Supporting Information

In vivo Visualization of γ-Glutamyl transpeptidase Activity with an Activatable Self-immobilizing Near-Infrared Probe

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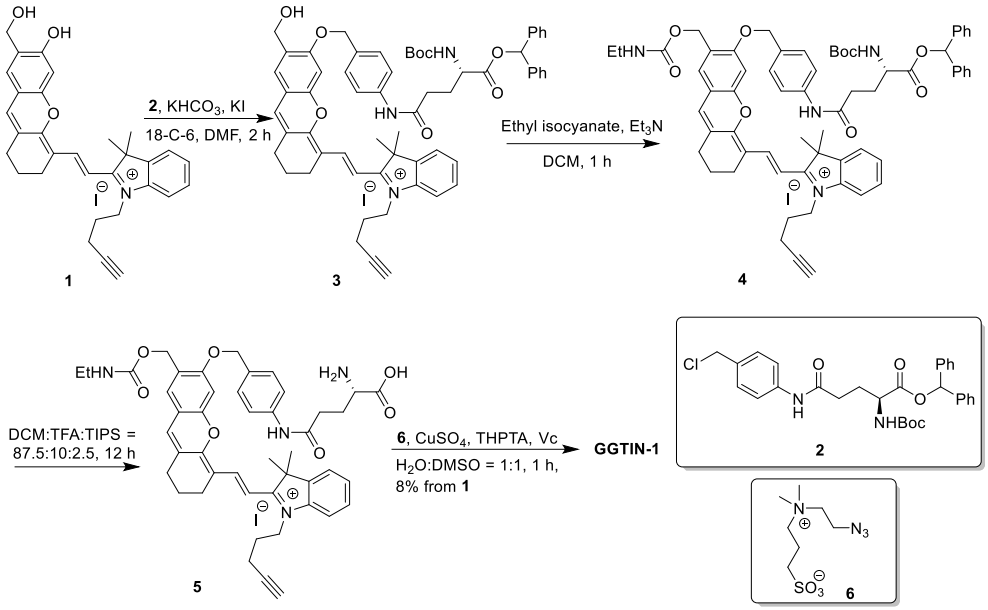


Figure S1. Synthesis of self-immobilizing probe **GGTIN-1**.

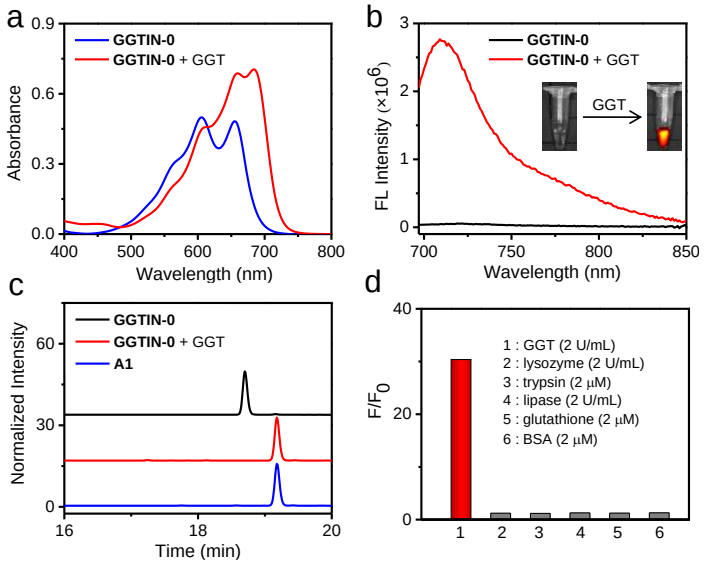


Figure S2. (a) UV-Vis absorption spectra of **GGTIN-0** (10 μM) in PBS (pH 7.4) before and after incubation with GGT (2 U/mL) at 37 ºC for 30 min. (b) Fluorescence spectra of **GGTIN-0** (10 μM) upon incubation with GGT (2 U/mL, 37 °C) for 30 min. (c) HPLC traces of **GGTIN-0** (10 μM) before and after incubation with GGT (2 U/mL). See Table S1 for the HPLC conditions. (d) Fluorescence response of **GGTIN-0** (10 μM) to various analytes in PBS. $\lambda_{ex/em}$ = 687/714 nm.

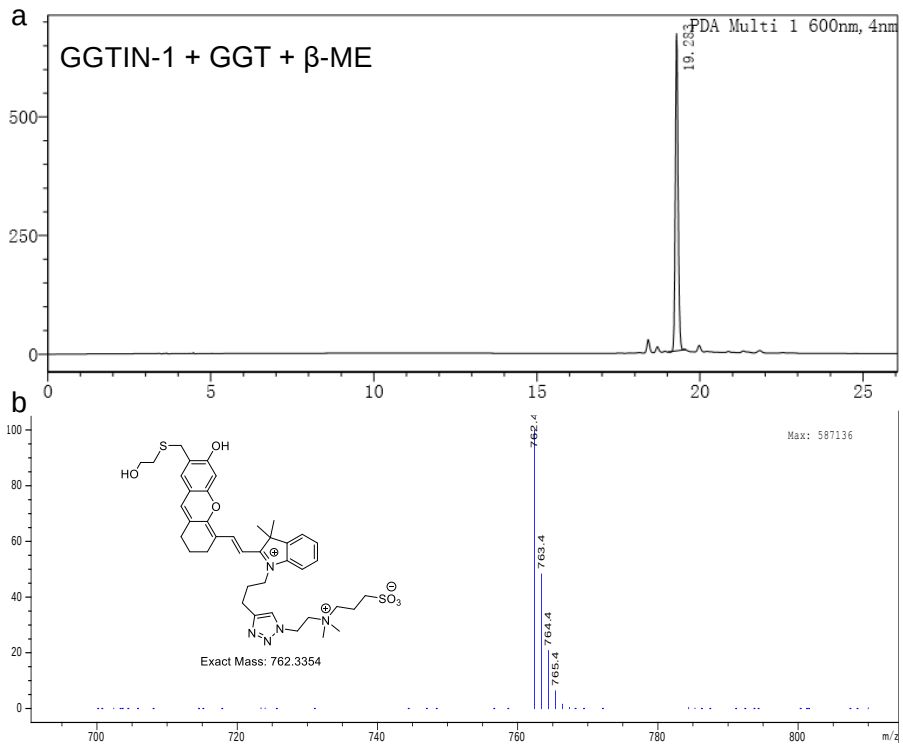


Figure S3. (a) HPLC trace of **GGTIN-1** (10 μM) after incubation with GGT (2 U/mL) and β-mercaptoethanol (β-ME, 140 mM) at 37 °C for 30 min at 600 nm. (b) Mass spectrum (ESI) analysis of peak at 19.283 min (a). See Table S1 for the HPLC conditions.

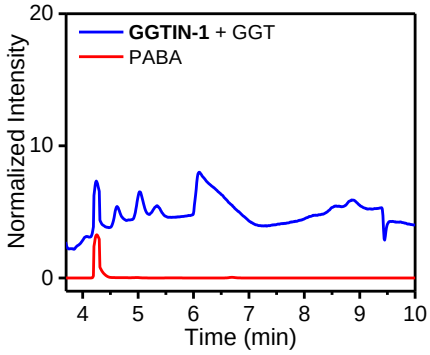


Figure S4. HPLC trace of **GGTIN-1** (10 μM) at 254 nm after incubation with GGT (2 U/mL) at 37 ºC for 30 minutes. PABA: *p*-aminobenzyl alcohol. See Table S2 for the HPLC conditions.

Table S1. HPLC conditions for the analysis of probes with or without GGT, β-ME. [a]

Time (minute)	Flow (mL/min)	H_2O (1‰ TFA)%	CH_3CN (1‰ TFA)%
0	1	95	5
2	1	95	5
2-25	1	0	100
25-30	1	0	100
30-31	1	95	5

[a] HPLC was performed on a reversed-phase C18 column (Inertsil ODS-SP, 5 μm, 4.6 × 250 mm).

Table S2. HPLC conditions for the analysis of **PABA** [a]

Time (minute)	Flow (mL/min)	H_2O (1‰ TFA)%	CH_3CN (1‰ TFA)%
0	1	100	0
0-15	1	90	10
12-22	1	0	100
22-25	1	95	5

[a] HPLC was performed on a reversed-phase C18 column (Inertsil ODS-SP, 5 μm, 4.6 × 250 mm).

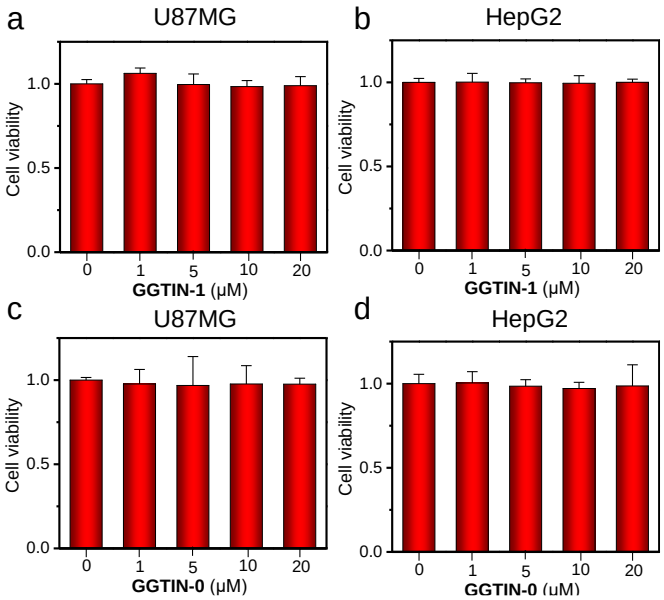


Figure S5. Test of cell viability. U87MG or HepG2 cells were incubated with **GGTIN-1** (a, b) and **GGTIN-0** (c, d) at 0, 1, 5, 10, 20 μM for 24 h, and the cell viability was determined by MTT assay. Error bars mean ± s.d. (n = 3).

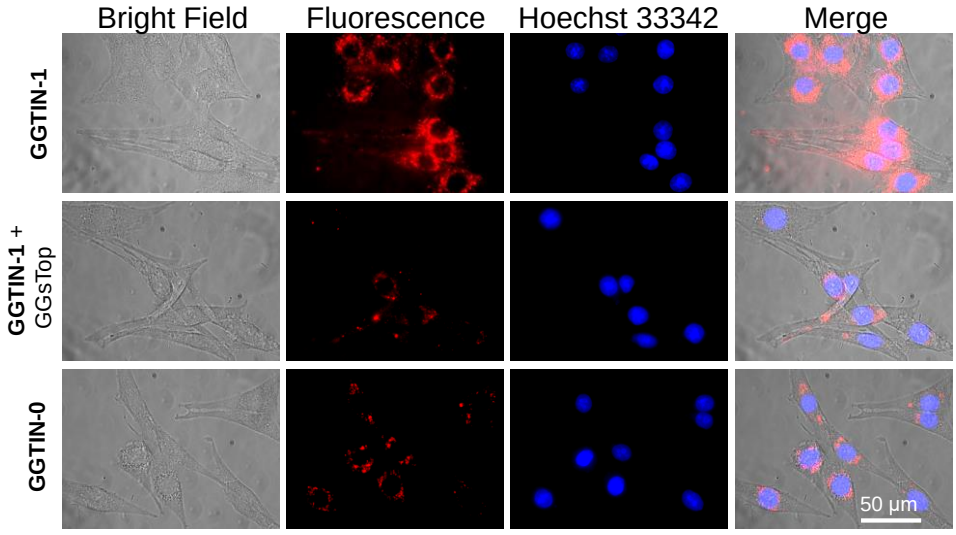


Figure S6. Fluorescence imaging of U87MG cells with **GGTIN-1. GGTIN-1** or **GGTIN-0** (5 μM) was incubated with U87MG cells at 37 °C for 1 h, or pretreated cells with GGsTop (0.5 mM), a GGT inhibitor, for 1 h before incubation with probe. Cells were washed with PBS before imaging with microscope.

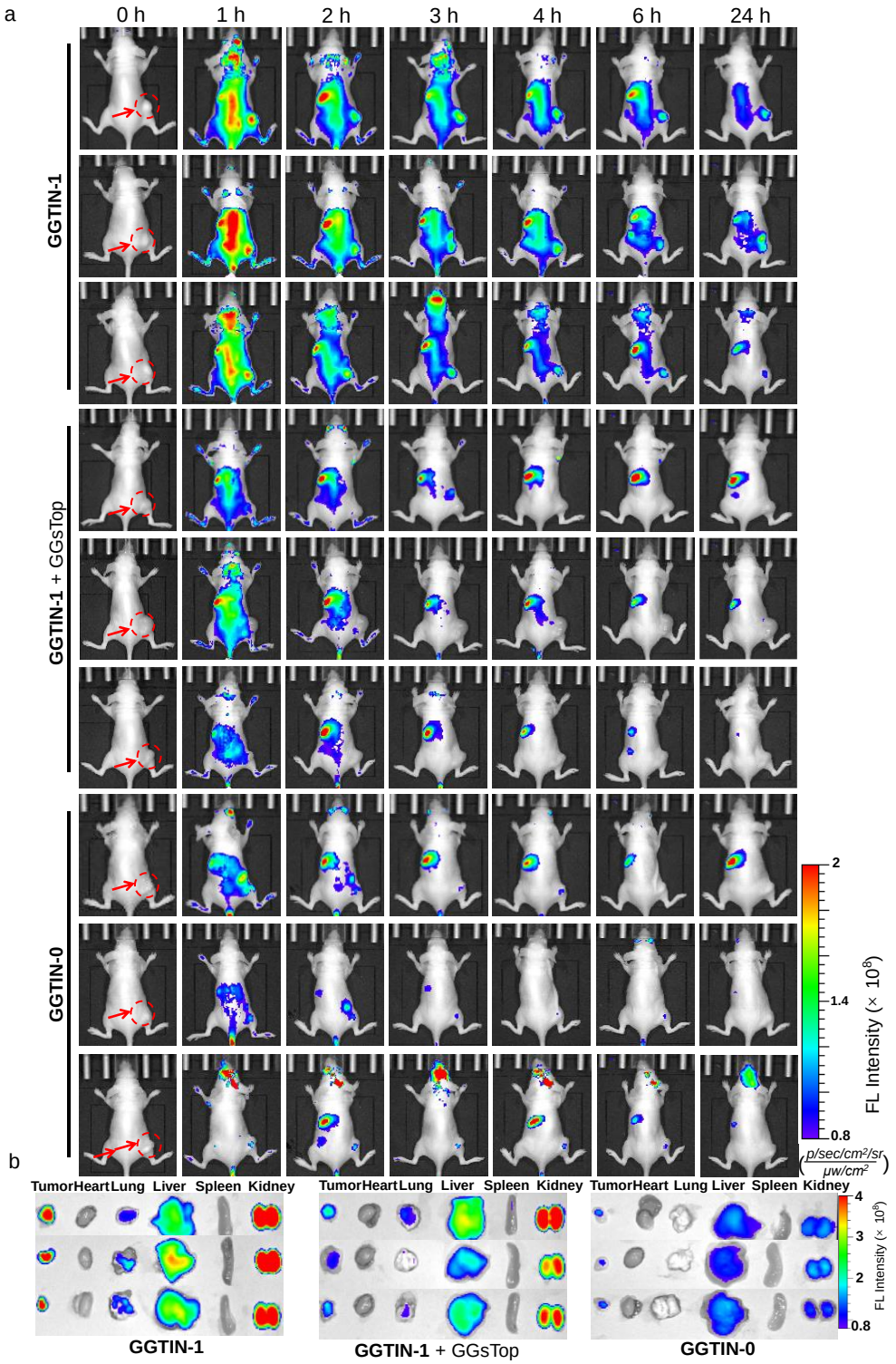


Figure S7. (a) Fluorescence images of endogenous GGT activity in U87MG tumor-bearing mice after i.v. injection of probes or together with i.t. injection of GGsTop (5 mM, 100 μL) before i.v. injection of **GGTIN-1**. (b) Fluorescence images of tumor and main organs at 1 h after i.v. injection of **GGTIN-1**, **GGTIN-0**, or **GGTIN-1** together with i.t. injection of GGsTop.

General information

Unless otherwise noted, all reagents were obtained commercially and used without further purification. The γ-glutamyl transpeptidase (GGT from equine kidney) was purchased from Sigma-Aldrich and BSA (bovine serum albumin) from Aladdin. U87MG cells and HepG2 cells were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Matrigel™ Basement Membrane Matirx for living tumor inoculation in mice were purchased from BD (UAS). The syntheses of compounds **1**[1], **6**[1], **A1**[1], **A2**[1], **2**[2], and **GGTIN-0**[2] are followed corresponding references.

The ^{1}H spectra was taken on Bruker nuclear magnetic resonance spectrometer (600 MHz). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane (δ = 0.00 ppm). Proton coupling patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). High-resolution mass spectra (HRMS) were recorded on a Bruker micro-TOF-QII time of flight mass spectrometer with electrospray ionization. Absorbance spectrum determined by UV1800 Series UV-Vis spectrophotometer (Shimadzu, Japan). HPLC was performed on a Shimadzu HPLC System equipped with a LC-20AT gradient pump and an inline diode array UV-Vis detector. A reversed-phase C18 (Inertsil ODS-SP, 5 μm, 4.6 × 250 mm or phenomenex, 5 μm, 21.2 x 250 mm) column was used with a MeCN/H_2O gradient mobile phase containing 0.1% trifluoroacetic acid at a flow of 1 or 12 mL/min for the analysis or purification. Fluorescence spectrum determined by a wavelength-calibrated FluoroMax-3 fluorometer (Horiba Jobin Yvon, France). In-gel fluorescence scanning was taken using Gel Documentation and Typhoon TRIO Variable Mode Imager System (GE Healthcare, USA). Absorbance for MTT assay was determined in a microplate reader (Molecular Devices, SpectraMax *i*3). Cell images were taken using fluorescence microscope (Leica, Germany). Image processing was made on image J software (National Institutes of Health, USA). In vivo images were obtained by IVIS Lumina XRMS Series III in Vivo Imaging System (PerkinElmer, Inc. USA).

Chemical synthesis and characterization of probe GGTIN-1

(*S*,*E*)-3-((2-(4-(3-(2-(2-(6-(4-(4-amino-4-carboxybutanamido)benzyloxy)-7-((ethylcarbamoyloxy)methyl)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3,3-dimethyl-3H-indolium-1-yl)propyl)-1H-1,2,3-triazol-1yl)ethyl)dimethylammonio)propane-1-sulf onate iodide (GGTIN-1)

A mixture of **1**[1] (50.0 mg，0.084 mmol), **2**[2] (136.1 mg，0.250 mmol), KI (144.7 mg，0.840 mmol), $KHCO_3$ (43.3 mg，0.420 mmol), and 18-crown-6 (23.7mg，0.084 mmol) in DMF (0.42 mL) were stirred at rt for 2 h. After dilution with DCM (10 mL), the solution was washed with water (10 mL x 3). The organic layer was dried over Na_2SO_4. Purified by chromatography on a short silica gel column afforded compound **3** (39.1 mg) as a crude product, which was used in the next step without further purification.

To a solution of **3** (39.1 mg，0.036 mmol) in DCM (0.36 mL) at rt were added ethyl isocyanate (29.3 μL，0.36 mmol), triethylamine (25 μL，0.18 mmol) and the resulting mixture were stirred for 1 h. Volatile solvent and excess reagents were then removed under vacuum to afford compound **4** as crude product, which was treated with a mixture of DCM:TFA:TIPS (87.5:10:2.5, 1 mL) at 0 ºC for 12 h. Diethyl ether (10 mL) was then added to the reaction mixture and the precipitate was collected by centrifugation to afford compound **5** as crude product.

A mixture of **5** prepared above, **6**[1] (8.7 mg，0.039 mmol), tris(3-hydroxypropyl triazolylmethyl)amine (1.6 mg，0.004 mmol), $CuSO_4$ (0.6 mg，0.004 mmol) and ascorbic acid (25.8 mg，0.146 mmol) in DMSO (0.05 mL) and H_2O (0.05 mL) were stirred at rt for 1 h. The title compound was obtained as blue solid (6.8 mg, 7.5% from compound **1**) after purification by preparative RP-HPLC on a C18 column. The purity of title was further confirmed by HPLC. ^{1}H NMR (600 MHz, d_6-DMSO) δ 10.15 (s, 1H), 8.58 (d, *J* = 10.0 Hz, 1H), 8.29 (s, 2H), 8.10 (s, 1H), 7.81 (d, *J* = 4.8 Hz, 1H), 7.71 (d, *J* = 5.2 Hz, 1H), 7.66 (d, *J* = 5.6 Hz, 2H), 7.56 (t, *J* = 5.2 Hz, 1H), 7.53 (s, 2H), 7.48 – 7.46 (m, 3H), 7.29 (t, *J* = 3.6 Hz, 1H), 7.21 (s, 1H), 6.61 (d, *J* = 10.0 Hz, 1H), 5.31 (s, 2H), 5.05 (s, 2H), 4.92 (t, *J* = 4.8 Hz, 2H), 4.50 (t, *J* = 4.6 Hz, 2H), 3.92 (s, 1H), 3.83 (t, *J* = 4.6 Hz, 2H), 3.53 – 3.50 (m, 2H), 3.06 (s, 6H), 3.05 – 2.98 (m, 2H), 2.86 (t, *J* = 5.0 Hz, 2H), 2.74 (t, *J* = 4.4 Hz, 2H), 2.66 (t, *J* = 4.4 Hz, 2H), 2.60 – 2.53 (m, 1H), 2.45 (t, *J* = 4.4 Hz, 2H), 2.19 – 2.15 (m, 2H), 2.11 – 2.02 (m, 2H), 2.02 – 1.96 (m, 2H), 1.87 – 1.81 (m, 2H), 1.77 (s, 6H), 1.02 (t, J = 4.8 Hz, 3H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 177.86, 170.85, 163.34, 161.11, 159.56, 158.26, 158.05, 156.28, 154.07, 146.56, 142.55, 141.91, 139.62, 131.09, 129.41, 128.87, 127.93, 127.56, 124.61, 123.59, 119.56, 118.85, 116.86, 115.34, 114.65, 113.59, 104.84, 100.72, 72.55, 67.49, 62.93, 60.74, 60.59, 51.27, 50.86, 49.21, 47.65, 46.18, 43.23, 40.89, 40.51, 35.59, 28.10, 27.31, 27.14, 25.60, 22.45, 19.32, 15.53. HR-MS (ESI) m/z calcd for $C_{53}H_{67}N_8O_{10}S$ $(M-I)^+$ 1007.4701, found 1007.4703.

Figure S8. ^{1}H NMR Spectrum of GGTIN-1

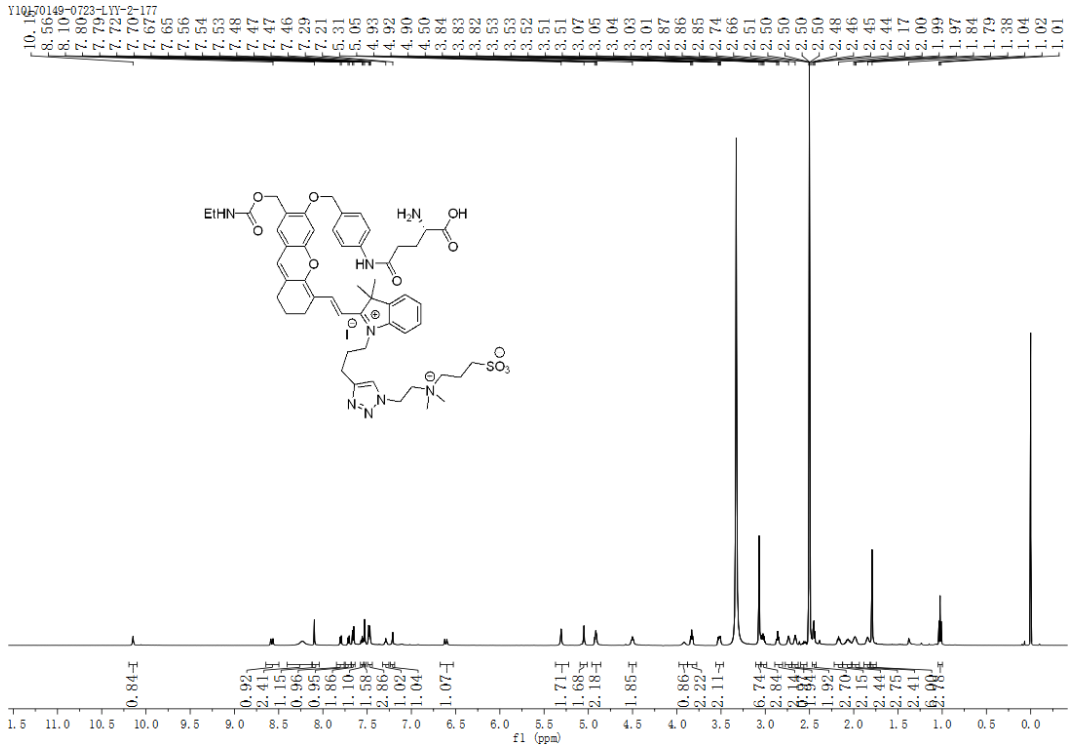


Figure S9. ^{13}C NMR Spectrum of GGTIN-1

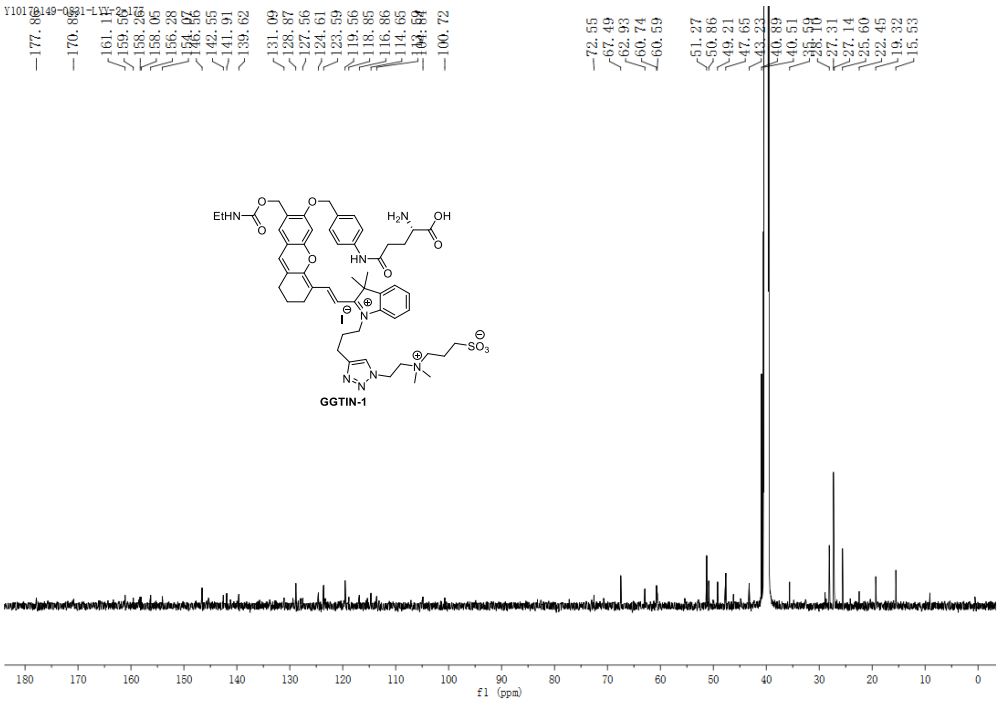


Figure S10. HR-MS spectrum of compound GGTIN-1

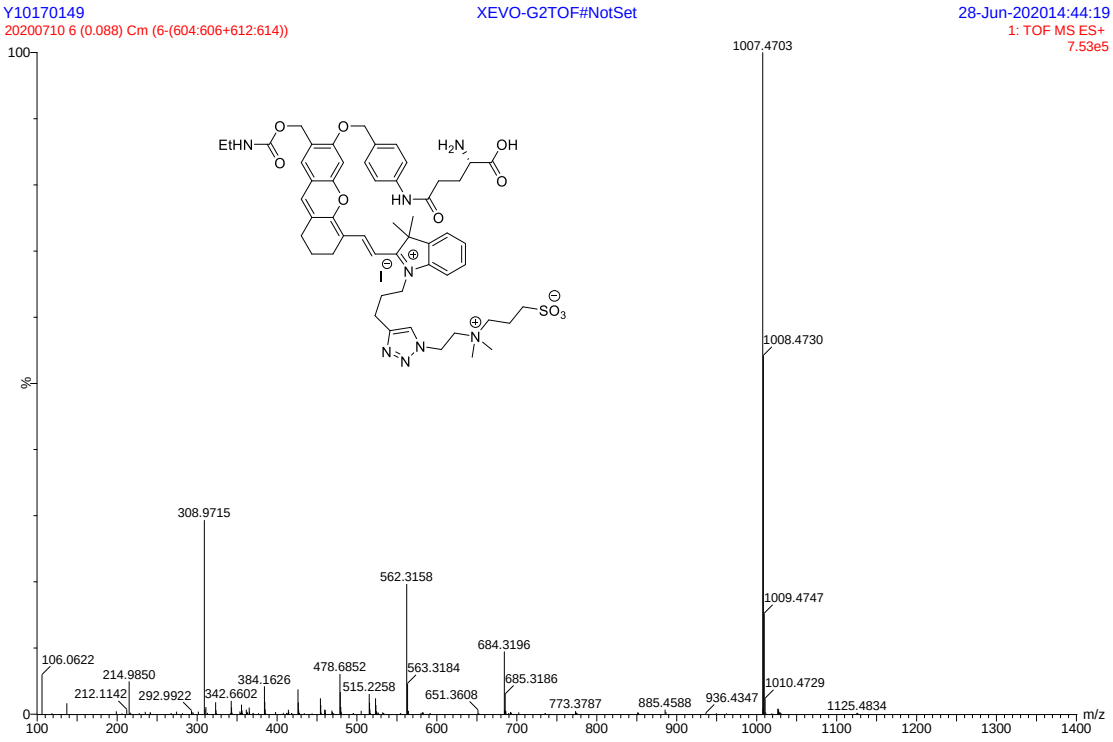
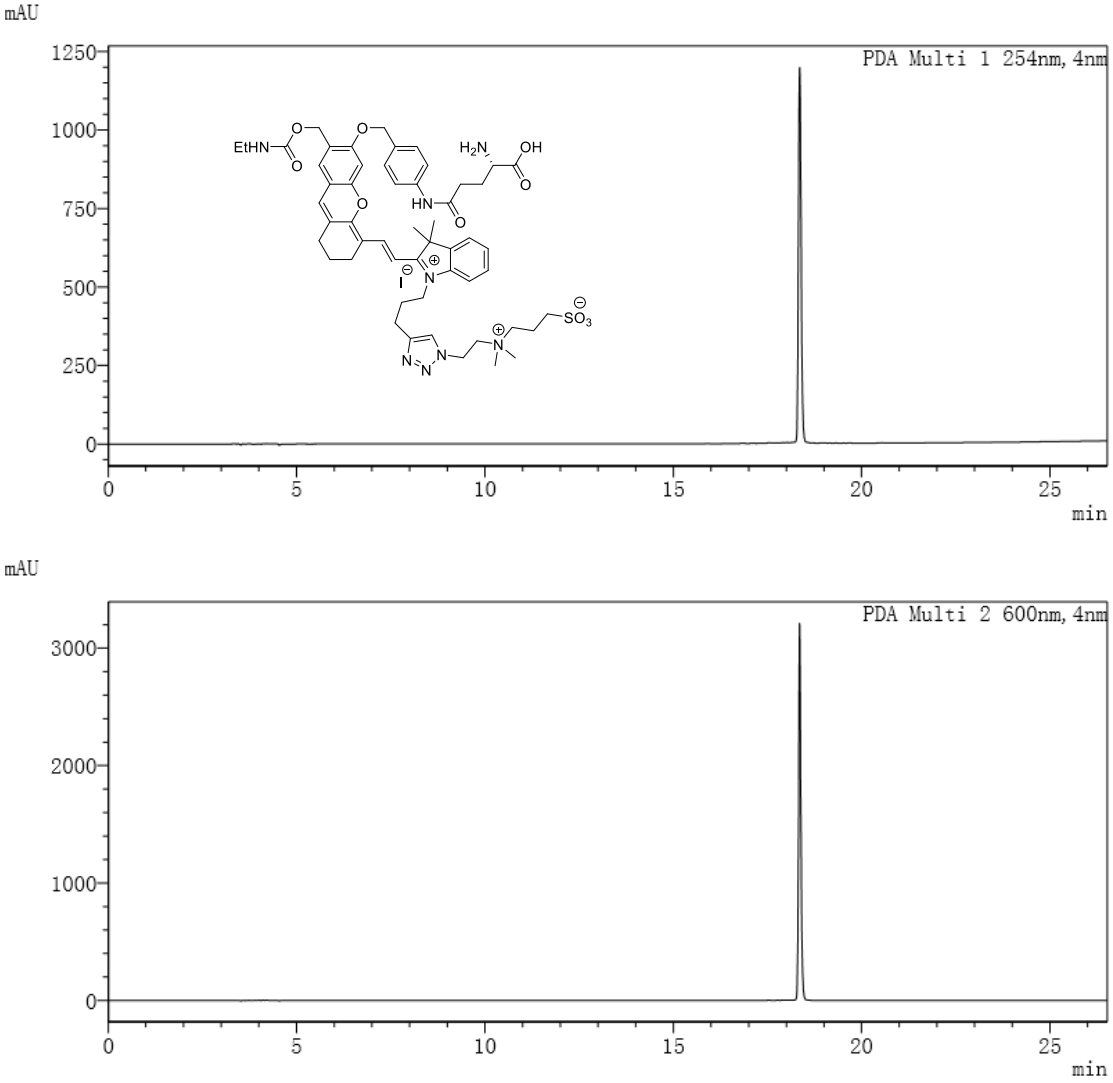


Figure S11. HPLC traces of GGTIN-1 at 254 nm and 600 nm[a]



[a] See Table S1 for the HPLC conditions.

References

(1) Li, Y.; Song, H.; Xue, C. H.; Fang, Z. J.; Xiong, L. Q.; Xie, H. A self-immobilizing near-infrared fluorogenic probe for sensitive imaging of extracellular enzyme activity in vivo. *Chem. Sci.* **2020**, 11, 5889-5894.

(2) Fang, Z. J.; Li, Y.; Xie, H. A 4-OTBS Benzyl-based Protective Group for Carboxylic Acids. *Tetrahedron Lett.*, **2019**, 60, 1658-1662.