β-Galactosidase-activated bioluminescent probe for *in vivo* tumor imaging

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Supporting Information

Contents

1. Materials and instruments

All reagents and solvents were purchased from Heowns Biochem. Tech., Adamas-β and Shanghai Bide Pharmatech. and without any extra-purification. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dimyristoyl-rac-glycerol-3-methoxypolyethylene glycol-2000 (DMG-PEG2000) were purchased from Xi'an Ruixi Biological Technology Co., Ltd. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.1 M citrate buffer (pH= 4.2) and cholesterol were purchased from Tianjin Kaimeihong Biological Technology Co., Ltd. Trypsin-EDTA (0.25%) were purchased from Sigma-Aldrich. fLuc mRNA was purchased from TriLink BioTechnologies. Dulbecco's modified phosphate-buffered saline (DPBS) and firefly luciferase (fLuc) were purchased from Adamas-β Life. β-galactosidase (GAL) was purchased from Pusitang Biotech. D-(+)-ribono-1,4-lactone (D-Ribon) was purchased from Heowns Biochem. Tech. The ^{1}H NMR spectra were recorded on a Bruker AM 400 MHz spectrometer in $CDCl_3$, CD_3OD and DMSO-d_6, respectively. The ^{13}C NMR spectra were recorded on a Bruker AM 100 MHz spectrometer in DMSO-d_6, respectively. The cell viability and the bioluminescence intensity of probe responses to enzymes were recorded by the Tecan Infinite F/M200 Pro microplate reader. The bioluminescence images of cells, mice, and *ex vivo* organs with their regions of interest were measured by an IVIS Lumina II imaging system. High performance liquid chromatography (HPLC) was performed on LC-20AT (Japan) equipped with an SPD-20A UV/Vis detector and a Venusil C18 chromatographic column (4.6 × 150 mm, 5 μm).

2. General procedure of bioluminescence measurements

For the response to GAL, 100 μM **β-Luc** was incubated with GAL (100 U/mL) in 90 μL DPBS solution containing 2 mM ATP and 10 mM Mg^{2+} in a 96-well plate at 37 °C for 4 h. Then 10 μL of 50 μg/mL fLuc was added into each well. The variations of bioluminescence intensity were recorded by the Tecan Infinite F/M200 Pro microplate reader.

3. HPLC analysis

The stability of **β-Luc** (100 μM) was first determined in DPBS for 48 h. To examine the responsiveness of **β-Luc** toward GAL, **β-Luc** was incubated with or without GAL (100 U/mL) for 4 h. D-luciferin (100 μM) dissolved in DPBS was used as a control. Each sample was then subjected to HPLC analysis with UV absorption at 330 nm (acetonitrile containing 0.1% trifluoroacetic acid/water containing 0.1% trifluoroacetic acid, 10% - 60%). The detailed parameters of mobile phase with time were shown in **Table S1**.

4. The sensitivity of β-Luc responding to enzymes

To obtain the linearity and limit of detection (LOD) of GAL, 100 μM **β-Luc** was incubated with various concentrations of GAL (0 - 50 mU/mL), together with 2 mM ATP, and 10 mM Mg^{2+} in 90

μL DPBS at 37 °C for 2 h, then 10 μL of 50 μg/mL fLuc was added to each well. The changes of bioluminescence intensity were recorded by the Tecan Infinite F/M200 Pro microplate reader.

The LOD of **β-Luc** was calculated by the following formula:

$$LOD = 3\sigma/K$$

σ is depicted as the standard deviation of blank measurement, while K is described as the slope of bioluminescence intensity toward various concentrations of GAL.

5. The selectivity of β-Luc

A white 96-well plate was chosen for the selectivity test, and the probe **β-Luc** (100 μM) was mixed with each of the following species (1 mM) in 90 μL DPBS containing 2 mM ATP and 10 mM Mg^{2+} at 37 °C for 4 h: Cu^{2+}, Fe^{3+}, Ca^{2+}, NO_3^-, SO_4^{2-}, $H_2PO_4^-$, $O_2^{\cdot-}$, NO_2^-, $S_2O_4^{2-}$, H_2O_2, glutathione, D-cystine, D-galactose, nicotinamide adenine dinucleotide phosphate, acetylcholinesterase (100 U/mL), alkaline phosphatase (100 U/mL), carboxylesterase (100 U/mL), and GAL (100 U/mL). Subsequently, 10 μL containing 50 μg/mL fLuc was added to each well. The change in bioluminescence intensity of each well was recorded by a Tecan Infinite F/M200 Pro microplate reader.

6. Cytotoxicity analysis

Cytotoxicity of **β-Luc** was evaluated by CCK-8 assay. fLuc-transfected human ovarian cancer cells (SKOV3-Luc) were inoculated in transparent 96-well plates at a density of ~1×10^4 cells per well, cultured in DMEM containing 10% FBS as well as 1% PS, and incubated in 5% CO_2 as well as in a thermostat humidified incubator at 37 °C overnight. The cultured cells were removed from the medium and rinsed three times with PBS. Subsequently, fresh medium containing probe **β-Luc** at a concentration of 0 - 100 μM was replaced and washed three times with PBS buffer after 12 h of incubation. 100 μL of fresh medium (containing 10 μL CCK-8) was added to the above wells and incubated for 1 h. The UV absorbance at 450 nm of each well was subsequently recorded by a Tecan Infinite F/M200 Pro microplate reader.

7. Bioluminescence imaging of GAL activity in fLuc-transfected cells

The fLuc-transfected SKOV3 cells were seeded in a black 96-well plate at a density of ~1×10^4 cells per well. After culture for 24 h at 37 °C, the original medium was replaced with 200 μL of fresh DMEM per well. Subsequently, different concentrations (0-100 μM) of **β-Luc** were added to SKOV3-Luc cells. For GAL inhibition experiment, SKOV3-Luc cells were pre-incubated with D-Ribon (50 mg/mL) in DMEM medium at 37 °C for 30 min, and then the old medium was removed, followed by the addition of **β-Luc** at a concentration of 100 μM. The bioluminescent signals were recorded by an IVIS Lumina II imaging system.

8. Preparation of 4A3-SCC-PH mRNA LNPs

The fLuc mRNA-loaded LNPs formulation was prepared referring to previously reported protocols.[1-3] 4A3-SCC-PH/DOPE/cholesterol/DMG-PEG2000 dissolved in ethanol (or N,N-dimethylformamide) at a molar ratios of 15:15:30:3, and fLuc mRNA was dissolved in 10 mM citrate buffer (pH = 4.2) at a weight molar of 40:1. The two solutions were pipet mixed rapidly with an aqueous/ethanol volume ratios of 3:1 and followed by incubation at room temperature for 20 min. For *in vitro* experiments, the fLuc mRNA-loaded 4A3-SCC-PH LNPs were diluted with DPBS to 0.5 ng/μL fLuc mRNA. For *in vivo* experiments, the fLuc mRNA-loaded 4A3-SCC-PH LNPs were dialyzed against DPBS for 2 h and diluted with DPBS to 20 ng/μL fLuc mRNA for intraperitoneal administration.

9. Bioluminescence imaging of GAL activity in nontransgenic cells

Non-transgenic SKOV3 cells were seeded in a black 96-well plate at a density of ~1 × 10^4 cells per well. After culture for 24 h at 37 ℃, the original medium was replaced with 150 μL of fresh DMEM per well. Subsequently, fLuc mRNA-loaded 4A3-SCC-PH LNPs (50 ng/well) were added and incubated at 37 ℃ for 24 h. Next, different concentrations of 0 - 100 μM **β-Luc** were added to each well. For GAL inhibition experiment, SKOV3 cells transfected by fLuc mRNA-loaded 4A3-SCC-PH LNPs were pre-treated D-Ribon (50 mg/mL) in 200 μL DMEM for 30 min at 37 °C, followed by the addition of 100 μM **β-Luc**. The bioluminescence signals were recorded by an IVIS Lumina II imaging system at 1 min intervals.

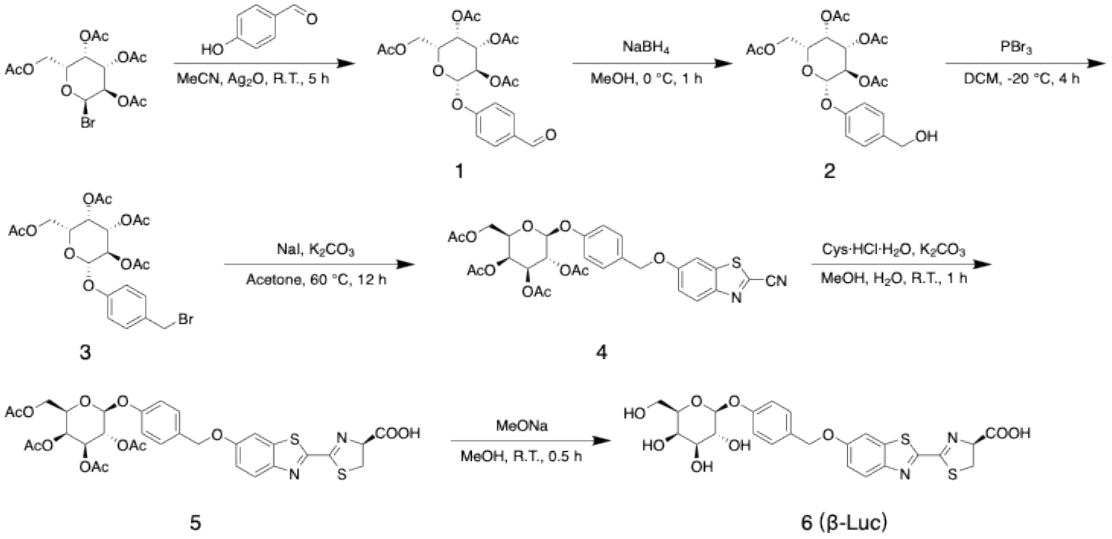
10. Bioluminescence imaging of GAL activity in tumor-bearing mice

Female BALB/c mice (4 - 6 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animal experiments were approved by the Ethical Committee of Nankai University and were conducted in accordance with the guidelines for animal experiments. Female BALB/c mice of 4 - 6 weeks old were used to construct the bilateral subcutaneous (s.c.) tumor-bearing mouse model by inoculation with 4T1-Luc cells (~2 × 10^6 cells) on both legs of mice. After 7 days, all left legs of the mice were injected intratumorally (i.t.) with 50 μL **β-Luc** at a concentration of 10 mM (DPBS solution containing 10% DMSO). In the inhibition group, all right legs of the mice were injected i.t. with 50 μL **β-Luc** (10 mM) after 1 h-injection of D-Ribon (100 mg/mL). Then the above mice were imaged using an IVIS Lumina II imaging system.

In addition, to establish the non-transgenic 4T1 breast cancer metastasis-bearing mouse model, female BALB/c mice (4-6 weeks) were first fed for 7 days in advance and then randomly divided into two groups. The mice in control group were injected intraperitoneally (i.p.) with PBS. The mice in experiment group were injected i.p. with 4T1 cells (~2 × 10^6 cells). After 7 days, all mice were injected i.p. with 100 μL fLuc mRNA-loaded 4A3-SCC-PH LNPs at a dose of 0.1 mg/kg mRNA. 6

hours later, 10 mM **β-Luc** (100 μL) was injected i.p. into the above mice and the mice were sacrificed, and all major organs were immediately collected for ex vivo bioluminescence imaging using an IVIS Lumina II imaging system.

11. Synthetic routes and characterizations



Scheme 1. The synthetic route of probe **β-Luc**.

Compound 1. The synthesis of compound **1** was referred from previous protocol.[4] 4-Hydroxybenzaldehyde (2.69 g, 22.0 mmol), Ag_2O (5.79 g, 25.0 mmol) and 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl-1-bromide (4.11 g, 10.0 mmol) were dissolved in acetonitrile (20 mL) and stirred at room temperature overnight. After reaction was completed, the resulting mixture was decanted into a funnel and washed the black precipitate twice by methanol, the filtrate was collected by vacuum filtration and concentrated. The crude product was purified via silica gel column chromatography with EtOAc/petroleum ether (1/10 to 1/1, v/v) as the eluent to afford a pale-yellow solid (2.61 g, yield 57.74%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.92 (s, 1H), 7.85 (d, J = 8.8 Hz, 2H), 7.11 (d, J = 8.8 Hz, 2H), 5.54 - 5.47 (m, 2H), 5.17 (d, J = 8.0 Hz, 1H), 5.13 (dd, J = 10.8, 3.6 Hz, 1H), 4.25-4.21 (m, 1H), 4.18-4.10 (m, 2H), 2.18 (s, 3H), 2.064 (s, 3H), 2.061 (s, 3H), 2.02 (s, 3H).

Compound 2. Sodium borohydride (0.19 g, 4.90 mmol) was added to a solution of compound 1 (1.11 g, 2.45 mmol) in methanol (40 mL) at 0 °C. The mixture was stirred at 0 °C until none bubbles produced. After reaction completion, 100 mL saturated ammonium chloride solution was poured into above resulting mixture, then extracted with 100 mL DCM for twice, the organic phase was washed with brine and water, then dried by anhydrous sodium sulfate, and the final filtrate was concentrated by evaporation under reduced pressure, while final crude product was purified by silica gel chromatography with EtOAc/petroleum ether (1/10 to 1/1, v/v) as the eluent to afford a clear light oil (1.00 g, yield 89.68%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.31 (d, J = 8.4 Hz, 2H), 7.00 (d, J = 8.8 Hz, 2H), 5.51-5.45 (m, 2H), 5.11 (dd, J = 10.4 Hz, 3.6 Hz, 1H), 5.03 (d, J = 7.6 Hz, 1H), 4.65

(d, *J* = 5.6 Hz, 2H), 4.26-4.21 (m, 1H), 4.18-4.14 (m, 1H), 4.08-4.04 (m, 1H), 2.19 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 2.02 (s, 3H).

Compound 3. 227 μL phosphorus tribromide was dissolved in anhydrous DCM (20 mL), then slowly dripped into a solution of compound 2 (1.00g, 2.20 mmol) in anhydrous DCM (20 mL) at -20 °C. After addition, the following mixture was stirred at 0 °C for 2 h. After reaction completion, the resulting mixture was poured into cold water (100 mL), then extracted with 100 mL DCM for twice, the organic phase was washed with brine and water, then dried by anhydrous sodium sulfate, and the final filtrate was concentrated by evaporation under reduced pressure to afford a white light oil (0.70 g, yield 61.49%).^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.33 (d, *J* = 8.8 Hz, 2H), 6.96 (d, *J* = 8.4 Hz, 2H), 5.50-5.45 (m, 2H), 5.10 (dd, *J* = 10.4 Hz, 3.2 Hz, 1H), 5.04 (d, *J* = 8.0 Hz, 1H), 4.48 (s, 2H), 4.24-4.20 (m, 1H), 4.17-4.13 (m, 1H), 4.08-4.04 (m, 1H), 2.18 (s, 3H), 2.06 (s, 6H), 2.01 (s, 3H).

Compound 4. A mixture of compound 3 (0.70 g, 1.35 mmol), sodium iodide (0.30 g, 2.03 mmol) and potassium carbonate (0.28 g, 2.03 mmol) were dissolved in 40 mL acetone. The reaction mixture was stirred for 24 h at 60 °C under a nitrogen atmosphere. After completion, the reaction mixture was concentrated by evaporation under reduced pressure. The crude product was purified by silica gel chromatography with EtOAc/petroleum ether (1/10 to 1/1, v/v) as the eluent to afford a white solid product (0.56 g, yield 67.56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.09 (d, *J* = 9.2 Hz, 1H), 7.42-7.38 (m, 3H), 7.29 (dd, *J* = 9.2 Hz, 2.4 Hz, 1H), 7.05 (d, *J* = 8.8 Hz, 2H), 5.52-5.46 (m, 2H), 5.13-5.10 (m, 3H), 5.06 (d, *J* = 8.0 Hz, 1H), 4.26-4.24 (m, 1H), 4.18-4.11 (m, 1H), 4.08-4.05 (m, 1H), 2.19 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 2.02 (s, 3H).

Compound 5. D-cysteine hydrochloride (0.28 g, 1.8 mmol) and compound 4 (0.56 g, 0.9 mmol) were suspended in DCM:MeOH:H_2O (2:2:1, v/v/v, 30 mL) in a 50 mL flask. Potassium carbonate (0.25 g, 1.8 mmol) aqueous solution was then added to the mixture, and the resulting solution was stirred under nitrogen for 1 h. The progress of the reaction was monitored by TLC analysis. The methanol was removed in vacuo and the remaining aqueous solution was acidified to pH = 3 with 2 M HCl, and then extracted with 100 mL EtOAc for twice, the organic phase was washed with 0.01 M HCl, then dried by anhydrous sodium sulfate, and the final filtrate was concentrated by evaporation under reduced pressure, while final crude product was purified by silica gel chromatography with DCM/Acetic Acid/Methanol (200/1/1 to 200/10/10, v/v/v) as the eluent to afford a pale-yellow powder (0.56 g, yield 85.47%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.06 (d, *J* = 9.2 Hz, 1H), 7.85 (d, *J* = 2.8 Hz, 1H), 7.47 (d, *J* = 8.8 Hz, 2H), 7.26 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.02 (d, *J* = 8.8 Hz, 2H), 5.48 (d, *J* = 7.6 Hz, 1H), 5.44-5.39 (m, 1H), 5.35-5.34 (m, 1H), 5.30-5.27 (m, 1H), 5.24-5.19 (m, 1H), 5.16 (s, 2H), 4.43 (t, *J* = 6.8 Hz, 1H), 4.14-4.06 (m, 2H), 3.80-3.66 (m, 2H),

2.15 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H), 1.95 (s, 3H) ^{13}C NMR (100 MHz, DMSO-d_6) δ: 171.14, 169.97, 169.82, 169.54, 169.23, 164.35, 157.95, 157.92, 156.33, 147.17, 137.07, 130.91, 129.73, 124.79, 117.51, 116.43, 105.87, 97.74, 78.12, 70.35, 70.14, 69.47, 68.35, 67.22, 61.28, 34.73, 20.46, 20.43, 20.38, 20.33.

Compound 6. (β-Luc) Compound 5 (0.56 g, 0.78 mmol) was dissolved in 20 mL of methanol, and MeONa (0.08 g, 1.56 mmol) was added, the mixture was stirred at room temperature for 1 h, then the remaining aqueous solution was acidified to pH 3 with 2 M HCl, and then extracted with 100 mL EtOAc and DCM for twice respectively, the organic phase was combined and washed with 0.01 M HCl, then dried by anhydrous sodium sulfate, and the final filtrate was concentrated by evaporation under reduced pressure, while final crude product was purified by silica gel chromatography with DCM/Acetic Acid (30/1 to 3/1, v/v) as the eluent to afford a pale-yellow powder (0.35 g, yield 81.66%). ^{1}H NMR (400 MHz, CD_3OD-d_4) δ: 7.97 (d, J = 9.2 Hz, 1H), 7.63 (d, J = 2.4 Hz, 1H), 7.41 (d, J = 8.4 Hz, 2H), 7.24 (dd, J = 8.8, 2.4 Hz, 1H), 7.14 (d, J = 8.8 Hz, 2H), 5.39 (t, J = 9.2 Hz, 1H), 5.13 (s, 2H), 4.88 (d, J = 3.6 Hz, 1H), 3.90 (d, J = 3.2 Hz, 1H), 3.83-3.72 (m, 5H), 3.70-3.67 (m, 1H), 3.58 (dd, J = 9.6, 3.2 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 171.64, 164.83, 158.49, 158.39, 157.86, 147.62, 137.57, 130.02, 125.26, 118.02, 116.72, 106.30, 101.41, 78.62, 75.98, 73.78, 70.74, 70.19, 68.61, 60.85, 35.22. HRMS-ESI (m/z) calcd for $C_{24}H_{24}N_2O_9S_2$ [M-H]$^-$: 547.0850, found: 547.0859.

12. Supplementary figures and table

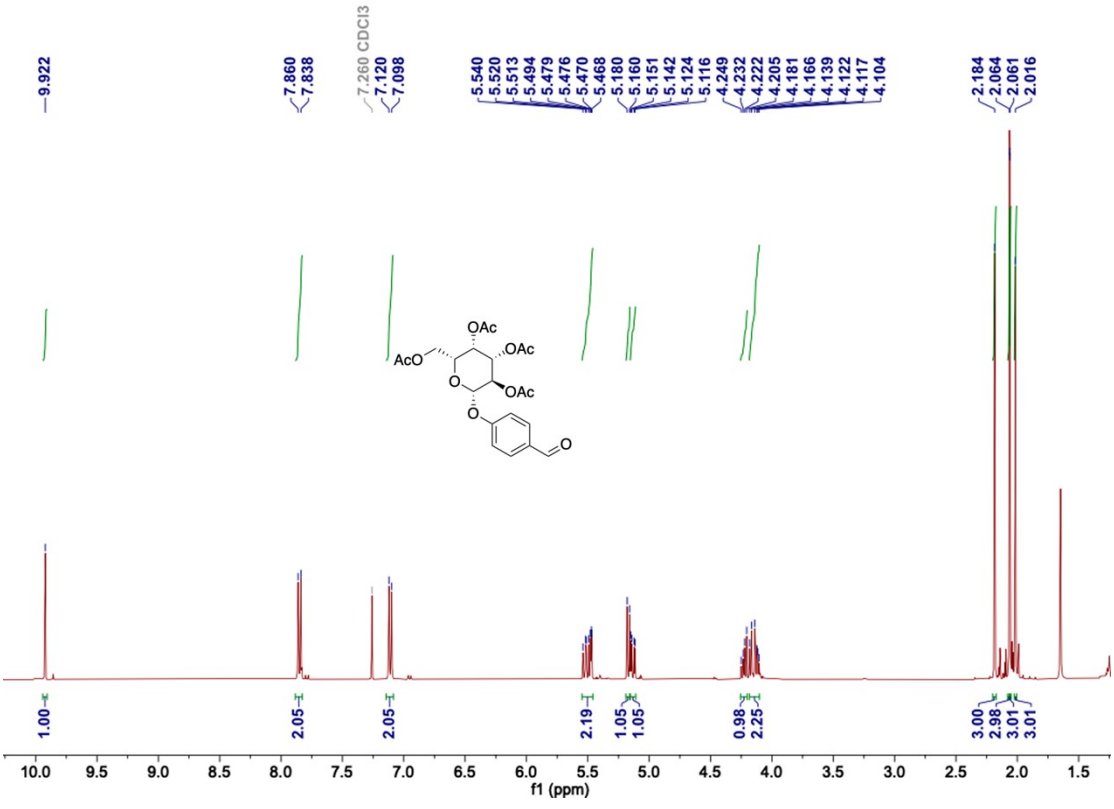


Figure S1. ^{1}H NMR spectrum of **Compound 1**

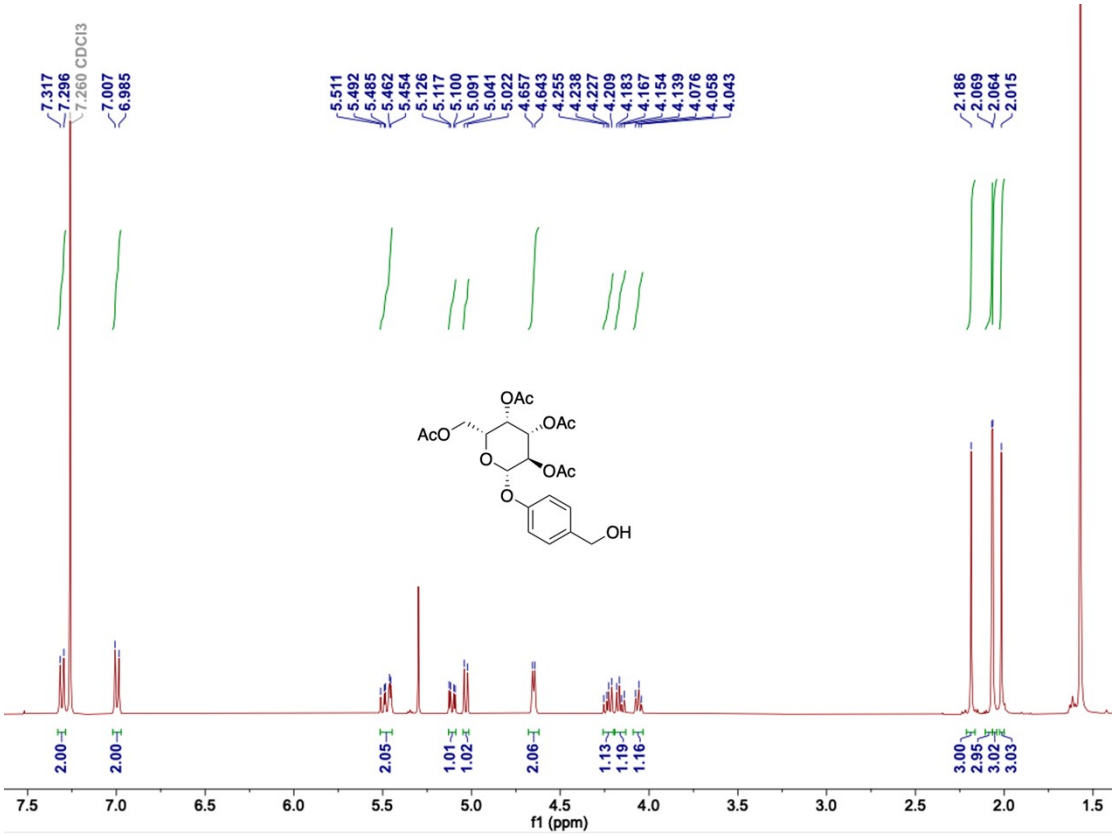


Figure S2. ^{1}H NMR spectrum of **Compound 2**

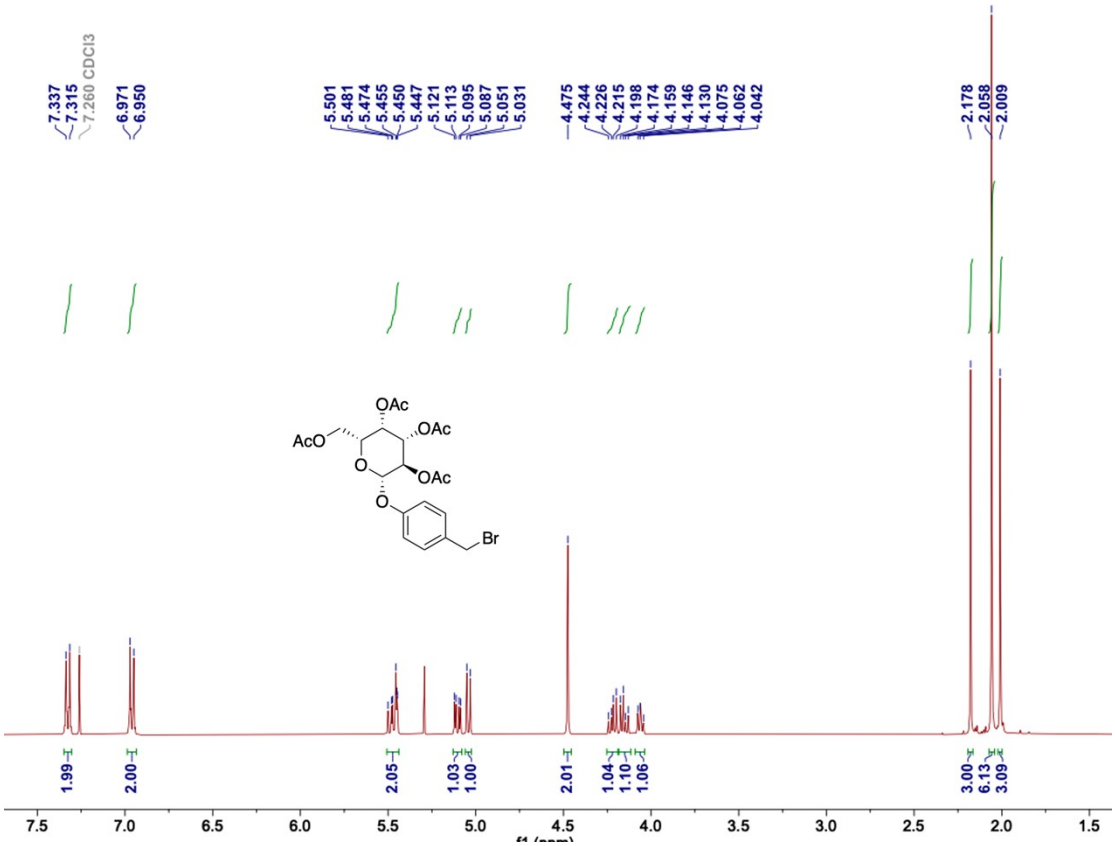


Figure S3. ^{1}H NMR spectrum of **Compound 3**

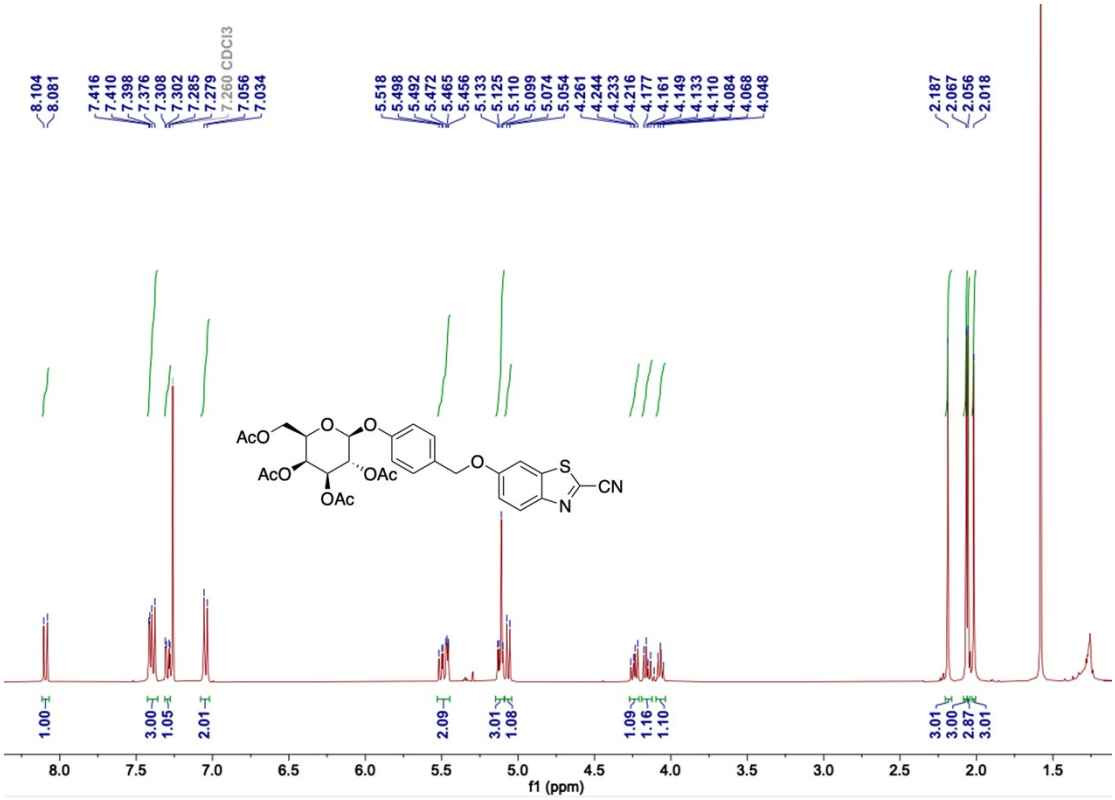


Figure S4. ^{1}H NMR spectrum of **Compound 4**

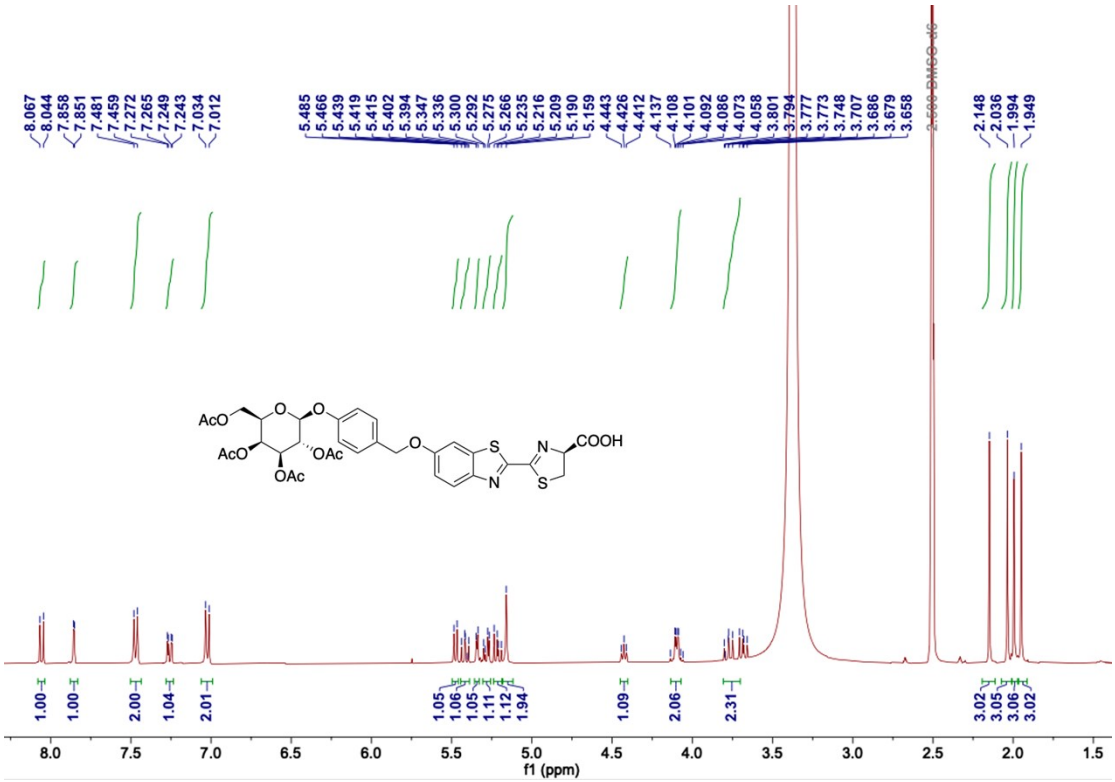


Figure S5. ^{1}H NMR spectrum of **Compound 5**

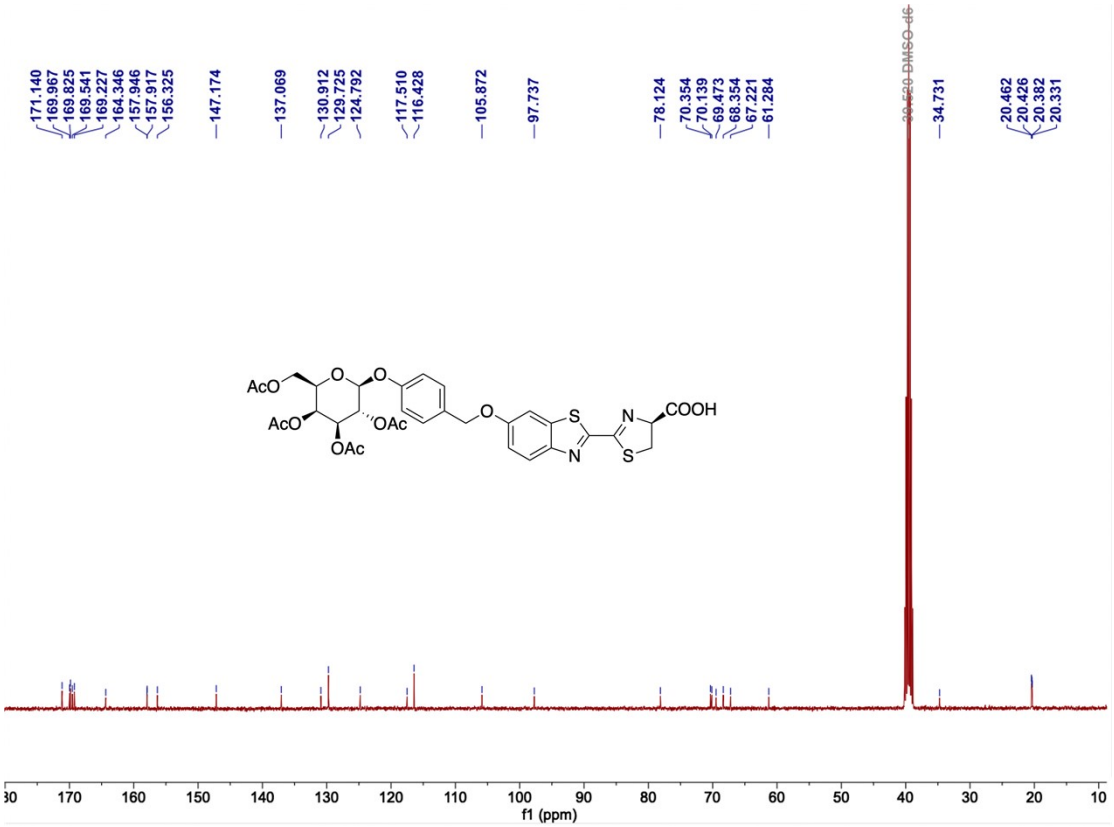


Figure S6. ^{13}C NMR spectrum of **Compound 5**

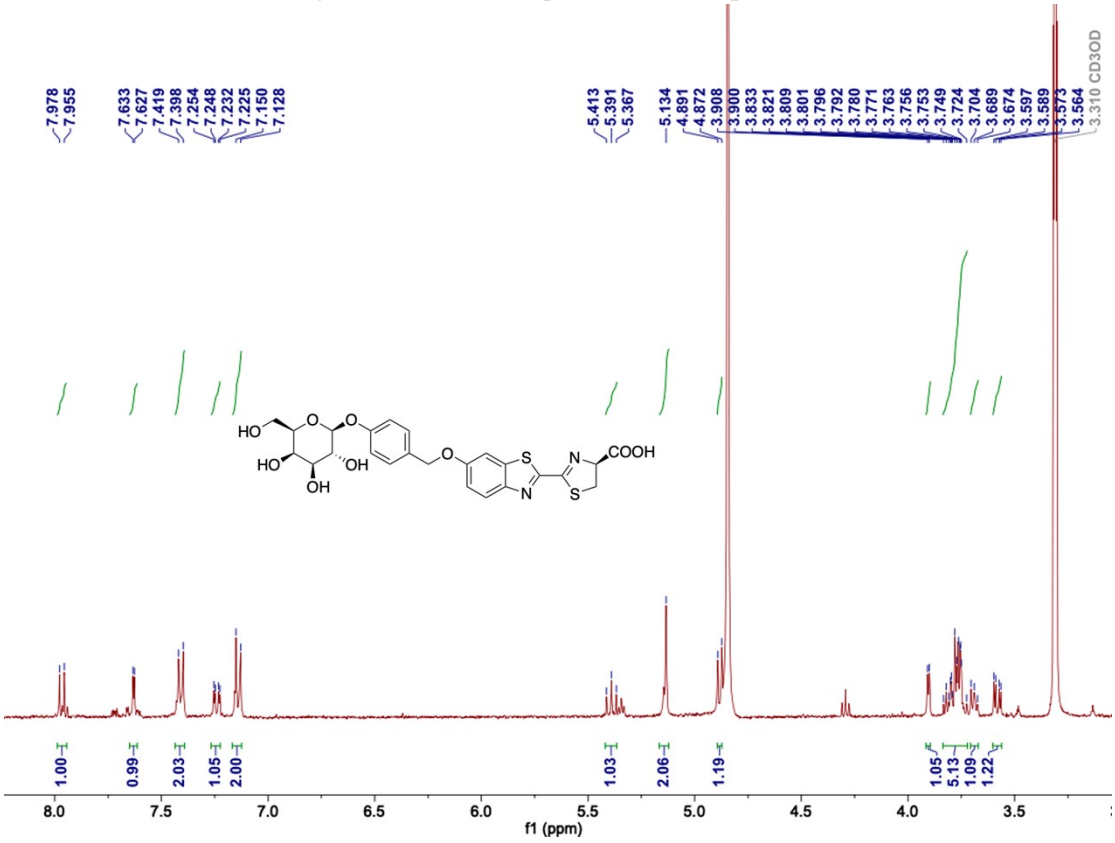


Figure S7. ^{1}H NMR spectrum of **β-Luc**

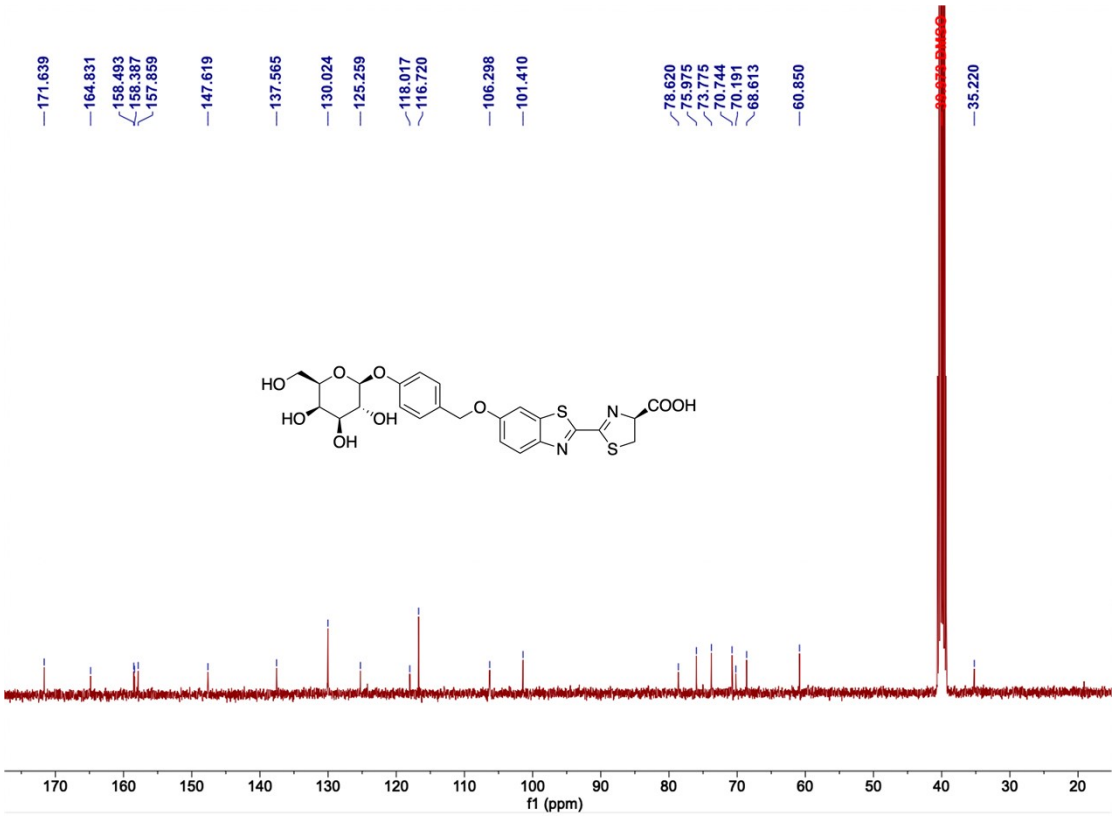


Figure S8. ^{13}C NMR spectrum of **β-Luc**

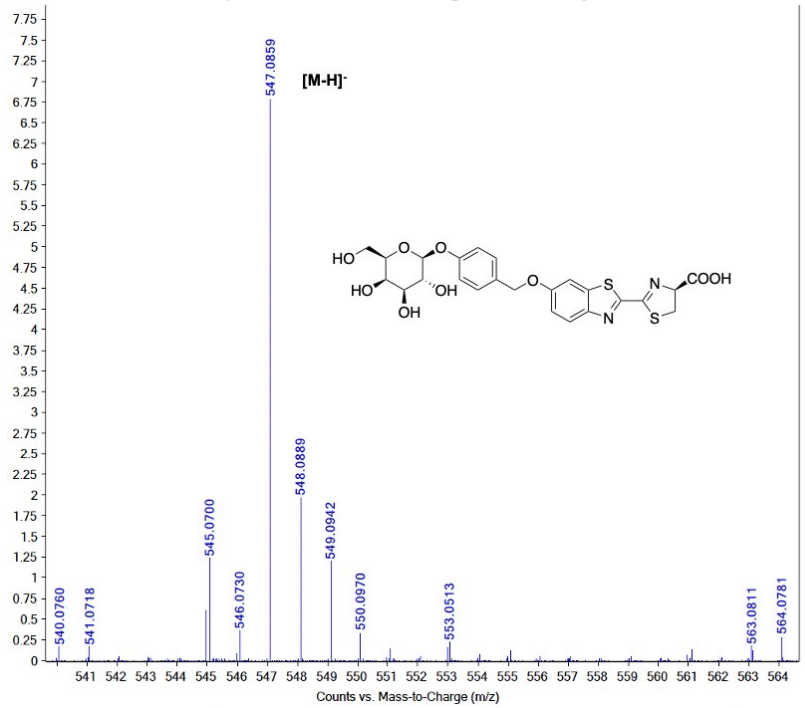


Figure S9. ESI-MS of **β-Luc**

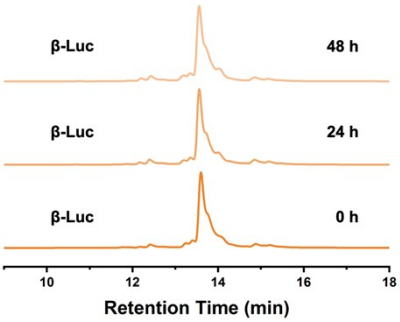


Figure S10. HPLC traces of **β-Luc** after incubation of various durations. Wavelength for detection: 330 nm.

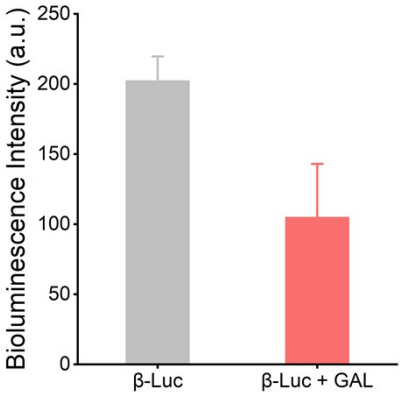


Figure S11. Bioluminescence intensity of 100 μM probe **β-Luc** after incubation in 1 × DPBS at 37 °C in the absence or presence of 100 U/mL GAL for 4 h, containing 2 mM ATP and 10 mM Mg^{2+}.

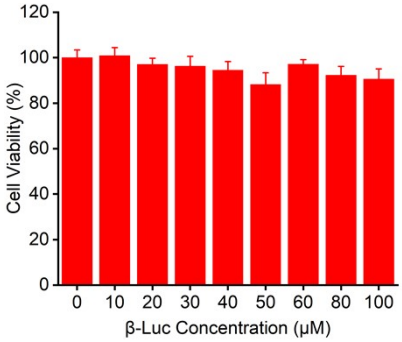


Figure S12. The cytotoxicity assay for the cell viability of SKOV3-Luc cells treated with various concentrations of **β-Luc** for 12 h, respectively. Error bars represent the standard deviations of 3 trials.

Table S1. The mobile phase parameters with time in HPLC-UV analysis.

Time (min)	Phase A (%)	Phase B (%)	The Flow Rate (mL / min)

0	10	90	
10	60	40	
15	70	30	0.6
20	80	20	
25	90	10	

Phase A: acetonitrile containing 0.1% trifluoroacetic acid; **Phase B**: water containing 0.1% trifluoroacetic acid.

Table S2. Representative works for detecting GAL activity

Probe	Imaging Mode	LOD (mU/mL)	$\lambda_{ex}/\lambda_{em}$ (nm)	Applicability Cells/Models	Ref.
TPh-PyBz-β-gal	Fluorescence	220	365/606	HepG2	5
DP-βgal	Fluorescence	3.2	550/675	SKOV3, HepG2	6
QM-TPA-Gal	Fluorescence	210	460/680	HeLa/SKOV3 tumor-bearing mice; HeLa, SKOV3;	7
HCyXA- βGal	Fluorescence	12	680/710	HeLa, SKOV3, WI-38	8
HBT-PXZ-Ga	Fluorescence	57.9	350/540	*S. pneumoniae*	9
P1	Fluorescence	2.7	470/533	PC-12, HepG2, RAW 264.7	10
HD-BTZ-gal	Fluorescence, Photoacoustic	5.9	710/756	OVCAR3, 4T1; 4T1 tumor-bearing mice	11
Lugal	Bioluminescence	N/A	/560	*Myf5-nLacZ*$^{\pm}$ transgenic mice; *lacZ-luc* C2C12	12
LuGal	Bioluminescence	2×10^{-4}	/560	coliforms	13
β-Luc	Bioluminescence	8.5	/560	4T1 tumor-bearing mice; SKOV3	This work

Table S3. Representative clinical methods for detecting GAL activity

Method	LOD	Time	Clinical Samples	Ref.
ELISA	1.57 fM or 4.5 fM	~30 min	blood or serum	14, 15
LFIA	50 pM or 10 nM	~25 min	saliva or serum	16, 17
β-Luc	8.5 mU/mL	~5 min	tumor tissues and cancer cells	This work

13. Reference

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