Supporting Information for

Ratiometric Detection of γ-Glutamyltransferase in Human Colon Cancer Tissues using a Two-photon Probe

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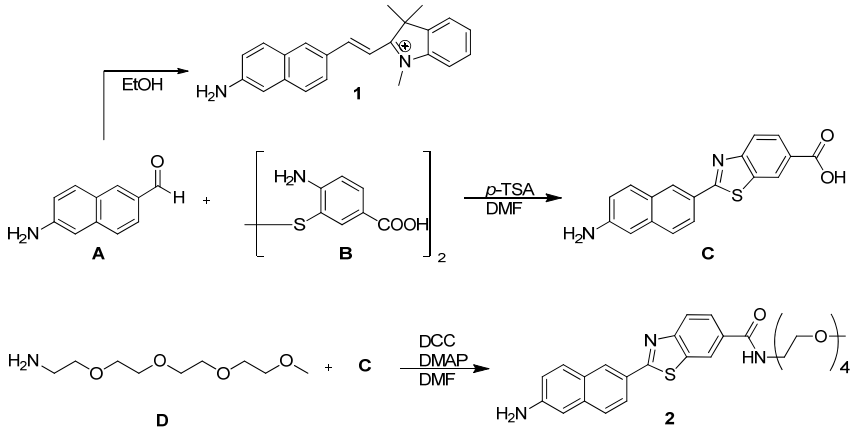
Synthesis of 1 and 2. Compound **A**, **B** and **D** were prepared by the literature method.[1]



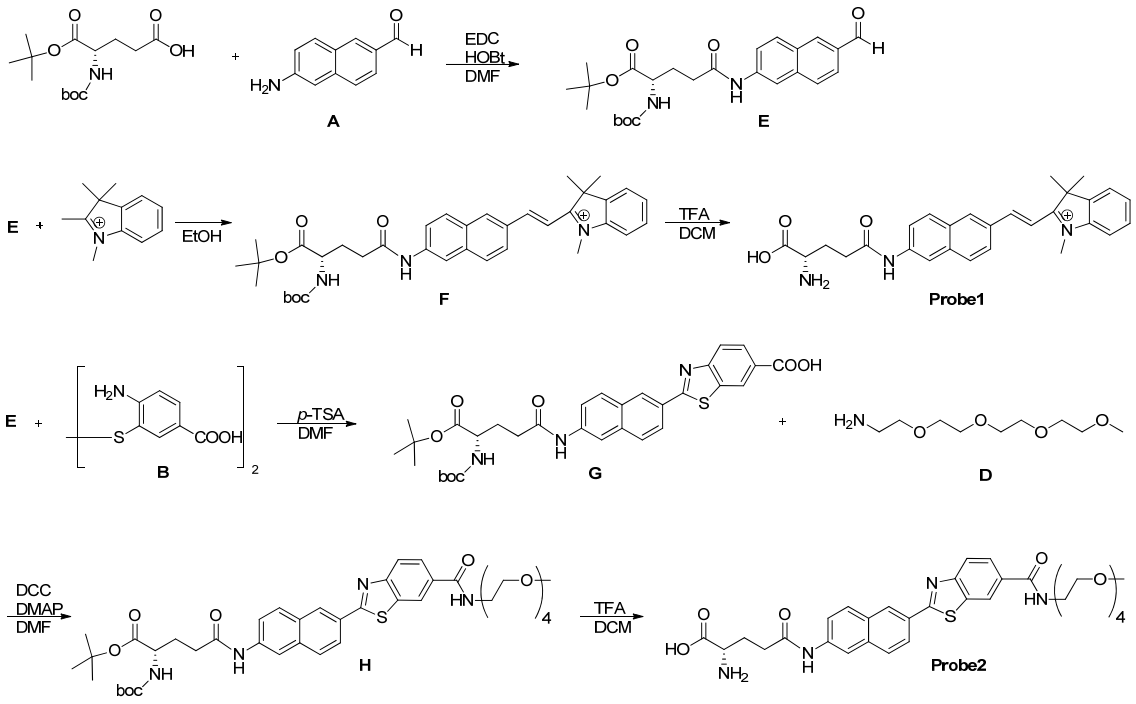
Compound 1 A mixture of **A** (100 mg, 0.58 mmol) and 1,2,3,3-Tetramethyl-3*H*-indolium iodide (127 mg, 0.73 mmol) was stirred overnight under nitrogen atmosphere at 90 °C. Then reaction mixture was concentrated under reduced pressure and purified with column chromatography (DCM: MeOH = 95:5), Yield: 80 mg (38 %), ^{1}H NMR (600 MHz, CD_3OD) δ 8.48 (d, 1H, *J* = 16.2 Hz), 8.28 (s, 1H), 7.96 (dd, 1H, *J* = 2.4 Hz, 7.8 Hz), 7.60 (d, 1H, *J* = 9 Hz), 7.79 (d, 1H, *J* = 9 Hz), 7.72 (m, 2H), 7.59 (m, 3H), 7.51 (s, 1H, *J* = 16.2 Hz), 7.03 (dd, 1H, *J* = 8.4 Hz, 2.4 Hz), 6.92 (d, 1H, *J* = 2.4 Hz), 4.09 (s, 3H), 1.84 (s, 6H). HRMS (ESI+): m/z calculated for [$C_{23}H_{23}N_2$] 327.1856, found: 327.1864

Compound C compound **A** (116 mg, 0.68 mmol), **B** (124 mg, 0.68 mmol) and *p*-TSA was dissolved in DMF. The mixture was refluxed overnight at 80 °C. After evaporation of solvent, the residue was purified with column chromatography (DCM: MeOH = 95:5) Yield: 22 mg (10 %), ^{1}H NMR (600 MHz, CD_3OD) δ 8.46 (s, 1H), 8.40 (s, 1H), 8.14 (d, 1H, *J* = 8.4 Hz), 8.01 (d, 2H, *J* = 7.8 Hz), 7.68 (d, 1H, *J* = 8.4 Hz), 7.64 (d, 1H, *J* = 9 Hz), 7.06 (d, 1H, *J* = 9 Hz), 6.98 (s, 1H), 4.55 (s, 2H)

Compound 2 The compound **C** (10 mg, 0.03 mmol) and 1-Ethyl-3-(3-dimethylaminopropyl)- carbodiimide (EDC) (5.8 mg, 0.03 mmol), 4-Dimethylaminopyridine (DMAP) (5.3 mg, 0.03 mmol) was dissolved in DMF and stirred for 1 hour. Dropwise compound **D** (12.4 mg, 0.06 mmol) dissolved in DMF at room temperature and stirred for

12 hours. The mixture was concentrated under reduced pressure and extracted with H_2O/DCM. The organic phase was concentrated under reduced pressure and purified with column chromatography (DCM: MeOH = 97:3). Yield 6 mg (40 %), ^{1}H NMR (600 MHz, $(CD_3)_2SO$) δ 8.56 (s, 1H), 8.35 (s, 1H), 8.02 (d, 1H, *J* = 8.4 Hz), 7.97 (d, 1H, *J* = 9 Hz), 7.79 (d, 1H, *J* = 9 Hz), 7.60 (d, 1H, *J* = 8.4 Hz), 6.98 (d, 1H, *J* =7.2 Hz), 6.82 (s, 1H), 5.81 (s, 2H), 3.53–3.42 (m, 16H), 3.35 (m, 3H). HRMS (ESI+): m/z calculated for $[C_{27}H_{32}O_5N_3S]^+$: 510.2057, found: 510.2069

Synthesis of Probe1 and Probe2



Compound E The mixture of N-Boc-Glu-OtBu (1.0 g, 3.29 mmol) and 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (830 mg, 4.33 mmol), Hydroxybenzotriazole (HOBt) (1.3 g, 8.49 mmol) was dissolved in Dimethylformamide (DMF) (3 mL) and stirred it for 1 hour. Dropwise the compound **A** (500 mg, 2.92 mmol) dissolved in DMF into the flask and stirred it overnight at 70 ℃. The mixture was concentrated in reduced pressure and extract with H_2O/DCM. The organic phase was concentrated under reduced pressure and purified with column

chromatography ($CHCl_3$: MeOH = 98:2). Yield: 675 mg (65 %), ^{1}H NMR (600 MHz, CD_3OD) δ 10.01 (s, 1H), 8.30 (d, 1H, *J* = 13.8 Hz), 7.93 (d, 1H, *J* = 9 Hz), 7.83 (m, 3H), 7.63 (d, 1H, *J* = 7.8 Hz), 4.10 (m, 1H), 2.52 (m, 1H), 2.20 (m, 1H), 1.98 (m, 1H), 1.46 (s, 9H), 1.40 (s, 9H)

Compound F The mixture of **E** (300 mg, 0.84 mmol) and 1,2,3,3-Tetramethyl-3*H*-indolium iodide (147 mg, 0.84 mmol) was stirred overnight. The mixture was concentrated in reduced pressure and purified with column chromatography (DCM: MeOH = 95:5). Yield 103 mg (20 %) ^{1}H NMR (600 MHz, CD_3OD) δ 8.55 (d, 1H, *J* = 16.2 Hz), 8.45 (s, 1H), 8.37 (s, 1H), 8.13 (d, 1H, *J* = 8.4 Hz), 7.97 (d, 1H, *J* = 9 Hz), 7.90 (d, 1H, *J* = 9 Hz), 7.81 (m, 1H), 7.76 (m, 1H), 7.73 (s, 1H), 7.70 (s, 1H), 7.66 (d, 1H, *J* = 9 Hz), 7.42 (t, 1H, *J* = 6.6 Hz), 4.21 (s, 1H), 4.09 (q, 1H, *J* = 4.8 Hz), 2.58 (t, 2H, *J* = 7.2 Hz), 2.23 (m, 1H), 2.01 (m, 1H), 1.90 (s, 1H), 1.49 (s, 9H), 1.42 (s, 9H)

Probe1 Compound **F** (50 mg, 0.082 mmol) was dissolved in DCM (1 mL) and stirred for 30 min. TFA (1 mL) was added at 0 ℃ and the mixture was stirred overnight. The mixture was concentrated in reduced pressure and recrystalized with acetone. Yield 15 mg (40 %) ^{1}H NMR (600 MHz, $(CD_3)_2SO$) δ 10.68 (s, 1H) 8.62 (s, 1H), 8.53 (d, 1H, *J* = 16.2 Hz), 8.41 (s, 1H), 8.28 (d, 1H, *J* = 8.4 Hz), 7.95 (d, 2H, *J* = 7.8 Hz), 7.86 (m, 2H), 7.72 (d, 1H, *J* = 16.2 Hz), 7.67 (d, 1H, *J* = 9 Hz), 7.60 (m, 2H), 5.19 (s. 3H), 4.14 (s, 3H), 3.99 (q, 1H, *J* = 7.2 Hz), 2.58 (m, 2H), 2.02 (m, 2H), 1.79 (s, 6H). HRMS (ESI+): m/z calculated for $[C_{28}H_{30}O_3N_3]$: 456.2293 and found 456.2282

Compound G Compound **E** (300 mg, 0.84 mmol), **B (**154 mg, 0.84 mmol) and *p*-TSA was dissolved in DMF. The mixture was refluxed and stirred overnight at 80 °C. After evaporation of solvent, the residue was purified with column chromatography (DCM: MeOH = 97:3), Yield 66 mg (13 %) ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.55 (s, 1H), 8.40 (s, 1H), 8.27 (s, 1H), 8.09 (d, 1H, *J* = 7.8 Hz), 8.02 (d, 1H, *J* = 8.4 Hz), 7.99 (d, 1H, *J* = 7.2 Hz) 7.82 (s, 2H), 7.51 (t, 1H, *J* = 7.2 Hz), 4.12 (d, 1H, *J* = 5.4 Hz), 2.44 (d, 2H, *J* = 5.4 Hz), 2.20 (m, 2H), 1.40 (s, 9H), 1.39 (s, 9H),

Compound H The mixture of **G** (110 mg, 0.22 mmol) and *N*,*N'*-Dicyclohexylcarbodiimide (DCC) (68 mg, 0.33 mmol), and Hydroxybenzotriazole (HOBt) (46 mg, 0.44 mmol) was stirred for 1 hour. Compound **D** (45 mg, 0.22 mmol) was slowly added and stirred overnight at room temperature. The mixture was concentrated in reduced vacuum and added MeCN. The solid was removed and the residue was concentrated in reduced vacuum and purified with column chromatography ($CHCl_3$: MeOH = 97:3) ,Yield 60 mg (40 %), ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.54 (s, 1H), 8.41 (d, 2H, *J* =7.2 Hz), 8.24 (s, 1H), 8.06 (d, 1H, *J* = 8.4 Hz), 7.98 (d, 1H, *J* = 8.4 Hz), 7.91 (d, 1H, *J* = 9 Hz), 7.81(d, 1H, *J* = 9 Hz), 7.57 (dd, 1H, *J* = 1.2 Hz, 9 Hz), 6.89 (d, 1H, *J* = 7.8 Hz), 4.06 (q, 1H, *J* = 7.8 Hz), 3.68–3.56 (m, 16H), 3.44 (m, 3H), 2.53 (m, 2H), 2.20(m, 1H), 1.99 (m, 1H), 1.46 (s, 9H), 1.44 (s, 9H)

Probe2 Compound **H** (25 mg, 0.05 mmol) was dissolved in DCM (1 mL) and stirred for 30min. TFA (1 mL) was added at 0 ℃ and the mixture was stirred overnight. The mixture was concentrated in reduced pressure and recrystalized with acetone. Yield 10 mg (40 %) ^{1}H NMR (600 MHz, CD_3OD) δ 8.38 (d, 2H, *J* = 13.8 Hz), 8.24 (d, 1H, *J* = 12 Hz), 8.04 (d, 1H, *J* = 9 Hz), 7.98 (m, 1H), 7.91 (dd, 1H, *J* = 3.6 Hz, 8.4 Hz), 7.87(d, 1H, *J* = 7.2 Hz), 7.79 (d, 1H, *J* = 9 Hz), 7.57(m, 1H), 4.12 (q, 1H, *J* = 6.5 Hz), 3.62–3.52(m, 16H), 3.45 (m, 3H), 2.74 (m, 2H), 2.29 (m, 2H) HRMS (ESI+): m/z calculated for [$C_{32}H_{39}O_8N_4S$]: 639.2483 found 639.2500

Spectroscopic measurements Absorption spectra were recorded on a S-3100 UV-Vis spectrophotometer and fluorescence spectra were obtained with FluoroMate FS-2 fluorescence spectrophotometer with a 1 cm standard quartz cell. The fluorescence quantum yield was determined by using Rhodamine (Φ = 0.95 in MeOH) for **Probe1** and 9, 10-diphenylanthrancene (Φ = 0.93 in cyclohexane) for **Probe2** as the reference by the literature method.[2,3]

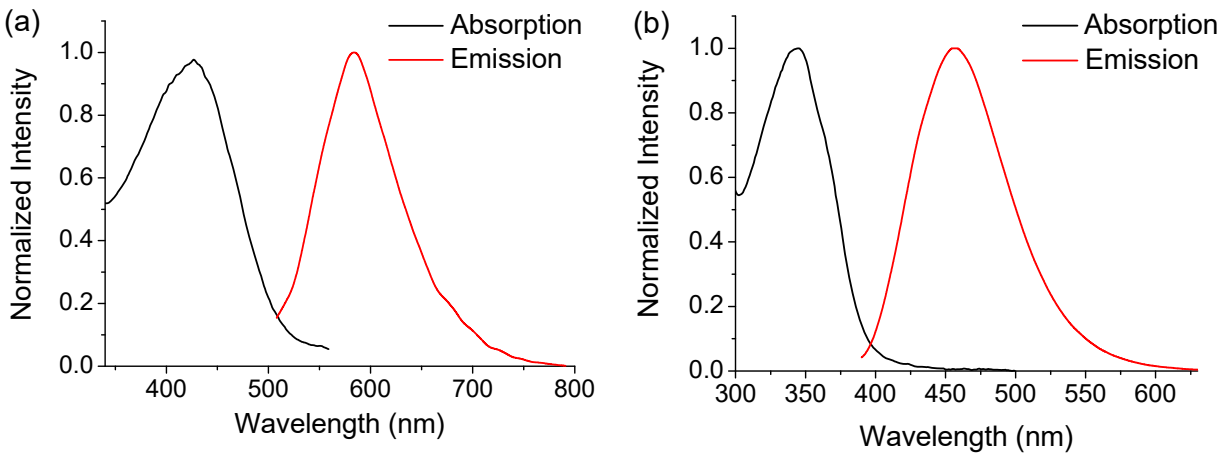


Figure S1. Normalized one-photon absorption and emission spectra of (a) **Probe1** and (b) **Probe2** in PBS buffer (10 mM, pH 7.4).

Water solubility Small amount of each probe was dissolved in DMSO to prepare the stock solutions (1.0×10^{-2} M). The solution was diluted to $1.0 \times 10^{-5} \sim 0.1 \times 10^{-6}$ M and added to a cuvette containing 3.0 mL of PBS buffer (10 mM, pH 7.4). In all cases, the concentration of DMSO in buffer was maintained to be 0.1 %.[4] The plot of absorbance against the dye concentration were linear at low concentration and showed downward curvature at higher concentration (Figure S2). The maximum concentration in the linear region was taken as the solubility. The solubility of **Probe1** was 1.0 μM and that of **Probe2** was 2.0 μM in PBS buffer

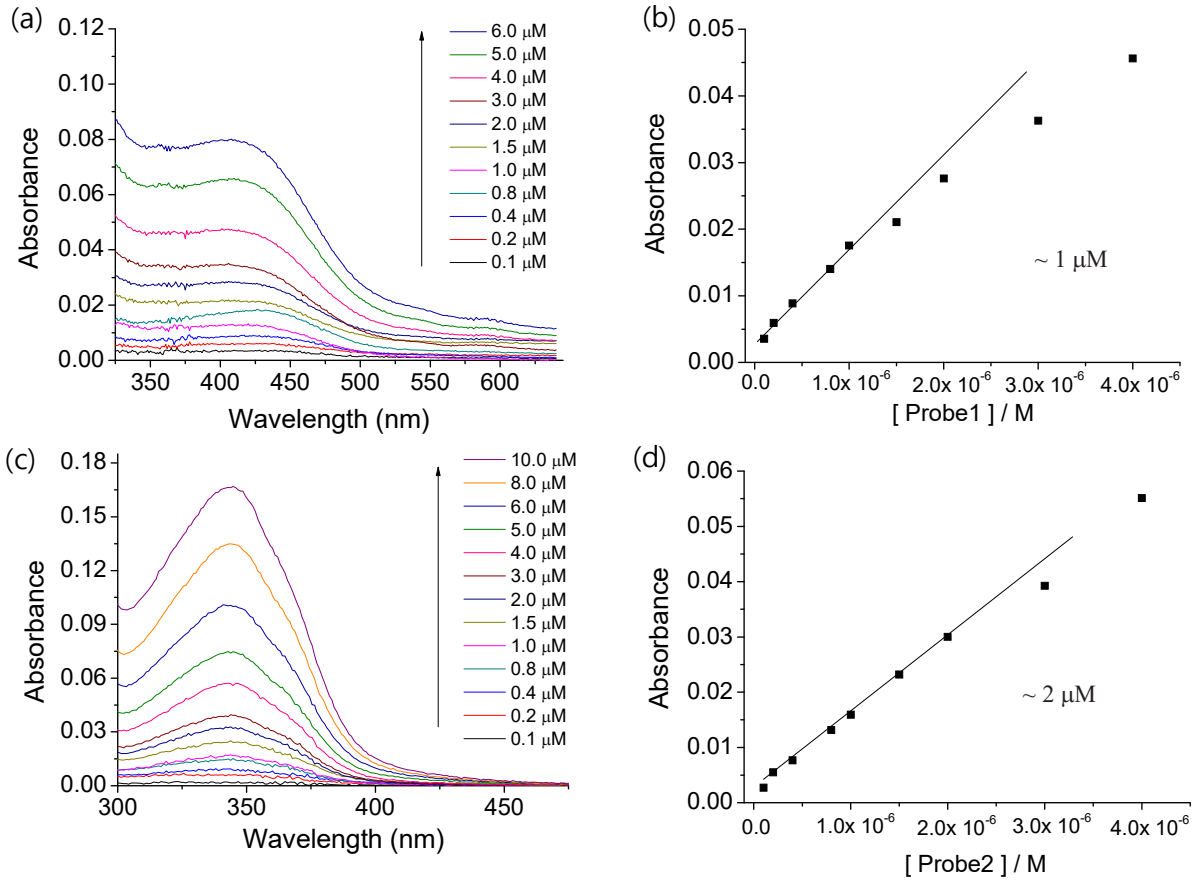


Figure S2. One-photon absorption spectra of (a) **Probe1** and (c) **Probe2** in 10 mM PBS buffer (pH 7.4). Graph of absorbance in different concentration of (b) **Probe1** and (d) **Probe2**.

Enzyme kinetic assays Enzymatic kinetic experiments were investigated using a Varioskan Flash micro plate reader (6–1536 well) with a 96-well plate. Various concentrations of **Probe2** (0.1–10 μM) were prepared in PBS buffer solution (10 mM, pH = 7.4). γ-Glutamyltransferase was added to a final concentration of 100 U/L, the fluorescence intensity was collected at 465 nm for **Probe2** (λ_{ex} = 385 nm) with 60 second intervals from 0 to 30 min at 37 ºC. The kinetic parameters of Michaelis-Menten equation were calculated with hyperbolic function by the nonlinear fitting algorithm (OriginPro 8.0). Kinetic parameter for **Probe1** was not determined because of very weak fluorescence in PBS buffer.

Table S1. Kinetic parameters for **Probe2** with GGT

Compound	K_m (μM)	k_{cat} (ms^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	V_{max} (μM s^{-1})
Probe1	n.d.[a]	n.d. [a]	n.d. [a]	n.d. [a]
Probe2	1.37	0.607	4.43 x 10^2	1.05

a) Not determined.

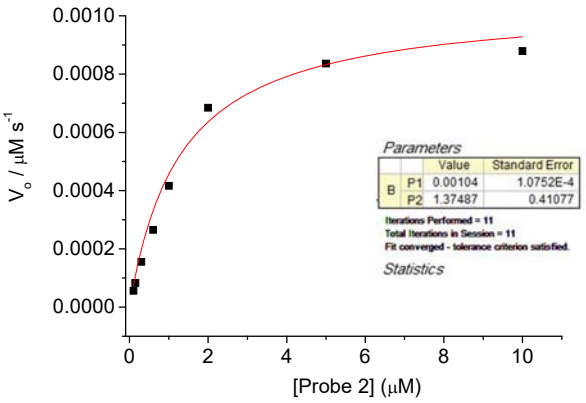


Figure S3. Kinetics of **Probe2** (0.1–10 μM) with GGT (100 U/L) in PBS buffer (10 mM, pH 7.4) at 385 nm excitation.

Measurement of two-photon cross section. The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique.[5] **Probe1**(1.0×10^{-6} M) and **Probe2** (1.0×10^{-6} M) was dissolved in PBS buffer (10 mM, pH = 7.4) respectively and the two-photon induced fluorescence intensity was measured at 760–1020 nm (**Probe1**) and 700–890 nm (**Probe2**) using rhodamine 6G as the reference, whose two-photon property has been well characterized in the literature.[6] The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using $\delta = \delta_r(S_s\Phi_r\varphi_r c_r)/(S_r\Phi_s\varphi_s c_s)$: where the subscripts s and r stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as S. Φ is the fluorescence quantum yield. φ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as c. δ_r is the TPA cross section of the reference molecule.

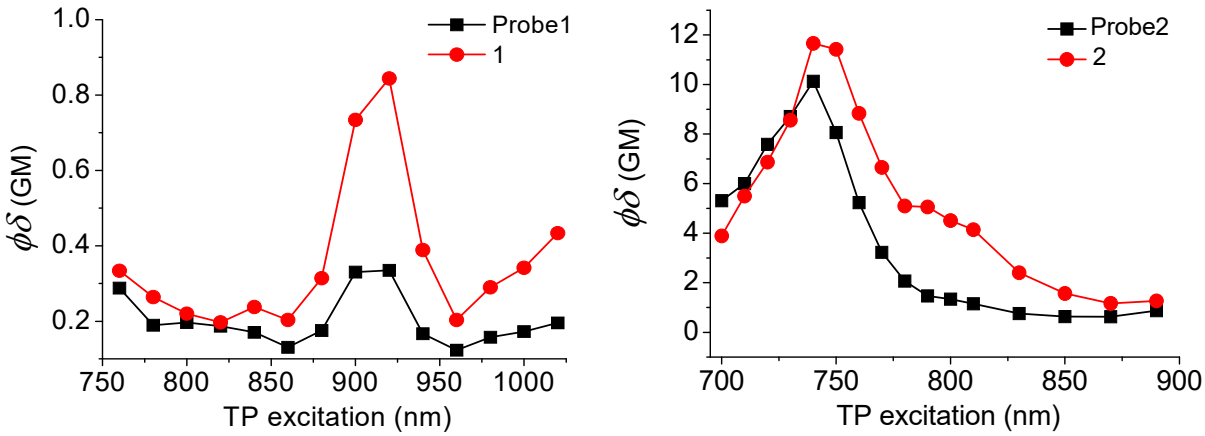


Figure S4. Two-photon action spectra of **Probe1**, **1**, **Probe2** and **2** in PBS buffer (10 mM, pH 7.4).

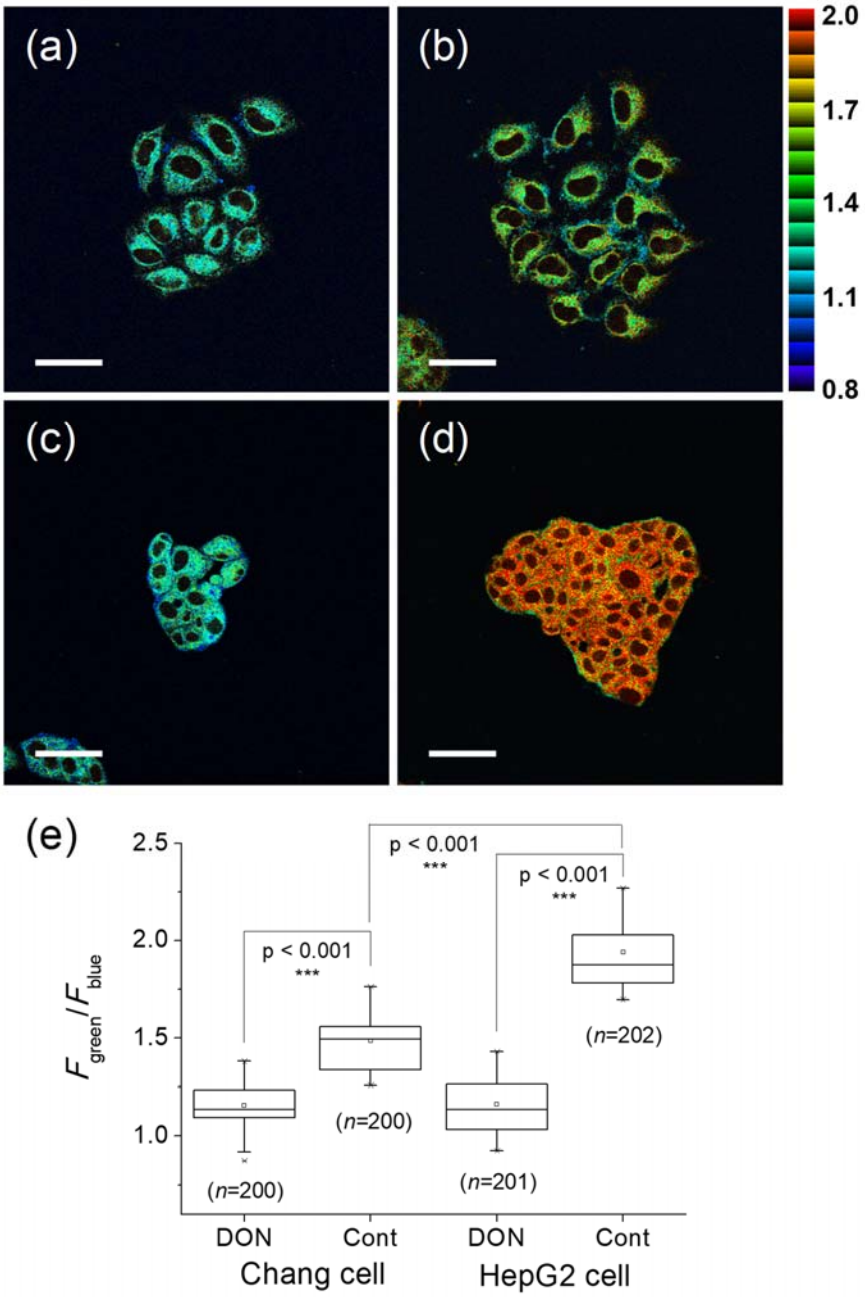


Figure S5. Pseudocolored ratiometric images of (a, b) Chang cells and (c, d) HepG2 cells incubated with **Probe2** (2.0 μM) for 90 min. DON (1 mM) was pretreated for 1 h and incubated with **Probe2**. (e) Average F_{green}/F_{blue} intensity ratios. Images were collected at 750 nm excitation and emission windows of 400–450 nm (blue) and 500–600 nm (green). Scale bars, 50 μm. *n* is number of counted cells.

Photostability. Photostability of **Probe2** was determined by monitoring the changes in two-photon emission fluorescence (TPEF) intensity with time at three designated positions of **Probe2** (2.0 μM) in PBS buffer (10 mM, pH 7.4). The TPEF intensity of **Probe2** remained nearly the same for 30 min, indicating high photostability.

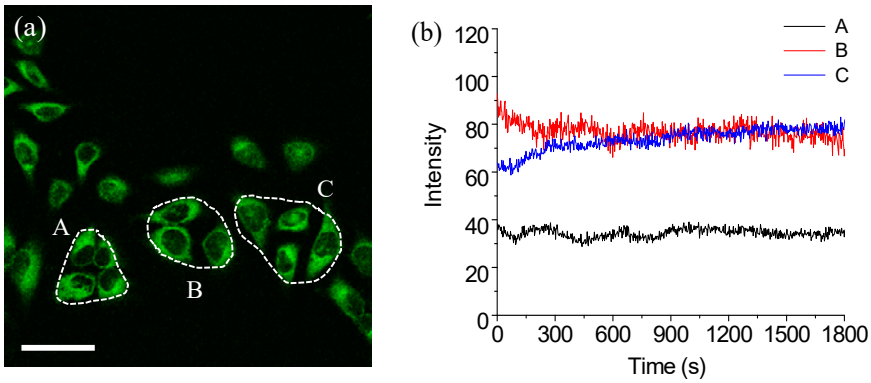


Figure S6. (a) TPM images of HeLa cells after staining of **Probe2** (2.0 μM). (b) Time-dependent two-photon fluorescence intensity of A–C in Figure (a). Detection window of TPEF intensities was 390–700 nm with femto-second pulses at 750 nm excitation. Scale bar, 50 μm.

Two-photon microscopy images in HCT116 with Probe1

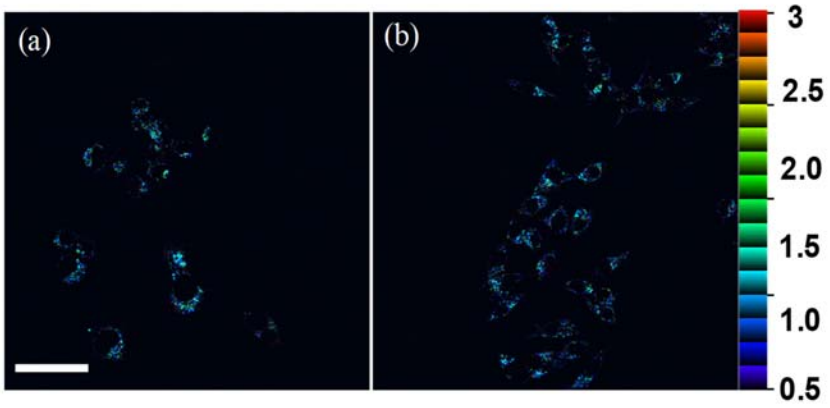


Figure S7. Pseudocolored images (F_{red}/F_{orange}) of HCT 116 cells incubated with (a) **Probe1** (2.0 μM) for 90 min and DON (1.0 mM), (b) **Probe1** (2.0 μM) for 90 min. HCT 116 cells in a were pretreated with the inhibitor and then incubated with **Probe1**. F_{orange} = 520–580 nm and F_{red} = 600–670 nm. The excitation wavelength was 750 nm. Scale bar, 50 μm.

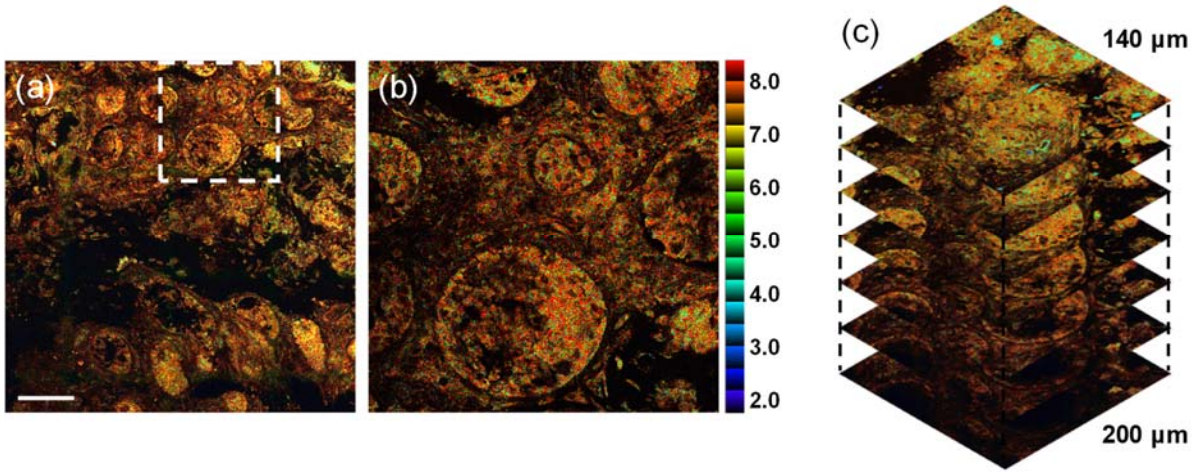


Figure S8. (a) Pseudocolored ratiometric image of human colon cancer tissues incubated with **Probe2** (20 μM) for 90 min. (b) Enlarged image and (c) Section images within the dotted white box in (A) acquired at depths of 140–200 μm. Images were collected at 750 nm excitation and emission windows of 400–450 nm (blue) and 500–600 nm (green). Scale bars, 150 μm.

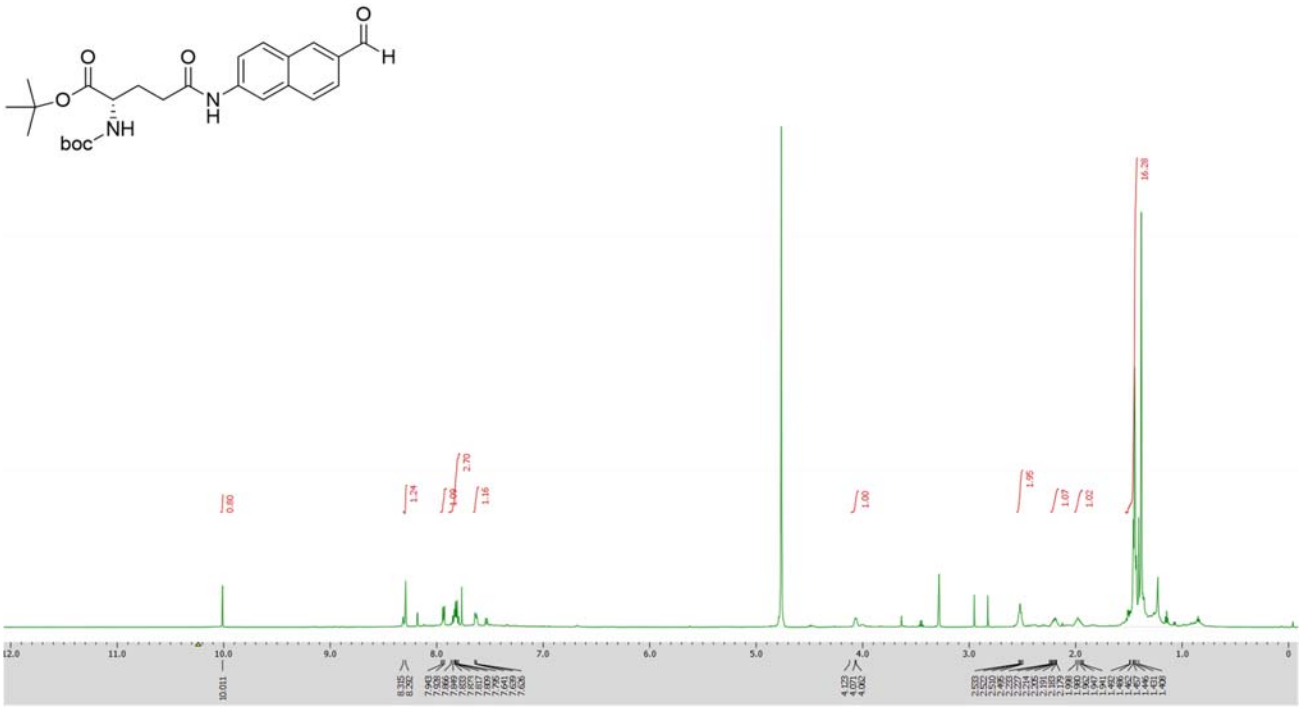


Figure S9. ^{1}H-NMR spectrum (600 MHz) of **E** in CD_3OD

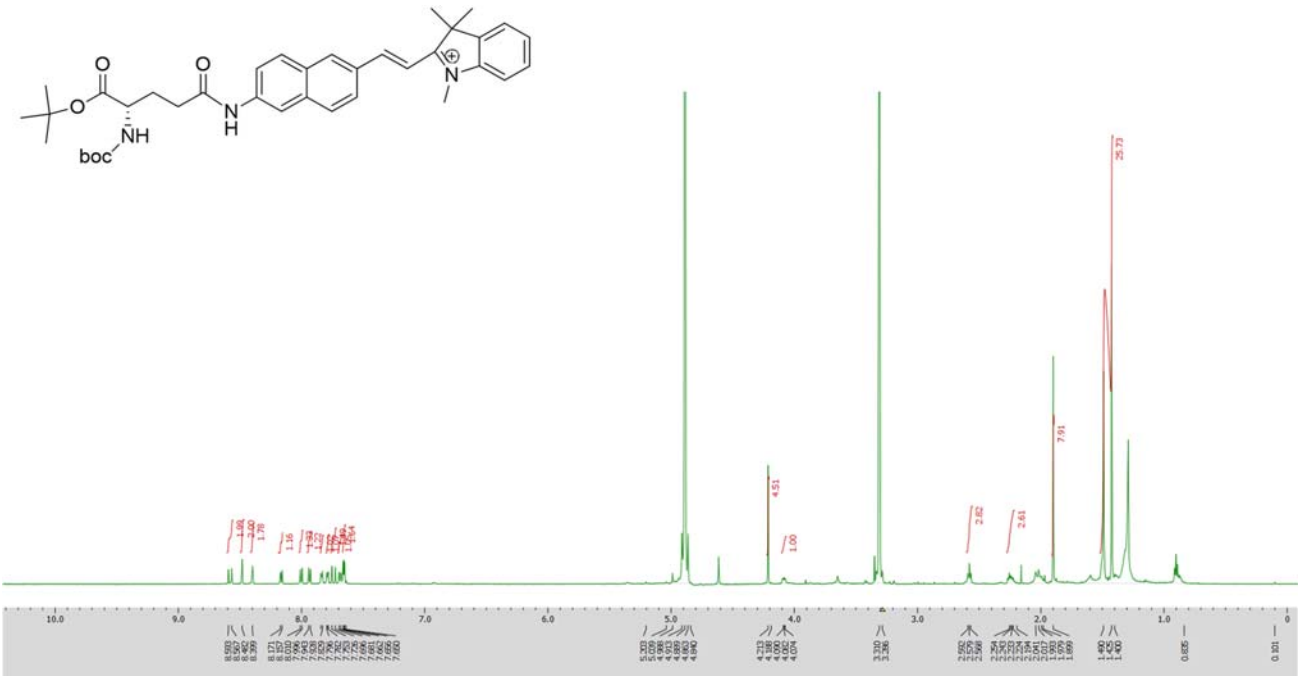


Figure S10. ^{1}H-NMR spectrum (600 MHz) of **F** in CD_3OD

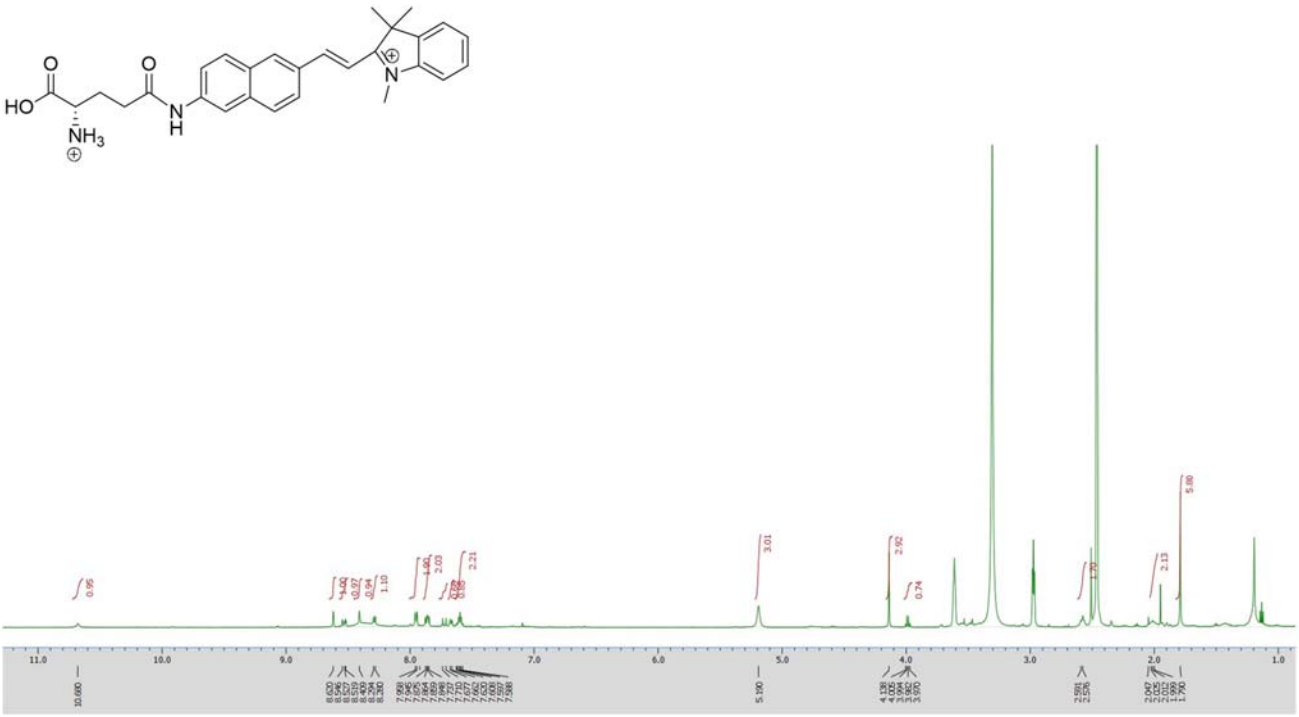


Figure S11. ^{1}H-NMR spectrum (600 MHz) of **Probe1** in $(CD_3)_2SO$

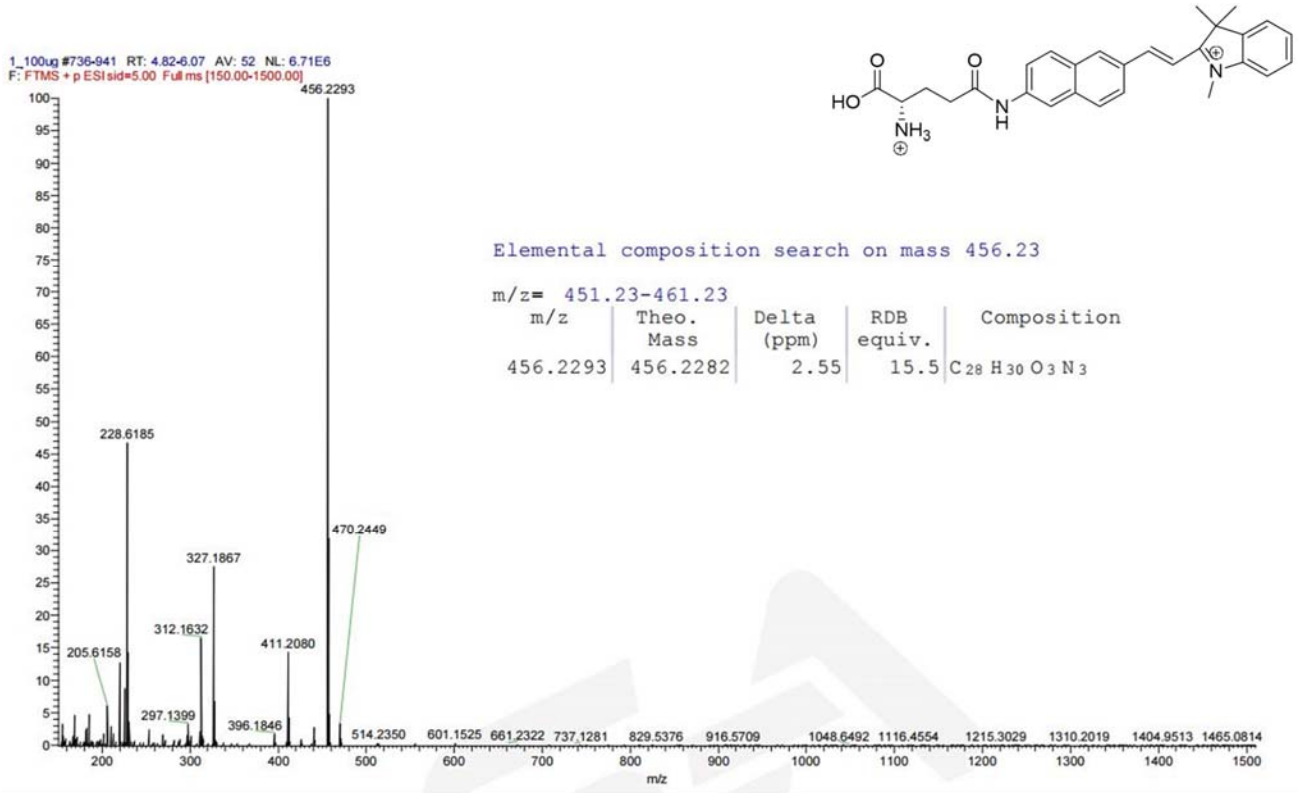


Figure S12. HRMS spectrum of **Probe1**

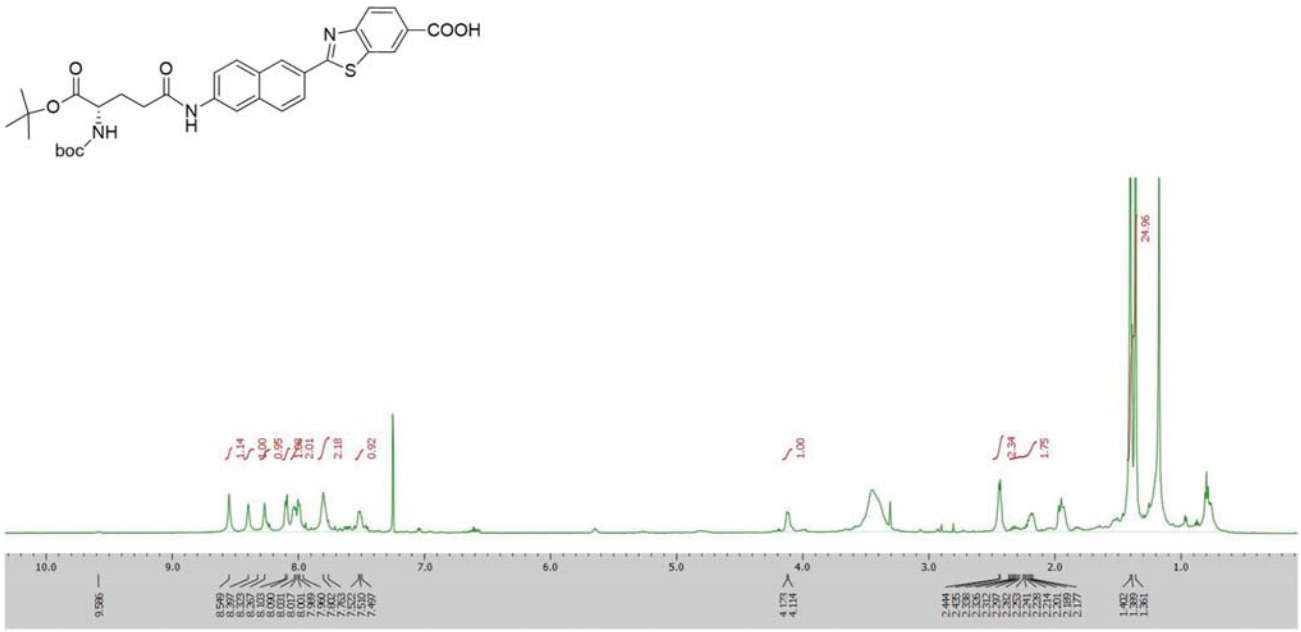


Figure S13. ^{1}H-NMR spectrum (600 MHz) of **G** in $CDCl_3$

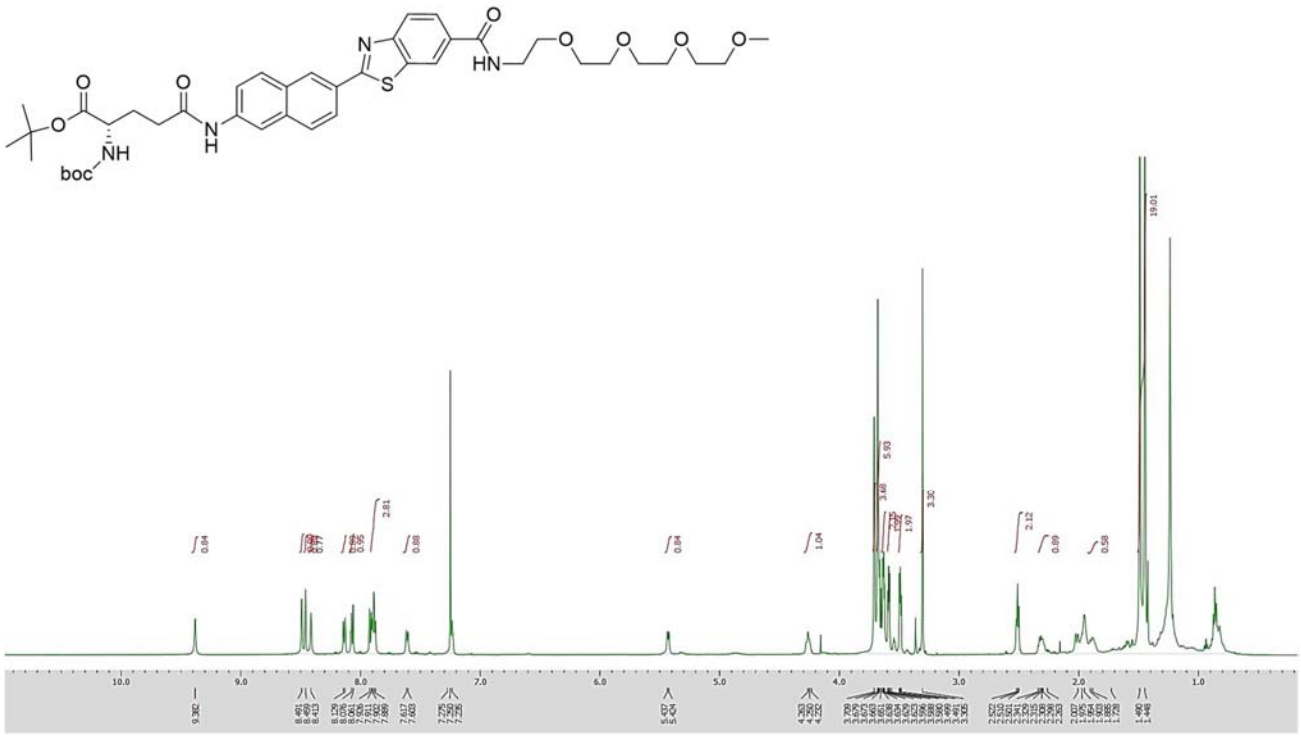


Figure S14. ^{1}H-NMR spectrum (600 MHz) of **H** in $CDCl_3$

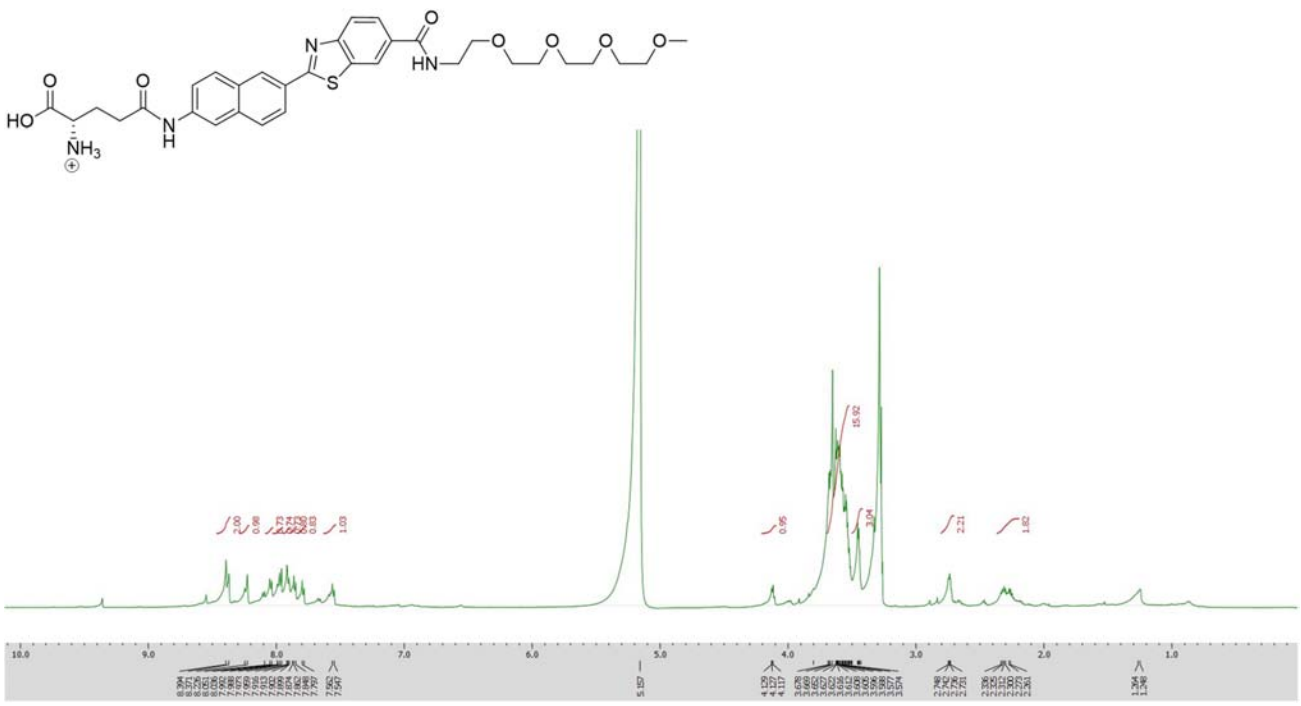
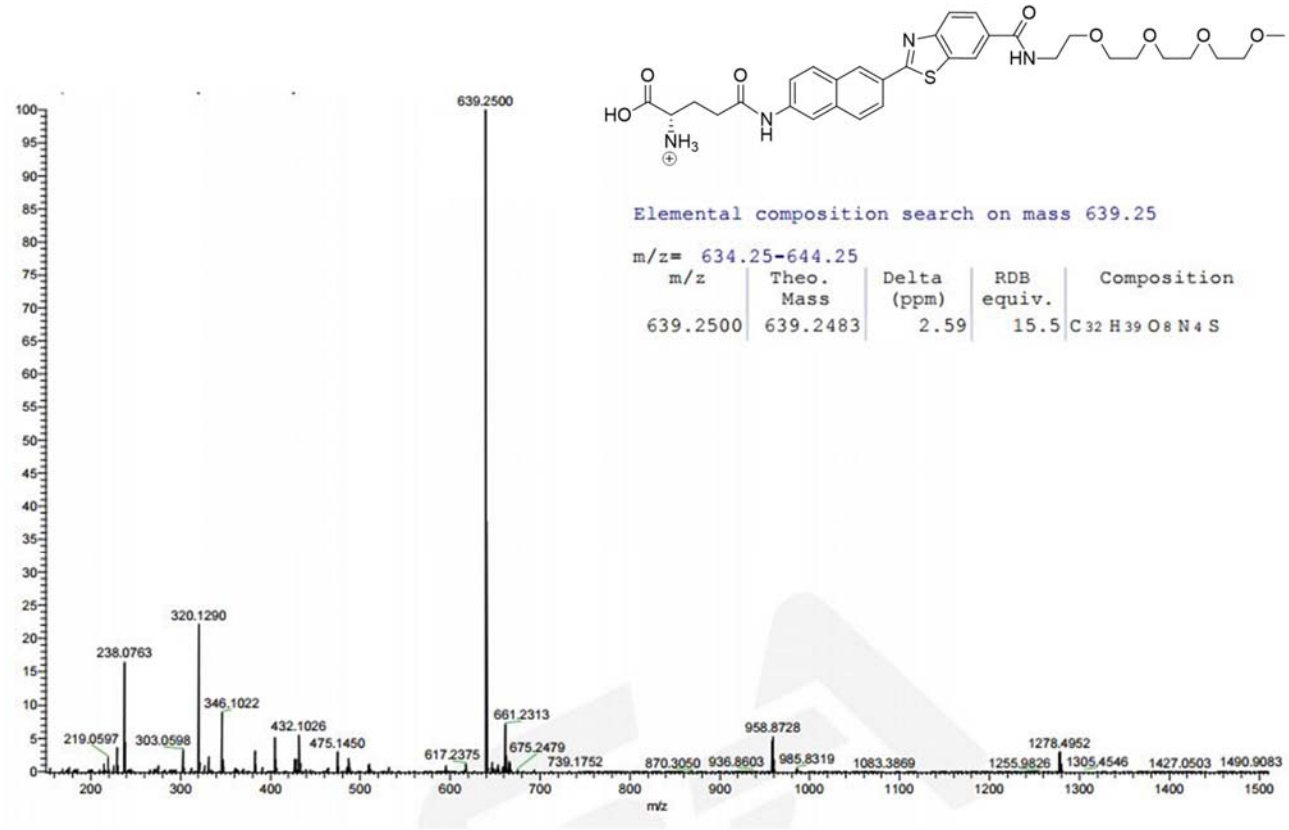


Figure S15. ^{1}H-NMR spectrum (600 MHz) of **Probe2** in CD_3OD



Elemental composition search on mass 639.25

m/z= 634.25-644.25

m/z	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
639.2500	639.2483	2.59	15.5	$C_{32}H_{39}O_8N_4S$

Figure S16. HRMS spectrum of **Probe2**

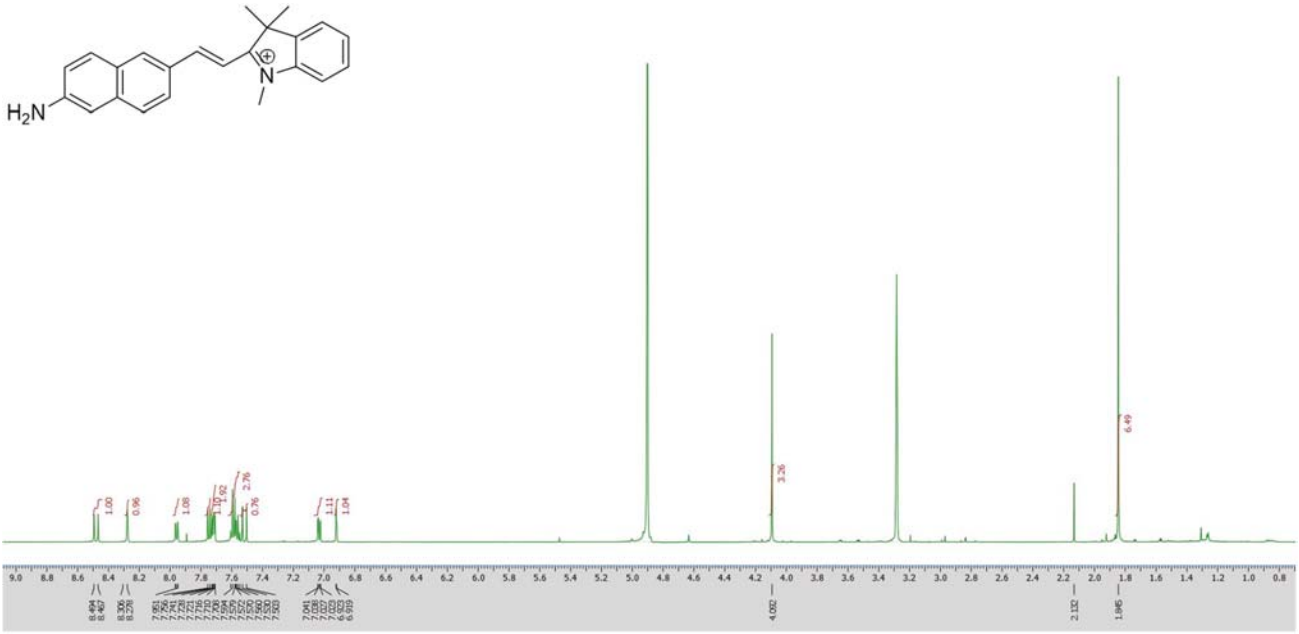
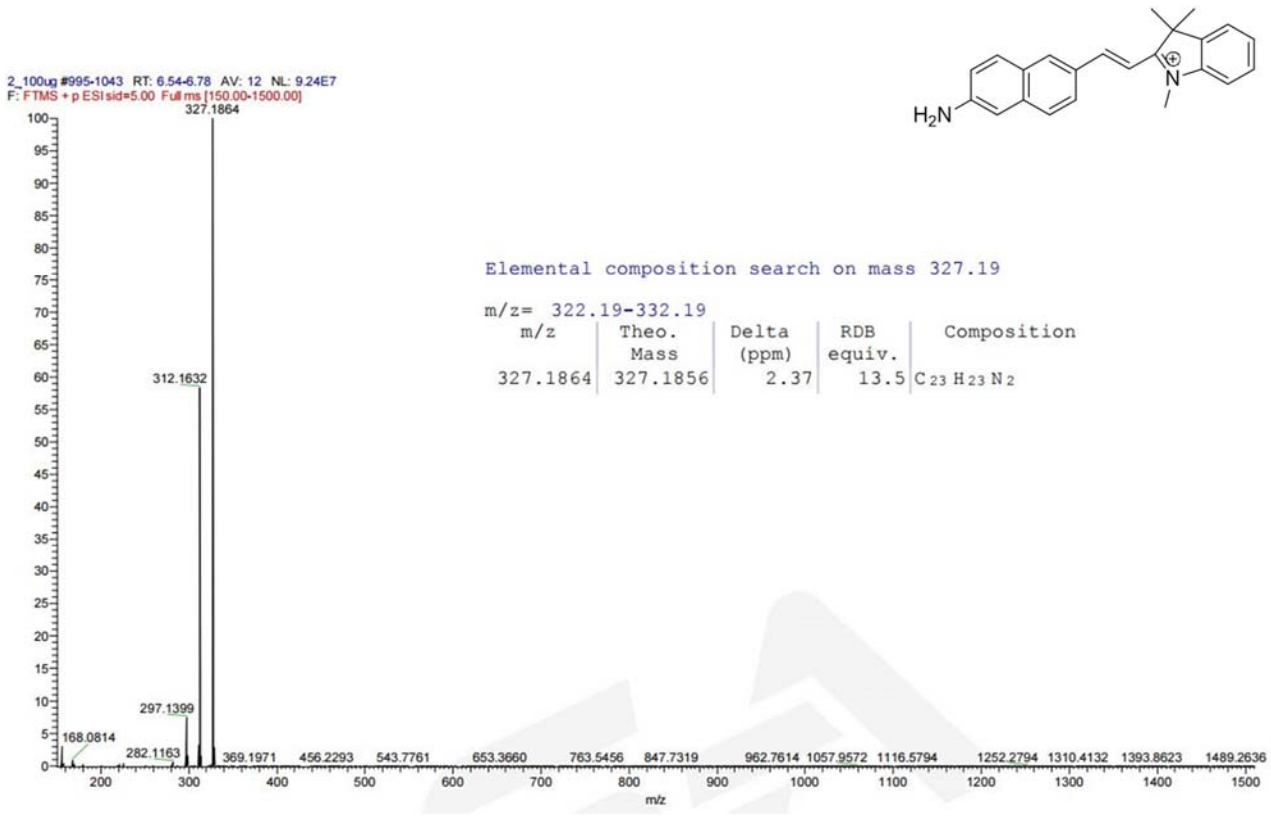


Figure S17. ^{1}H-NMR spectrum (600 MHz) of **1** in CD_3OD



Elemental composition search on mass 327.19

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m/z	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
327.1864	327.1856	2.37	13.5	$C_{23}H_{23}N_2$

Figure S18. HRMS spectrum of **1**

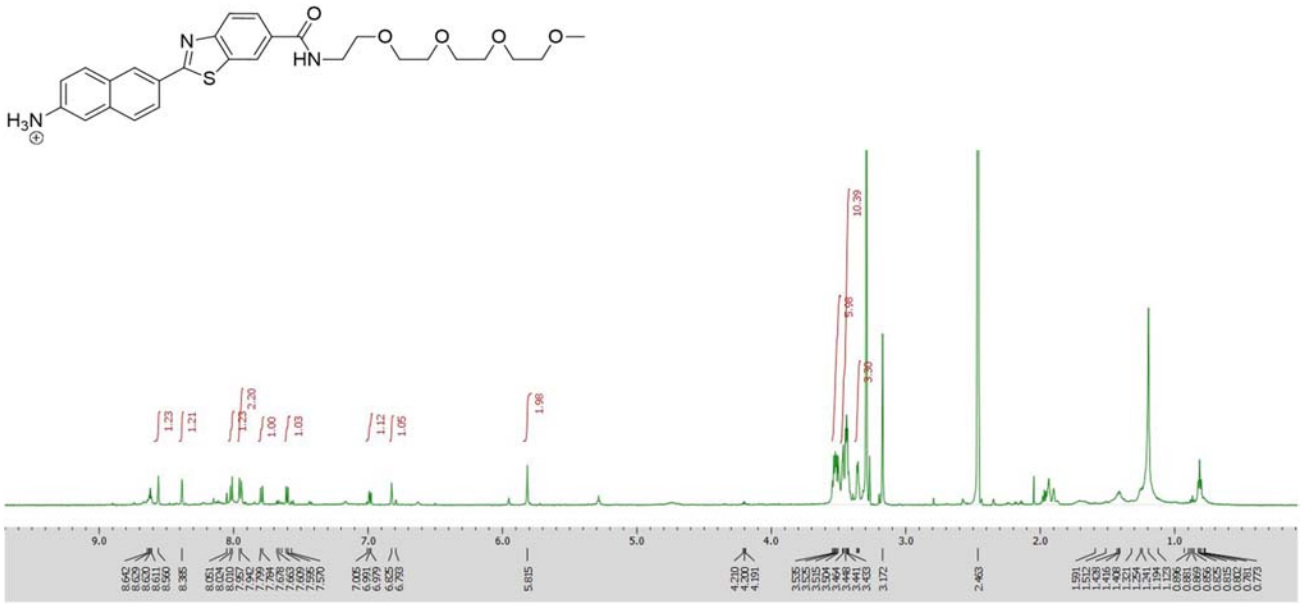
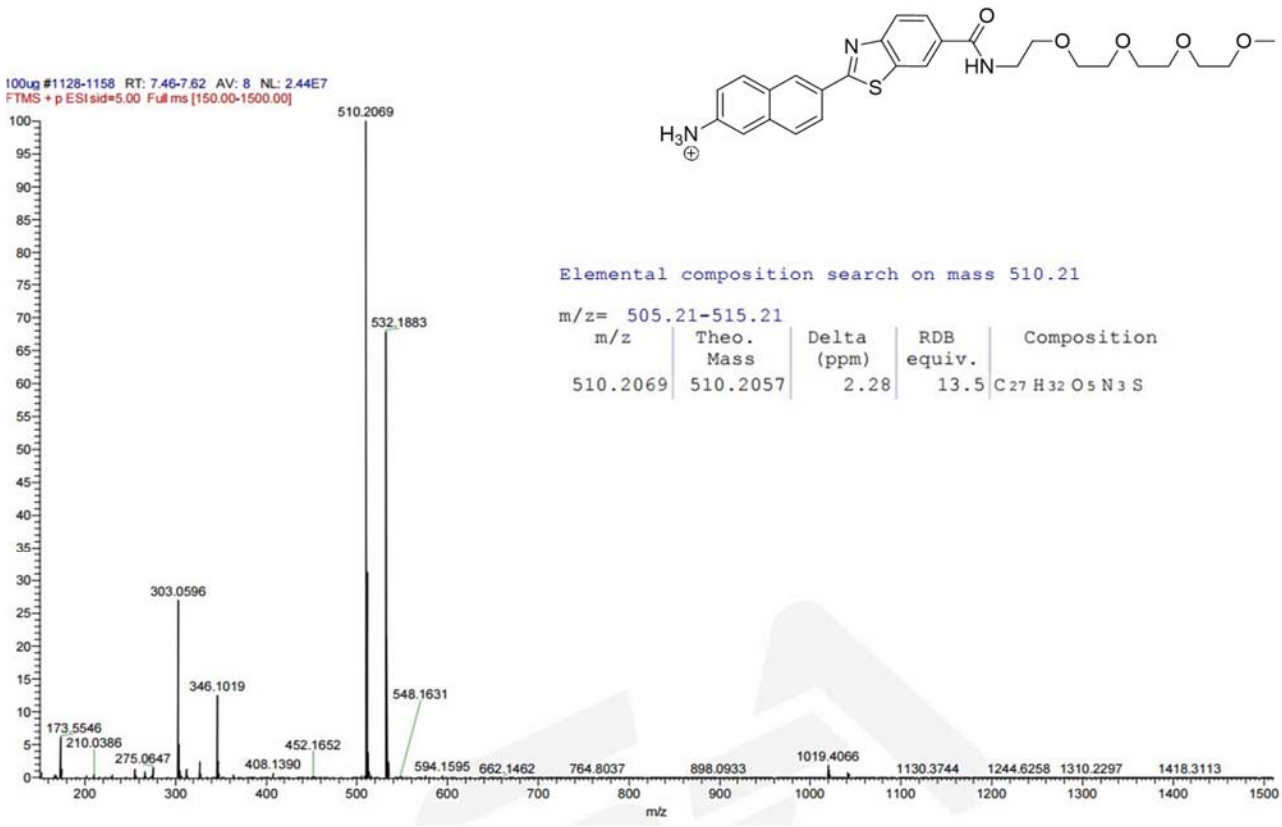


Figure S19. ^{1}H-NMR spectrum (600 MHz) of **2** in $(CD_3)_2SO$



Elemental composition search on mass 510.21

m/z= 505.21-515.21

m/z	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
510.2069	510.2057	2.28	13.5	$C_{27}H_{32}O_5N_3S$

Figure S20. HRMS spectrum of **2**

References

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