Supporting Information

A Novel Mitochondrial-Targeting Near-Infrared Fluorescence Probe for Imaging γ-Glutamyl Transpeptidase Activity in Living Cells

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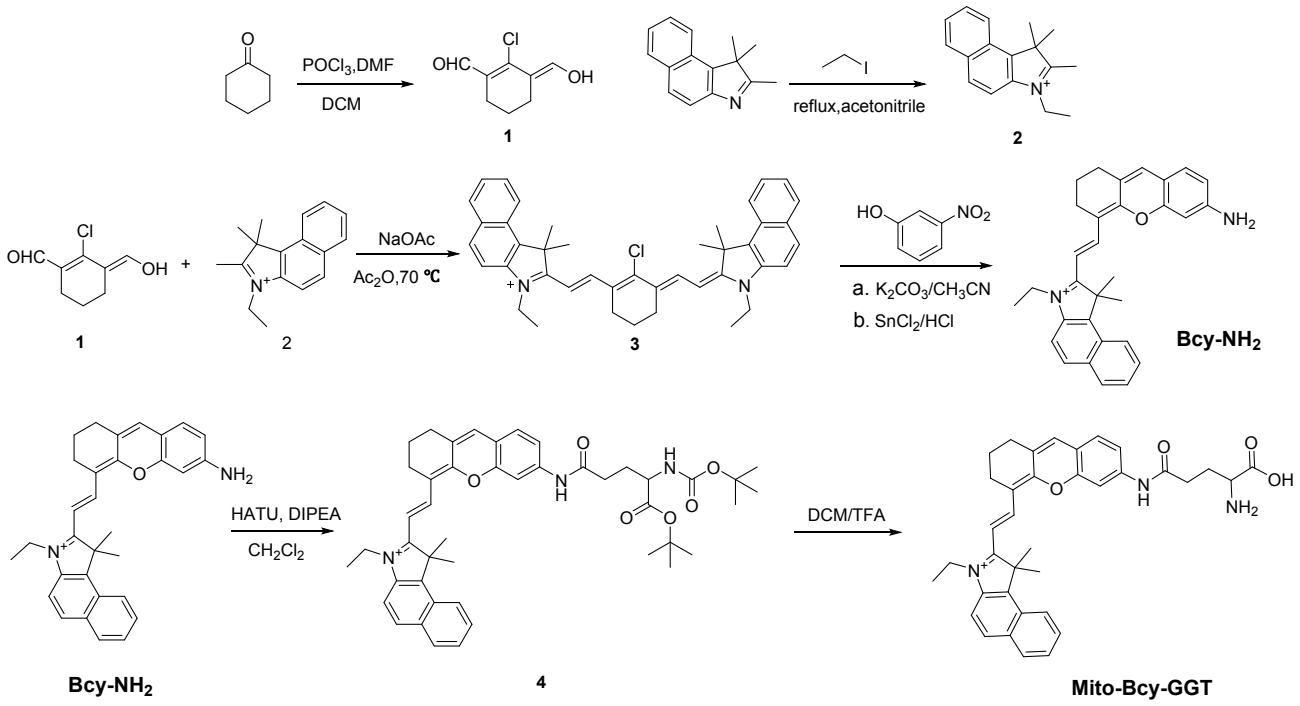
1. Synthetic procedures for Mito-Bcy-GGT and important intermediates



Scheme S1. Synthetic routes for Mito-Bcy-GGT probe.

2-((E)-2-((E)-2-chloro-3-((E)-2-(3-ethyl-1,1-dimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3-ethyl-1,1-dimethyl-1H-benzo[e]indol-3-ium iodide (3)

First, Compound 1 and 2 were synthesized according to procedures in the literature with slight modifications [1-3]. Then, Compound 1 (2.1 g, 5.78 mmol), Compound 2 (0.5 g, 2.89 mmol), NaOAc (0.5 g, 5.78 mmol) were mixed in acetic anhydride (10 ml) and refluxed at 70 °C for 30 min. Afterwards, saturated $NaHCO_3$ solution was slowly added to the mixture to obtain the green solid. Then, the crude product was recrystallized, cooled and crystallized and filtered. The filtrate was washed with saturated $NaHCO_3$, and the solution was extracted by DCM and dried with anhydrous Na_2SO_4. Then the solvent was removed under reduced pressure. The crude product was purified by chromatography on a silica gel (eluent: CH_2Cl_2 /CH_3OH=100/1-20/1, v/v) to give **3** as a green solid. Yield: 1.0 g (76.6 %). ^1H NMR (400MHz, 298 K, $CDCl_3$): ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm) 8.49-8.46 (d, J=12.0 Hz , 2H), 8.15-8.13 (d, J=8.0 Hz , 2H), 7.98-7.94 (t, J=16.0 Hz , 4H), 7.64-7.61 (t, J=12.0 Hz, 2H), 7.50-7.46 (t, J=16.0 Hz, 4H), 7.29 (s, 1H), 6.26-6.23 (d, J=12.0 Hz, 2H), 4.39-4.38 (d, J=4.0 Hz, 4H), 2.78 (s, 4H), 2.03 (s, 15H), 1.55-1.51 (t, J=16.0 Hz, 6H). ^{13}C NMR ($CDCl_3$, 100 MHz) , δ (ppm) 173.25, 150.10, 143.40, 139.22, 133.96, 132.16, 131.11, 130.24, 128.16, 127.80,

125.15, 122.18, 110.70, 100.86, 51.23, 40.50, 27.73, 26.80, 21.02, 12.94, HRMS (ESI): m/z calcd for $C_{42}H_{44}N_2O^+$ $[M]^+$ 611.3188; found: 611.4000.

(E)-2-(2-(6-amino-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3-ethyl-1,1-dimethyl-1H-benzo[e]indol-3-ium iodide (Bcy-NH_2)

First, 3-nitrophenol (417.33 mg, 3 mmol) and K_2CO_3 (414 mg, 3 mmol) were dissolved in acetonitrile (20 mL) and reacted at room temperature for 30 min under N_2 atmosphere. Then, Compound 3 (888.3 mg, 1.2 mmol) was added and the mixture was reacted at 50 ^oC for 4 h. After the reaction was completed, the solvent was removed under reduced pressure. The crude product was extracted with DCM, washed with water, and dried with anhydrous sodium sulfate. The crude product was dissolved in 30 mL methanol. Then, $SnCl_2$ dissolved in 4 mL concentrated hydrochloric acid was added under N_2 protection and stirred overnight at 70 ^oC. Afterwards, the solution was neutralized with saturated Na_2CO_3 solution and filtered. The precipitate was dried with anhydrous sodium sulfate and purified by chromatography on a silica gel (eluent: CH_2Cl_2/CH_3OH=100/1-20/1, v/v) to give **Bcy-NH_2** as dark blue solid. Yield: 1.0 g (76.6 %). ^1H NMR (400 MHz, 298 K, DMSO-d_6): ^1H NMR (DMSO-d_6, 400 MHz) δ (ppm) 8.57-8.53 (d, J=16.0 Hz , 1H), 8.26-8.24 (d, J=8.0 Hz , 1H), 8.15-8.09 (m, 2H), 7.84-7.82 (d, J=8.0 Hz ,1H), 7.73-7.69 (t, J=16.0 Hz ,1H), 7.64 (s, 1H), 7.57-7.54 (t, J=12.0 Hz,1H), 7.42-7.40 (d, J=8.0 Hz, 1H), 6.94 (s, 2H), 6.79-6.74 (t, J=20.0 Hz, 2H), 6.34-6.30 (d, J=16.0 Hz, 1H), 4.43-4.41 (d, J=8.0 Hz, 2H), 2.72-2.70 (d, 4H), 1.98 (s, 6H), 1.84-1.83 (d, J=4.0 Hz, 2H), 1.74-1.64 (m, 3H), ^{13}C NMR (DMSO-d_6, 100 MHz) , δ (ppm) 174.98, 162.59, 156.10, 155.48, 141.57, 139.75, 134.06, 132.06, 131.04, 130.51, 130.20, 128.34, 127.90, 125.55, 122.88, 122.50, 114.95, 114.30, 113.23, 112.15, 100.04, 97.84, 78.89, 66.80, 51.35, 40.62, 40.41, 40.20, 40.00, 39.79, 39.58, 39.37, 33.37, 31.54, 28.45, 27.85, 20.92, 12.93, HRMS (ESI): m/z calcd for $C_{31}H_{31}N_2O^+$[M]$^+$447.2431; found: 447.3000.

2. Cell lysate experiment

First, cell lysate with a volume of 100 μL was extracted from 6 × 10^5 cells. DON was mixed with cell lysates (2.5 μL) and incubated at 37 °C for 1 h. For comparison, cell lysates (2.5 μL) were also mixed with PBS and incubated at 37 °C for 1 h. Then, Mito-Bcy-GGT (10 μM) was added and the solution was incubated at 37 °C for another 3 h. All the fluorescence spectra were recorded in the range from 700 nm to 880 nm with an excitation wavelength of 680 nm, using slit widths of 10 nm for both excitation and emission.

3. The effect of pH on the response of GGT towards Mito-Bcy-GGT

To investigate the effect of pH on the response of GGT towards Mito-Bcy-GGT, GGT and Mito-Bcy-GGT were first mixed in buffers of different pH values (4.0, 5.0, 6.0, 6.2, 6.6, 7.0, 7.4, 7.8, 8.0, 8.4, 8.8, 9.2, 10.0). Then the mixture was incubated at 37 °C for 3 h. Fluorescence spectra were then recorded. Fluorescence intensities at 727 nm were plotted against different pH values. The effect of pH on the stability of Mito-Bcy-GGT was also studied under the same conditions.

4. HPLC and ESI-MS analysis of Mito-Bcy-GGT mediated reactions

The HPLC chromatograms of Mito-Bcy-GGT, Bcy-NH_2, and the reaction products of Mito-Bcy-GGT and Bcy-NH_2 were performed with a C18 column (150 nm × 4.6 mm), using the following conditions: methanol/H_2O = 100/0 (v/v); flow rate: 1 mL/min; detection wavelength: 254 nm. The HPLC chromatograms of Mito-Bcy-GGT, Bcy-GGT, and the reaction products of GGT and Mito-Bcy-GGT were performed on a system with a C18 column (250 nm × 4.6 mm) and the conditions were as follows: methanol/H_2O = 100/0 (v/v); flow rate: 1 mL/min; detection wavelength: 680 nm. For further demonstration of the reaction mechanism, ESI-MS was introduced to analyze the products of Mito-Bcy-GGT after reaction with GGT in positive mode.

5. Kinetic Studies

Various concentrations of Mito-Bcy-GGT (2, 5, 10, 15, 20, 30 μM) were incubated with GGT (800 U/L) at 37 °C for 3 h in PBS buffer (10 mM, pH = 7.4), containing 1.5% DMSO. Then, fluorescence spectra were measured for analysis. The data points were fitted to a Michaelis-Menten curve. The initial reaction velocity (μM S^{-1}) and kinetic parameters were calculated according to the Michaelis Menten equation: $V = V_{max*}[S] (K_m + [S])$, where V is initial velocity, and [S] is substrate concentration.

6. Aqueous solubility

First, stock solution of Bcy-NH_2 (30 μM) was prepared in DMSO. Then Bcy-NH_2 was diluted to different concentrations (0 ~ 10 μM) with PBS and fluorescence spectra were recorded. In all cases, the volume percentage of DMSO was maintained to be 1.5 %. Fluorescence intensity at 727 nm was plotted against the concentrations of Bcy-NH_2. The maximum concentration in the linear region was described as solubility.

7. Photostability

Bcy-NH_2 (5 μM) was irradiated for 0.5 hour using an FS5 spectrofluorometer (Edinburgh, UK) and fluorescence intensity at 727 nm was recorded in real time. For comparison, indocyanine green (ICG) was also studied under the same conditions.

8. Determination of fluorescence quantum yield

$$\emptyset_F = I/I_R \times A_R/A \times (n/n_R)^2 \times \emptyset_R \qquad (1)$$

The quantum yield of Bcy-NH_2 was determined according to Equation 1, using ICG as a reference, where $\emptyset_R$ is the quantum yield of the reference, I is the area under the emission spectra, A is the absorbance at the excitation wavelength, and n is the refractive index of the solvent used. R refers to reference.

9. Real time response study

For real time response study, Mito-Bcy-GGT was mixed with or without GGT (800 U/L) in PBS, supplemented with 1.5% DMSO and 0.1% polyvinylpyrrolidone. Fluorescence intensity was recorded in real time for 4 h on a microplate reader (Tecan, 5082 Grodig, Austria).

10. References

1. Prasad. P. R, Selvakumar. K, Singh. H. B, *J. Org. Chem.,* 2016, **81**, 3214-3226.
2. Kang. N. Y, Park. S. J, Ang. X. W. E, *Chem. Commun.*, 2014, **50**, 6589-6591.
3. Roberts. S, Seeger. M, Jiang. Y, *J. Am. Chem. Soc.*, 2017, **140**, 2718-2721.

11. Figures S1-S26

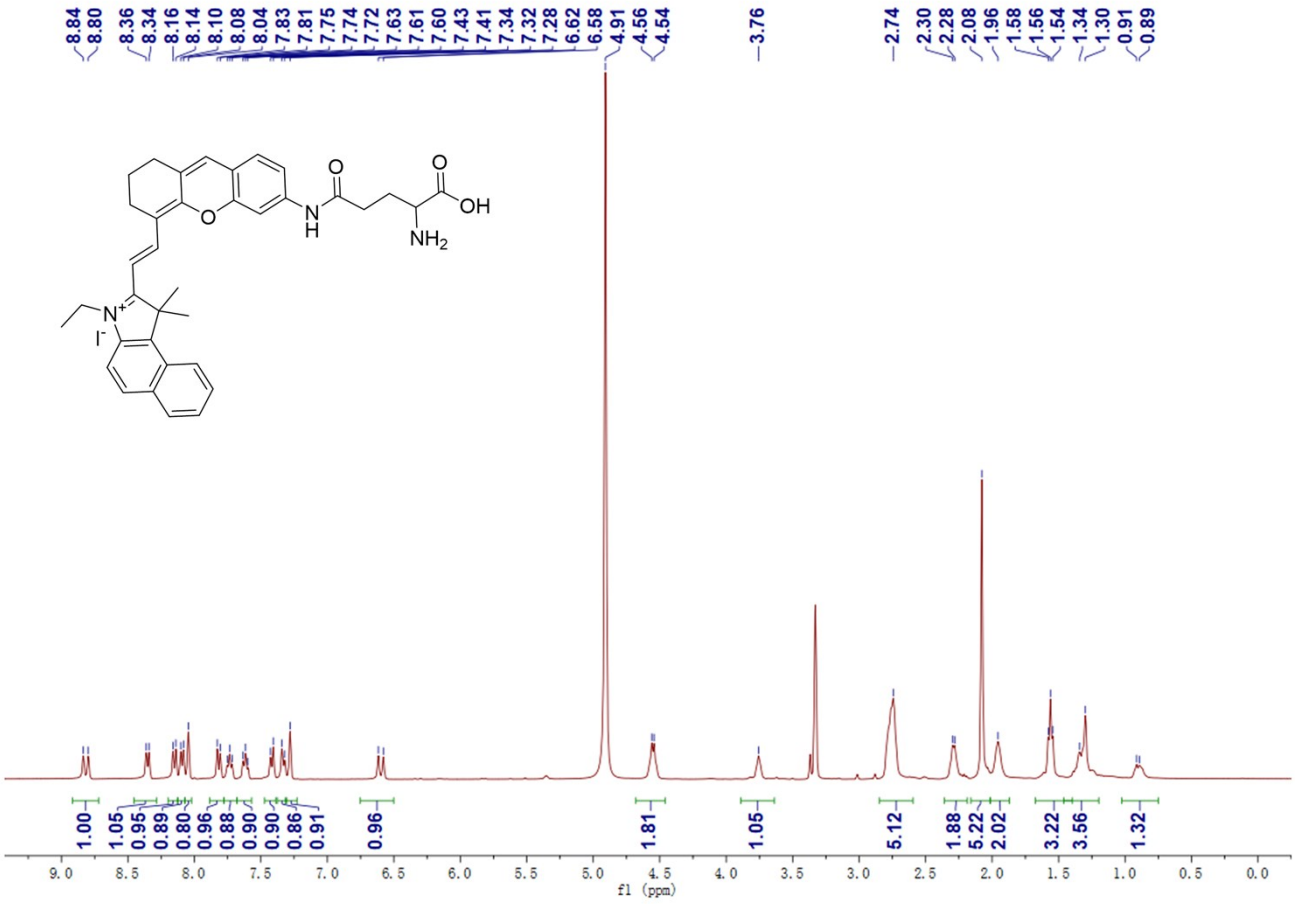


Fig. S1 ^{1}H NMR spectrum of Mito-Bcy-GGT in D-CD_3OD

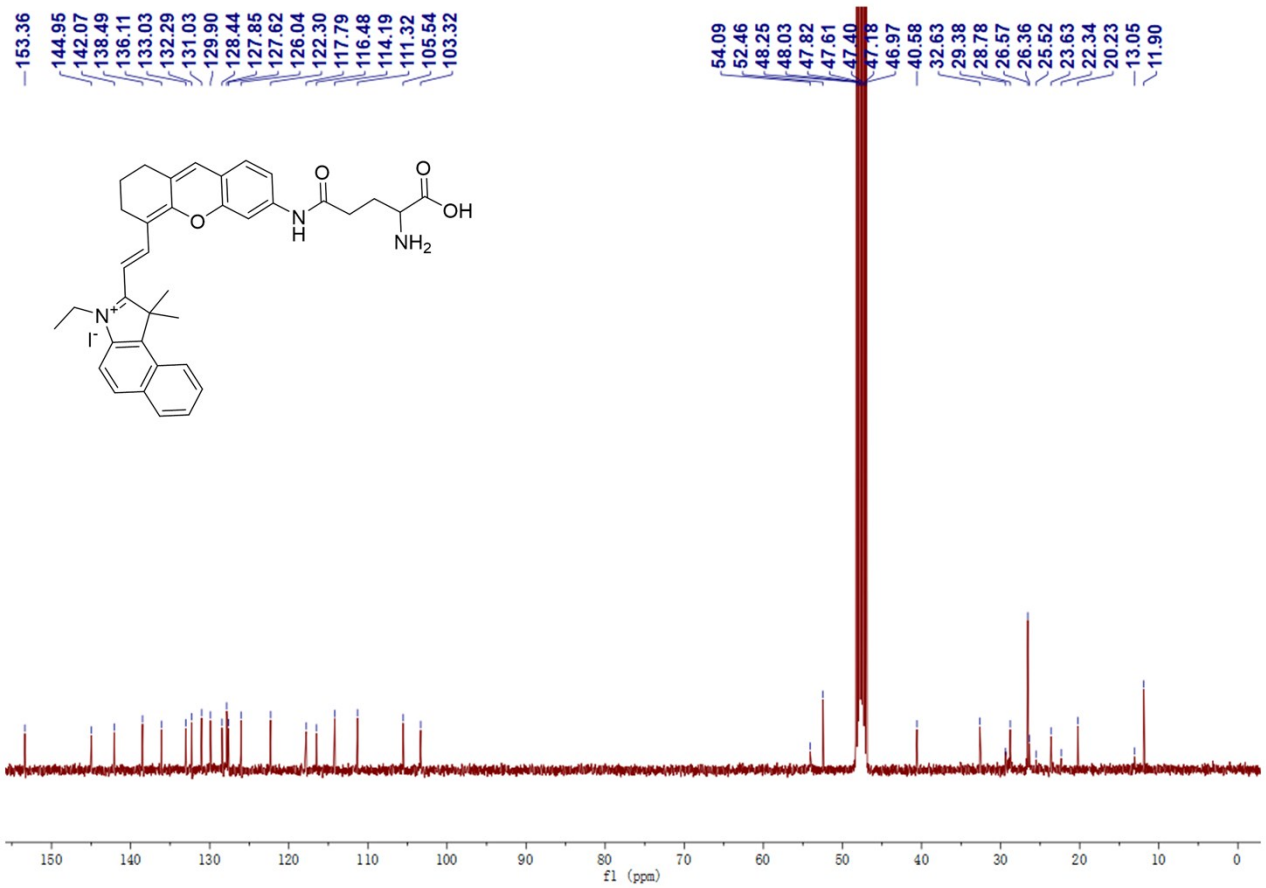


Fig. S2 ^{13}C NMR spectrum of Mito-Bcy-GGT in D-CD_3OD.

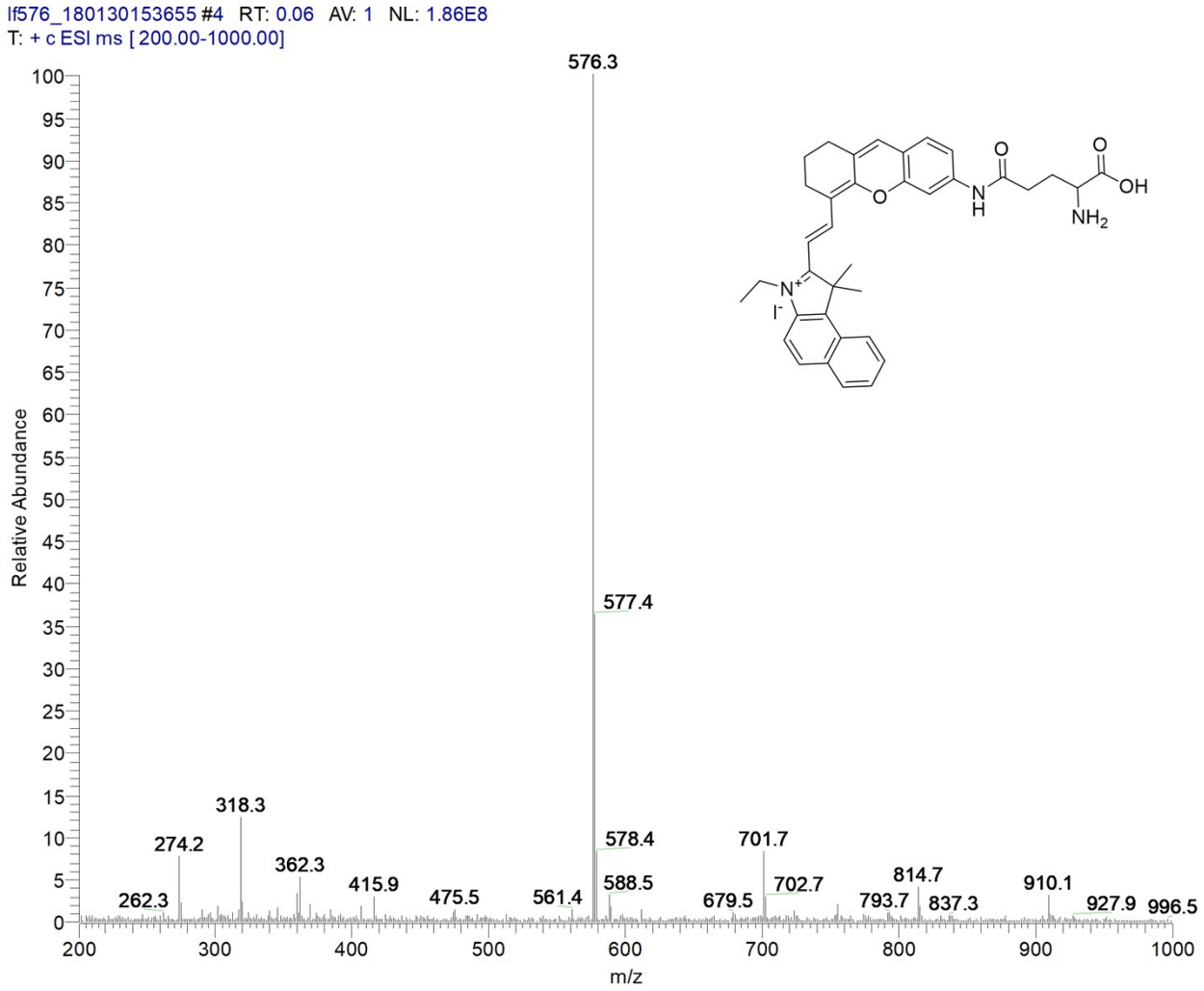


Fig. S3 ESI spectrum of Mito-Bcy-GGT in methanol.

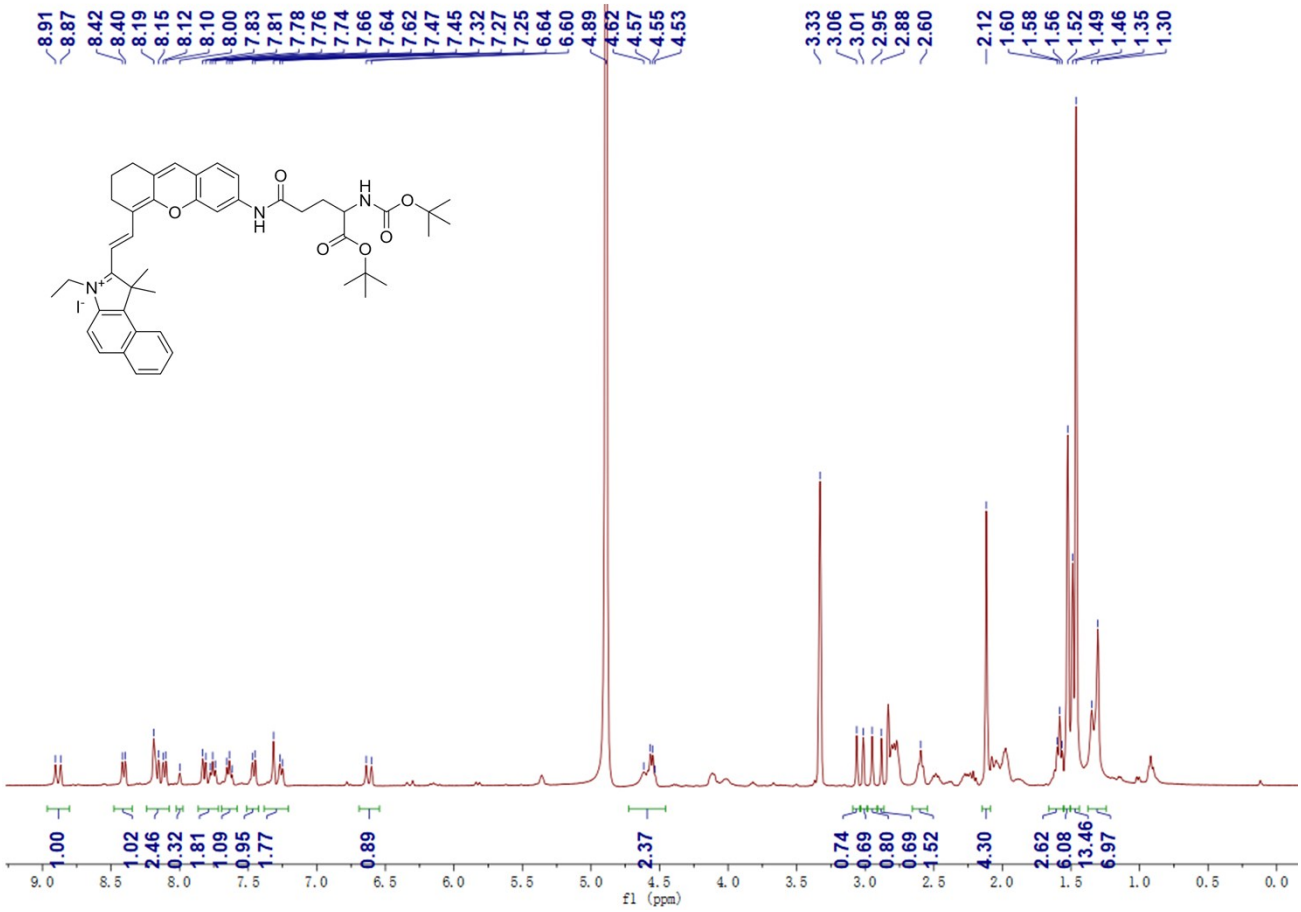


Fig. S4 ^{1}H NMR spectrum of Compound 4 in D-CD_3OD.

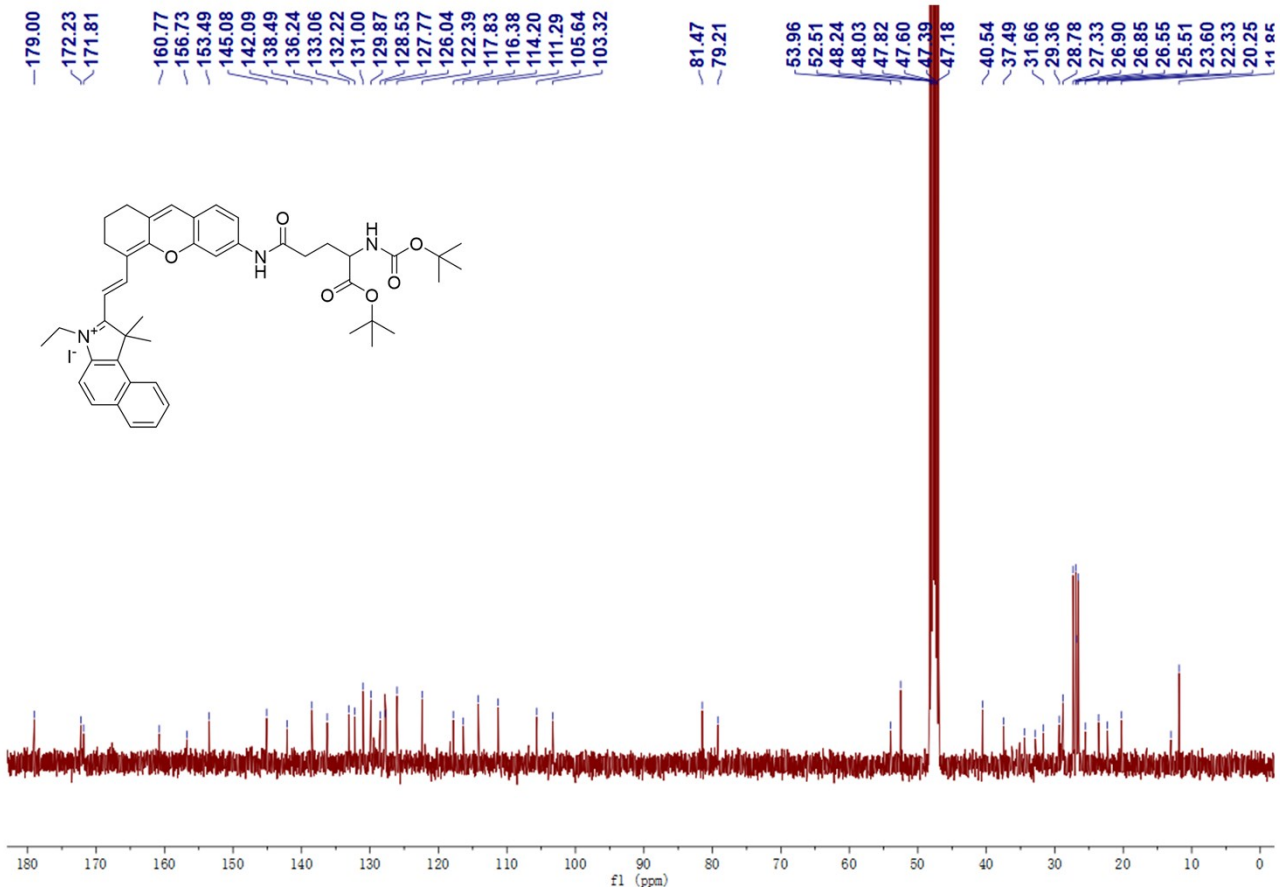


Fig. S5 ^{13}C NMR spectrum of Compound 4 in CD_3OD.

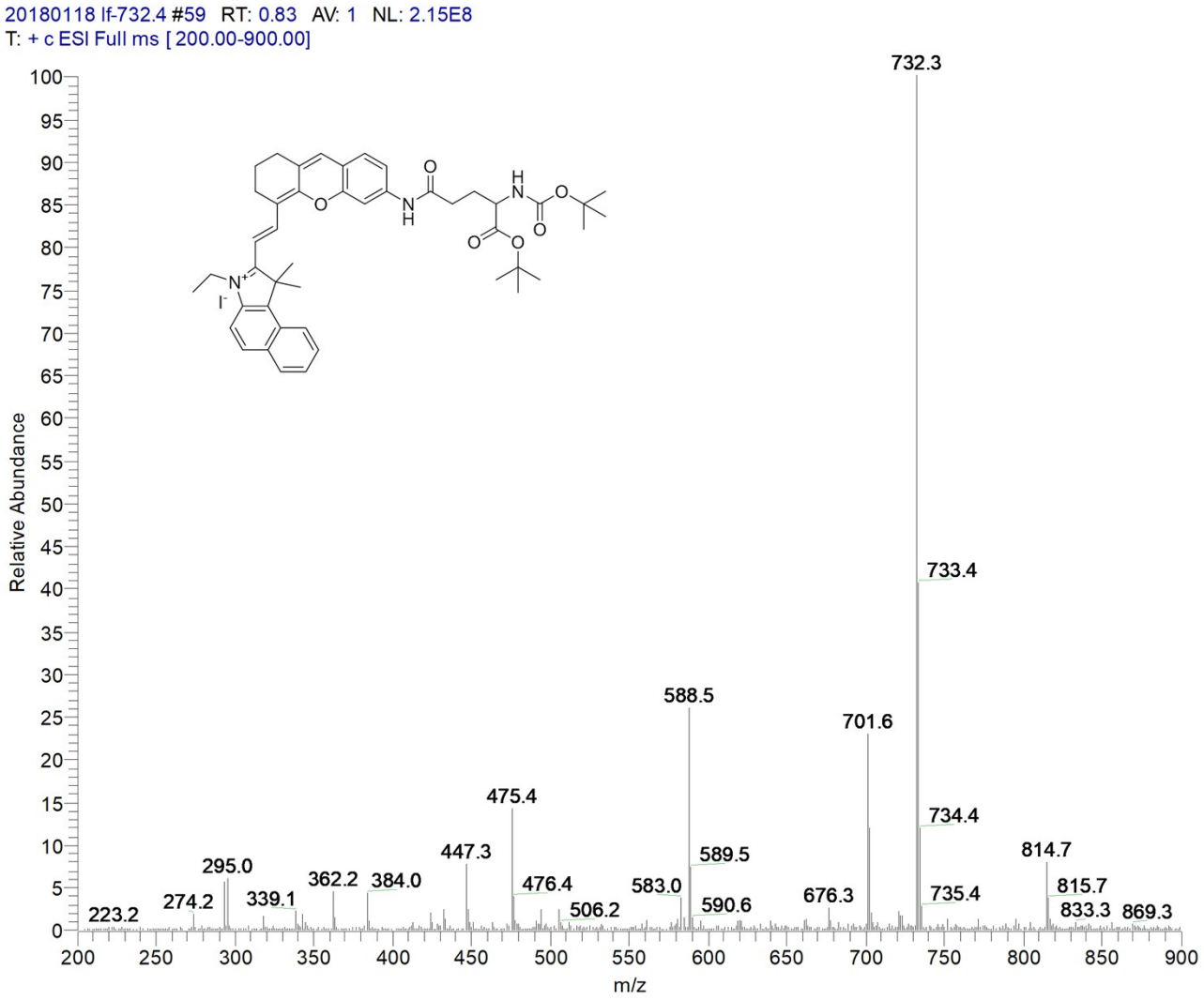


Fig. S6 ESI spectrum of Compound 4 in methanol.

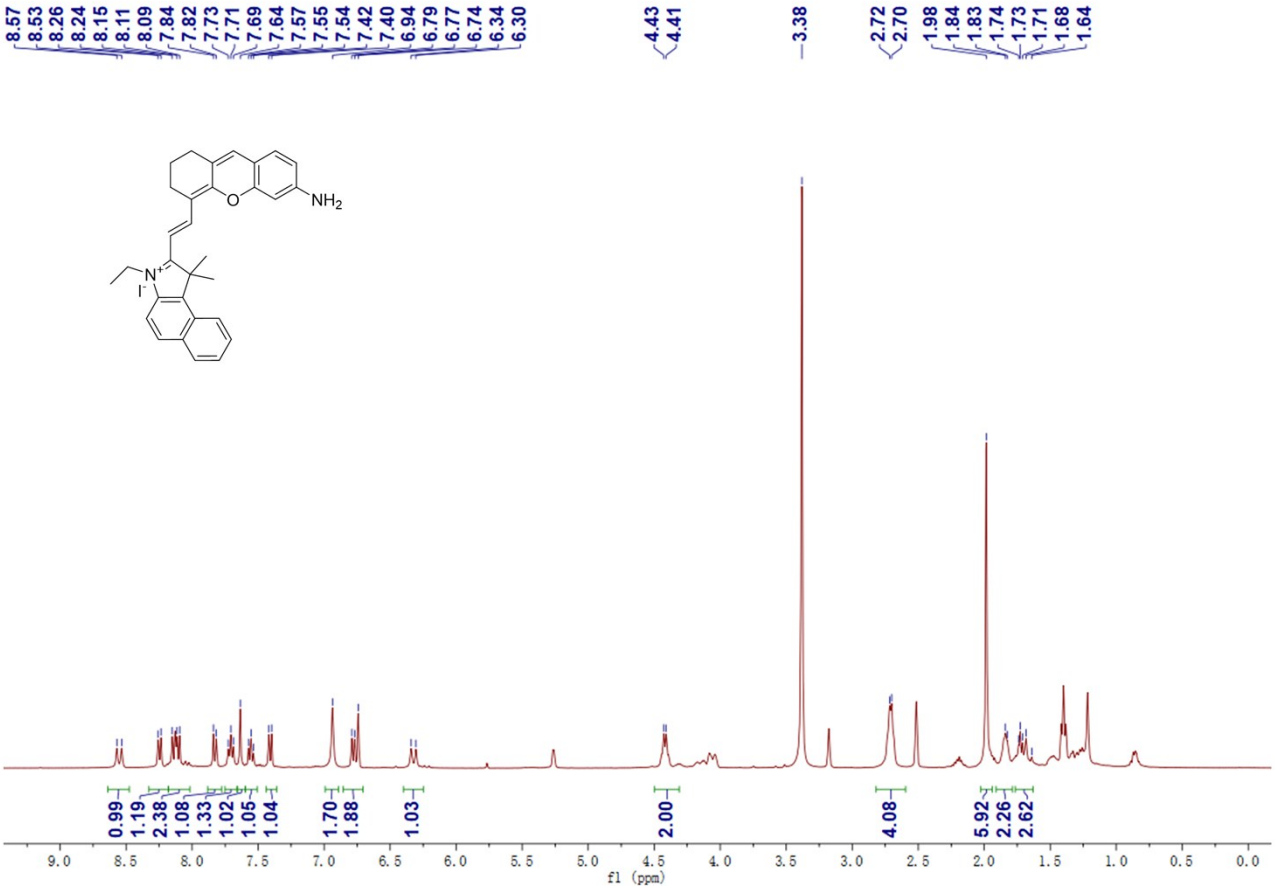


Fig. S7 ^{1}H NMR spectrum of Bcy-NH_2 in D-DMSO.

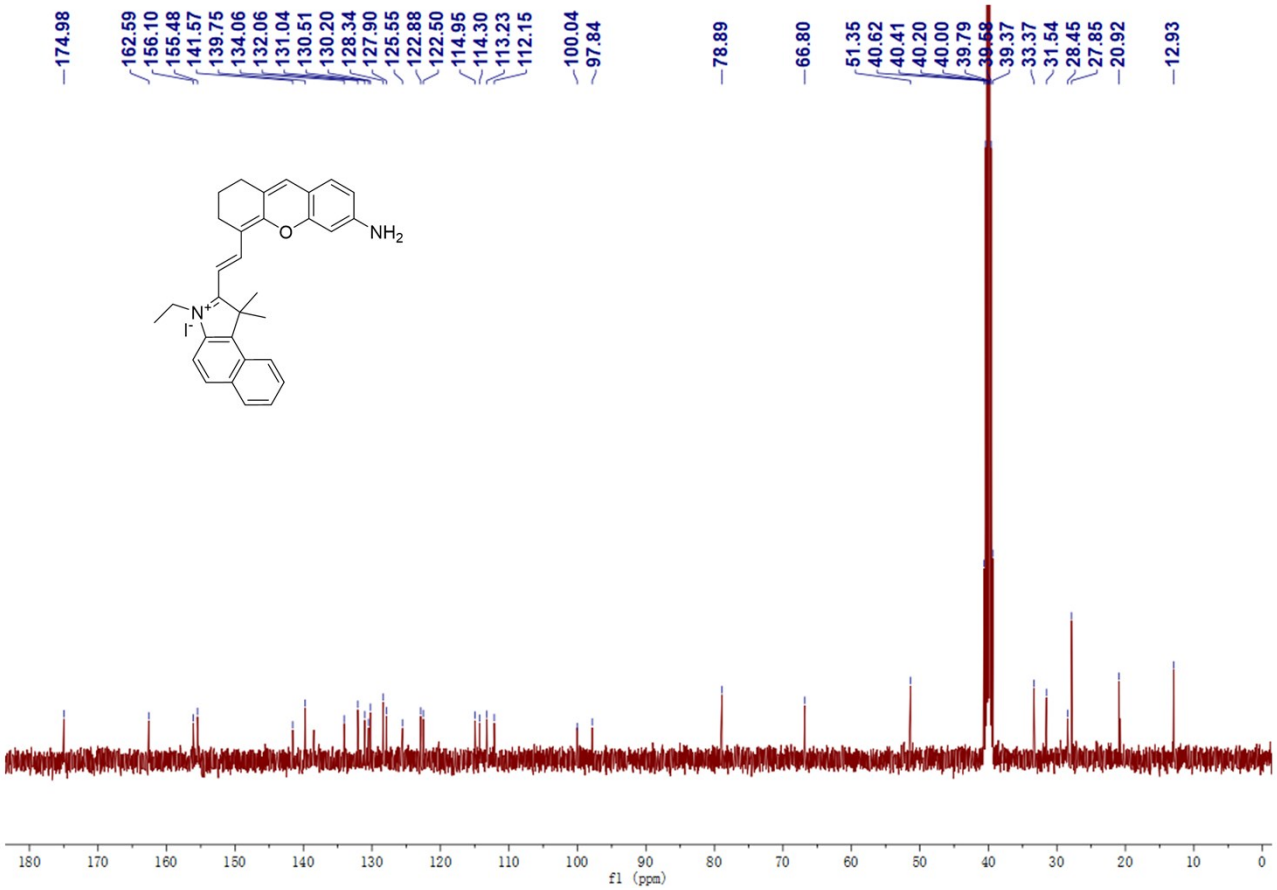


Figure S8. ^{13}C NMR spectrum of Bcy-NH_2 in D-DMSO.

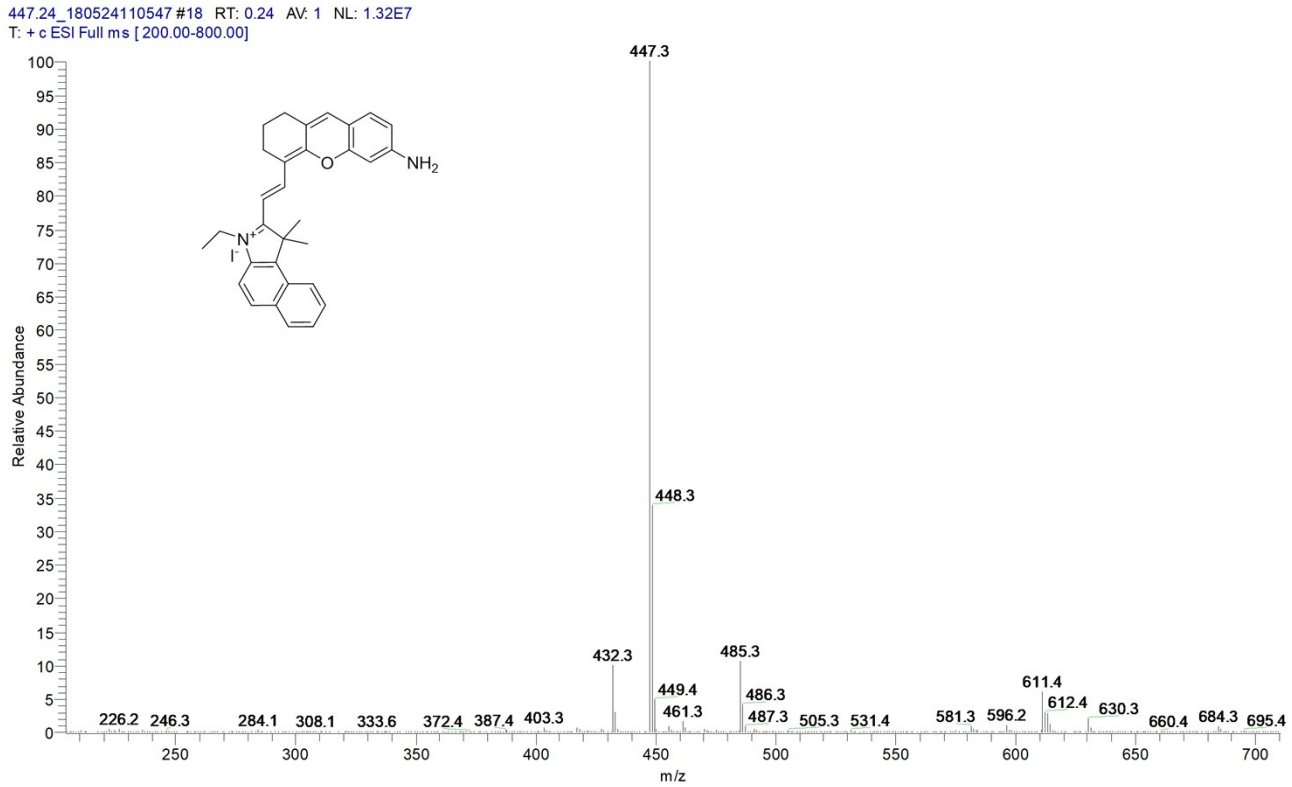


Fig. S9 HRMS (ESI) spectrum of Bcy-NH_2 in methanol.

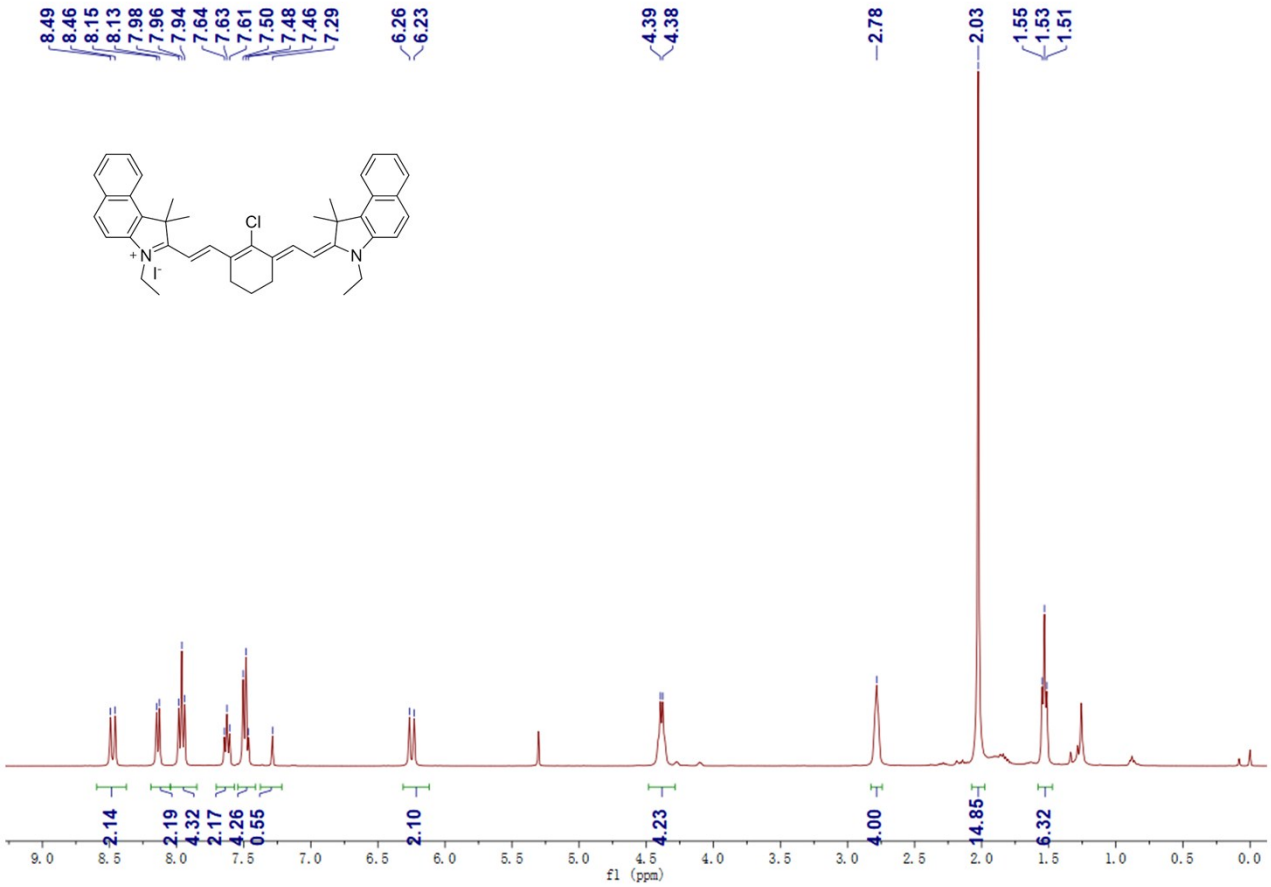


Fig. S10 ^{1}H NMR spectrum of Compound 3 in D-$CDCl_3$.

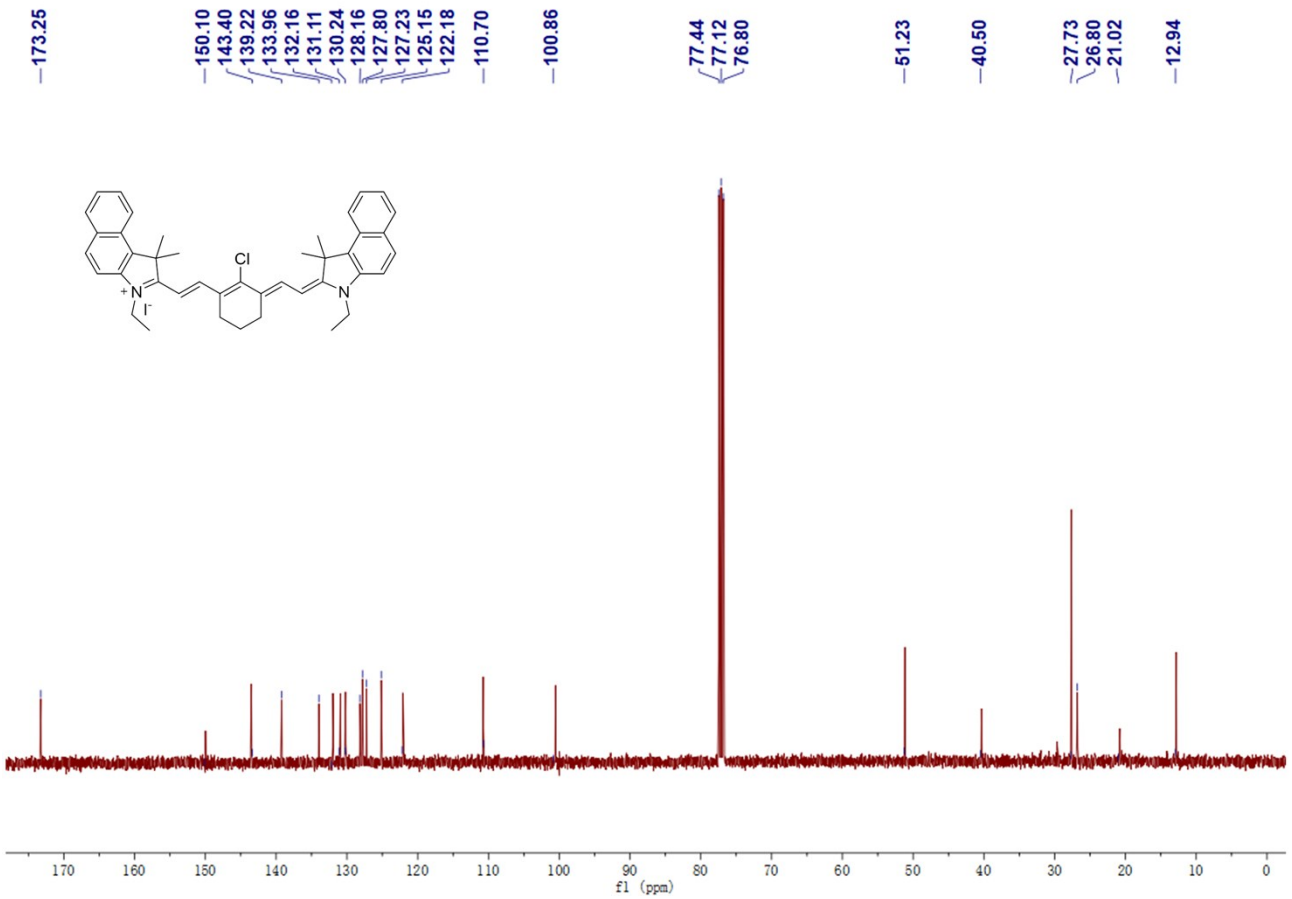


Fig. S11 ^{13}C NMR spectrum of Compound 3 in D-$CDCl_3$.

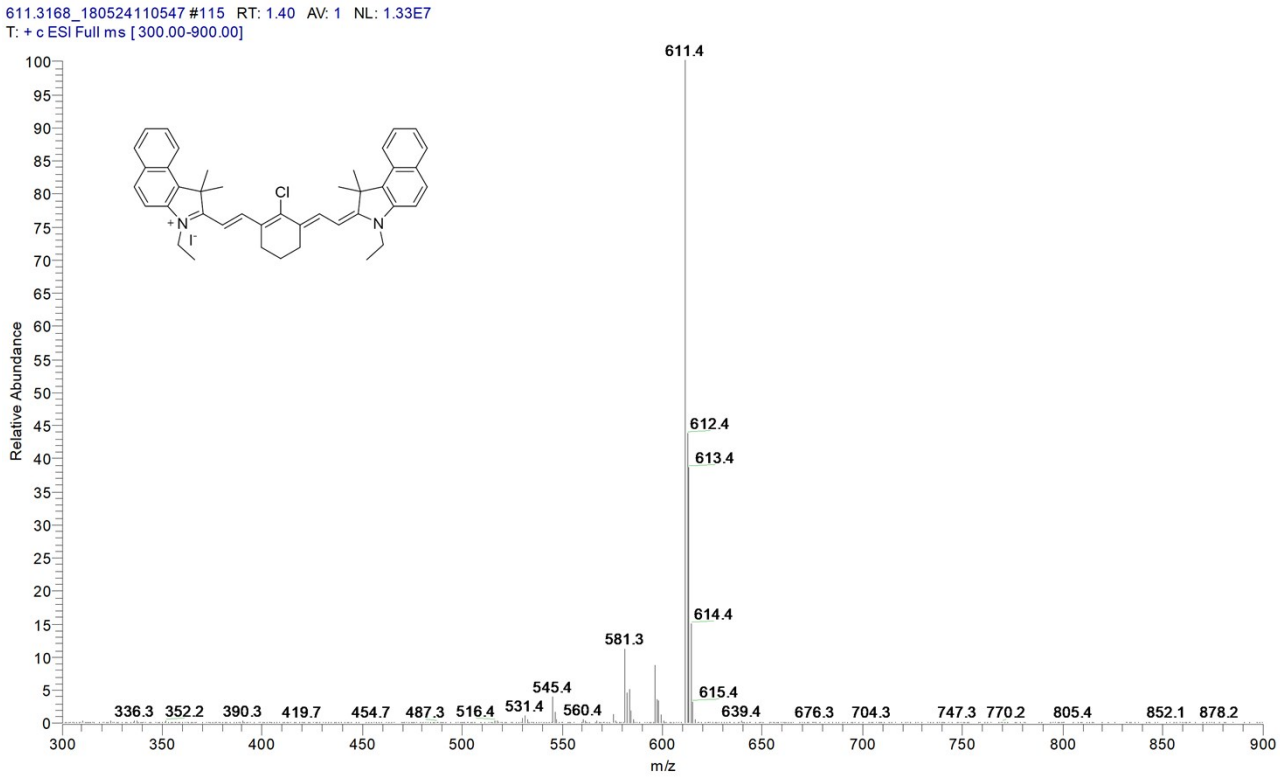


Fig. S12 ESI-MS spectrum of Compound 3 in methanol.

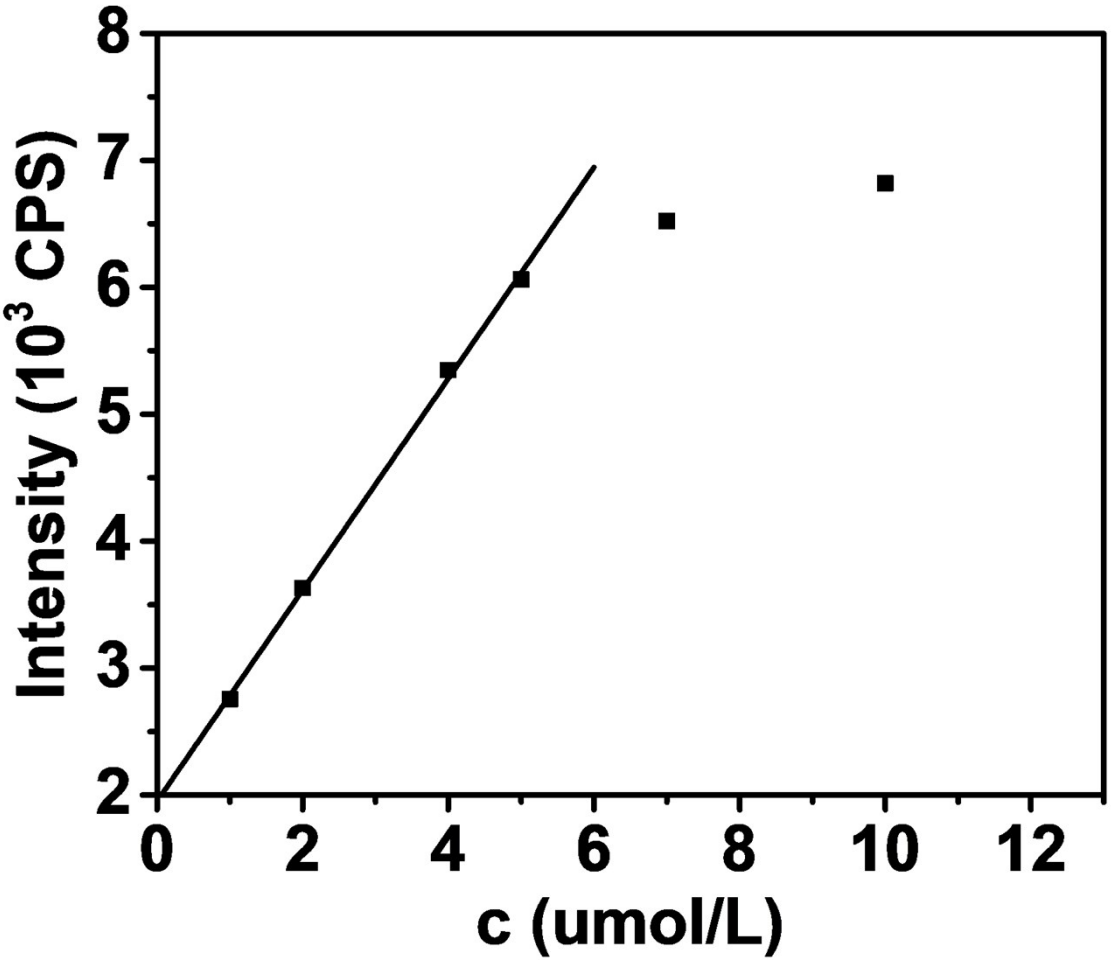


Fig. S13 Plot of fluorescence intensity against the concentrations of Bcy-NH_2. λ_{ex}=680 nm.

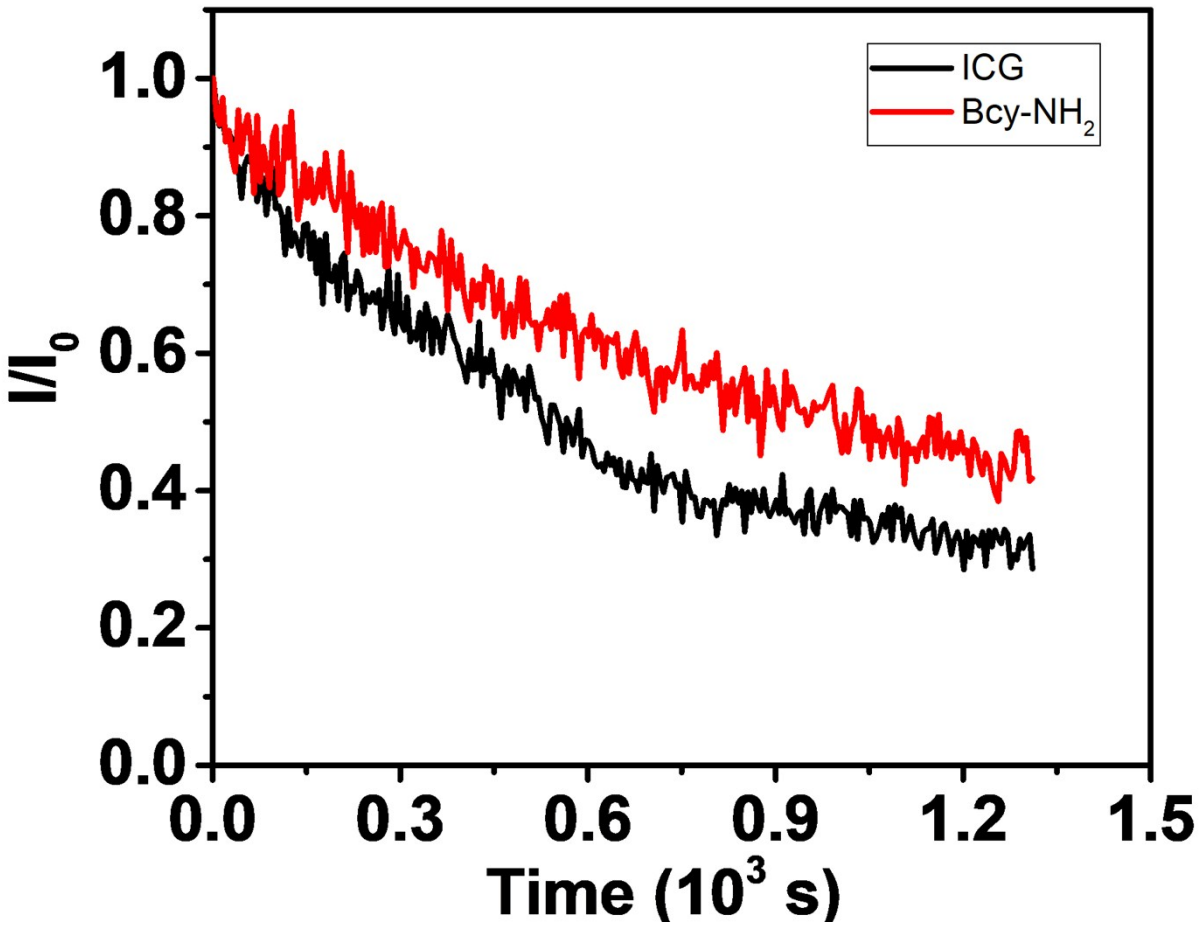


Fig. S14 Photostability study of Bcy-NH2. ICG was chosen as a reference. λ_{ex}=680 nm.

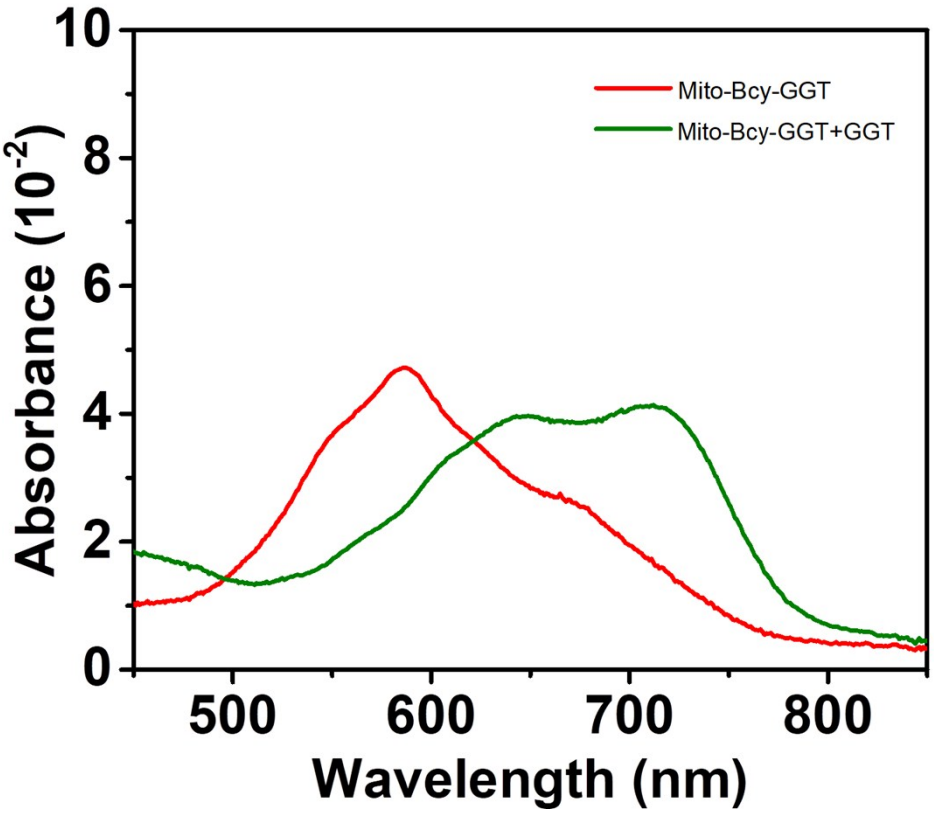


Fig. S15 Absorption spectra of Mito-Bcy-GGT (10 μM) before and after reaction with GGT (800 U/L) at 37 °C for 3 h in PBS (10 mM，pH 7.4), supplemented with 1.5% DMSO. λ_{ex} = 680 nm.

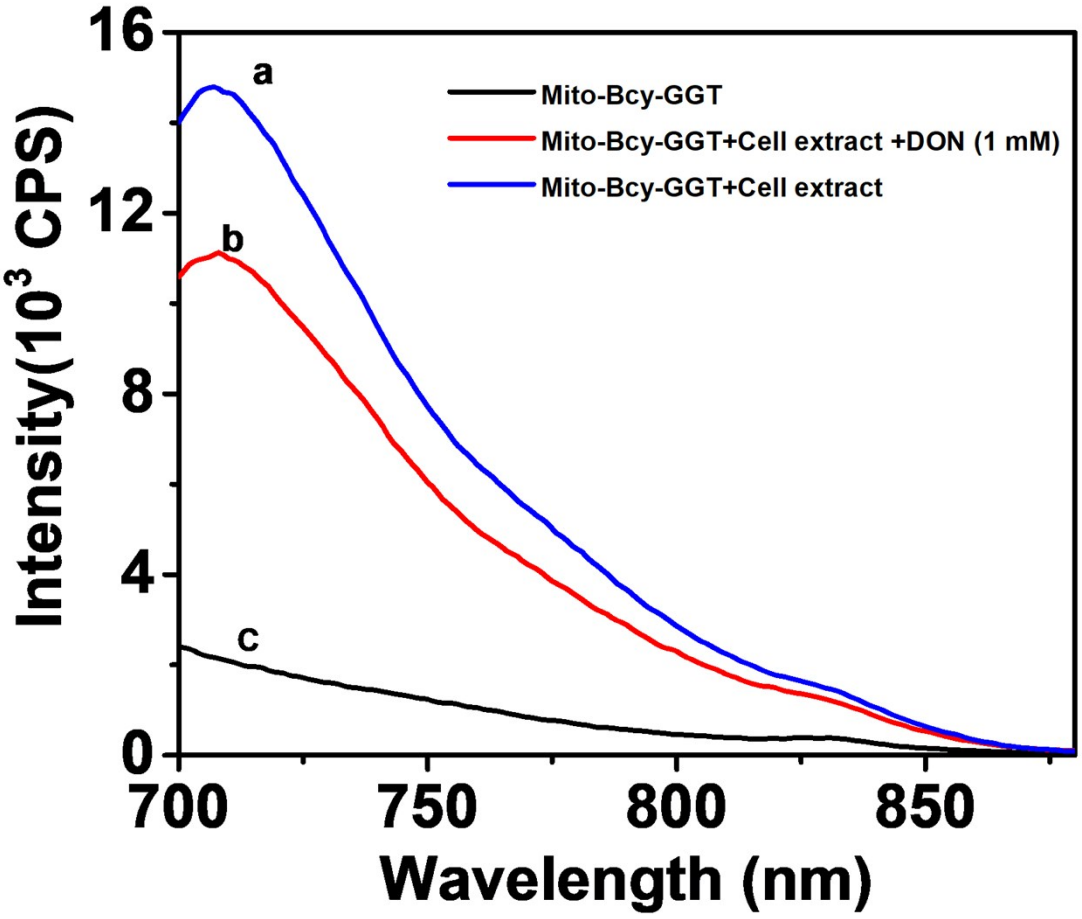


Fig. S16 Fluorescence responses of Mito-Bcy-GGT to cell lysates. (a) Mito-Bcy-GGT (10 μM) + cell extract (2.5 μL); (b) Mito-Bcy-GGT (10 μM) + cell extract (2.5 μL) + DON (1 mM); (c) Mito-Bcy-GGT (10 μM) alone.

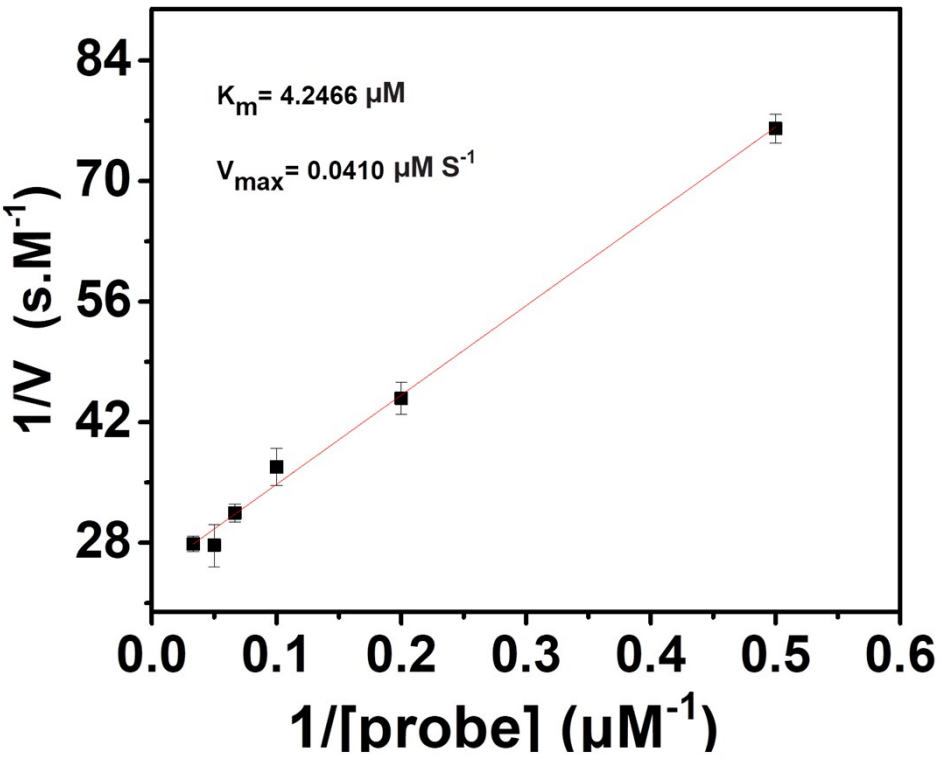


Fig. S17 Lineweaver-Burk plot for the GGT-catalyzed reaction. The Michaelis-Menten equation was described as: $V= V_{max}[probe]/(K_m+ [probe])$, where V is the reaction rate, [probe] is the Mito-Bcy-GGT concentration (substrate), and K_m is the Michaelis constant. Conditions: 800 U/L GGT, 2.0-30 μM of Mito-Bcy-GGT, $\lambda_{ex/em}$= 680/727 nm. Data points were fitted using a linear regression model (correlation coefficient R= 0.991).

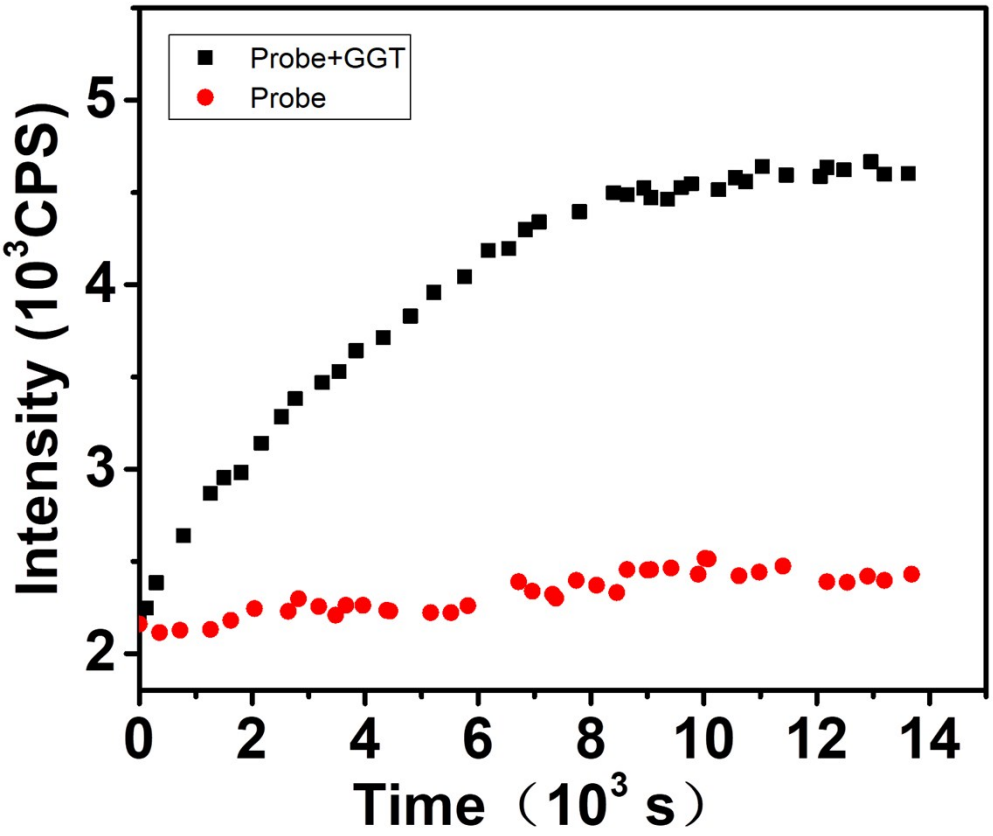


Fig. S18 Real time fluorescence intensity of Mito-Bcy-GGT (10 μM) with and without addition of GGT (800 U/L). $\lambda_{ex/em}$= 680/727 nm.

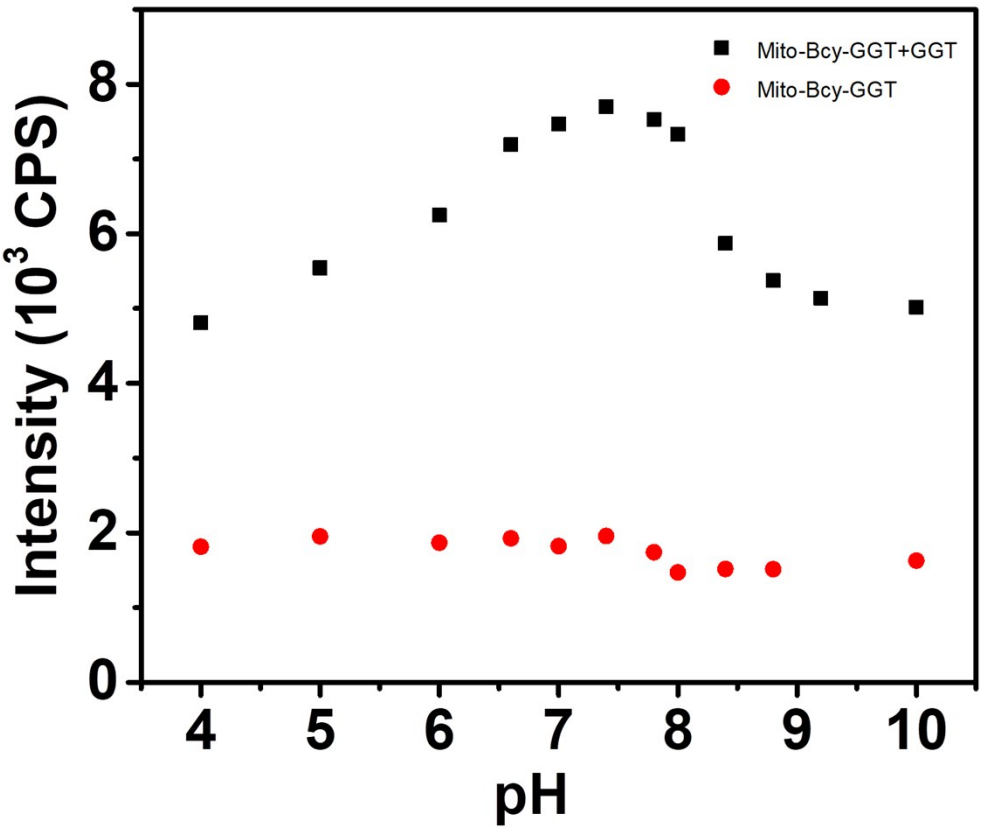


Fig. S19 Effects of pH on the fluorescence signal of Mito-Bcy-GGT (10 μM) without (red dots) and with (black squares) GGT (800 U/L). $\lambda_{ex}/_{em}$= 680/727 nm.

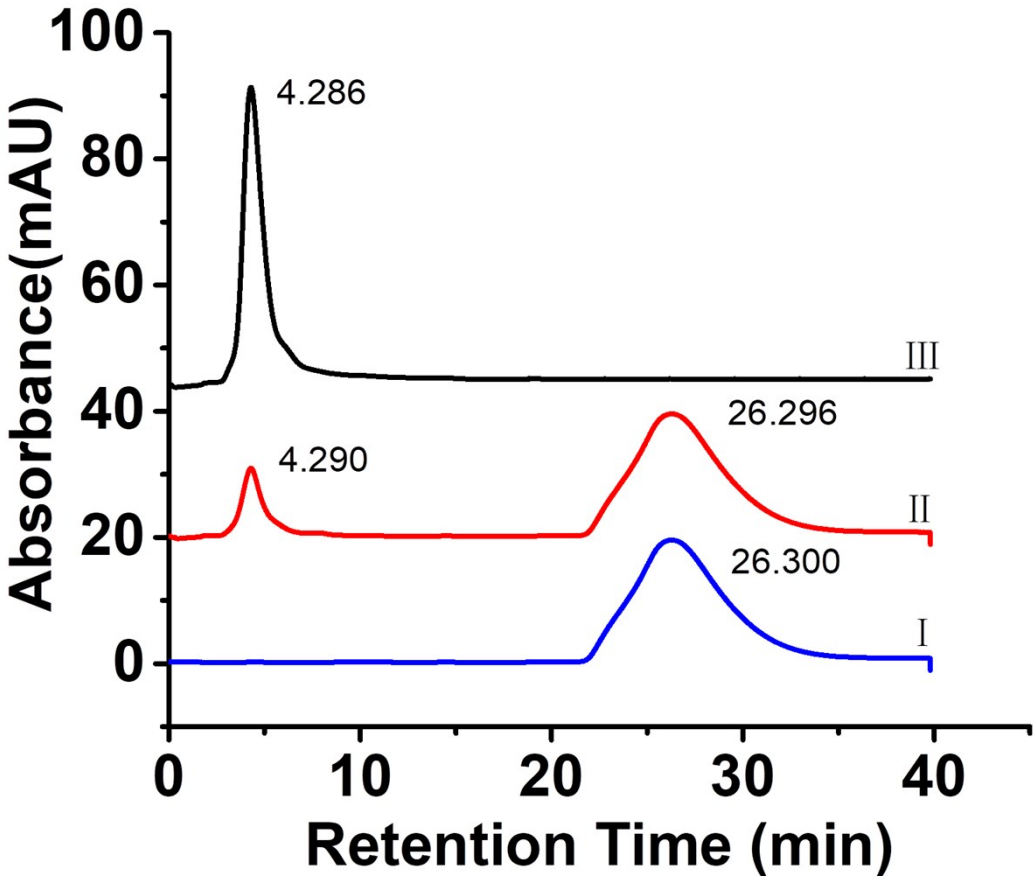


Fig. S20 HPLC profiles of (I) Bcy-NH_2, (II) Mito-Bcy-GGT reacted with GGT for 2 h at 37 ^oC, (III) Mito-Bcy-GGT. Detection wavelength=254 nm.

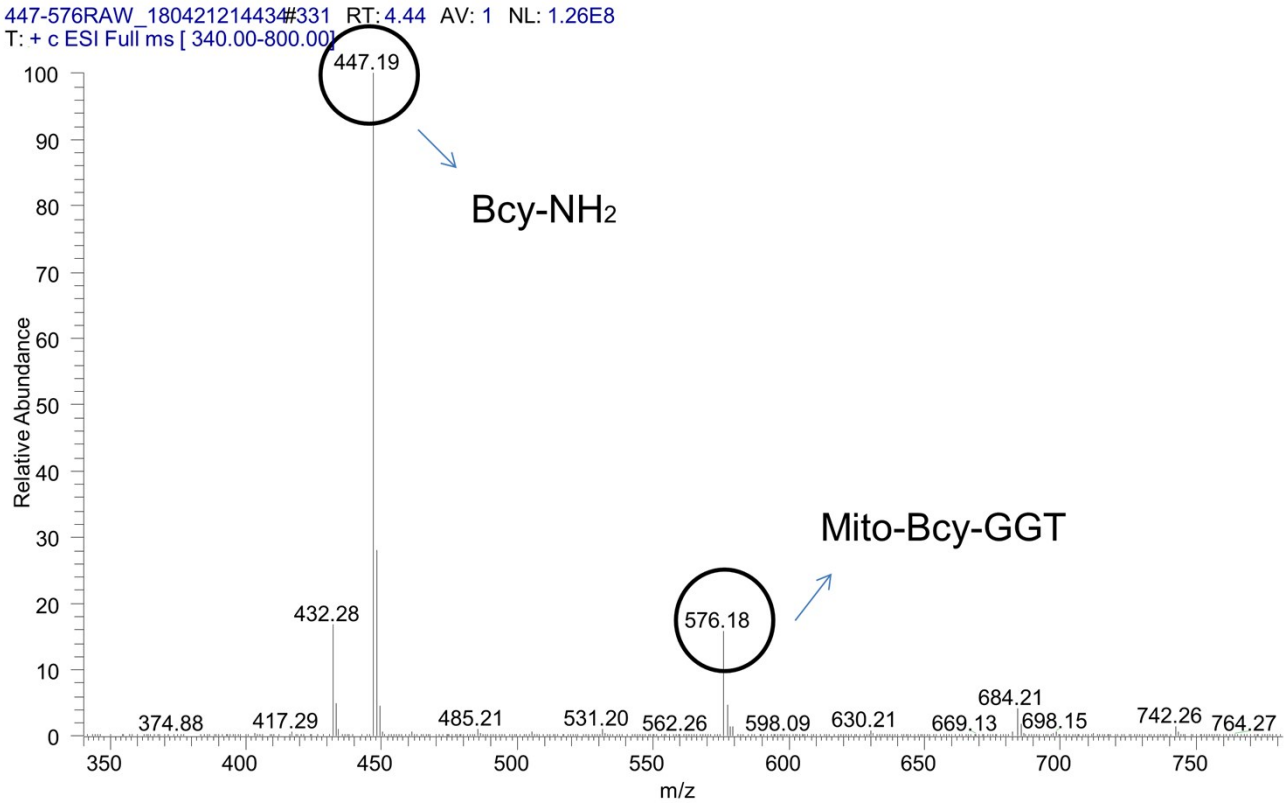


Fig. S21 HRMS (ESI) spectrum of Mito-Bcy-GGT probe reacted with GGT for 3 h at 37 ^{o}C.

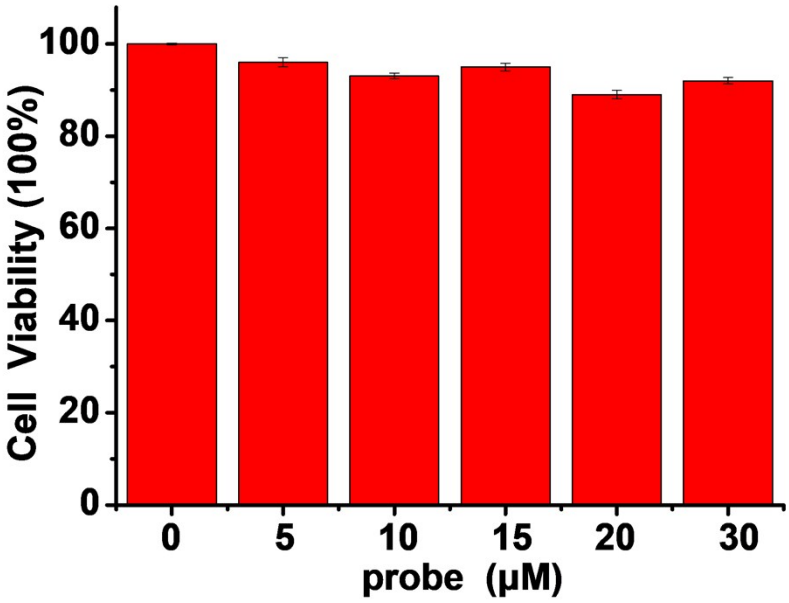


Fig. S22 Cell viability estimated by MTT assay. HepG2 cells were incubated with different concentrations of Mito-Bcy-GGT (0-30 μM) for 3 h. Error bars represent standard deviation of three repeated experiments.

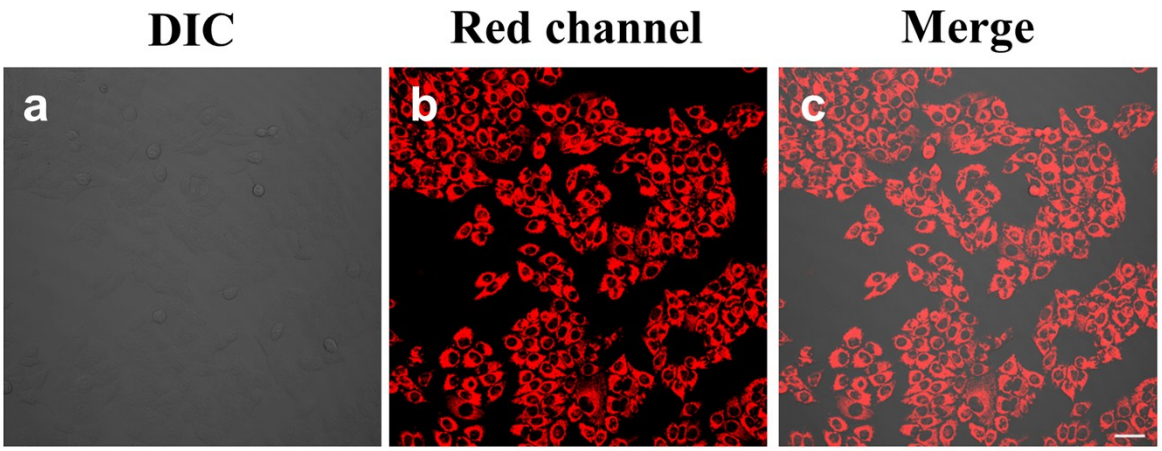


Fig. S23 Confocal images of HepG2 cells. (a) Differential interference contrast (DIC) image. (b) Cells incubated with Mito-Bcy-GGT (5 μM) at 37 °C for 2 h. (c) overlay of (a) and (b). Scale bar=50 μm

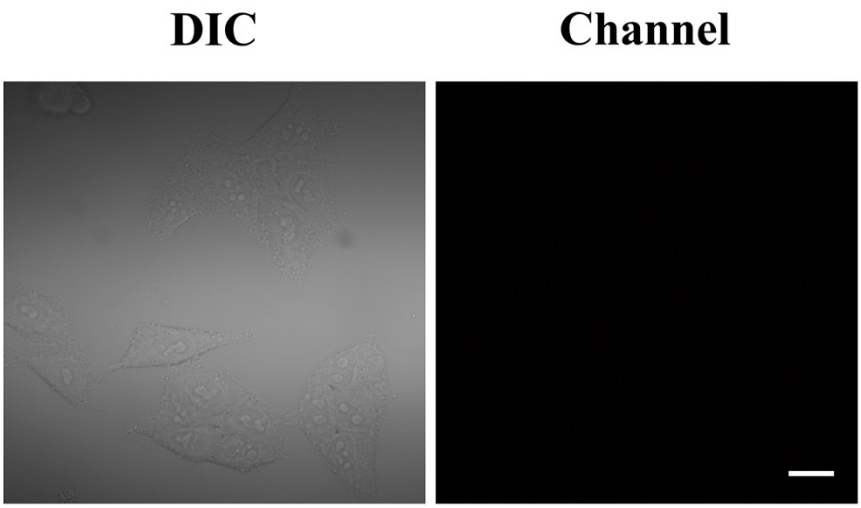


Fig. S24 Confocal image of HepG2 cells. (a) DIC image of HepG2. (b) Fluorescence images of cells alone. Scale bar=20 μm

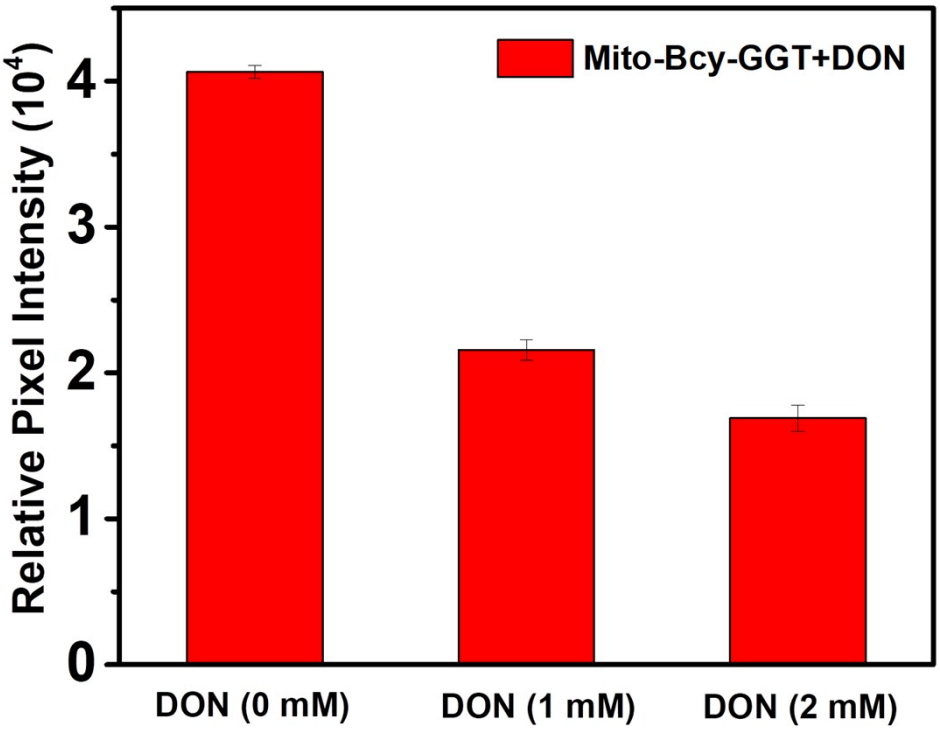


Fig. S25 Relative pixel intensity of the corresponding fluorescence images in (Fig. 4B). The results are the mean ± standard deviation of three separate measurements.

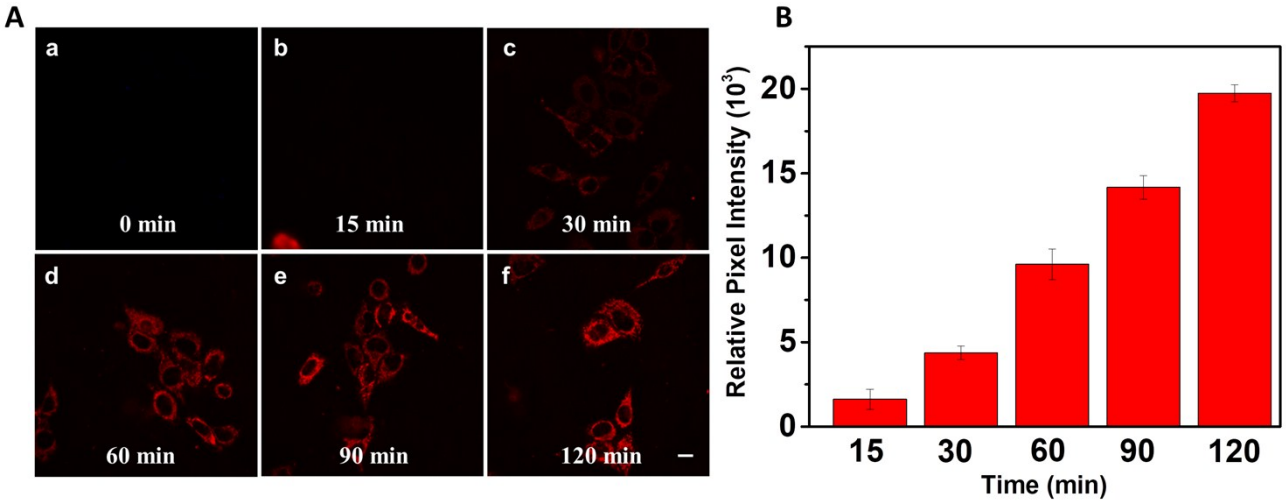


Fig S26 (A) Confocal fluorescence images of HepG2 cells incubated with Mito-Bcy-GGT (5 μM) obtained at different time intervals (0, 15, 30, 60, 90 and 120 min). (B) Relative pixel intensity of the corresponding fluorescence images in (A). The results are the mean ± standard deviation of three separate regions. Scale bar=20 μm