Supporting Information for

Development of a two-photon fluorescent probe for selective detection of β-galactosidase in living cells and tissues †

Zihong Li, Mingguang Ren, Li Wang, Lixuan Dai and Weiying Lin*

Institute of Fluorescent Probes for Biological Imaging, School of Materials Science and Engineering, School of Chemistry and Chemical Engineering, University of Jinan, Jinan, Shandong 250022, P. R. China.

Email: weiyinglin2013@163.com

*Correspondence to: Weiying Lin, Institute of Fluorescent Probes for Biological Imaging, School of Materials Science and Engineering, School of Chemistry and Chemical Engineering, University of Jinan, Jinan, Shandong 250022, P. R. China. Email: weiyinglin2013@163.com.

Table of contents

Preparation of Solutions of probe G-GAL and Analytes

Without other noted, all the tests were operated according to the following procedure. A stock solution (1.0 mM) of **G-GAL** was prepared in DMSO. After adjusting the final volume to 10 mL with 0.1 M PBS buffer, standing at 37 °C 20 min, 3 mL portion of it was transferred to a 1 cm quartz cell to measure absorbance or fluorescence. All fluorescence measurements were conducted at room temperature on a Hitachi F4600 Fluorescence Spectrophotometer. The slight pH variations of the solutions were achieved by adding the minimum volumes of NaOH (0.1 M) or HCl (0.2 M).

Culture and preparation of HeLa cells and OVCAR-3 cells

HeLa cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C. Before the experiments, seed the HeLa cells in 35-mm glass-bottomed dishes at a density of 2×10^5 cells per dish in 2 mL of culture medium and incubate them inside an incubator containing 5% CO_2 and 95% air at 37 °C. Incubate the cells for 24 h. Cells will attach to the glass surface during this time.

OVCAR-3 cells grown in modified RPMI-1640 (Roswell Park Memorial Institute 1640) with 20% FBS and Insulin 0.01 mg/ml with the atmosphere of 5% CO_2 and 95% air at 37 °C for 24 h. The OVCAR-3 cells were washed with PBS when used.

OVCAR-3 cells and Hela cells treated with **G-GAL** (20.0 μM) for 50 min at 37 °C. The ideal fluorescence images were acquired with a Nikon A1MP confocal microscopy with the equipment of a CCD camera.

Cytotoxicity assays

The living cells line were treated in RPMI 1640 supplied with fetal bovine serum (20%, FBS), penicillin (100 U/mL), streptomycin (100 μg/mL) and Insulin (0.01 mg/mL) under the atmosphere of CO_2 (5%) and air (95%) at 37 °C. The OVCAR-3 cells were then seeded into 96-well plates, and 0, 1, 5, 10, 20, 30 μM (final concentration) of the probe **G-GAL** (99.9% RPMI 1640 and 0.1% DMSO) were

added respectively. Subsequently, the cells were cultured at 37 °C in an atmosphere of CO_2 (5%) and air (95%) for 24 hours. Then the OVCAR-3 cells were washed with PBS buffer, and RPMI 1640 medium (100 μL) was added. Next, MTT (10 μL, 5 mg/mL) was injected to every well and incubated for 4 h. Violet formazan was treated with sodium dodecyl sulfate solution (100 μL) in the H_2O-DMF mixture. Absorbance of the solution was measured at 570 nm by the way of a microplate reader. The cell viability was determined by assuming 100% cell viability for cells without **G-GAL**.

Synthesis procedures of compound 1

Compound **GCTPOC** was prepared according to the reported reference. A mixture of 2,4-dihydroxybenzaldehyde (5 mmol, 691 mg, 2 eq), cyclohex-2-en-1-one (7.5 mmol, 721 mg, 3 eq) and 1,4-diazabicyclo[2.2.2]octane (Dabco, 12.5 mmol, 1.4 mg, 5 eq) in water/1,4-dioxane (20 mL, 1: 1, V/V) was heated to 95 °C for 2 days at N_2 atmosphere. The mixture was extracted with 100 mL ethyl acetate thrice. The combined extracted were washed with water, brine and dried over Na_2SO_4. The solvent was removed under reduced pressure to give the crude product, and then purified by silica gel flash chromatography using from CH_2Cl_2/petroleum ether (1:1 V/V) to ethyl acetate/petroleum ether (1:2 V/V) as eluent to afford compounds 1 as a colorless or primrose yellow solid as compound **1** (400 mg, 80% yield).

Synthesis procedures of compound 2

6-(acetoxymethyl)tetrahydro-2H-pyran-2,3,4,5-tetrayl tetraacetate (3.90 g, 10 mmol 1eq) was suspended in HBr (30% in AcOH, 10 mL) and acetic acid (5 mL) was added. The reaction was stirred for 1h. Then, DCM (20 mL) and ice (20 g) were added and separate the organic layers. The aqueous later was extracted with DCM (2×30 mL). The combined organic layers were washed with water (3×30 mL), $NaHCO_3$ (30 mL), brine (20 mL), dried over Na_2SO_4. The solvent was then removed under reduced pressure to give the product as compound **2** (3 g, 88% yield).

Calculation of fluorescence quantum yield of G-GAL

The fluorescence quantum yield of compounds is obtained by the following equation

$$\Phi_s = \Phi_r \left(\frac{A_r(\lambda_r)}{A_s(\lambda_s)} \right) \left(\frac{n_s^2}{n_r^2} \right) \frac{F_s}{F_r}$$

s and *r* are the sample and the reference values, respectively. Fluorescence quantum yield is *Φ*; The integrated intensityis expressed by *F*; *A* and *n* stand for the absorbance and refractive index, rescpectively.

Preparation of living organs and tumor for imaging experiments

The animals were purchased from School of Pharmaceutical Sciences, Shandong University, and the studies were approved by the Animal Ethical Experimentation Committee of Shandong University. All animals were kindly kept during experiment according to the requirements of the National Act on the use of experimental animals (China).

Five-week old nude mice were purchased from School of Pharmaceutical Sciences, Shandong University and the nude mice were kindly kept during the experiments. The nude mice were inoculated with Hela and OVCAR-3 cells and after 15 days a tumor was obtained, respectively. Then the organs (heart, liver, spleen, lung and kidney) and tumor were isolated from the mice. After washing by PBS (pH = 7.4) for three times, these isolated organs and tumor were loaded with **G-GAL** (20 μM), respectively, and finally subjected to imaging by using an IVIS Lumina XR in vivo imaging system with an excitation filter of 520 nm and an emission filter of 570 nm.

Preparation of mouse tumor slices for imaging experiments

The animals were purchased from School of Pharmaceutical Sciences, Shandong University, and the studies were approved by the Animal Ethical Experimentation Committee of Shandong University. All animals were kindly kept during experiment according to the requirements of the National Act on the use of experimental animals (China).

The slices were prepared from the tumor of 15-day inoculated mice, and they were cut to 200 mm thickness by using a vibrating-blade microtome in 25 mM PBS (pH =

7.4). The slices were incubated with 15 μM **G-GAL** in PBS buffer bubbled with 95% O_2 and 5% CO_2 for 2 h at 37 °C, and then washed three times with PBS, transferred to the glass bottomed dishes, and observed under one-photon and two-photon confocal microscope (Nikon AMP1), respectively. The fluorescence images of the slices were acquired using 800 excitation and fluorescence emission windows of 500-550 nm.

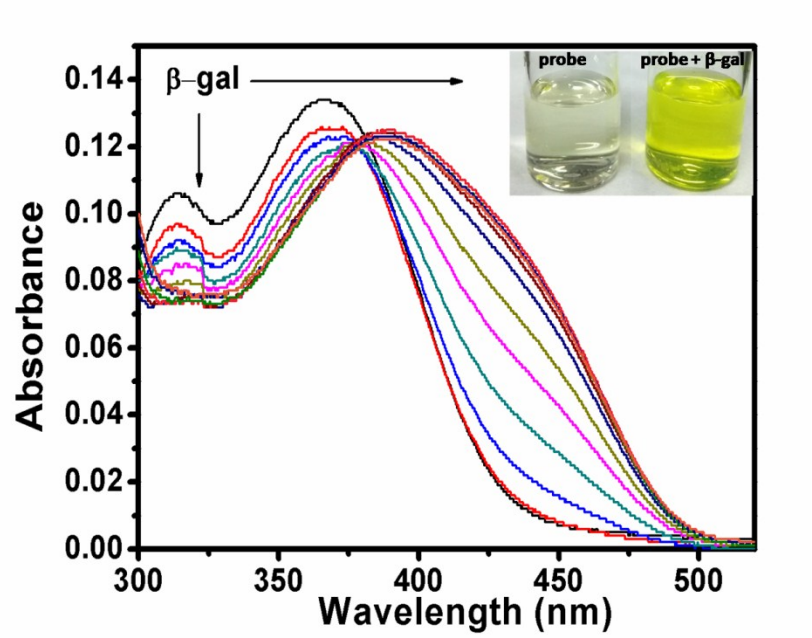

Fig. S1 Absorption spectra of 20 μM **G-GAL** in presence and out presence of β-gal in PBS buffer. (pH = 7.4)

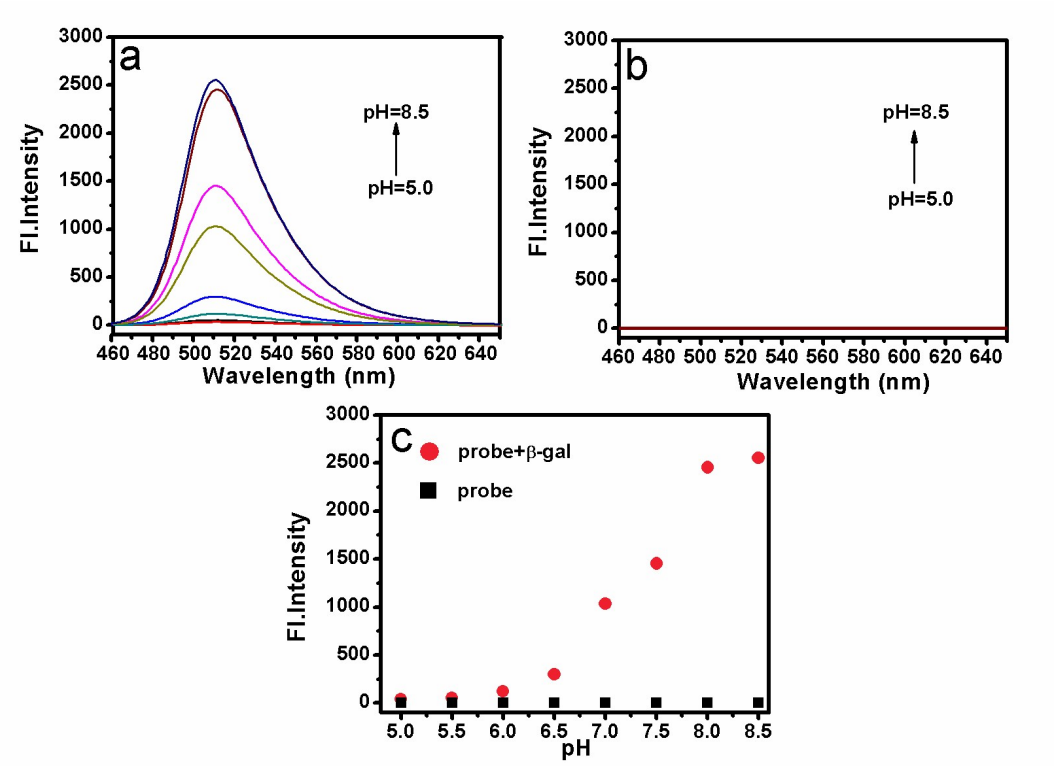

Fig. S2 (a) The emission intensity changes at 510 nm of compound **G-GAL** with β-

gal in different pH PBS buffer. (b) The emission intensity changes at 510 nm of compound **G-GAL** in different pH PBS buffer. (c) Overlay of a and b (λ_{ex} = 450 nm).

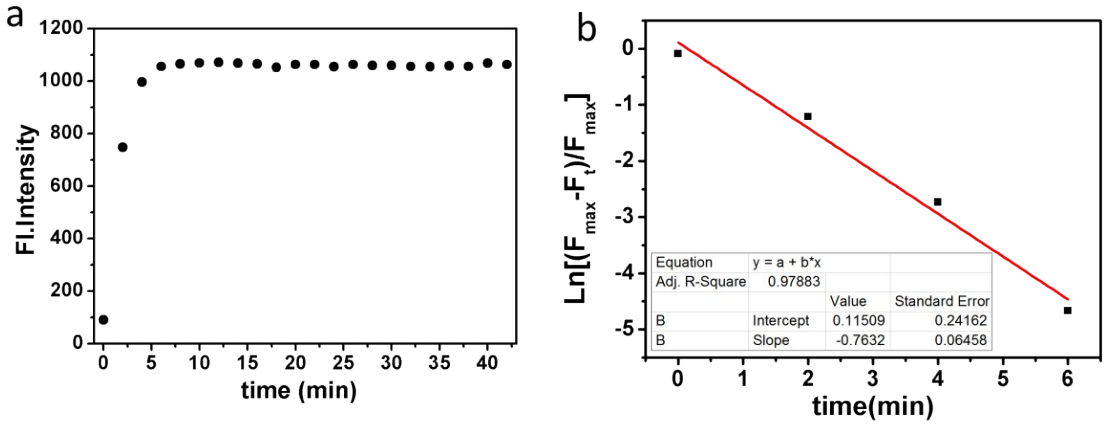

Fig. S3 (a) The same as Fig 2b. (b) Pseudo first-order kinetic plot of the reaction of **G-GAL** in the presence of β-gal at 37 °C.

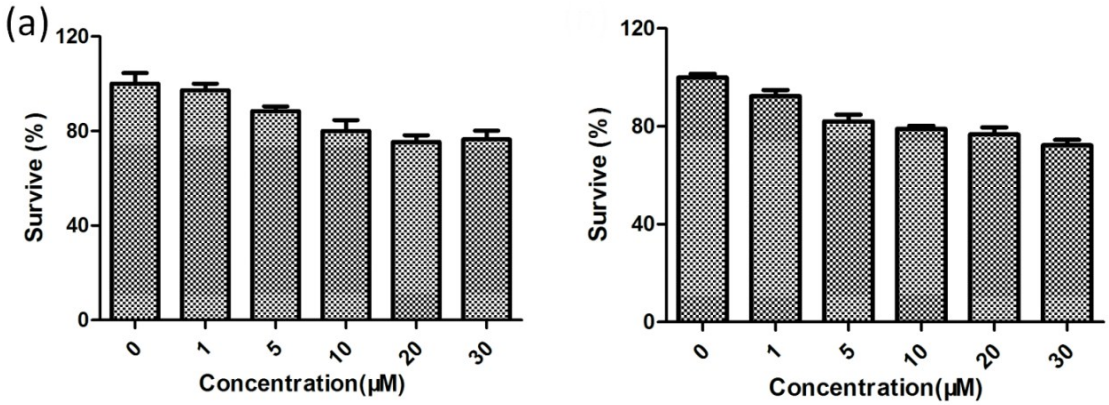

Fig. S4 Cytotoxicity assays of **G-GAL** at different concentrations for (a) OVCAR-3 and (b) HeLa cells.

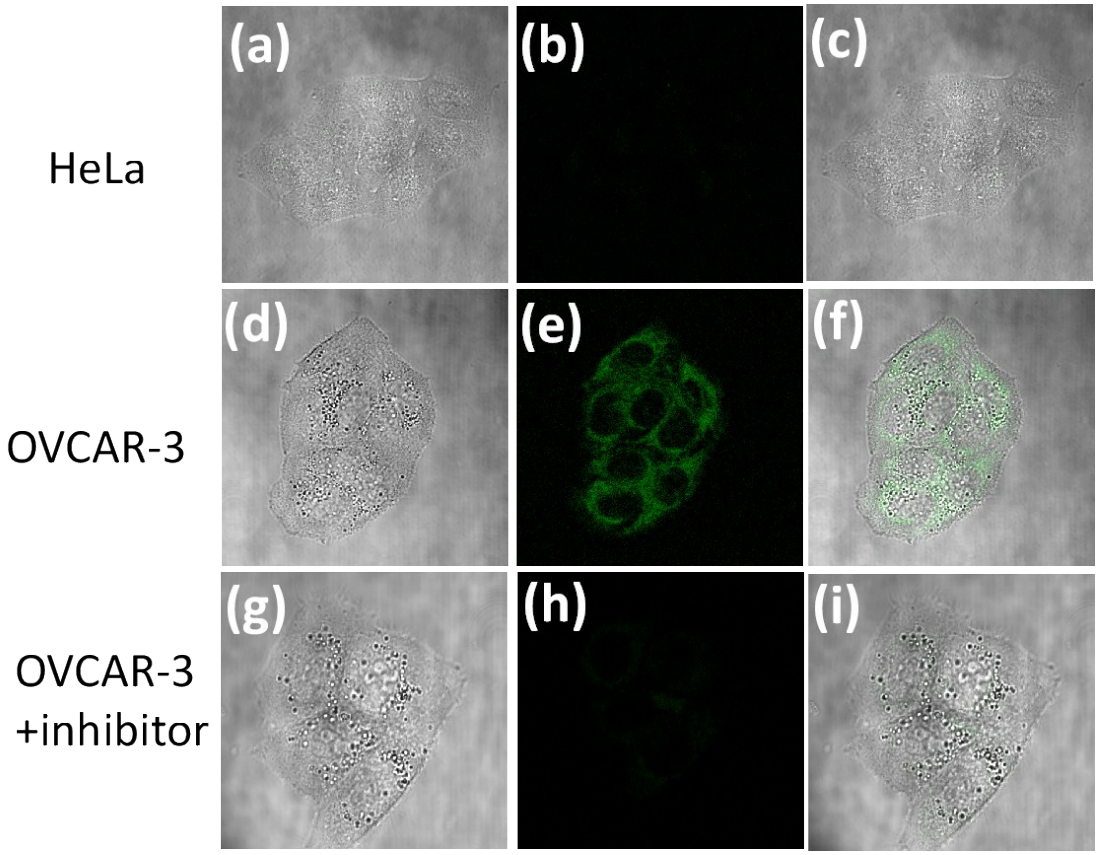

Fig. S5 Confocal fluorescence microscope images of HeLa and OVCAR-3 cells incubated with G-GAL (20 μM) for 50 min: (a) - (c) HeLa cells, (d) - (f) OVCAR-3 cells, and (g) - (i) OVCAR-3 cells pretreated with inhibitor (50 μM) for 1 h. (Eex = 405 nm).

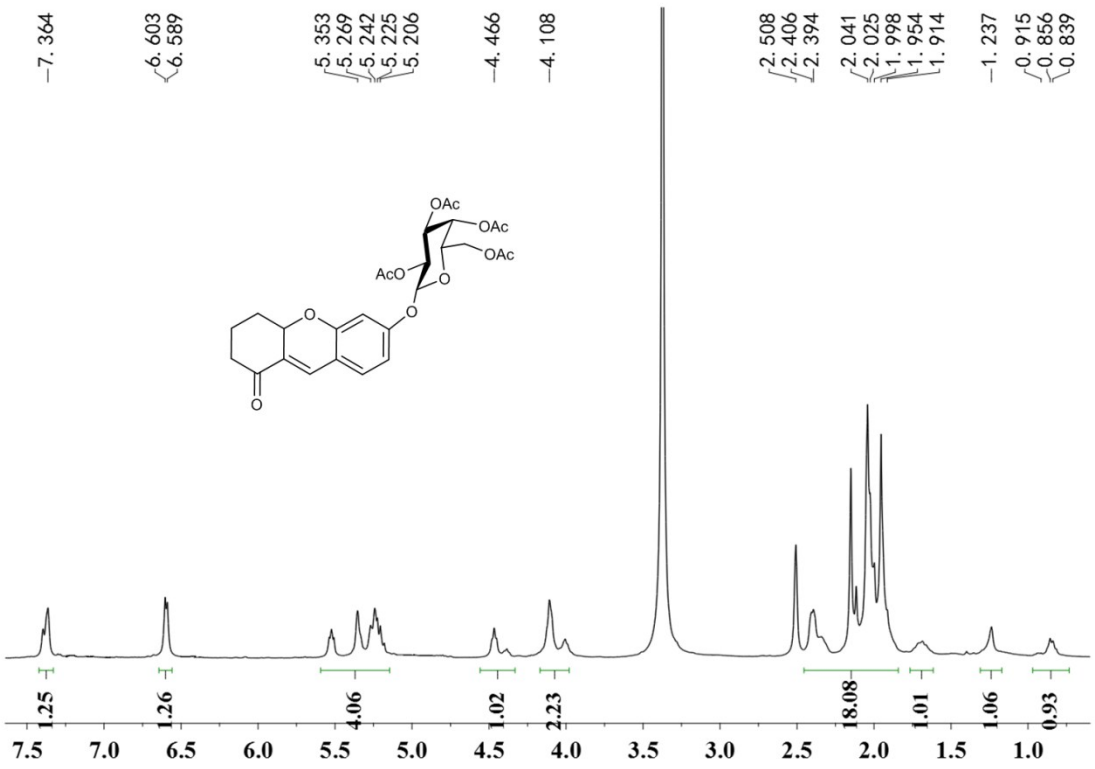

Fig. S6 ^{1}H-NMR (DMSO) spectrum of **Compound 3**.

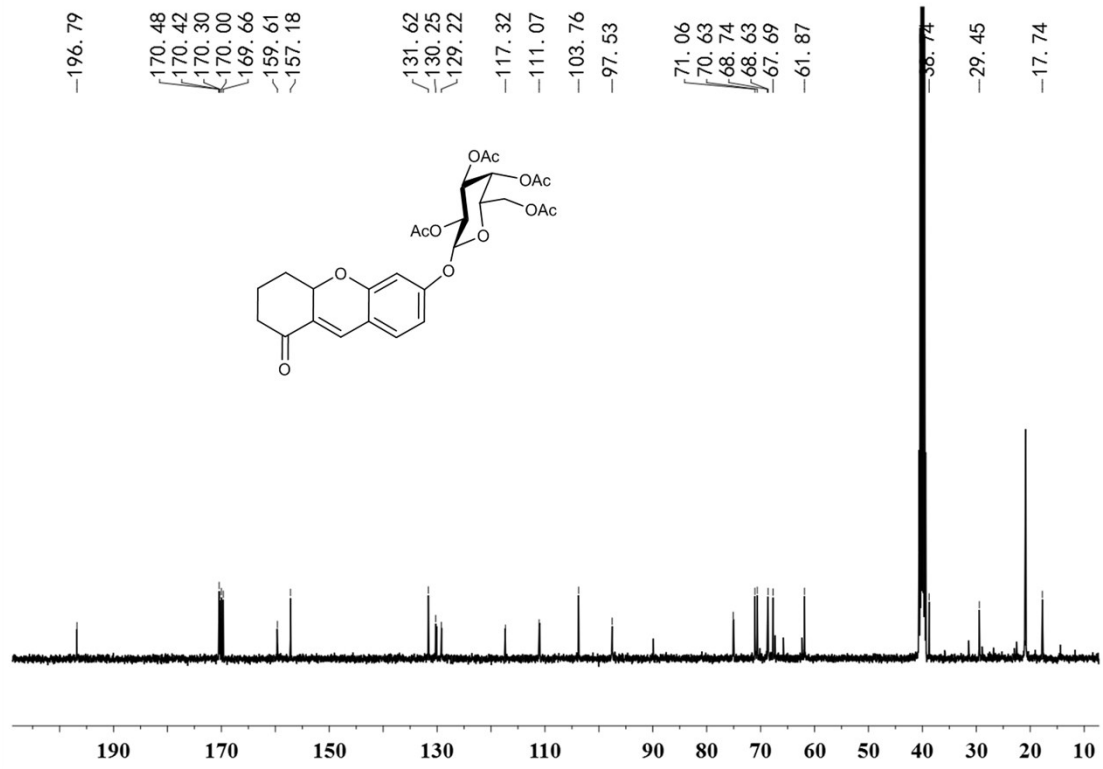

Fig. S7 ^{13}C-NMR (DMSO) spectrum of **Compound 3**.

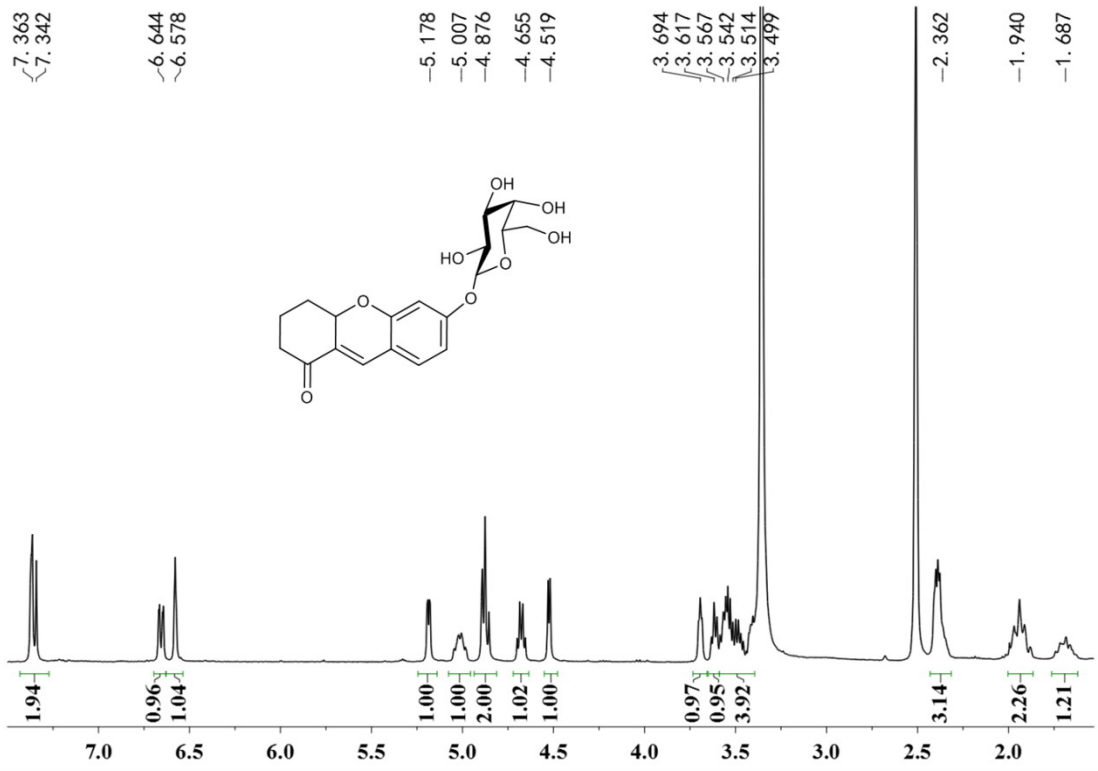

Fig. S8 ^{1}H-NMR (DMSO) spectrum of **G-GAL**.

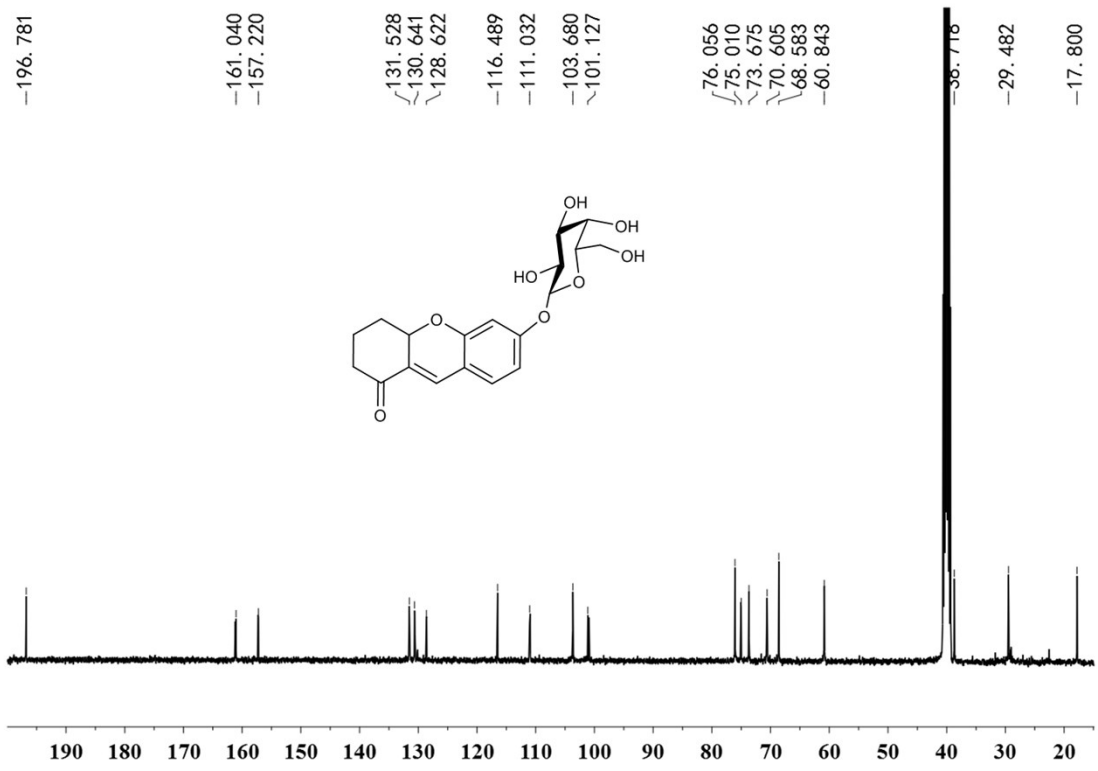

Fig. S9 ^{13}C-NMR (DMSO) spectrum of **G-GAL**.

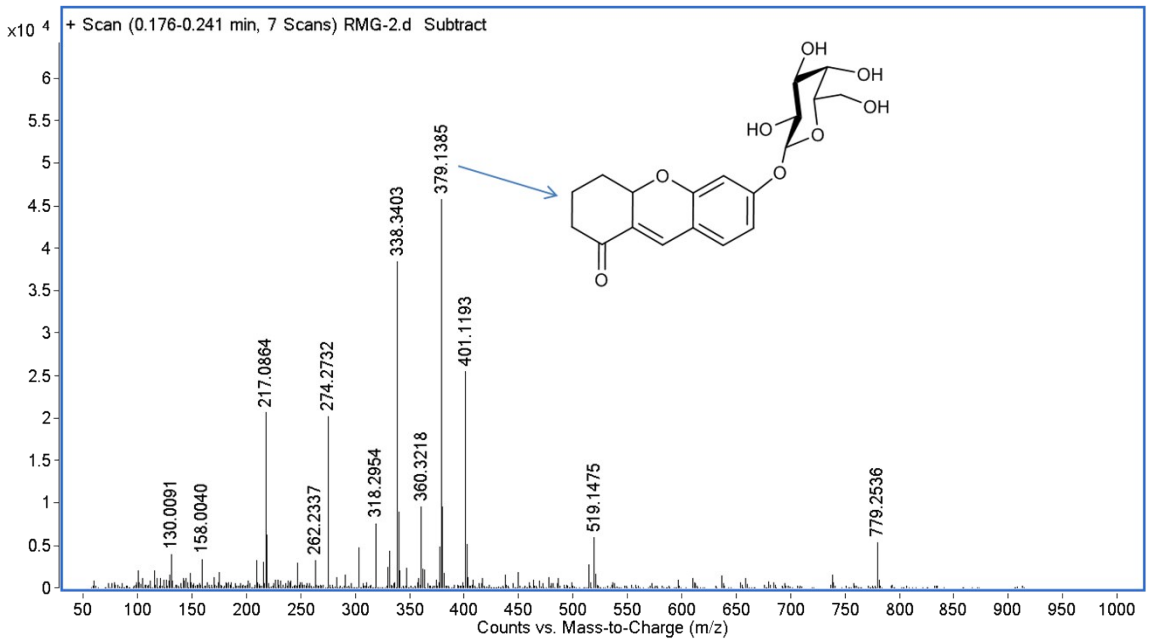

Fig. S10 HRMS (ESI) spectrum of **G-GAL**, $(M+H)^+$, 379.1387.

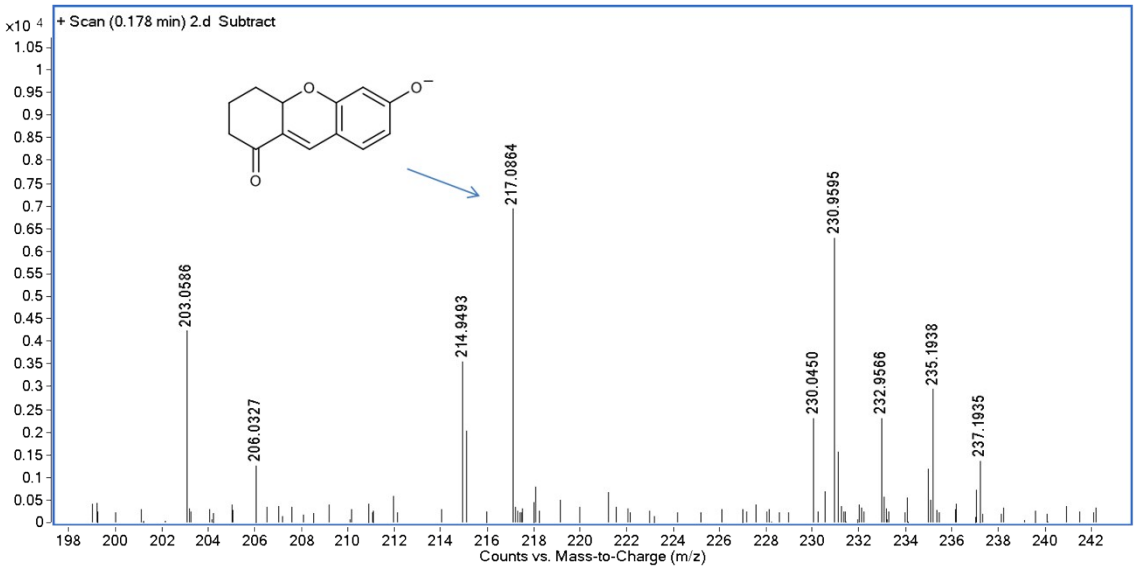

Fig. S11 HRMS (ESI) spectrum of the reaction products of **G-GAL** with β-gal, $(M+H)^+$, 217.0859.

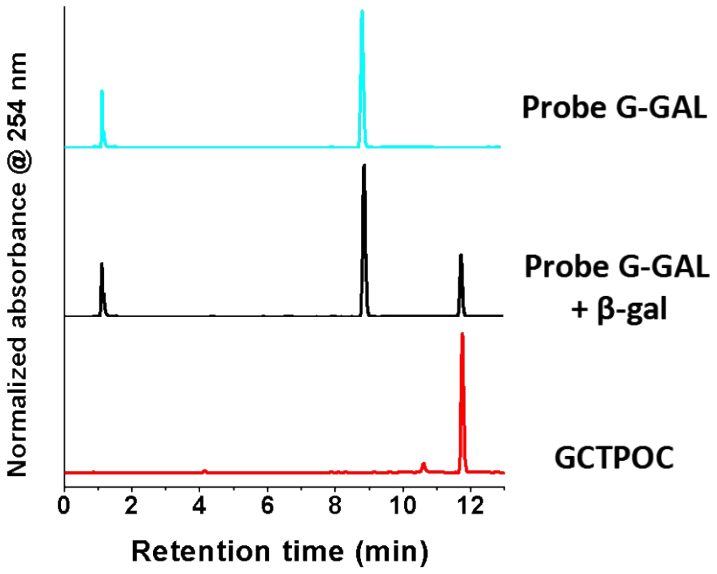

Fig. S12 Tracing the conversion of G-GAL to GCTPOC by HPLC analysis. An isocratic condition was used with eluent A (methyl alcohol) and eluent B (water). Absorbance at 254 nm was monitored. Peaks at 8.870 minutes and 11.747 minutes correspond to G-GAL and GCTPOC, respectively.

Cell type	One-Photon Intensity			Two-Photon Intensity		
	1	2	3	1	2	3
HeLa	173	112	135	3	11	23
OVCAR-3	754	744	727	150	175	165

Table 1. The relative fluorescence intensity in HeLa and OVCAR-3 cells of One-Photon and Two-Photon.

Tumor fluorescence ($\times 10^8$)	1	2	3
--	0.05	0.06	0.05
β-gal. overexpressed	2.24	2.95	2.3

Table 2. The related fluorescence intensity in the tumors of mice, respectively.