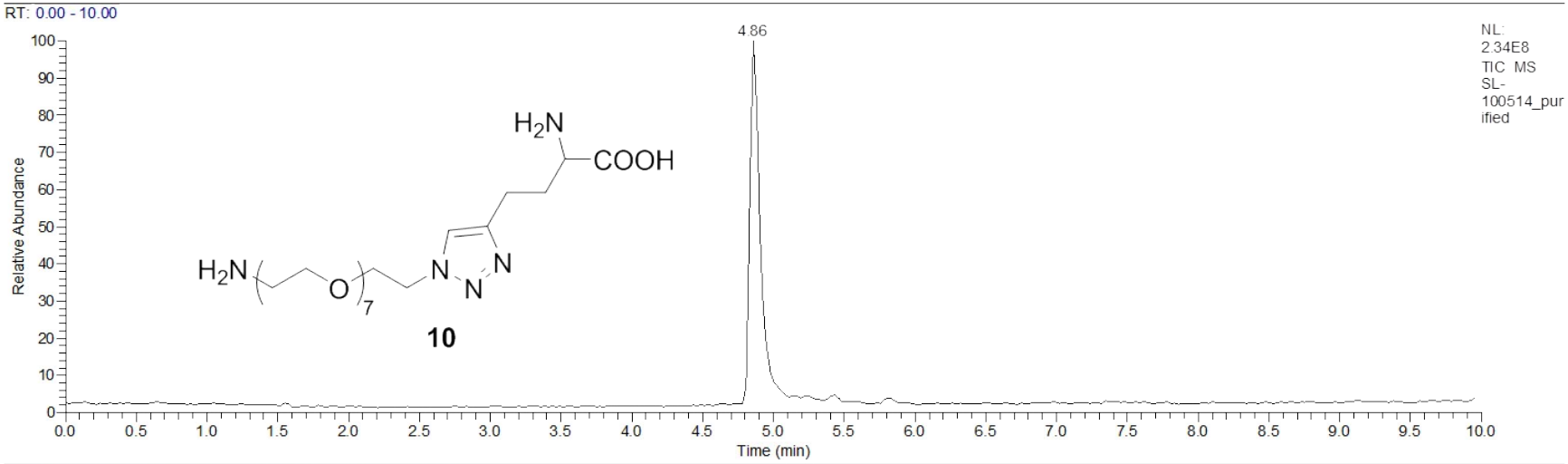
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Relative Abundance
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H_2N
O
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N
N
N
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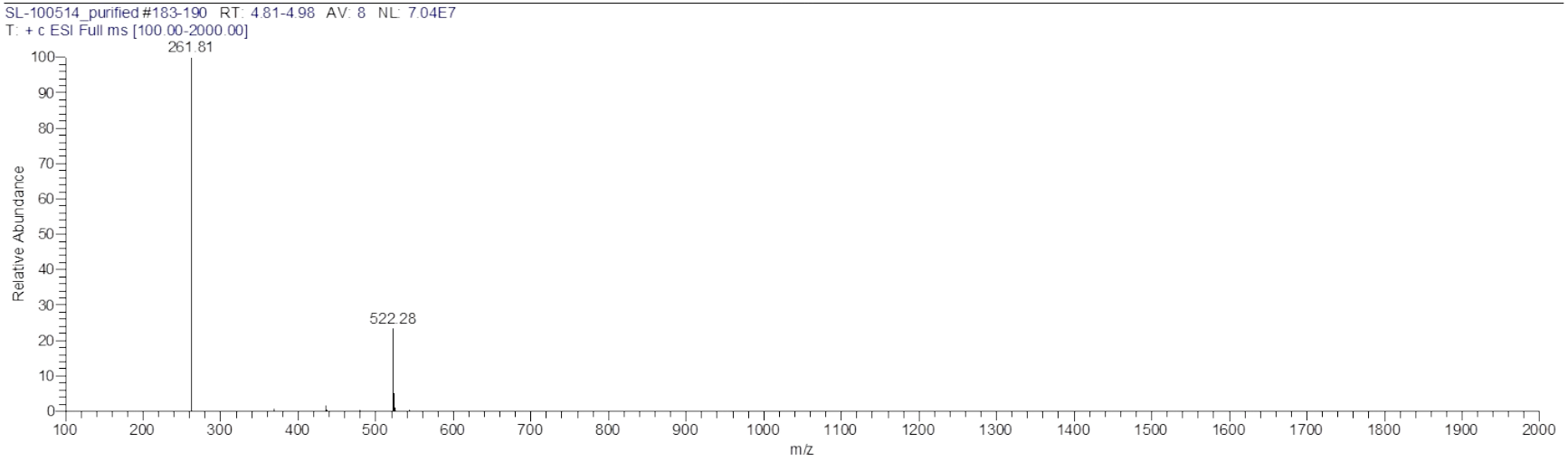
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Relative Abundance
m/z