Supplementary Information

Selective photo-ablation of glioma cells using an enzyme activatable photosensitizer

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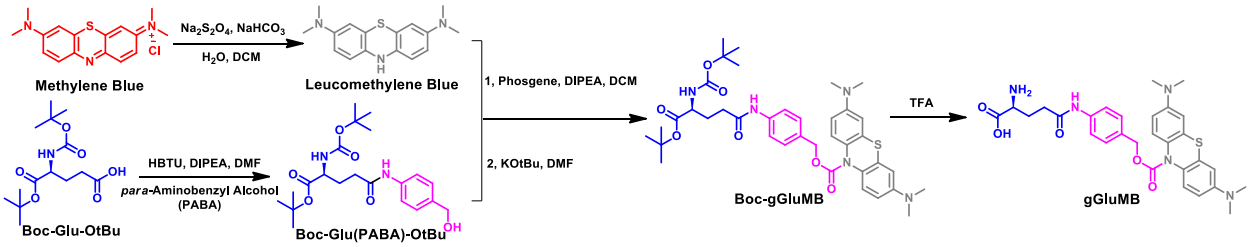


Scheme S1. Synthetic route of gGluMB.

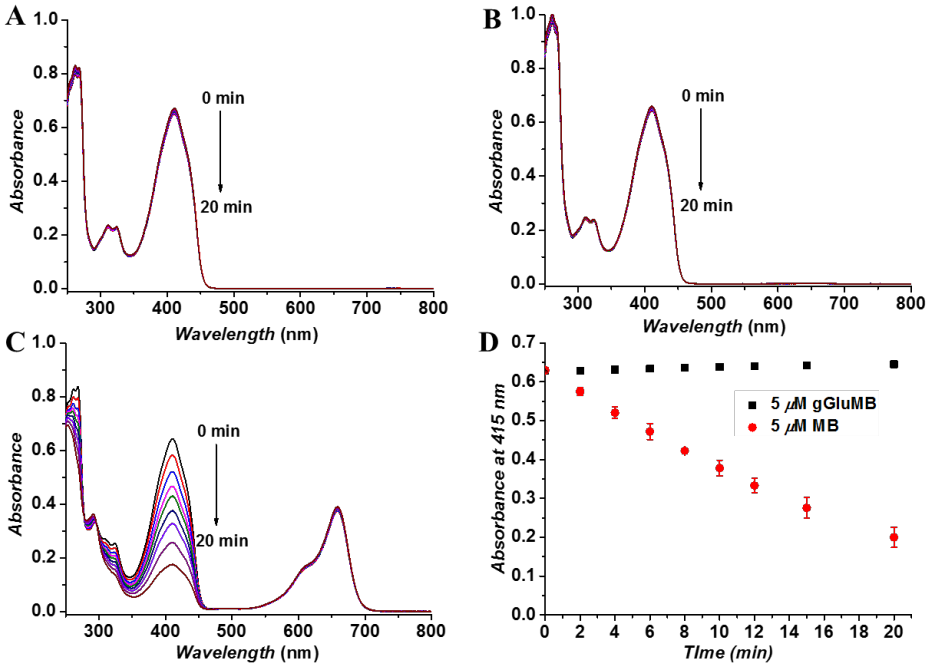


Fig. S1. $^1O_2$ production yield of gGluMB and MB using 1,3-diphenylisobenzofuran (DPBF) as a $^1O_2$ tracker. (**A**) DPBF (30 μM) alone; (**B**) DPBF is mixed with gGluMB (5 μM); (**C**) DPBF is mixed with MB (5 μM) in acetonitrile:water (v:v, 50:50) solution. The solutions were illuminated with 665/20 nm xenon light, and its absorption spectra were recorded every 2 min. (**D**) The absorbance change of DPBF at 415 nm in the presence of gGluMB and MB. Error bar represents the standard deviation of three independent measurements.

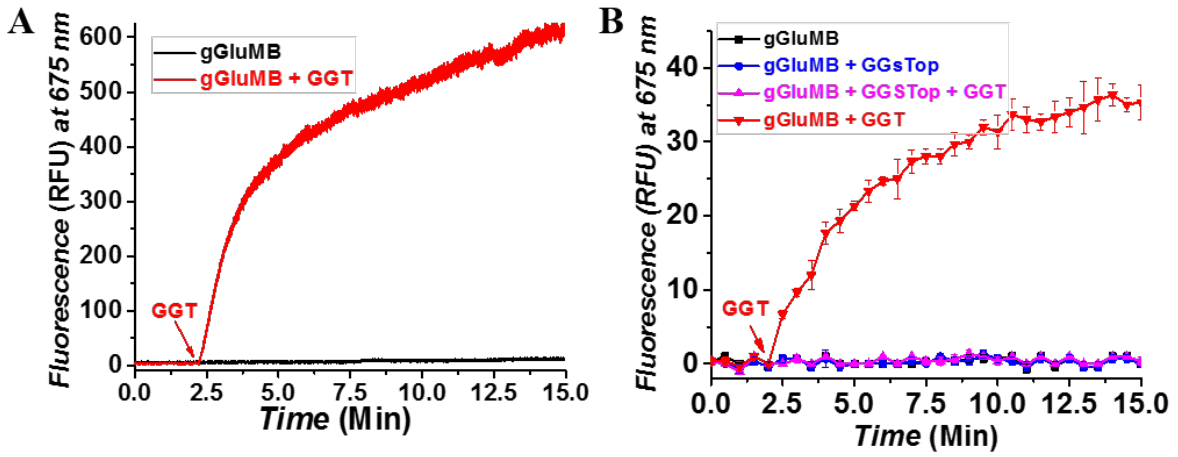


Fig. S2. GGT dependent MB release. (**A**) gGluMB (5 μM) was treated with or without GGT (100 U/L) at 2-min (indicated by arrow) in DMEM cell culture medium. The MB production was followed by its fluorescence (λ_{ex} = 655 nm, λ_{em} = 675 nm) using a fluorescence spectrometer. (**B**) The MB production is inhibited by a GGT-specific inhibitor (GGsTop) in PBS. gGluMB (5 μM) alone, or treated by GGsTop (100 μM), or treated by GGsTop (100 μM) and GGT (100 U/L), or treated by GGT (100 U/L) were monitored by its fluorescence (λ_{ex} = 655 nm, λ_{em} = 675 nm) using a plate reader. Error bars represent the standard deviation of three separate measurements.

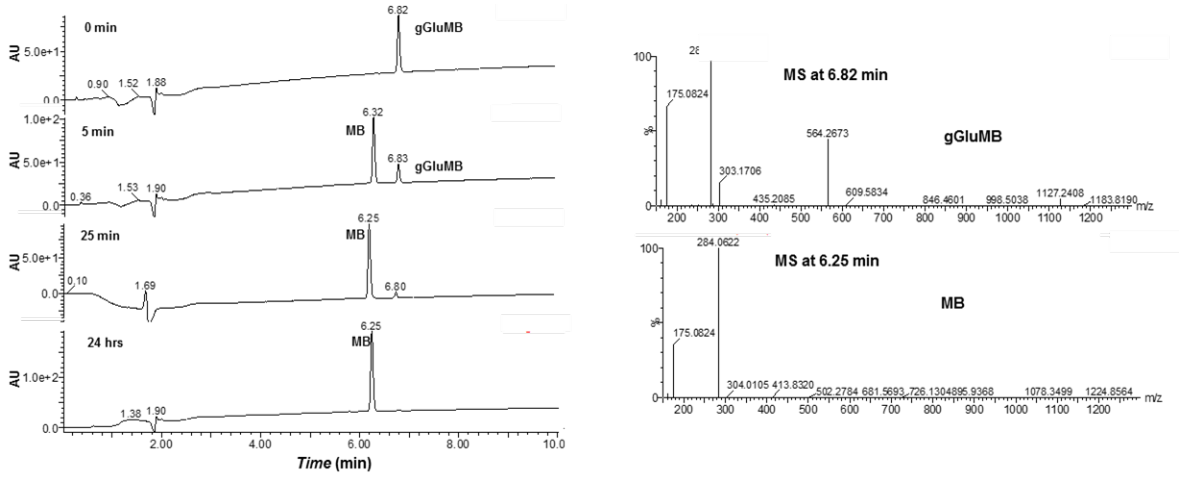


Fig. S3. LC-MS spectra of GGT-added gGluMB solution. gGluMB (100 μM, 1 mL) in PBS buffer (pH = 7.4) was treated with GGT (500 U/L) at room temperature. The HPLC spectra (*left*) were recorded at different time points (0, 5 min, 25 min, and 24 hrs). MS spectra (*right*) confirmed the peak at 6.82 min was gGluMB (m/z = 564.3) and peak at 6.25 min was MB (m/z = 284.1).

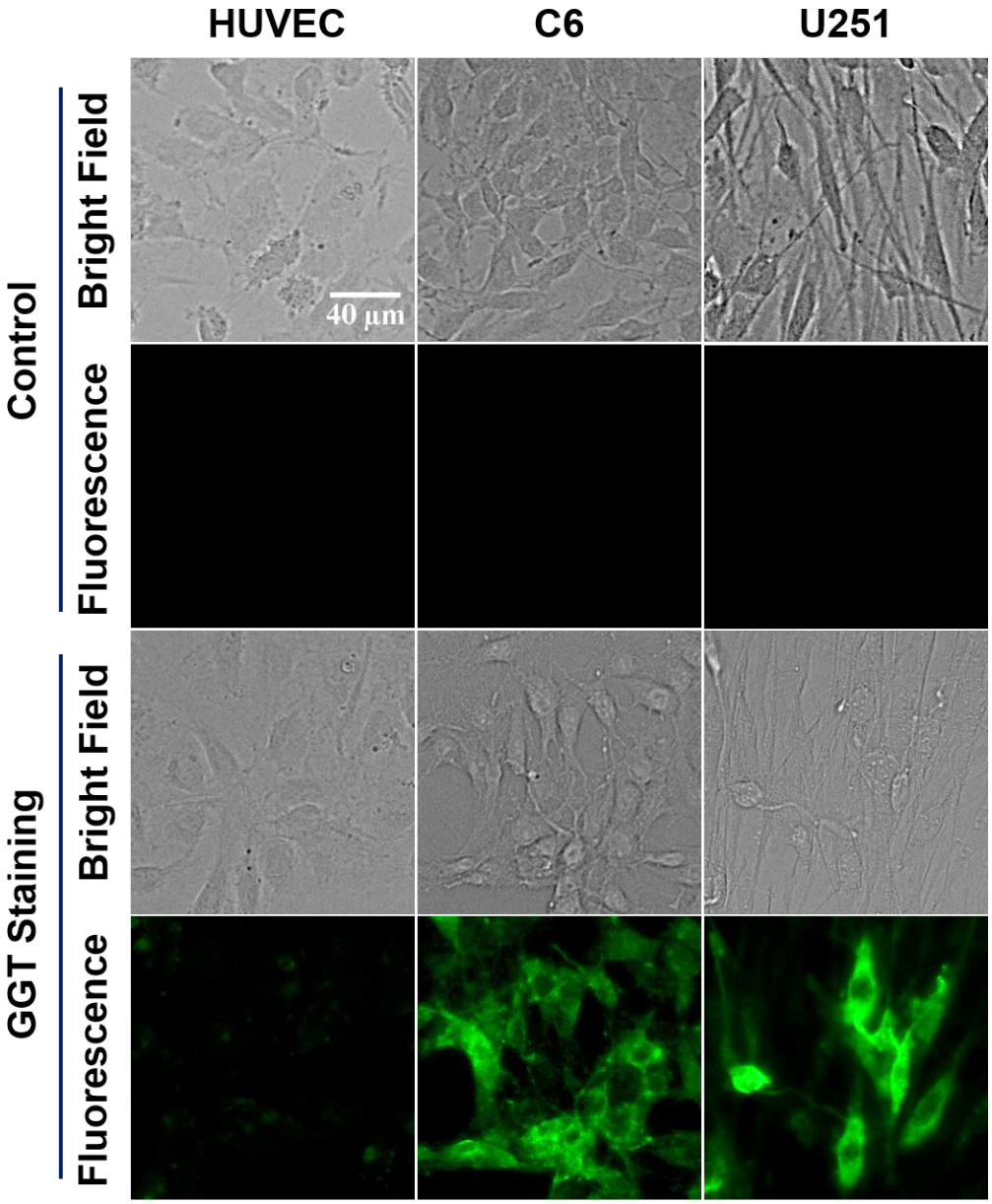


Fig. S4. GGT expression in HUVEC, C6, and U251 cells. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min, then incubated with 1% BSA in PBST (0.1% tween PBS) for 1 hr to permeabilize the cells and block non-specific protein-protein interactions. Cells showed no intrinsic fluorescence before antibody treatment. Cells were incubated with the primary mouse monoclonal anti-GGT1/GGT antibody (2 μg/mL) overnight at 4 ^{o}C. The secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, 2 μg/mL) was then applied for 1 hr at ambient temperature. Cell images were captured with a GFP filter set (λ_{ex}= 457 – 487 nm; λ_{em} = 502 – 538 nm). Scale bar = 40 μm. C6 and U251 glioma cells expressed GGT, but not HUVEC cells.

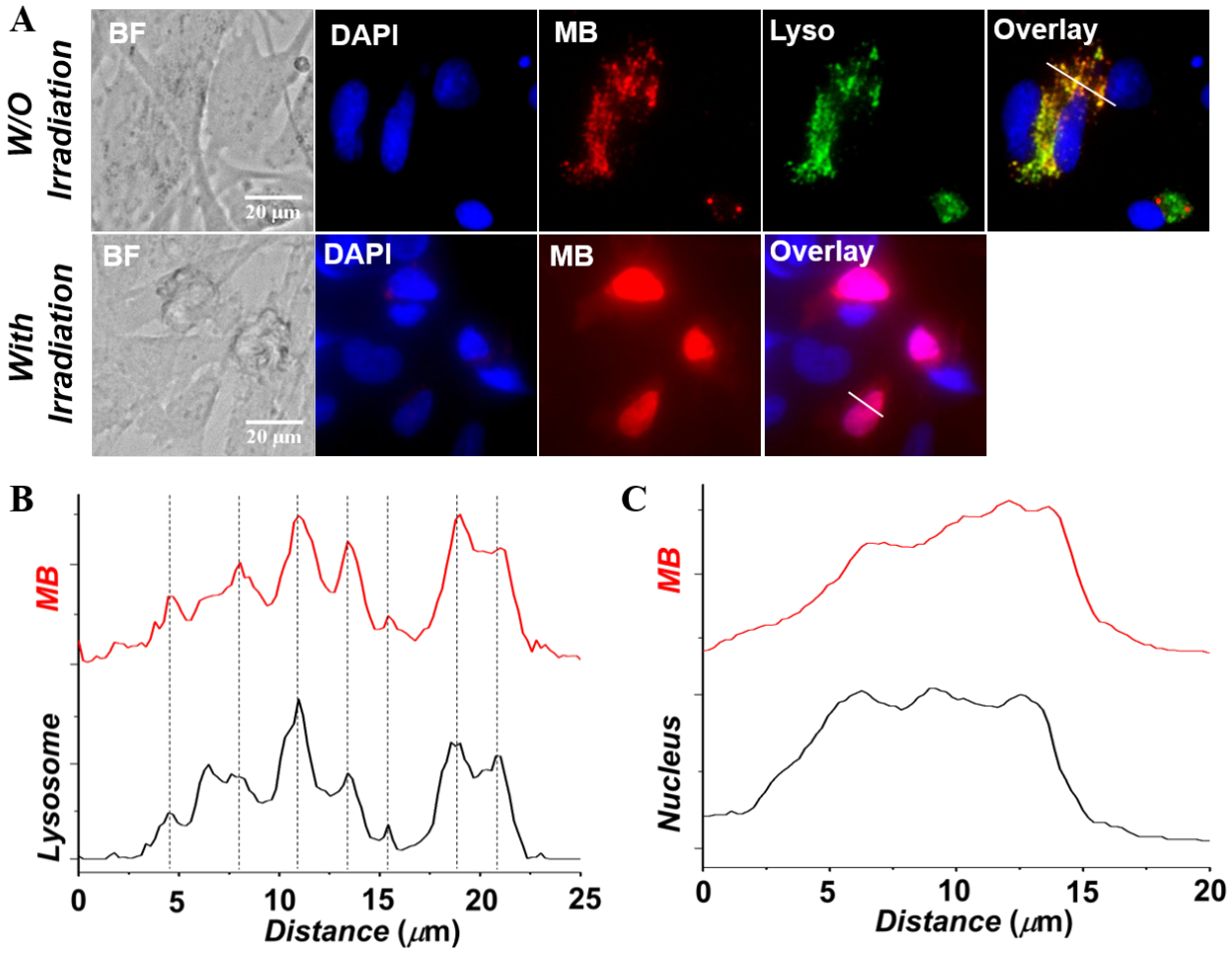


Fig. S5. Colocalization of the GGT converted MB with various organelle stains (**A**, lysosome and nucleus). U251 cells were treated with gGluMB (20 μM) for 5 hrs, then treated with respective organelle stain for 10 – 30 minutes. Cell images were captured with a fluorescence microscope before (*top row*) and after (*bottom row*) light irradiation (590 – 650 nm, 1 min). Lysosome (Lyso Green) images were captured with a GFP filter (λ_{ex}= 457 – 487 nm; λ_{em} = 502 – 538 nm), nuclei images (Hoechst) with a DAPI filter (λ_{ex}= 352 – 402 nm; λ_{em}= 417 – 477 nm), and MB images with a Cy5 filter (λ_{ex}= 590 – 650 nm; λ_{em}= 663 – 738 nm). Scale bar = 20 μm. Spatial fluorescence intensity of MB and lysosome stain across the marked line in the respective lysosome overlay image (**B**) and the nucleus overlay image (**C**).

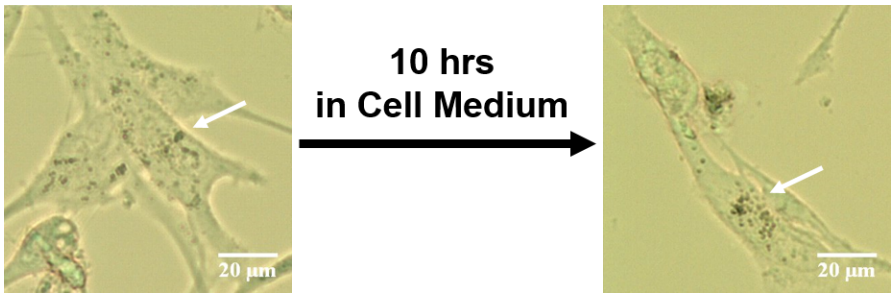


Fig. S6. Cellular retention of the GGT converted MB in the U251 cells. U251 cells were treated with gGluMB (40 μM) for 5 hrs, and a number of MB aggregates (arrow) were produced, *left real-color image*. The cells were incubated for an additional 10 hrs in a fresh cell culture medium, a large number of MB aggregates are still existing in cells, *right real-color image*. Scale bar = 20 μm.

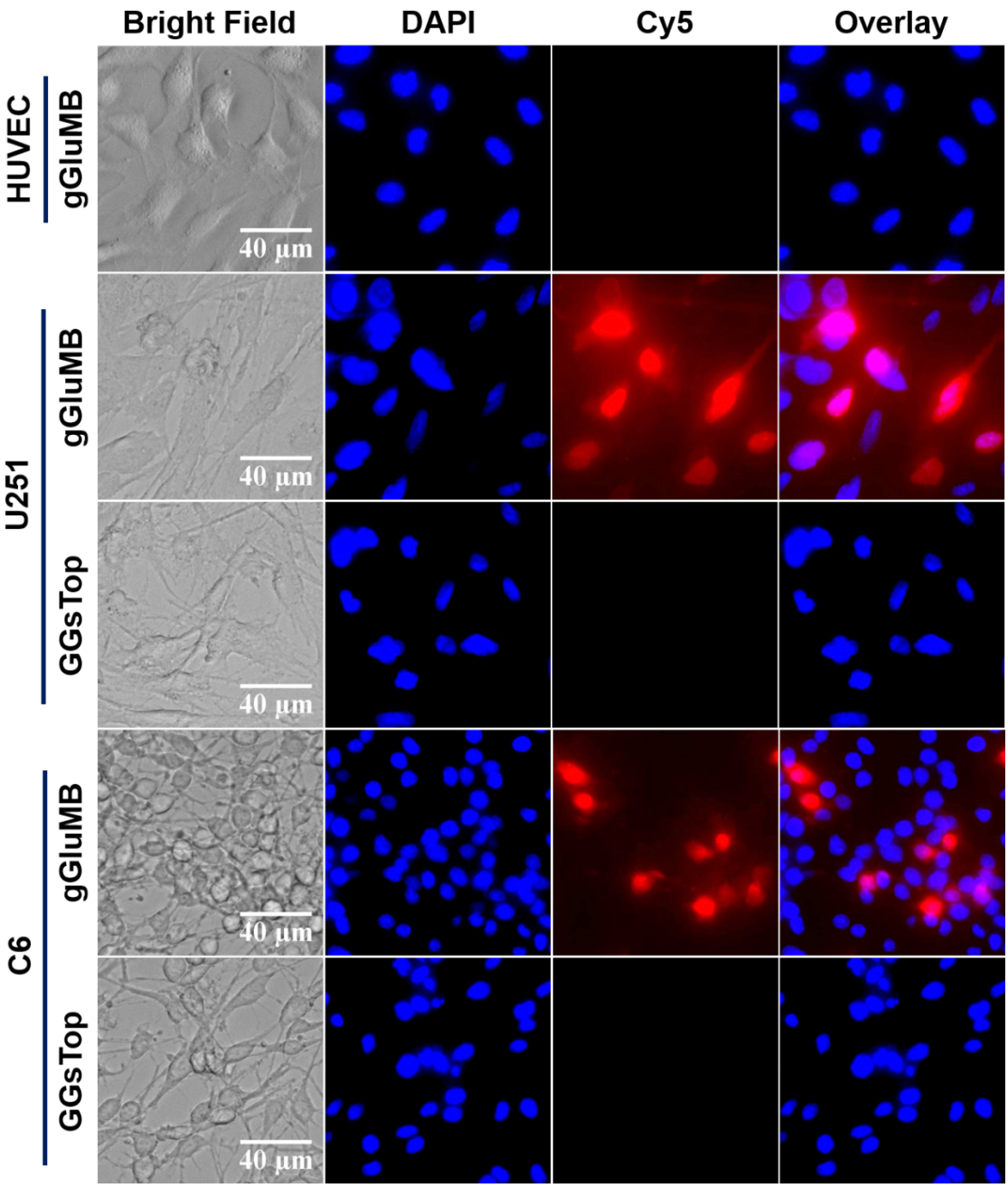


Fig. S7. Light and inhibitor effects of GGT-expressing glioma cells (U251 and C6) and GGT-devoid normal cells (HUVEC). Cells were pretreated with or without GGsTop (250 μM) for 2 hrs, then treated with gGluMB (20 μM) for 5 hrs. Cell images were captured with a fluorescence microscope after light irradiation (590 – 650 nm, 1 min). Nuclei stain (Hoechst) images were captured with a DAPI filter (λ_{ex}= 352 – 402 nm; λ_{em}= 417 – 477 nm), and MB images with a Cy5 filter (λ_{ex}= 590 – 650 nm; λ_{em}= 663 – 738 nm). Scale bar = 40 μm.

Movie S1. Live imaging of light-induced MB dispersion and relocation.

U251 cells were treated with gGluMB (20 μM) for 5 hrs and then irradiated (590 – 650 nm, 1 min, 95 mW/cm^2). The MB fluorescence images was recorded in Cy5 channel using a 40X objective.

Experimental Details

Synthetic Materials and Methods. All chemicals and solvents in synthesis were purchased from commercial vendors (Sigma-Aldrich and Fisher Scientific) unless otherwise specified. Chemicals were used without further purification. The reaction progress was monitored with liquid chromatography coupled mass spectrometer (LC-MS, Waters) or thin-layer chromatography (TLC). Compounds were separated and purified with silica gel flash chromatography or high performance liquid chromatography (HPLC, preparative C-18 column). ^{1}H and ^{13}C NMR spectra were collected in deuterated methanol (CD_3OD) on Bruker Ascend-500 spectrometer. Chemical shifts are reported in the standard δ notation of parts per million using residual protio-solvent of CD_3OD (3.31 for ^{1}H and 49.00 for ^{13}C) as an internal reference. High resolution mass spectroscopy (HRMS) data were collected on a PE Sciex API 100 mass spectrometer.

Synthesis of Boc-Glu(PABA)-OtBu: In a 25-mL round bottom flask, Boc-Glu-OtBu (150 mg, 0.5 mmol, Combi-Blocks), *para*-aminobenzyl alcohol (74 mg, 0.6 mmol), 3-[bis(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate (HBTU, 285 mg, 0.75 mmol), and *N*,*N*-diisopropylethylamine (DIPEA, 193 mg, 1.5 mmol) were dissolved in DMF (5 mL). The solution stirred at room temperature overnight. The reaction was diluted with ethyl acetate (EtOAc), then washed with water and brine. The EtOAc phase was dried with $MgSO_4$ and concentrated in a rotary evaporator. The crude product was purified on a silica gel column with hexane/EtOAc (v/v, 4/1). A white solid product Boc-Glu(PABA)-OtBu (185 mg, yield: 91%) was acquired. ^{1}H NMR (500 MHz, CD_3OD) δ 7.52 (d, J = 8.4 Hz, 2H), 7.29 (d, J = 8.4 Hz, 2H), 4.55 (s, 2H), 4.06 – 4.02 (m, 1H), 2.47 (t, J = 7.5 Hz, 2H), 2.20 – 2.15 (m, 1H), 1.97 – 1.92 (m, 1H), 1.47 (s, 9H), 1.43 (s, 9H). ^{13}C NMR (126 MHz, CD_3OD) δ 173.22, 173.14, 158.10, 139.00, 138.48, 128.56, 121.17, 82.78, 80.56, 64.85, 55.49, 34.16, 28.71, 28.41, 28.25. ESI-HRMS: for $C_{21}H_{32}N_2O_6$: expected m/z = 431.2158 $[M+Na]^+$; found m/z = 431.2157 $[M+Na]^+$; 0.2 ppm error.

Synthesis of Boc-gGluMB: Methylene blue (MB, 165 mg, 0.5 mmol) was dissolved in dichloromethane (DCM, 3 mL) in a 10-mL round bottom flask. Sodium bicarbonate (84 mg, 1.0 mmol) in water (2 mL) and sodium dithionite ($Na_2S_2O_4$, 174 mg, 1.0 mmol) in water (2 mL) was gradually added to the previous solution under nitrogen condition. After stirring for 30 min, the solution color changed from dark blue to brown, indicating the formation of leucomethylene blue (LMB). The DCM phase was dried and used for next step without further purification.

Phosgene in toluene (***caution: toxic*****,** 15% w/w, 0.7 mL, 1.0 mmol) was added to DCM (5 mL) in a 25-mL round-bottom flask and cooled to -10 ºC. LMB (0.5 mmol) and DIPEA (129 mg, 1.0 mmol) were added to the phosgene solution. The reaction was warmed to room temperature. After 15 min, the extra phosgene was bubbled out, and the solvent was replaced with dimethylformamide (DMF). To this solution, a mixture of Boc-Glu(PABA)-OtBu (125 mg, 0.25 mmol) and potassium *tert*-butoxide (0.75 mmol) in DMF was drop-wisely added. The reaction stirred for 3 hrs, then was quenched with saturated aqueous ammonium chloride solution. After extraction, the crude product was purified with preparative C-18 column, and Boc-gGluMB was obtained as a solid (38 mg, yield: 21%). ^{1}H NMR (500 MHz, CD_3OD) δ 7.53 (d, J = 8.4 Hz, 2H), 7.47 (d, J = 8.9 Hz, 2H), 7.26 (d, J = 8.5 Hz, 2H), 7.10 (d, J = 2.7 Hz, 2H), 7.02 (dd, J = 8.9, 2.7 Hz, 2H), 5.15 (s, 2H), 4.04 (m, 1H), 3.05 (s, 12H), 2.47 (t, J = 7.4 Hz, 2H), 2.20 – 2.14 (m, 1H), 1.98 – 1.90 (m, 1H), 1.46 (s,

9H), 1.42 (s, 9H). ^{13}C NMR (126 MHz, CD_3OD) δ 173.22, 173.20, 158.08, 155.47, 147.21, 139.96, 134.67, 133.64, 132.84, 129.77, 128.93, 121.14, 115.51, 114.88, 82.78, 80.56, 69.05, 55.44, 43.28, 34.14, 28.71, 28.35, 28.25. ESI-HRMS: for $C_{21}H_{32}N_2O_6$: expected m/z = 720.3431 $[M+H]^+$; found m/z = 720.3422$[M+H]^+$; 1.2 ppm error.

Synthesis of gGluMB: Boc-gGluMB (20 mg, 0.028 mmol) was dissolved in pure trifluoroacetic acid (TFA, 1.0 mL) at 0 ^{o}C. After 15 min, the reaction was purified with preparative C-18 column. gGluMB was obtained as a solid (12 mg, yield: 76%). ^{1}H NMR (500 MHz, CD_3OD) δ 7.55 (d, J = 8.5 Hz, 2H), 7.41 (d, J = 8.9 Hz, 2H), 7.29 (d, J = 8.5 Hz, 2H), 6.97 (d, J = 2.6 Hz, 2H), 6.91 (dd, J = 8.9, 2.6 Hz, 2H), 5.16 (s, 2H), 4.08 (t, J = 6.5 Hz, 1H), 3.02 (s, 12H), 2.69 (t, J = 7.1 Hz, 2H), 2.33 – 2.18 (m, 2H). ^{13}C NMR (126 MHz, CD_3OD) δ 172.51, 171.50, 155.82, 148.43, 139.70, 134.57, 133.25, 132.16, 129.70, 128.63, 121.16, 114.35, 113.59, 68.83, 53.52, 42.42, 33.18, 26.96. ESI-HRMS: for $C_{21}H_{32}N_2O_6$: expected m/z = 564.2281 $[M+H]^+$; found m/z = 564.2254 $[M+H]^+$; 4.8 ppm error.

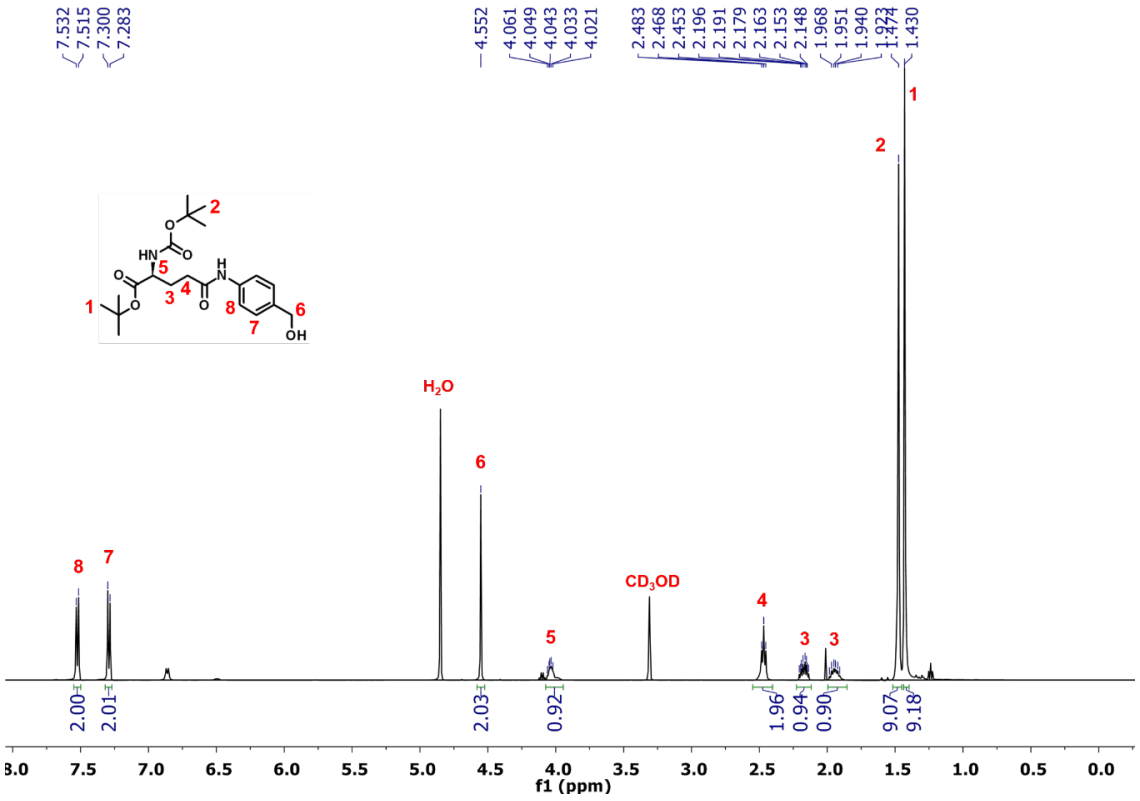
Optical characterization: gGluMB, methylene blue (MB), and the GGT inhibitor GGsTop (Fisher Scientific) stock solutions (1 mM) were prepared in water. γ-Glutamyl transpeptidase (GGT, Sigma-Aldrich) was diluted to 50 U/mL in PBS buffer (pH = 7.4). All stock solutions were stored at – 30 ^{o}C freezer and used for the following experiment. The optical characterization experiment was carried out using Cary 60 UV-Vis spectrophotometer and Cary Eclipse fluorescence spectrophotometer (Agilent).

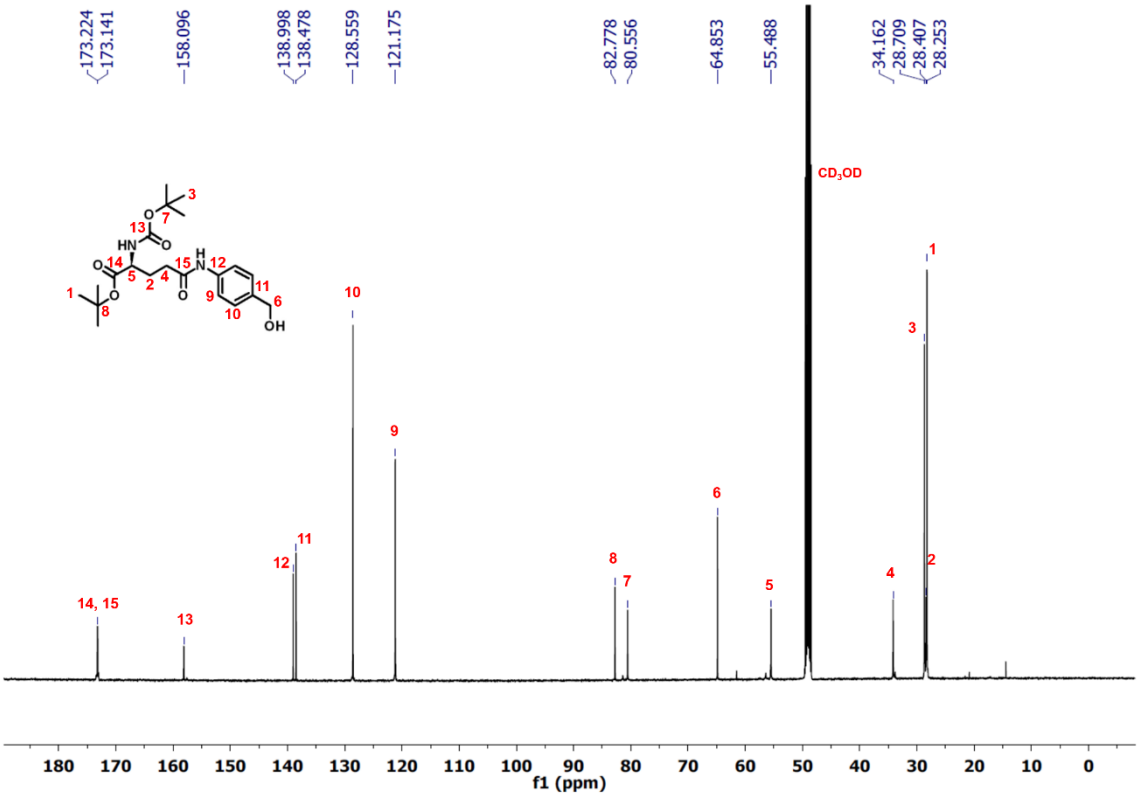
Cell Culture: U251 (human glioblastodma astrocytoma), C6 (*Rattus norvegicus* brain glioma), and HUVEC (human umbilical vein cells) were purchased from American Type Culture Collection (ATCC; Manassas, VA). U251 cells were maintained in EMEM medium supplemented with 2mM glutamine, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate (NaP), 10% fetal bovine serum (FBS), and 100 U/mL penicillin and streptomycin; C6 cells with F-12K medium supplemented with 10% FBS, and 100 U/mL penicillin and streptomycin; HUVEC cells with EBM-Plus medium supplemented with growth supplements. All cells were cultured in an incubator with humidified air containing 5% CO_2 at 37 ^{o}C.

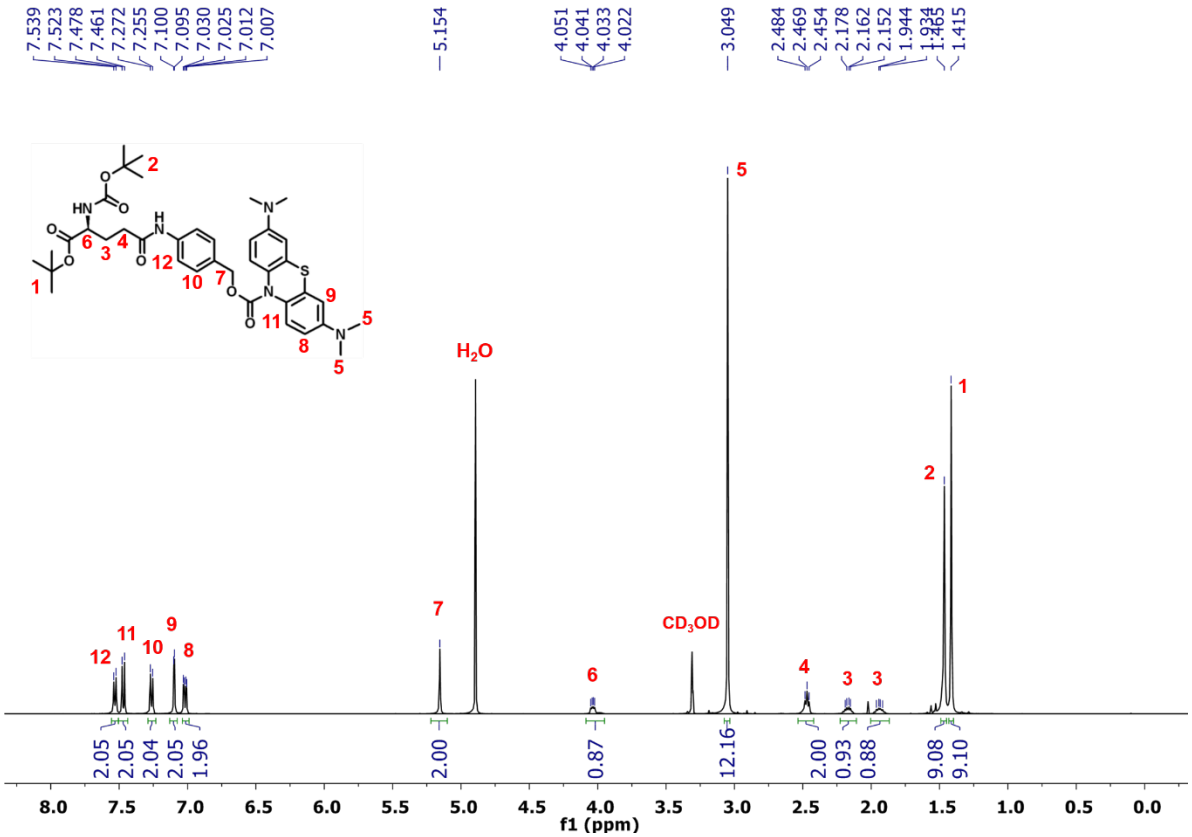
Cell imaging: Cells (25×10^3/well) were seeded on a glass-bottom chamber slide and incubated for 24 hrs with respective cell culture medium to allow cell adherence. Then, the old medium was replaced with 0.1% FBS supplemented medium for another 48 hrs. For fluorescence microscopic imaging, cells were pretreated with/without GGsTop (250 μM) for 2 hrs, then incubated with gGluMB (20 μM) for 5 hrs. Hoechst nuclei stain was further added for 5 min. After removal of the old medium, cells were washed with warm PBS and phenol-free DMEM medium was added for live-cell imaging. Fluorescence images were captured using an EVOS fluorescence microscope (Life Technologies) with an objective (40×) in bright field, DAPI channel for nuclei images, and Cy5 channel for gGluMB and MB images. Light irradiation was performed with Cy5-filtered red light (590 – 650 nm) for 1 min. For the colocalization experiment, cells were incubated with gGluMB (20 μM) for 5 hours, and then lysosome green tracker, mitochondria green tracker, or nuclei stain was added for 15 – 30 min. After washing, cells were imaged. For immunofluorescence imaging, the primary anti-GGT1/GGT antibody and the secondary antibody (Alexa Fluor 488 goat anti-mouse IgG) were purchased from Abcam, and cells were stained by following its protocol.

Photodynamic therapy: Cells (10×10^3/well) were incubated on a black 96-well plate with 0.1% FBS medium for 48 hrs. For inhibitor-treated cells, GGsTop (250 μM) was added for 2 hrs. Different concentrations of gGluMB (0, 5, 10, 20, 30, 40 μM) were added and incubated for 10 hrs. The old medium was removed and cells were washed with PBS. For light-irradiation, cells were irradiated with 665 nm LED light (30 mW/cm^2) for 30 min. After an additional 24 hrs incubation, the cell viability was measured using CCK assay.

^{1}H and ^{13}C NMR spectra



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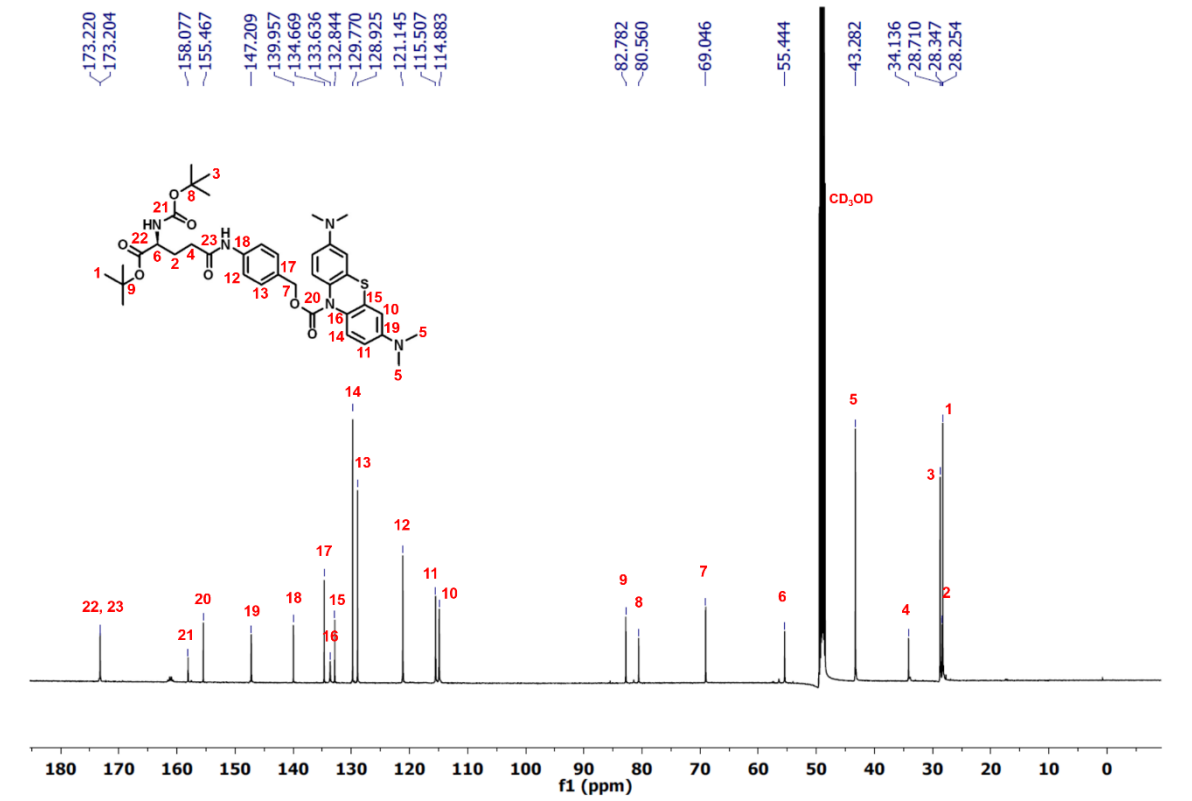
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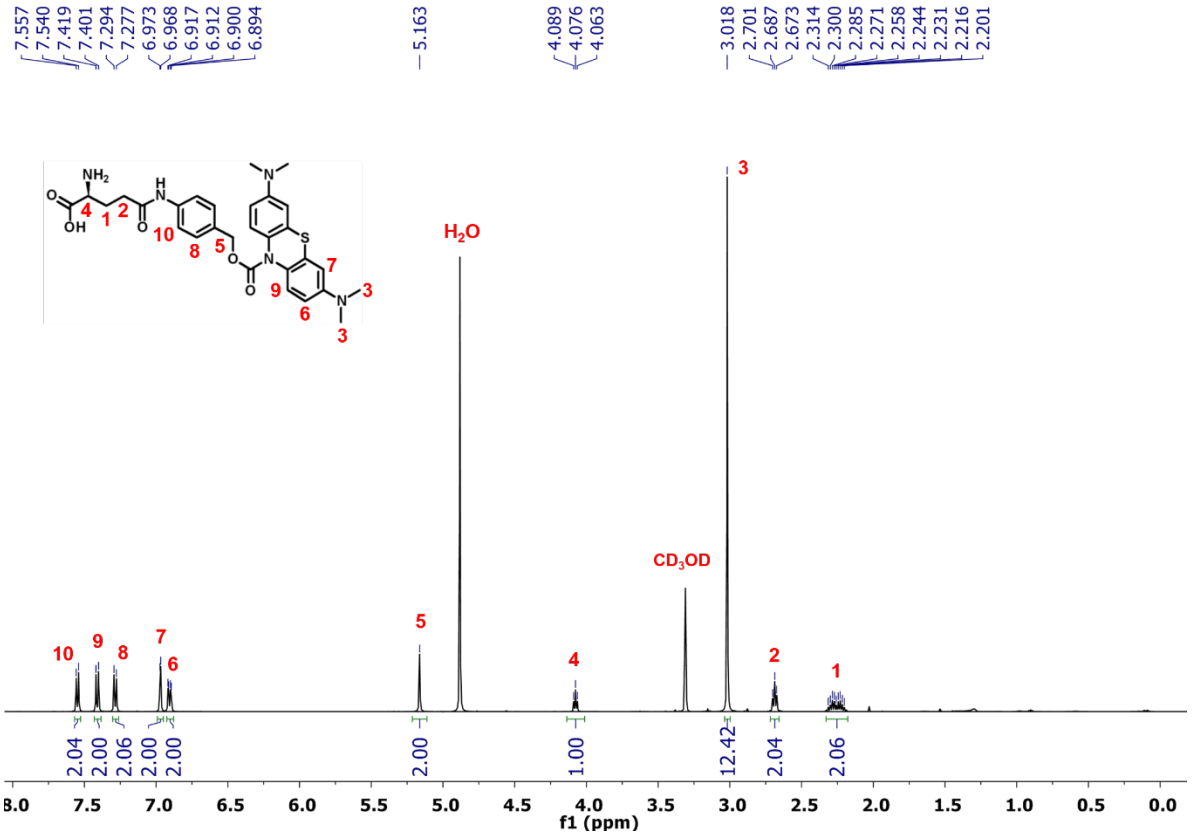
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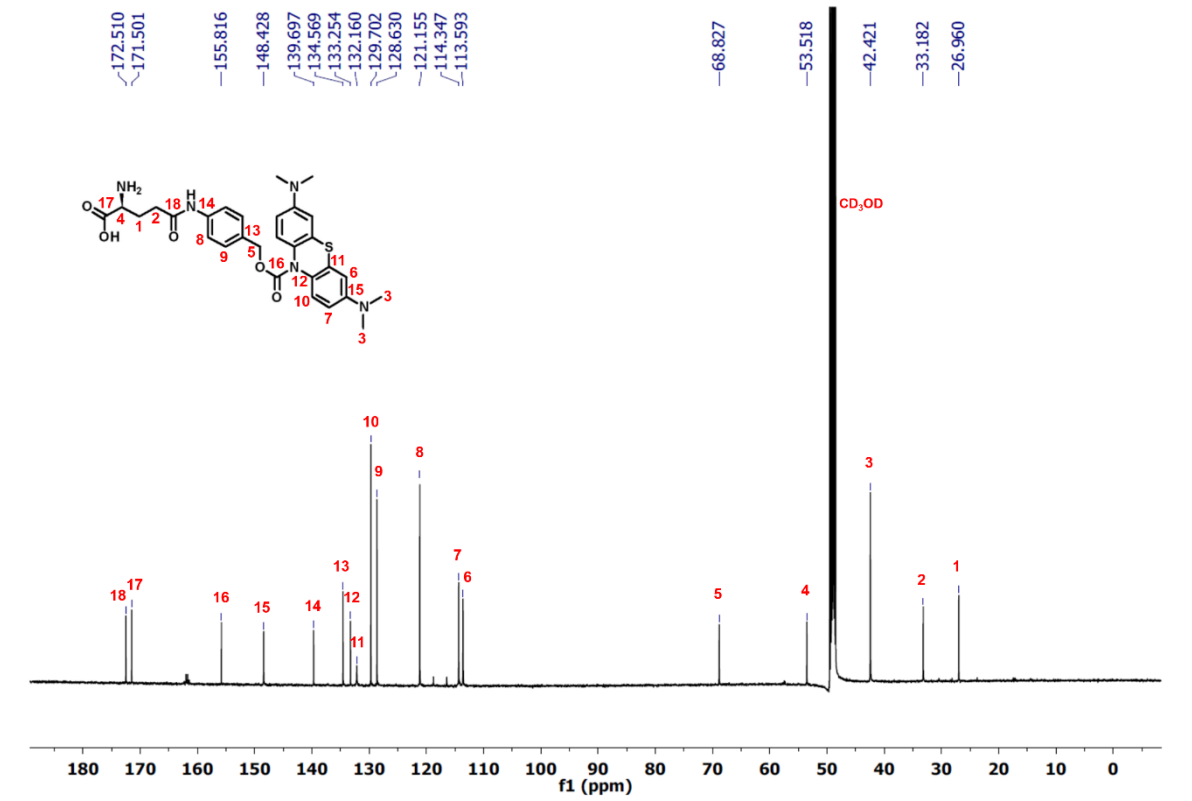
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