Electronic Supplementary Information (ESI)

In vivo ratiometric tracking of endogenous *β*-galactosidase activity using an activatable near-infrared fluorescent probe

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1. Experimental section

Materials and general methods

Unless special stated, all solvents and chemicals were purchased from commercial suppliers in analytical grade and used without further purification. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM 400 spectrometer, using TMS as an internal standard. High resolution mass spectrometry data were obtained with a Waters LCT Premier XE spectrometer. Absorption spectra were collected on a Varian Cary 500 spectrophotometer, and fluorescence spectra measurements were performed on a Varian Cary Eclipse fluorescence spectrophotometer. HPLC analysis was performed on an Agilent 1100 series. Confocal fluorescence images were taken on a Leica TCS SP8 (63 × oil lens). *In vivo* fluorescence images were measured with a PerkinElmer IVIS Lumina Kinetic Series III imaging system.

Cell experiments

Cell lines

The cell lines were purchased from the Institute of Cell Biology (Shanghai, China). Cells were all propagated in T-75 flasks cultured at 37 ºC under a humidified 5% CO_2 atmosphere in RPMI-1640 medium or DMEM medium (GIBCO/Invitrogen, Camarillo, CA, USA), which were supplemented with 10 % fetal bovine serum (FBS, Biological Industry, Kibbutz Beit Haemek, Israel) and 1% penicillin-streptomycin (10,000 U mL^{-1} penicillin and 10 mg/mL streptomycin, Solarbio life science, Beijing, China).

In vitro cytotoxicity assay

The cell cytotoxicity of BODIPY-*β*gal to SKOV-3 cells were measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The cytotoxicity was evaluated by Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to the factory's instruction. Cells were plated in 96-well plates in 0.1 mL volume of DMEM or RPMI-1640 medium with 10 % FBS, at a density of 1×10^4 cells/well and added with desired concentrations of BODIPY-*β*gal. After incubation for 24 h, absorbance was measured at 450 nm with a Tecan GENios Pro multifunction reader (Tecan Group Ltd., Maennedorf, Switzerland). Each concentration was measured in triplicate and used in three independent experiments. The relative cell viability was calculated by the equation: cell viability (%) = $(OD_{treated}/OD_{control}) \times 100\%$.

In vitro cellular imaging

The cells at 1×10^5 cells/well were seeded onto glass-bottom petri dishes with complete medium (1.5 mL) for 12 h. Then the cells pre-incubated with and without D-galactose were exposed to desired concentrations of compounds for 0.5 h. PBS was used to washed cells for three times to clean the background. 4 % paraformaldehyde was added at

room temperature for 20 min. The fixed cells were rinsed with PBS twice. The images were then photographed by using a Confocal laser scanning microscope Leica TCS SP8 (63 × oil lens).

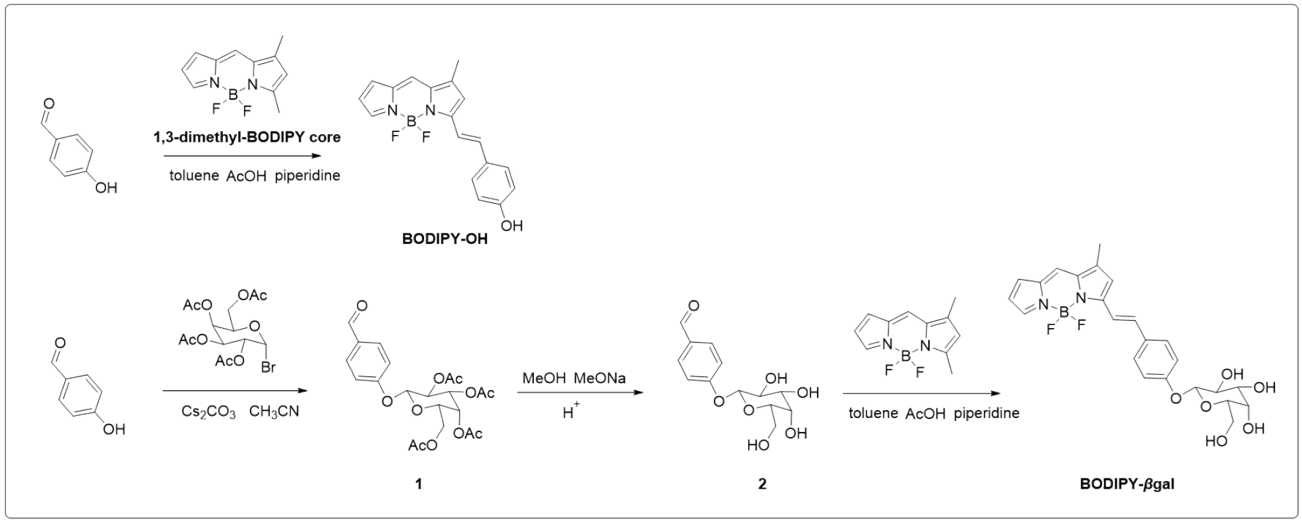
Animals

All animal studies were approved by the Animal Care and Use Committee in accordance with the guidelines for the care and use of laboratory animals. The 3-4-week-old female BALB/cA nude mice were produced from Shanghai Genechem Co.,Ltd., and maintained under standard conditions. The animals were housed in sterile cages within laminar airflow hoods in a specific pathogen-free room with a 12-h light/12-h dark schedule and fed autoclaved chow and water *ad* libitum. Number of qualitative qualification: No.311613700000141. Production Permit No.: SCXK (Shanghai) 2013-0017. SYXK No. of Shanghai Institute of Materia Medica: SYXK (Shanghai) 2013-0049.

Real-time *in vivo* imaging in tumor-bearing mice

The nude mice were inoculated with A549 cells on their right flanks by injecting 10^6 cells subcutaneously. When the tumors grew up to 10 mm in diameter, BODIPY-βgal (0.048 mg kg^{-1}) in PBS were injected orthotopically into the A549 cell tumor-bearing nude mice. The real-time *in vivo* imaging was recorded at different time internals after BODIPY-βgal injection. *In vivo* fluorescence images were measured with a PerkinElmer IVIS Lumina Kinetic Series III imaging system. After injection, the mice were sacrificed at 3 h. The grafted tumor tissues and major organs, including kidney, lung, spleen, liver, heart were excised and washed with 0.9% saline. The optical images of the organs and tissues were taken using a PE *in vivo* Professional Imaging System as described above.

Synthesis of BODIPY compounds



Scheme S1 Synthetic route of BODIPY-OH and BODIPY-βgal

Synthesis of BODIPY-OH

1,3-dimethyl-BODIPY core (100 mg, 0.45 mmol) and **4-hydroxybenzaldehyde** (83 mg, 0.68 mmol) were dissolved in toluene (40 mL) along with acetic acid (0.5 mL) and piperidine (1.0 mL), the system was under argon protection and then refluxed for 8 h. Toluene was removed by evaporation, and the residue was purified by silica gel chromatography (CH_2Cl_2/MeOH 100:1) to get the desired product **BODIPY-OH** (50 mg, 0.15 mmol), a dark purple solid. Yield was 33%. ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): δ 2.32 (s, 3H, -*CH*$_3$), 6.45 (m, 1H), 6.74 (s, 1H), 6.86 – 6.91 (m, 3H), 7.15 (s, 1H), 7.34 (d, J = 16.24 Hz, 1H, alkene-H), 7.51 (m, 3H), 7.66 (s, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6, ppm): δ 11.14, 114.06, 115.91, 116.24, 117.79, 123.40, 125.20, 126.66, 129.80, 132.55, 137.20, 137.60, 141.81, 145.45, 159.41, 159.99. Mass spectrometry (ESI negative ion mode for $[M - H]^-$): calcd for $[C_{18}H_{14}BF_2N_2O^-]$: 323.1167; found: 323.1165.

Synthesis of BODIPY-*β*gal

4-hydroxybenzaldehyde (250 mg, 2.05 mmol), **tetra-O-acetyl-α-D-galactopyranosyl-1-bromide** (1.20 g, 2.92 mmol) and Cs_2CO_3 (1.90 g, 5.84 mmol) were dissolved in anhydrous acetonitrile (25 mL). The system was stirred at room temperature for 16 h under argon protection. After reaction was over, filtration was performed. The solvent was removed under reduced pressure, and the residue was taken up in sat.NH_4Cl, next extracted with CH_2Cl_2. After dried over anhydrous Na_2SO_4, the solvent was removed by evaporation again, and the crude product was purified by silica gel chromatography (CH_2Cl_2/MeOH 100:1) to obtain the desired product **1** (500 mg, 1.11 mmol), a white solid. Yield was 54% yield. ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): δ 2.03 (s, 3H, -*CH*$_3$), 2.07 (s, 6H, -*CH*$_3$), 2.20 (s, 3H, -*CH*$_3$), 4.11 – 4.26 (m, 3H), 5.12 – 5.18 (m, 2H), 5.48 – 5.55 (m, 2H), 7.12 (d, J = 8.68 Hz, 2H, Ph-H), 7.86 (d, J = 8.68 Hz, 2H, Ph-H), 9.93 (s, 1H, -C*H*O). Mass spectrometry (ESI negative ion mode for $[M + Na]^+$): calcd for $[C_{21}H_{24}O_{11}Na^+]$: 475.1216; found: 475.1222.

1 (200 mg, 0.44 mmol) was dissolved in dry methanol (20 mL). Sodium methylate (216 mg, 4.00 mmol) in dry methanol (2 mL) was added dropwise to the solution, and the solution was stirred for 3 h at room temperature. When reaction was completed, the reaction mixture was neutralized by adding Amberlite IR-120 plus (H^+) until pH value was about 7. The residue was purified by silica gel chromatography (CH_2Cl_2/MeOH 10:1) to get the desired product **2** (112 mg, 0.39 mmol), a white solid. Yield was 88%. ^{1}H-NMR (400 MHz, CD_3OD, ppm): δ 3.61 (dd, J_1 = 3.40 Hz, J_2 = 9.76 Hz, 1H), 3.73 – 3.79 (m, 3H), 3.84 (dd, J_1 = 7.72 Hz, J_2 = 9.76 Hz, 1H), 3.92 (d, J = 3.40 Hz, 1H), 5.01 (d, J = 7.72 Hz, 1H, Ph-H), 7.25 (d, J = 8.72 Hz, 2H, Ph-H), 7.87 (d, J = 8.72 Hz, 2H, Ph-H), 9.86 (s, 1H, -C*H*O). ^{13}C-NMR (100 MHz, CD_3OD, ppm): δ 64.95, 72.73, 74.62, 77.30, 79.72, 104.69, 120.42, 135.04, 135.43, 166.68. Mass spectrometry (ESI negative ion mode for $[M - H]^-$): calcd for $[C_{13}H_{15}O_7^-]$: 283.0818; found: 283.0823.

1,3-dimethyl-BODIPY core (100 mg, 0.45 mmol) and **2** (40 mg, 0.14 mmol) were dissolved in toluene (40 mL) along with acetic acid (0.5 mL) and piperidine (1.0 mL), the system was under argon protection and then refluxed

for 8 h. Toluene was removed by evaporation, and the residue was purified by silica gel chromatography (CH_2Cl_2/MeOH 10:1) to get the desired product **BODIPY-*β*gal** (20 mg, 0.04 mmol), a dark purple solid. Yield was 29%. ^{1}H-NMR (400 MHz, CD_3OD, ppm): δ 2.34 (s, 3H, -CH_3), 3.60 (dd, J_1 = 3.40 Hz, J_2 = 9.72 Hz, 1H), 3.74 – 3.84 (m, 4H), 3.92 (d, J = 3.40 Hz, 1H), 4.95 (d, J = 7.72 Hz, 1H), 6.46 (m, 1H), 6.95 (s, 1H), 7.00 (d, J = 3.68 Hz, 1H), 7.16 (d, J = 8.80 Hz, 2H), 7.46 (m, 2H), 7.58 (m, 4H). Mass spectrometry (ESI negative ion mode for $[M - H]^-$): calcd for $[C_{24}H_{24}BF_2N_2O_6^-]$: 485.1695; found: 485.1710.

2. Photophysical properties and cells measurement

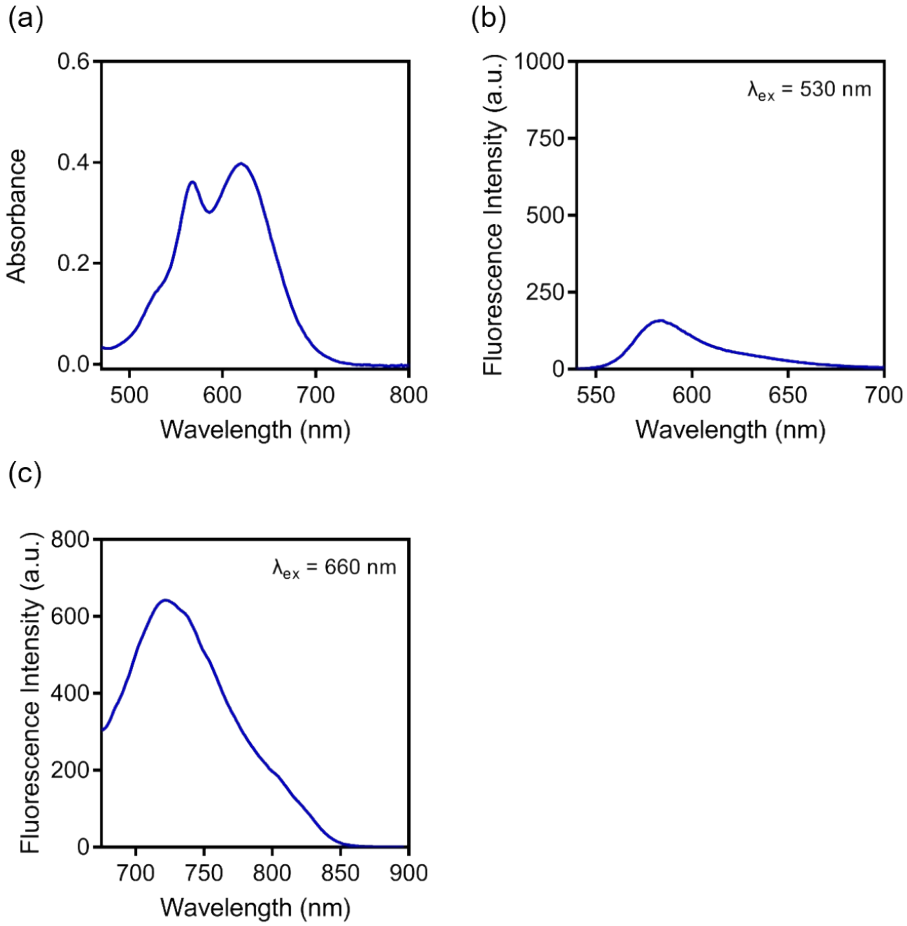


Fig. S1 (a) Absorption and (b and c) fluorescence spectra of BODIPY-OH (10 μM) in PBS/DMSO solution (1:1, v/v, pH = 7.4).

Table S1 Fluorescence quantum yield of the probe.

	BODIPY-*β*gal (575 nm)
Φ_F	0.93

The relative fluorescence quantum yield Φ_F value was determined using rhodamine B as a reference.

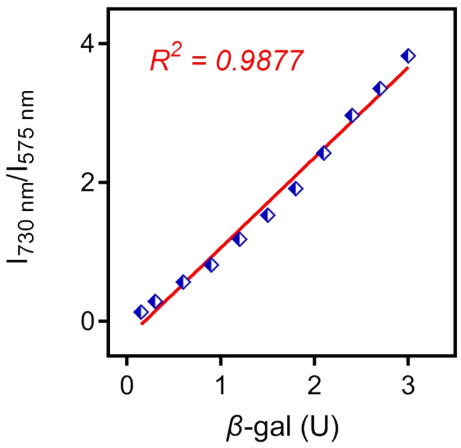


Fig. S2 The linear relationship between $I_{730\ nm}/I_{575\ nm}$ and β-gal concentration.

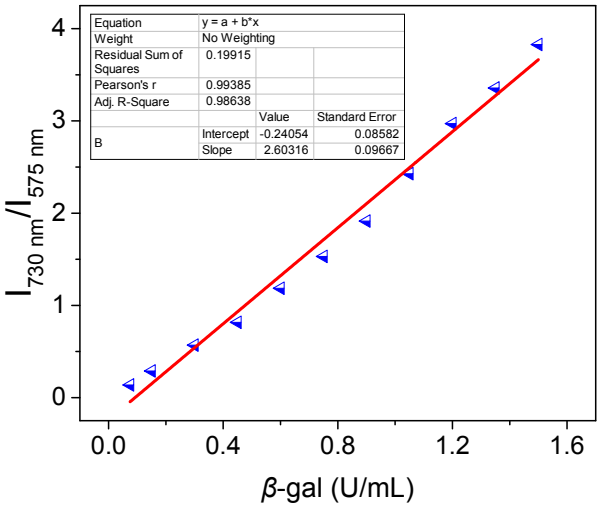


Fig. S3 Fluorescence intensity ratio changes with the concentration of β-gal. Note: the detection limit was calculated based on the fluorescence titration. The limit of detection for β-gal is 4.6×10^{-3} U/mL.

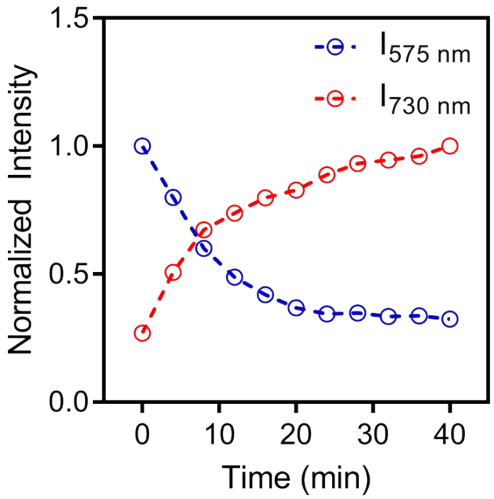


Fig. S4 Time-dependent of fluorescence intensity $I_{730\ nm}$ and $I_{575\ nm}$ incubation with β-gal (5 U) in Britton-Robinson buffer/DMSO solution (1:1, v/v, pH = 6.0).

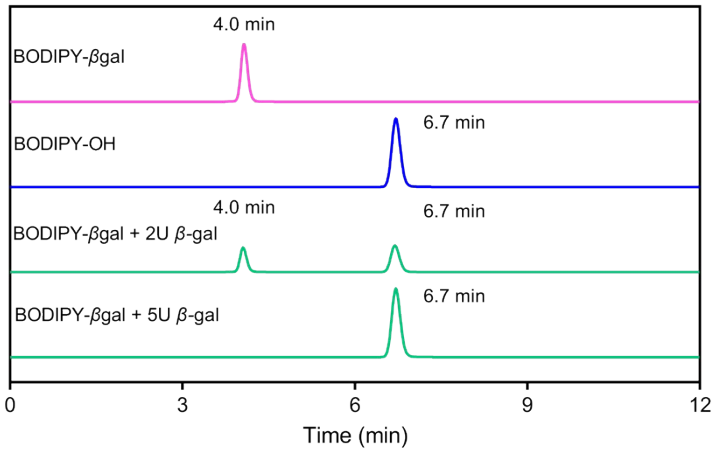


Fig. S5 Reverse-phase HPLC chromatogram of BODIPY-βgal, BODIPY-OH and BODIPY-βgal reaction with β-gal (2U and 5U) for 30 min. The eluent is methanol/H_2O (v/v, 8 : 2) mixed solvent. The flow rate is 1.0 mL min^{-1}, and the detection wavelength is 560 nm.

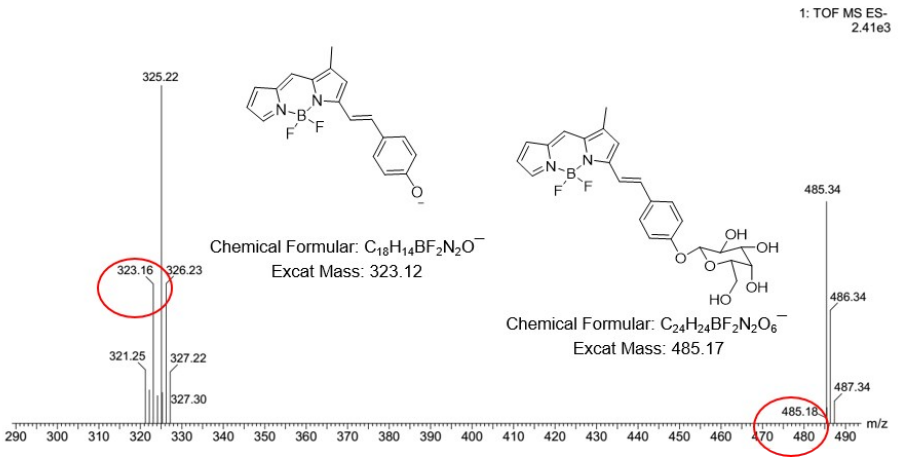


Fig. S6 HRMS demonstrating the enzyme-activatable mechanism and showing the cleavage product of BODIPY-βgal.

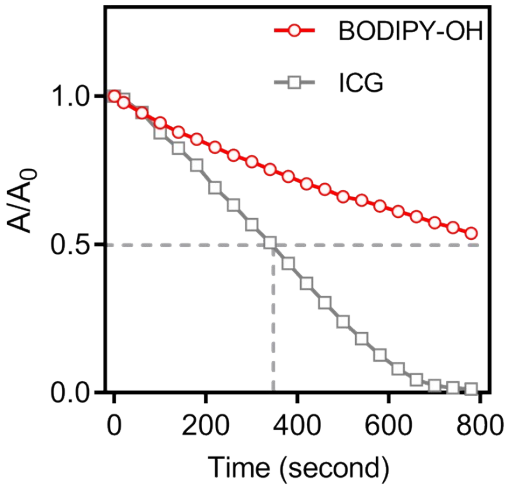


Fig. S7 Time-dependent absorption of BODIPY-OH (10 μM, monitored at 620 nm) and ICG (10 μM, monitored at 780 nm) under sustained illumination.

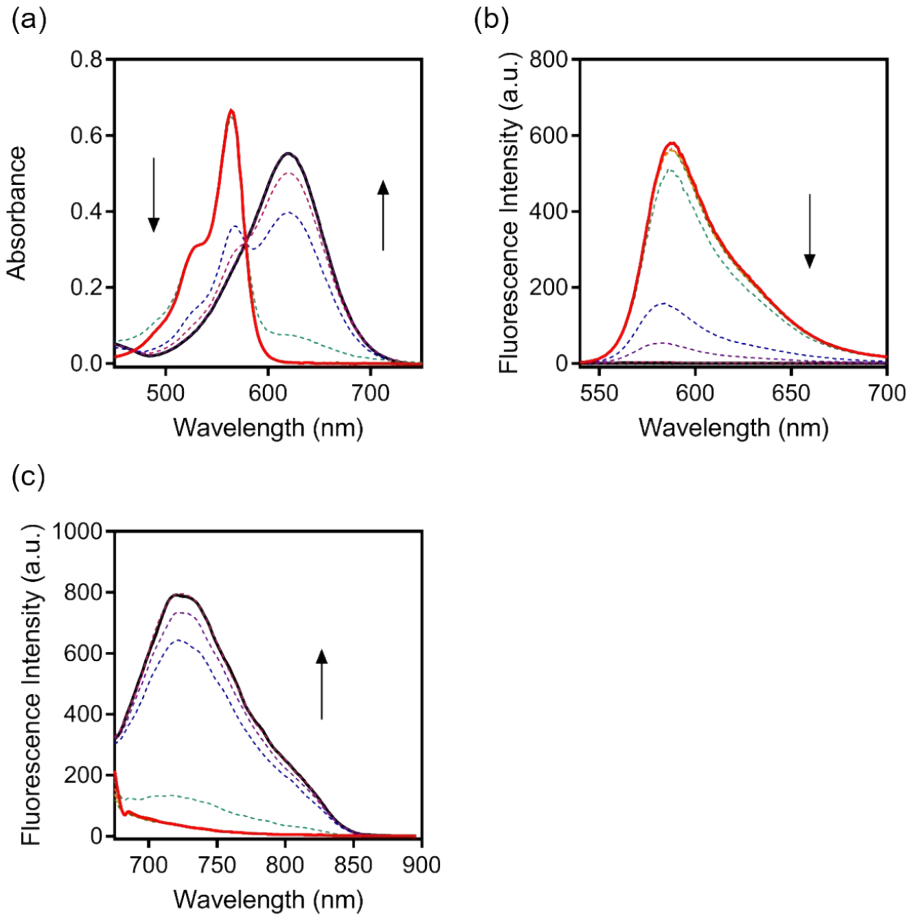


Fig. S8 (a) Absorption and (b and c) fluorescence spectra of BODIPY-OH (10 μM) in Britton-Robinson buffer/DMSO solution (1:1, v/v) with pH changing from 1.7 to 10.0, λ_{ex} = 530 nm (b) and λ_{ex} = 660 nm (c).

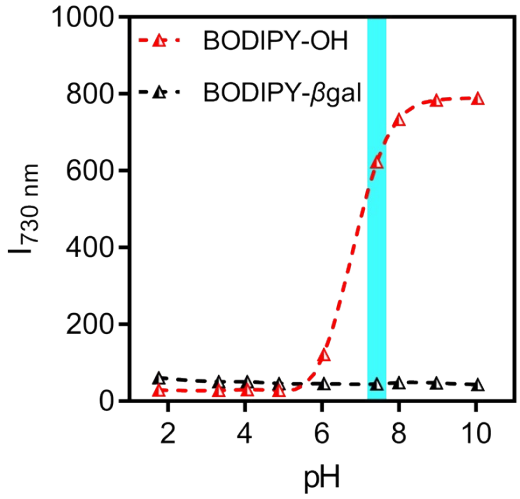


Fig. S9 Fluorescence intensity at 730 nm of BODIPY-βgal and BODIPY-OH with pH changing from 1.7 to 10.0, λ_{ex} = 660 nm.

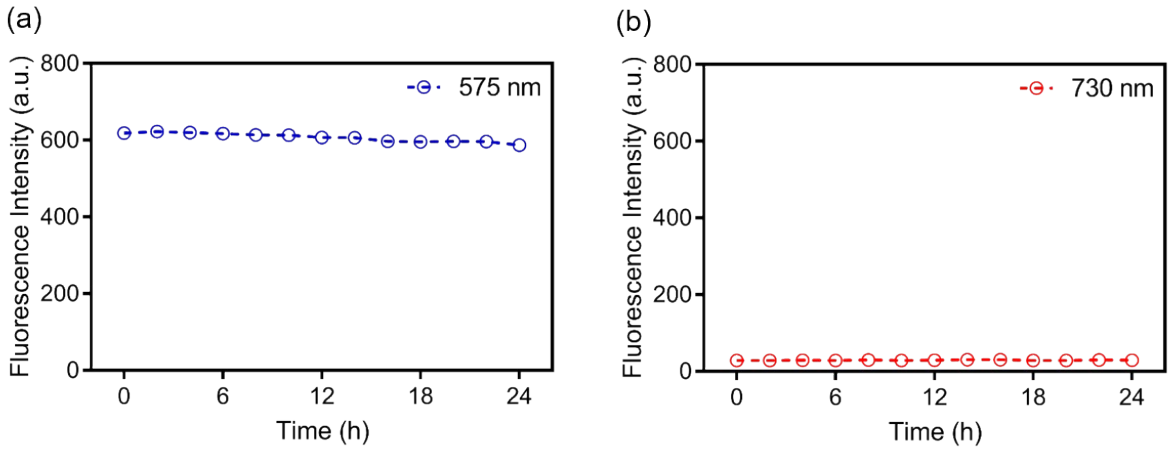


Fig. S10 Fluorescence intensity of BODIPY-βgal remain stable in fresh human serum over 24 h at 37 ^{o}C, λ_{ex} = 530 nm (a) and λ_{ex} = 660 nm (b).

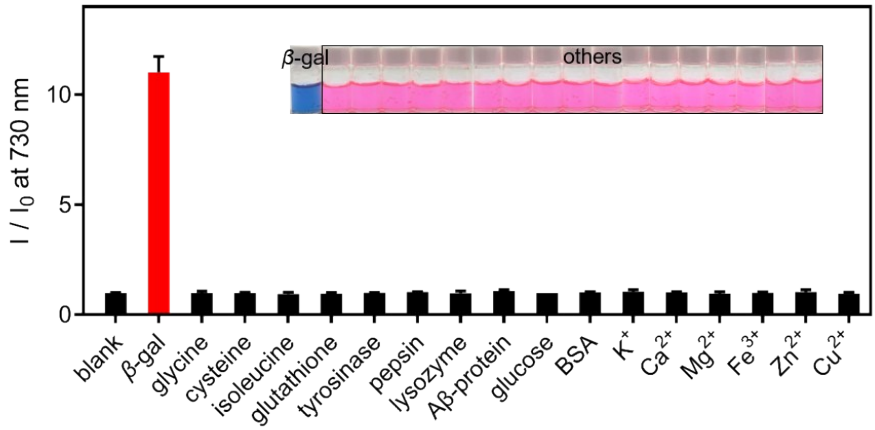


Fig. S11 Fluorescence responses of BODIPY-*β*gal (10 μM) upon incubation with *β*-gal (5 U) and various other analytes in aqueous solution (PBS/DMSO = 1:1 v:v, pH = 7.4) , λ_{ex} = 660 nm. I/I_0 represents the fluorescence intensity ratio at 730 nm, and I_0 is the fluorescence intensity of free BODIPY-*β*gal.

Cytotoxicity of BODIPY-*β*gal

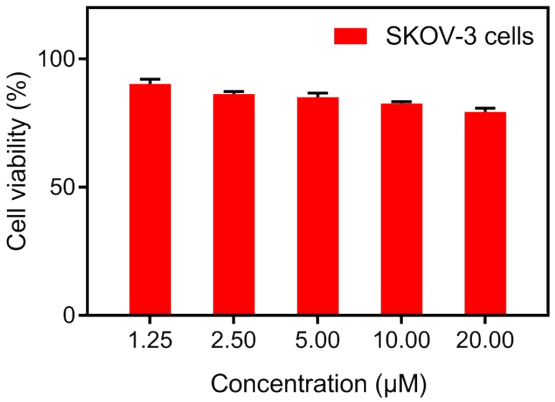


Fig. S12 Relative viability of SKOV-3 cells *in vitro* after incubation with BODIPY-*β*gal at various concentrations for 24 h. Data are shown as mean ± s.d., with n = 3.

3. Characterization of intermediate compounds and BODIPY-*β*gal

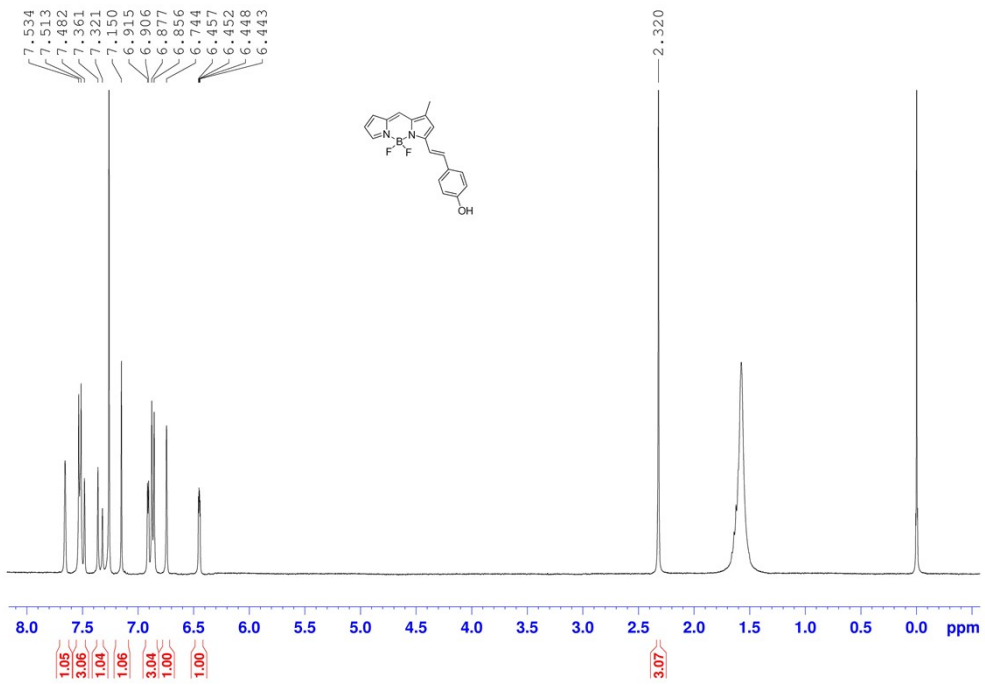


Fig. S13 ^{1}H NMR spectrum of **BODIPY-OH** in $CDCl_3$

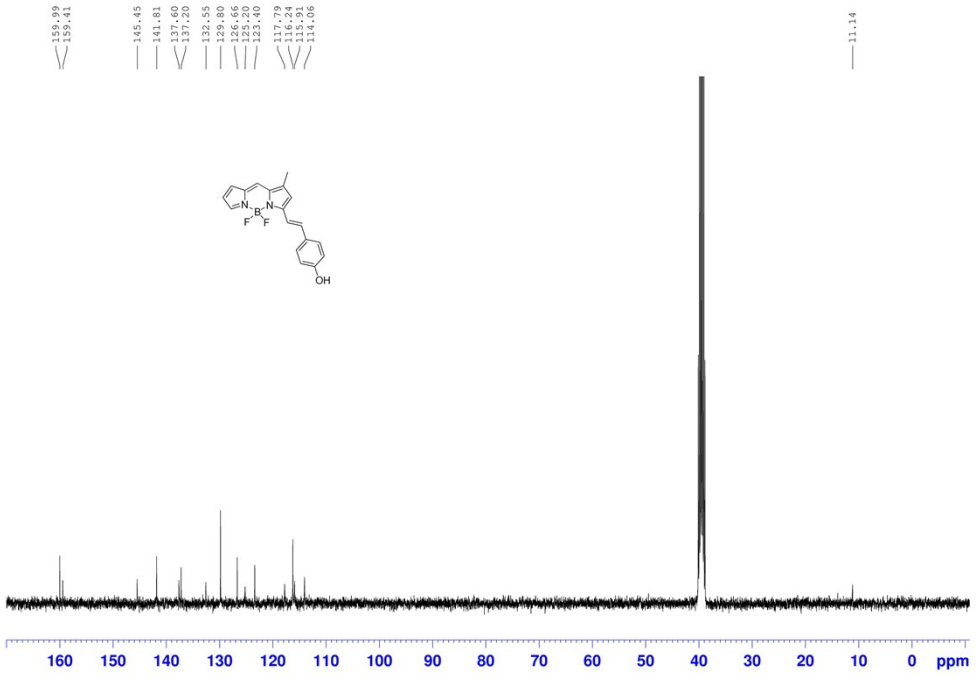


Fig. S14 ^{13}C NMR spectrum of **BODIPY-OH** in DMSO-d_6

Elemental Composition Report **Page 1**

Single Mass Analysis
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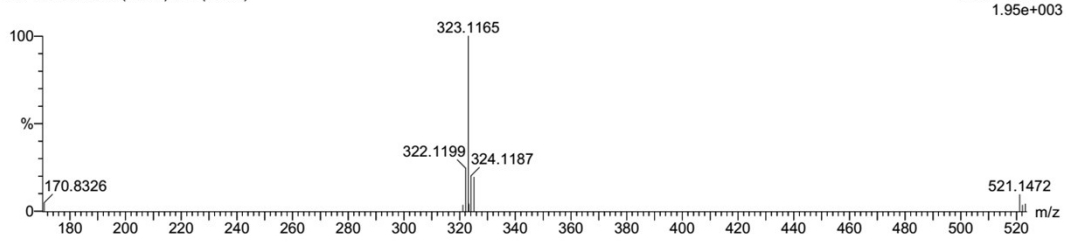
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
29 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 0-18 H: 0-30 N: 0-2 O: 0-1 B: 0-1 F: 0-2

WH-ZHU
ZW-SLM-075 80 (2.013) Cm (77:80)

1: TOF MS ES-
1.95e+003



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Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
323.1165	323.1167	-0.2	-0.6	12.5	37.4	0.0	C18 H14 N2 O B F2

Fig. S15 HRMS spectrum of **BODIPY-OH**

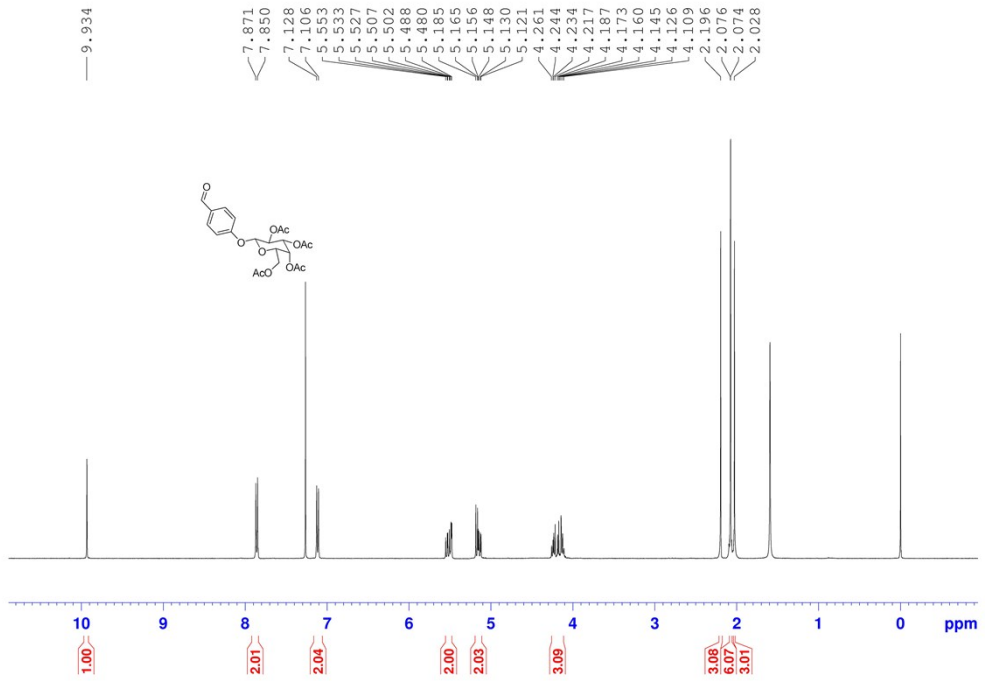

Fig. S16 ^{1}H NMR spectrum of **1** in $CDCl_3$

Elemental Composition Report **Page 1**

Single Mass Analysis
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Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
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WH-ZHU
ZW-SLM-077 37 (0.405) Cm (35:37)

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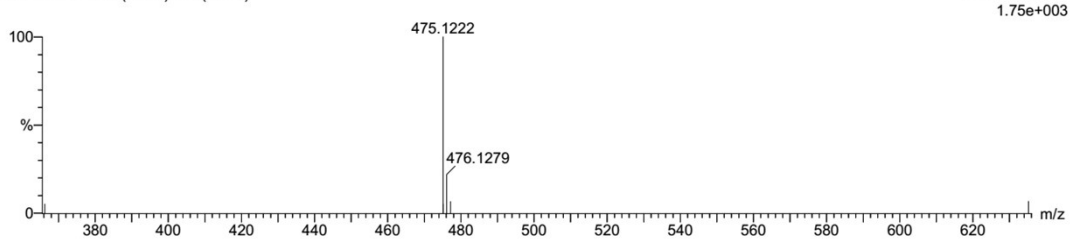
1.75e+003



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Maximum:		5.0	5.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
475.1222	475.1216	0.6	1.3	9.5	18.6	0.0	C21 H24 O11 Na

Fig S17 HRMS spectrum of **1**

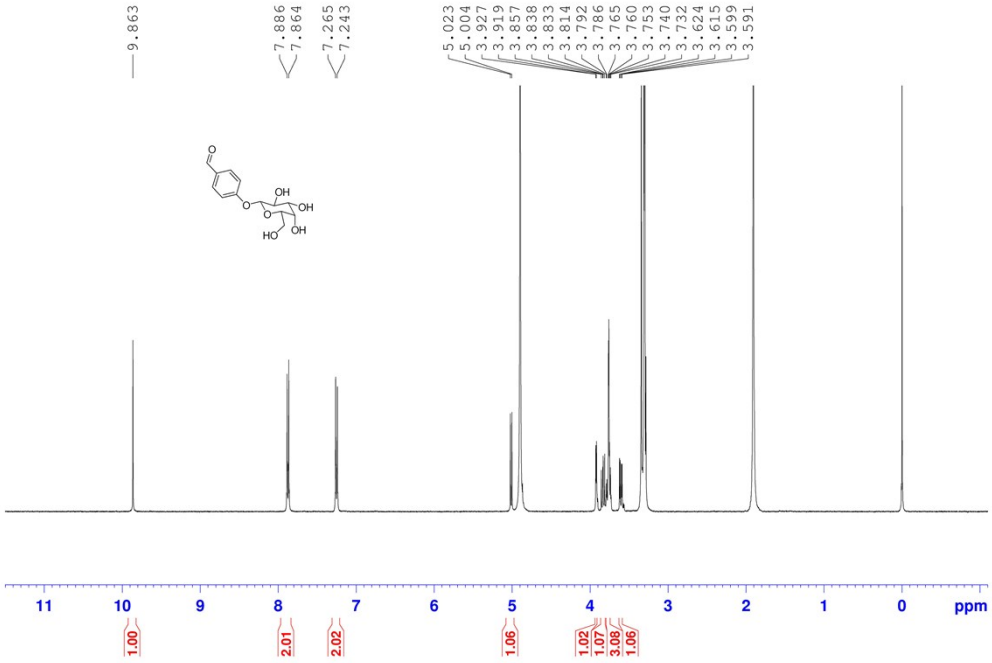

Fig. S18 ^{1}H NMR spectrum of **2** in CD_3OD

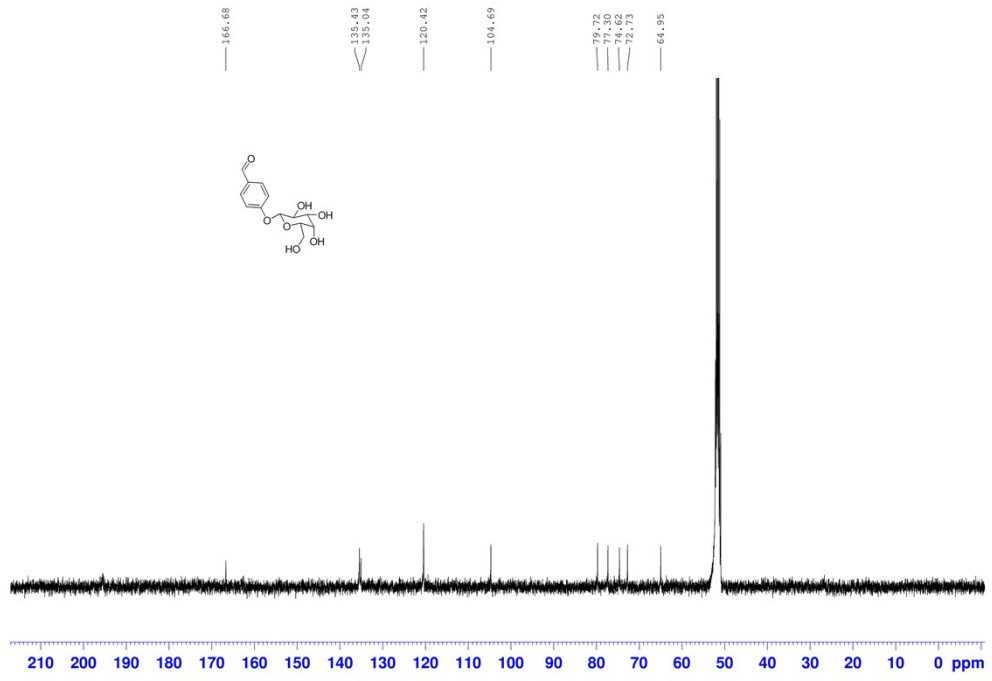

Fig. S19 ^{13}C NMR spectrum of **2** in CD_3OD

Elemental Composition Report **Page 1**

Single Mass Analysis
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Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
9 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
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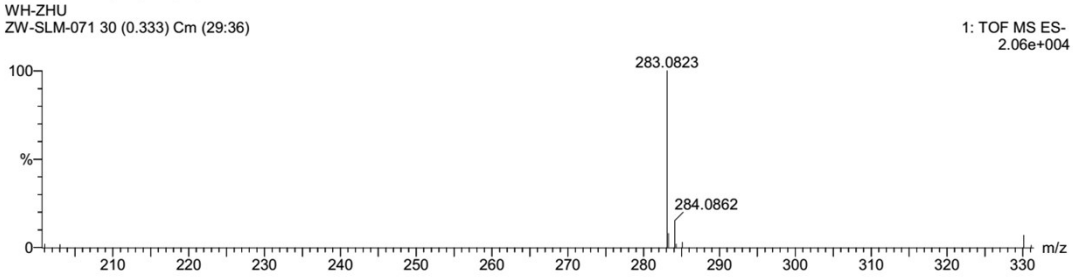
ZW-SLM-071 30 (0.333) Cm (29:36)



Minimum:				-1.5			
Maximum:		5.0	5.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
283.0823	283.0818	0.5	1.8	6.5	34.1	0.0	C13 H15 O7

Fig. S20 HRMS spectrum of **2**

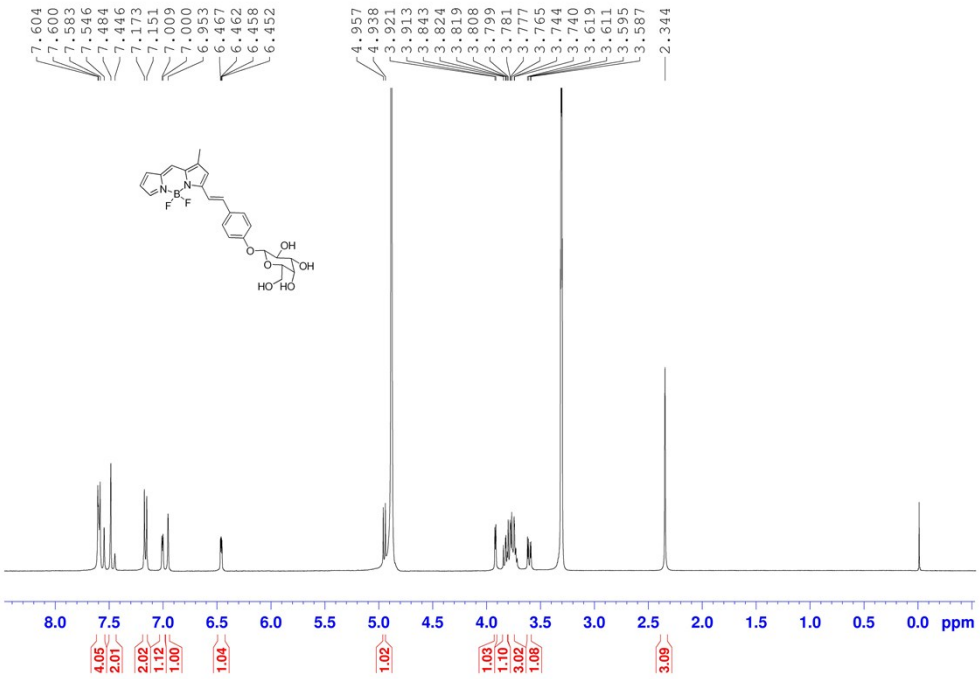


Fig. S21 ^{1}H NMR spectrum of **BODIPY-*β*gal** in CD_3OD

Elemental Composition Report **Page 1**

Single Mass Analysis
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Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
132 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
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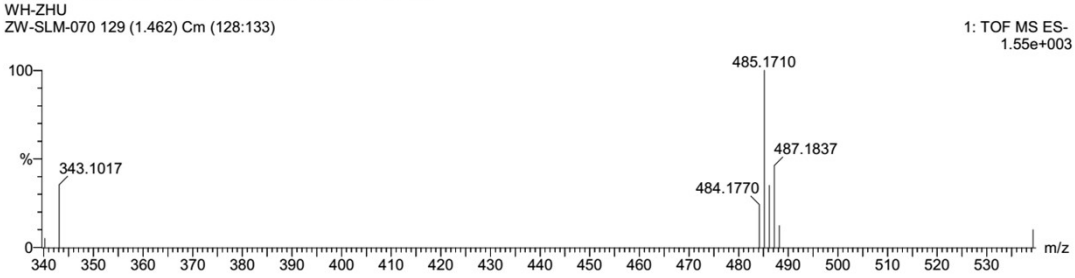
WH-ZHU
ZW-SLM-070 129 (1.462) Cm (128:133)



Minimum:				-1.5			
Maximum:		5.0	5.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
485.1710	485.1695	1.5	3.1	13.5	21.1	0.0	C24 H24 B N2 O6 F2

Fig. S22 HRMS spectrum of **BODIPY-*β*gal**