Supporting Information

Real-Time Tracking and In Vivo Visualization of *β*-Galactosidase Activity in Colorectal Tumor with a Ratiometric NIR Fluorescent Probe

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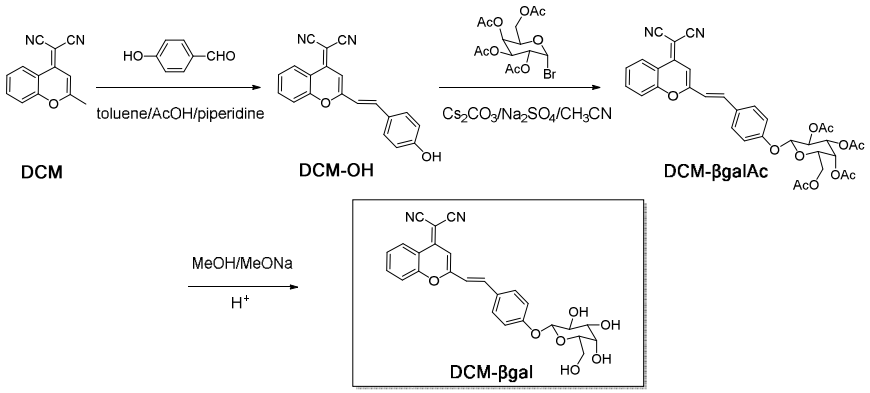
1. Experimental section

Materials and general methods

Unless special stated, all solvents and chemicals were purchased from commercial suppliers in analytical grade and used without further purification. *β*-Galactosidase (*β*-gal) was supplied by J&K Scientific Ltd. *LacZ* gene kit and *X*-gal were purchased from Invitrogen Company (San Diego, CA, USA). Avidin-*β*-gal was purchased from Sigma-Aldrich. 4-Hydroxybenzaldehyde was purchased from Adamas-beta. 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. Life time was measured with Edinburgh Lifespec-Ps spectrofluorometer (FL920). Confocal fluorescence images were taken on confocal laser scanning microscope (CLSM, Nikon A1R/A1). *In vivo* fluorescence images and three-dimensional (3D) *in vivo* fluorescence images were measured with a PerkinElmer IVIS Lumina Kinetic Series III imaging system and IVIS Spectrum CT imaging system, respectively.

Synthesis of DCM-*β*gal



Scheme S1 Synthetic route of DCM-*β*gal

Synthesis of DCM-OH

DCM (1.0 g, 4.8 mmol) and 4-hydroxybenzaldehyde (704 mg, 5.8 mmol) were dissolved in toluene (30 mL) along with acetic acid (0.5 mL) and piperidine (1.0 mL), the system was under argon protection and then refluxed for 10 h. Toluene was removed by evaporation, and the residue was purified by silica gel chromatography with dichloromethane to get the desired product **DCM-OH** (900 mg, 2.9 mmol), a dark orange solid. Yield was 60%. ^{1}H NMR(400 MHz, DMSO-d_6, ppm): δ = 6.85 (d, J = 8.8 Hz, 2H), 6.96 (s, 1H), 7.28 (d, J = 16.0 Hz, 1H), 7.60 (m, 3H), 7.70 (d, J = 16.0 Hz, 1H), 7.79 (d, J = 7.6 Hz, 1H), 7.91 (m, 1H), 8.73 (dd, J_1 = 8.4 Hz, J_2 = 1.2 Hz, 1H),

10.16 (s, 1H). ^{13}C NMR(100 MHz, DMSO-d_6, ppm): δ = 64.27, 110.96, 121.15, 121.26, 121.30, 122.35, 122.65, 124.25, 129.84, 131.33, 135.59, 140.53, 144.51, 157.26, 158.15, 164.14, 165.25. Mass spectrometry (ESI negative ion mode for $[M - H]^-$): Calcd. for $C_{20}H_{11}N_2O_2$: 311.0821; found: 311.0820.

Synthesis of DCM-*β*galAc

DCM-OH (150 mg，0.48 mmol), Na_2SO_4 (171.8 mg, 1.21 mmol) and Cs_2CO_3 (793 mg，2.43 mmol) were dissolved in acetonitrile (15 mL), and then tetra-*O*-acetyl-*α*-*D*-galactopyranosyl-1-bromide (297 mg，0.72 mmol) was added into the solution, 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 with dichloromethane to obtain the desired product **DCM-*β*galAc** (110 mg, 0.17 mmol), a brown solid. Yield was 36%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ = 1.98 (s, 3 H), 2.06 (s, 3 H), 2.10 (s, 3 H), 2.16 (s, 3 H), 4.12 (d, *J* = 6.4 Hz, 2 H), 4.49 (s, 1 H), 5.24-5.30 (m, 1 H), 5.38 (d, *J* = 4.0 Hz, 1 H), 5.61 (d, *J* = 7.2 Hz, 1 H), 5.82 (d, *J* = 4.8 Hz, 1 H), 7.02 (s, 1 H), 7.09 (d, *J* = 8.8 Hz, 2 H), 7.44 (d, *J* = 16.6 Hz, 1 H), 7.63 (t, *J* = 7.6 Hz, 1 H), 7.76 (d, *J* = 16 Hz, 1 H), 7.79 (d, *J* = 8.0 Hz, 2 H), 7.81 (d, *J* = 7.6 Hz, 1 H), 7.94 (t, *J* = 7.6 Hz, 1 H), 8.75 (d, *J* = 8.4 Hz, 1 H). ^{13}C NMR(100 MHz, DMSO-d_6, ppm): δ = 20.81, 20.86, 20.91, 20.99, 60.49, 67.71, 68.75, 70.61, 71.01, 73.48, 97.66, 106.90, 116.35, 117.22, 117.58, 118.77, 119.51, 125.13, 126.64, 127.41, 130.43, 135.90, 138.54, 139.08, 152.51, 153.43, 158.42, 158.78, 169.69, 170.01, 170.31, 170.43. Mass spectrometry (ESI positive ion mode for $[M + Na]^+$): Calcd. for $C_{34}H_{30}N_2O_{11}Na$: 665.1747; found: 665.1751.

Synthesis of DCM-*β*gal

DCM-*β*galAc (100 mg, 0.16 mmol) was dissolved in dry methanol (8 mL), and sodium methylate (54 mg, 1.0 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. Purification by silica gel chromatography with dichloromethane/methanol (100:1) provided pure product **DCM-*β*gal** (25 mg, 0.05 mmol), a dark red solid. Yield was 33%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ = 3.51-3.72 (m, 6 H), 4.55 (d, *J* = 4.4 Hz, 1 H), 4.69 (t, *J* = 5.6 Hz, 1 H), 4.91 (d, *J* =5.6 Hz, 1 H), 4.95 (d, *J* =8.0 Hz, 1 H), 5.22 (d, *J* =5.2 Hz, 1 H), 7.01 (s, 1 H), 7.12 (d, *J* = 8.4 Hz, 2 H), 7.41 (d, *J* = 16.0 Hz, 1 H), 7.63 (t, *J* = 7.6 Hz, 1 H), 7.75 (d, *J* = 8.8 Hz, 2 H), 7.76 (d, *J* = 15.2 Hz, 1 H), 7.81 (d, *J* = 8.4 Hz, 1 H), 7.94 (t, *J* = 7.6 Hz, 1 H), 8.75 (d, *J* = 8.0 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ = 60.12, 60.84, 68.60, 70.66, 73.72, 76.08, 101.00, 106.69, 116.43, 117.14, 117.58, 117.77, 118.03, 119.54, 125.11, 126.62, 129.08, 130.35, 135.87, 139.01, 152.51, 153.43, 159.01, 159.76. Mass spectrometry (ESI positive ion mode for $[M + Na]^+$): Calcd. for $C_{26}H_{22}N_2O_7Na$: 497.1325; found: 497.1320.

In vitro enzymatic assay

DCM-βgal was used at a final concentration of 10 μM. Absorption and fluorescence spectra of DCM-βgal with β-gal enzymatic reactions were performed at 37 °C in a 2 mL total volume of PBS buffer (0.1 M, pH 7.4, 30% DMSO) in a 1 cm cuvette.

Cell experiment

Cell lines

Human embryonic kidney 293T cells was supplied by the Institute of Cell Biology (Shanghai, China). 293T cells were cultured at 37 ºC under a humidified 5% CO_2 atmosphere in DMEM (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). *LacZ* gene was introduced into 293T cells to obtain *lacZ*-(+) 293T cells by gene transfection method according standard protocol.

Human ovarian cancer OVCAR-3 cells was supplied by the CoBioer biosciences Co., Ltd (Nanjing, China). OVCAR-3 cells were cultured at 37 ºC under a humidified 5% CO_2 atmosphere in RPMI-1640 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).

LacZ gene transient transfection

293T cells were centrifuged and resuspended in fresh serum-free DMEM at a density of 1.0×10^6 cells mL^{-1} before transfection. 500 μL cell suspension was distributed per well in a 12-well plate. DNA was diluted in fresh serum-free DMEM (in a volume equivalent to one-tenth of the culture to be transfected), PEI was added, and the mixture immediately vortexed and incubated for 10 min at room temperature prior to its addition to the cells. Following a 3 h incubation with DNA-PEI complexes, culture medium was completed to 1 mL by the addition of DMEM supplemented with 10% FBS.

In vitro cytotoxicity assay

The cytotoxicity of DCM-βgal or DCM-OH towards 293T and OVCAR-3 cell lines was 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 RPMI1640 medium with 10% FBS, at a density of 1×10^4 cells/well and added with desired concentrations of DCM-βgal or DCM-OH (0.1% DMSO). After incubation for 24 h, absorbance was measured at 450 nm with a Genios multifunction-reader (Tecan GENios Pro, Tecan Group Ltd. Maennedorf, Switzerland). Each concentration was measured in triplicate and used in three independent experiments.

Animals

Ethics statement Animal procedures were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The animal study was approved by the Institutional Animal Care and Use Committees of the China Medical Association (Permit Number: CCMA 2012–2397). The 6-week-old BALB/c homozygous athymic nude mice which weight was about 23 g were purchased from Shanghai Slac Laboratory Animal Company, and raised in the standard conditions. The animals were kept in specific pathogen-free environment with a 12-h light/12-h dark schedule and housed in sterile cages with airflow hoods. They were fed with autoclaved chow and water randomly.

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

The nude mice were inoculated with a colorectal cancer cell LoVo on their right flanks by injecting 10^6 cells subcutaneously. Diameter of tumors is *ca.* 1 cm. Avidin-β-gal (100 μg) in PBS was intravenously injected via tail vein into the human colorectal tumor-bearing nude mice to upregulate β-gal concentration in tumors. The unpretreated tumor-bearing mice serve as control. The real-time *in vivo* imaging and three-dimensional (3D) *in vivo* imaging was recorded at different time internals (0-3 h) after DCM-βgal injecton using PerkinElmer IVIS Lumina Kinetic Series III imaging system and IVIS Spectrum CT imaging system, respectively. The number of injected animals (nude mice) is 3×10 (parallel experiments: blank 2, intratumoral injection 6, intravenous injection 2). The concentration of the injected solution is 0.0375 mg kg^{-1} (PBS/DMSO = 95:5, pH = 7.4). In the *in vivo* imaging, λ_{ex} = 540 ± 10 nm, λ_{em} = 710 ± 15 nm. *In situ* injection is intratumoral injection.

2. Absorption and fluorescence spectra of DCM-O$^-$

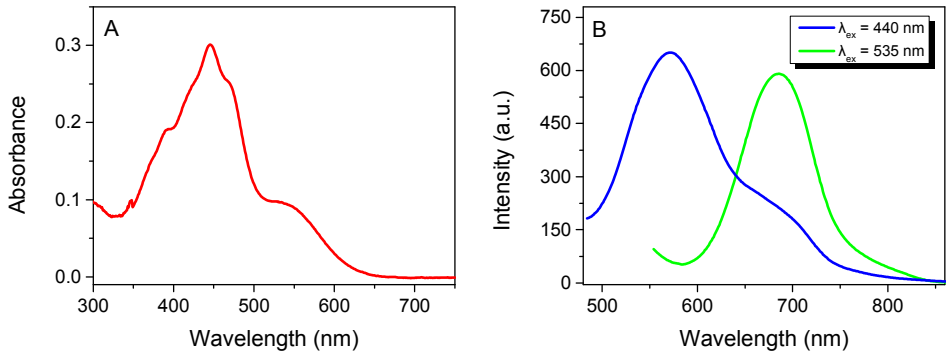


Figure S1. (A) Absorption and (B) fluorescence spectra of DCM-O$^-$ (10 μM) in PBS/DMSO solution (7:3, v/v, pH = 7.4, 37 °C).

3. Absorption and fluorescence spectra of DCM-*β*gal incubation with *β*-gal

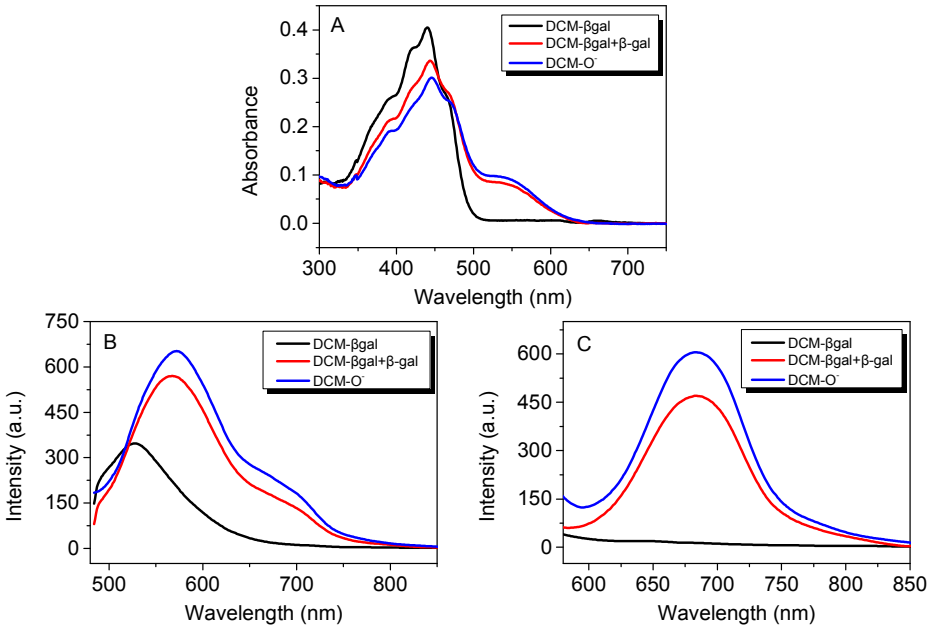


Figure S2. (A) Absorption and (B, C) fluorescence spectra of DCM-*β*gal in the absence and presence of *β*-gal and DCM-O$^-$ in PBS/DMSO solution (7:3, v/v, pH = 7.4, 37 °C). Note: Excitation wavelength of B is 450 nm; excitation wavelength of C is 535 nm.

4. Kinetics curve of DCM-*β*gal reaction with *β*-gal at 685 nm

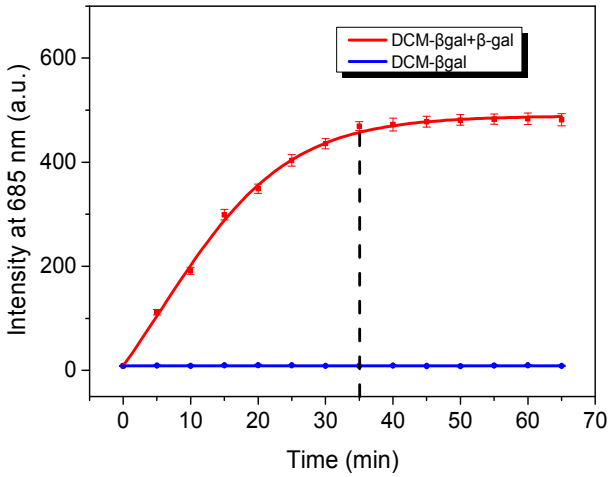


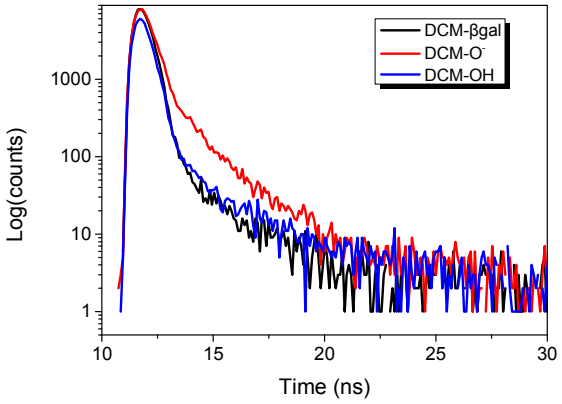
Figure S3. Time dependence of fluorescence intensity at 685 nm for DCM-*β*gal (10 μM) in PBS/DMSO solution (7:3, v:v, pH = 7.4, 37 ^{o}C) in the presence (red) and absence (blue) of *β*-gal. λ_{ex} = 535 nm.

5. Photophysical properties of DCM-*β*gal, DCM-O$^-$, and DCM-OH

Table S1 Photophysical Properties of DCM-*β*gal, DCM-O$^-$, and DCM-OH

	Φ_F[a]	ε (L mol^{-1} cm^{-1})	τ (ns)	χ^2
DCM-*β*gal	0.01	4.05×10^4	0.99	1.157
DCM-O$^-$	0.58	3.38×10^4	1.37	1.265
DCM-OH	0.05	3.23×10^4	1.38	1.308

[a] The relative fluorescence quantum yield Φ_F value was determined using fluorescein as a reference



Fluorescence decay of DCM-*β*gal, DCM-O$^-$, and DCM-OH. λ_{ex} = 447 nm, λ_{em} = 525 nm.

6. Enzyme kinetics parameters of DCM-*β*gal and *X*-gal

Table S2. Enzyme Kinetics Parameters of DCM-*β*gal and *X*-gal

substrate	K_m[a] (μM)	V_{max} (μM s^{-1})	K_{cat} (s^{-1})	K_{cat} / K_m ($s^{-1}μM^{-1}$)
DCM-*β*gal	60.07	0.54	29.19	0.48
X-gal	260.58	0.04	2.16	8.29×10^{-3}

[a] K_m is Michealis constant, obtained from Michaelis-Menten equation ($V = V_{max}[S]/(K_m + [S])$, where V is initial velocity, V_{max} is maximum velocity, [S] is substrate concentration, and K_m is Michealis constant).

7. The detection limit of DCM-*β*gal

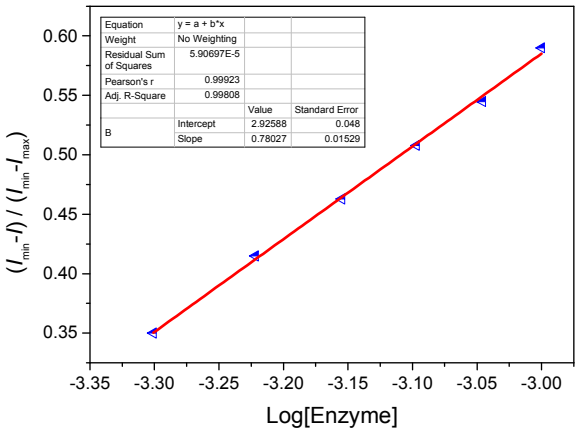


Figure S4. Fluorescence intensity ratio changes with the concentration of *β*-gal. Note: the detection limit was calculated based on the fluorescence titration. The limit of detection for *β*-gal is 1.7×10^{-4} U/mL.

8. HPLC chromatogram of DCM-*β*gal and DCM-O$^-$

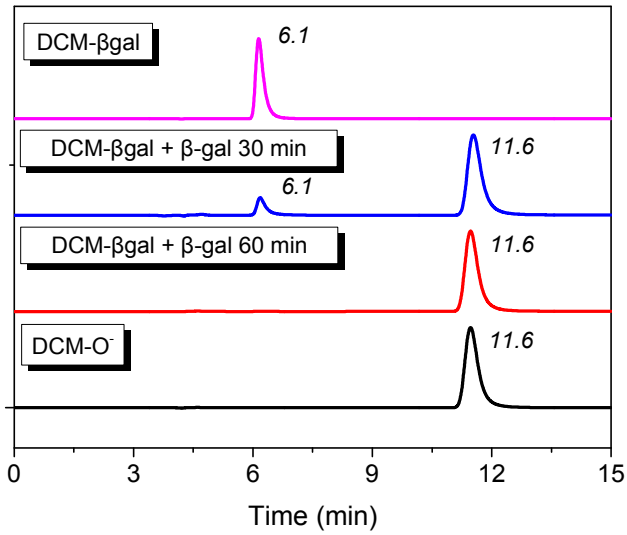


Figure S5. HPLC chromatogram of DCM-*β*gal before and after 30 and 60 min reaction with *β*-gal, and DCM-O$^-$. Eluent solvent: methanol/H_2O (v : v, 8 : 2), flow rate = 0.6 mL min^{-1}, detection wavelength at 450 nm.

9. ESI-MS spectra characterization of DCM-*β*gal reaction with *β*-gal

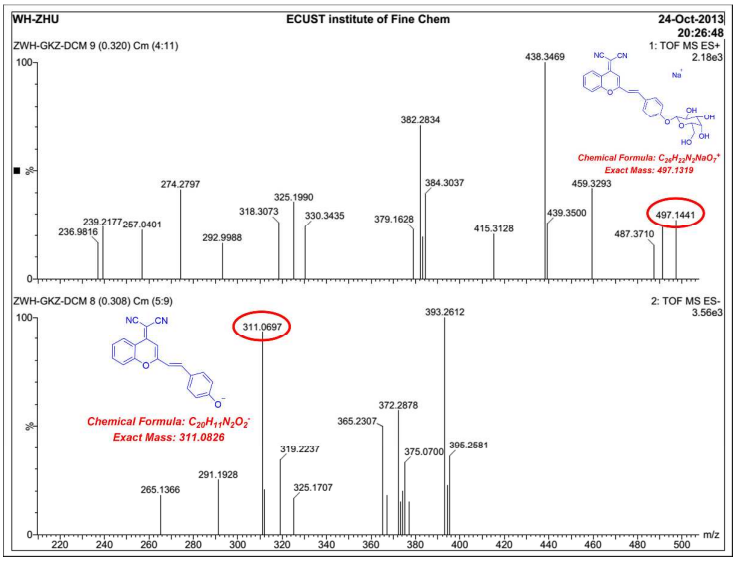


Figure S6. ESI-MS spectra characterization of DCM-*β*gal reaction with *β*-gal.

10. pH-dependent fluorescence intensity of DCM-*β*gal and DCM-OH

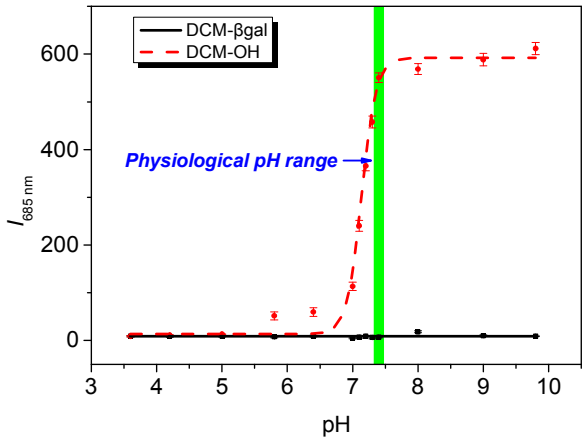


Figure S7. pH-dependent fluorescence intensity of DCM-*β*gal and DCM-OH (10 μM in citrate phosphate buffer, PBS, and glycine-NaOH buffer solution for various pH, monitored at 685 nm and λ_{ex} = 535 nm).

11. Cytotoxicity of DCM-*β*gal and DCM-OH

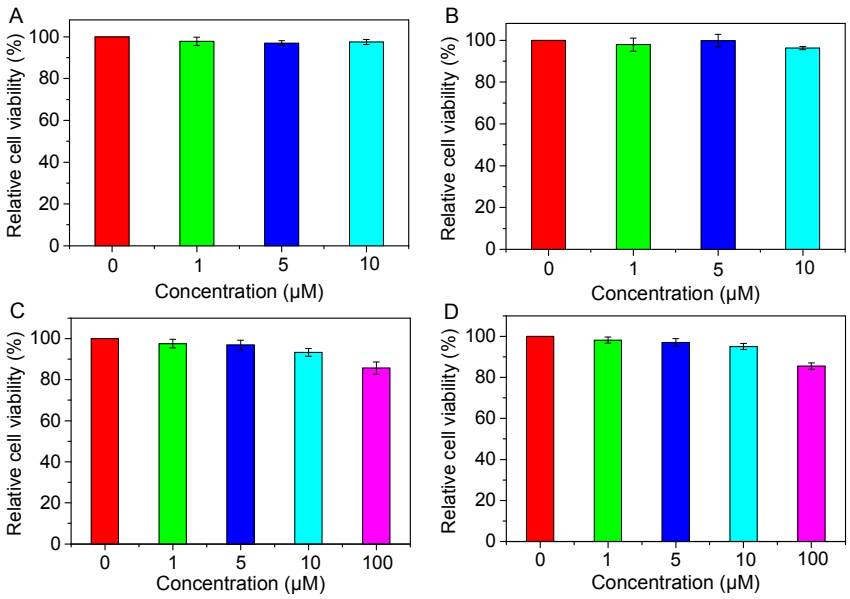


Figure S8. Relative viability of (A-B) 293T or (C-D) OVCAR-3 cells *in vitro* after incubation for 24 h with (A and C) DCM-*β*gal and (B and D) DCM-OH at various concentrations. Note: both DCM-*β*gal and its hydrolysate DCM-OH have minimal toxicity and enjoy superior biocompatibility toward cultured cell lines.

12. Semiquantitative determination of endogenous *β*-gal activity in living cells

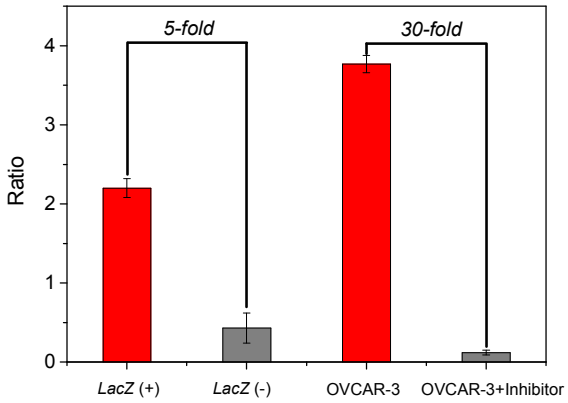


Figure S9. Semiquantitative determination of endogenous *β*-gal activity in *lacZ*-(+)/*lacZ*-(-) 293T cells and OVCAR-3 cells according to the ratio of averaged fluorescence intensity of red channel (650-720 nm) to green channel (490-530 nm). The semiquantitative calculation was conducted by Image Pro-plus 6.0. Error bars represent standard deviation.

13. *In vivo* imaging of *β*-gal activity in tumor-bearing nude mice

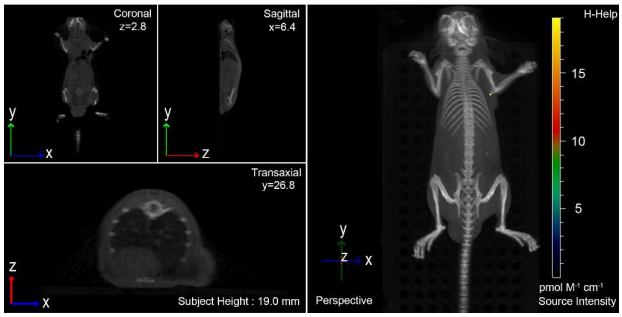


Figure S10. Three-dimensional *in vivo* imaging of *β*-gal activity in unpretreated tumor-bearing nude mice after tumor-injection of DCM-*β*gal for 3 h.

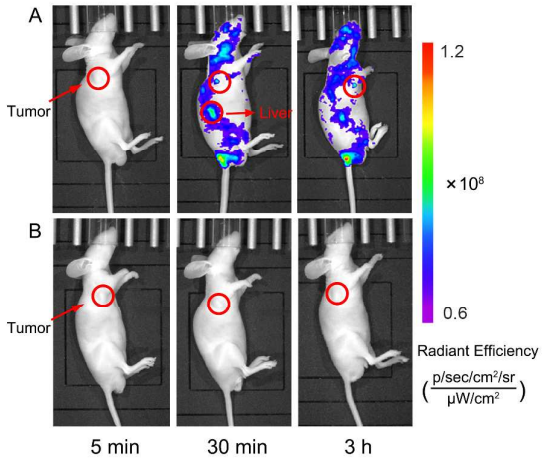


Figure S11. *In vivo* imaging of *β*-gal activity in tumor-bearing nude mice after intravenous injection: (A) avidin-*β*-gal (100 μg) in PBS was intravenously injected into LoVo-implanted mice, and after 18 h DCM-*β*gal was then injected into the mice, (B) tumor-bearing mice were not pretreated with avidin-*β*-gal before injection of DCM-*β*gal acting as the control. Note: As shown in Figure S11A, after 30 min post intravenous injection, NIR fluorescent signals were captured in the tumor site and liver, which was attributed to the evolution of DCM-O^- from DCM-*β*gal hydrolyzed by *β*-gal. Dispersive fluorescent signals rather than targeted to the tumor were also observed, which may attribute that the probe lacks active-targeting ability. At 3 h post-injection, the NIR fluorescent signals was still founded clearly. In sharp contrast, there was no fluorescent signals for the control mice even after 3 h post injection.

14. Stereoview crystal structure of *β*-gal

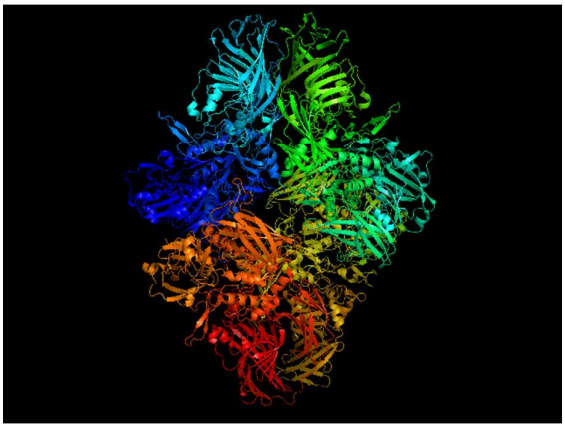


Figure S12. A stereoview crystal structure of *β*-gal. Note: *β*-gal (PDB code 3MUY) was obtained from the PDB database (http:// www.rcsb.org/pdb). The stereoview crystal structure was created by Pymol software.

15. Characterization of intermediate compounds and DCM-*β*gal

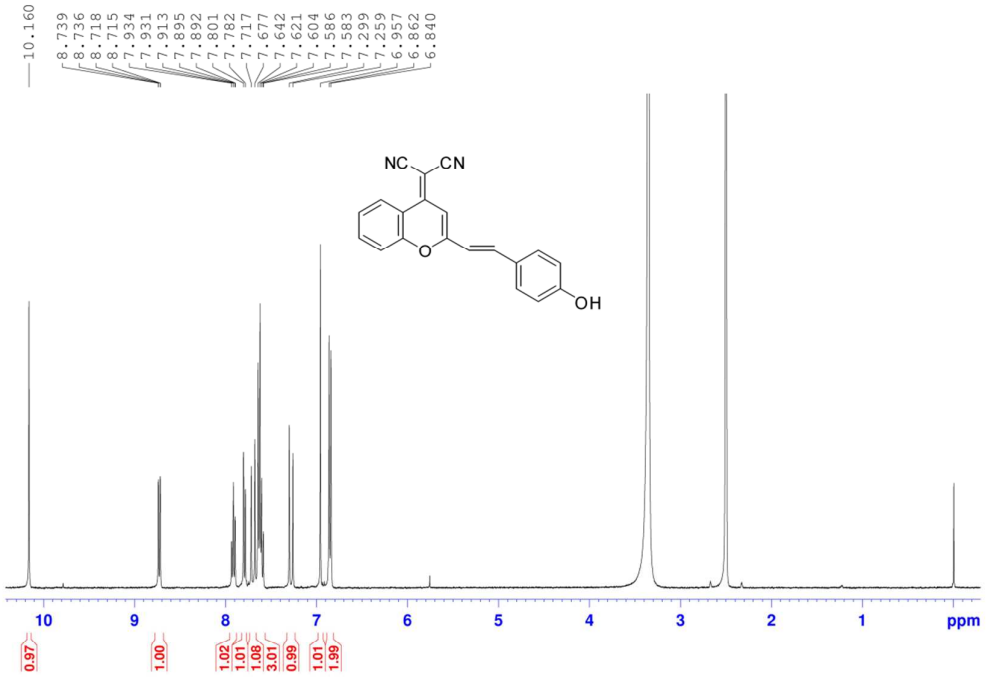


Figure S13. ^{1}H NMR spectrum of DCM-OH DMSO-d_6

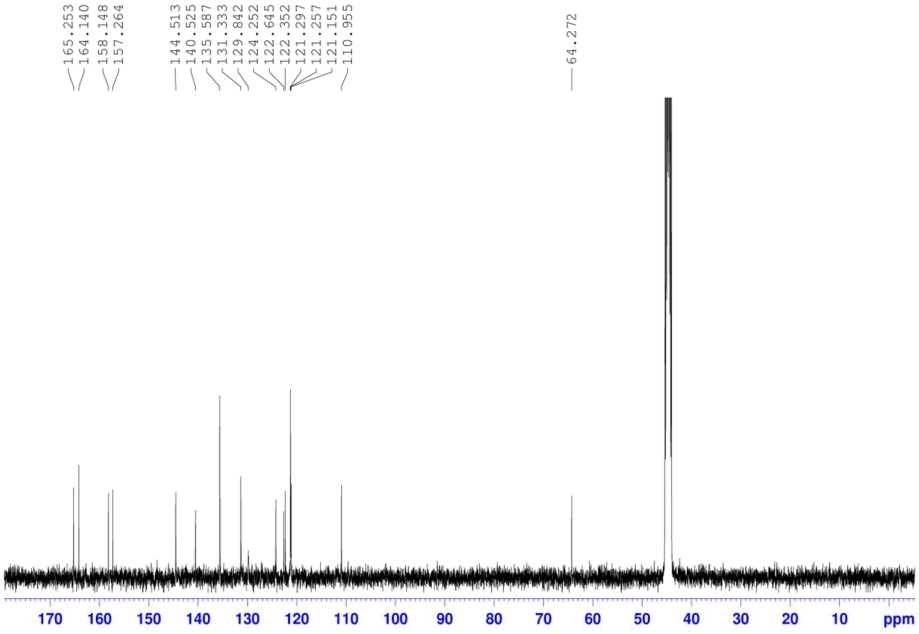


Figure S14. ^{13}C NMR spectrum of DCM-OH in DMSO-d_6

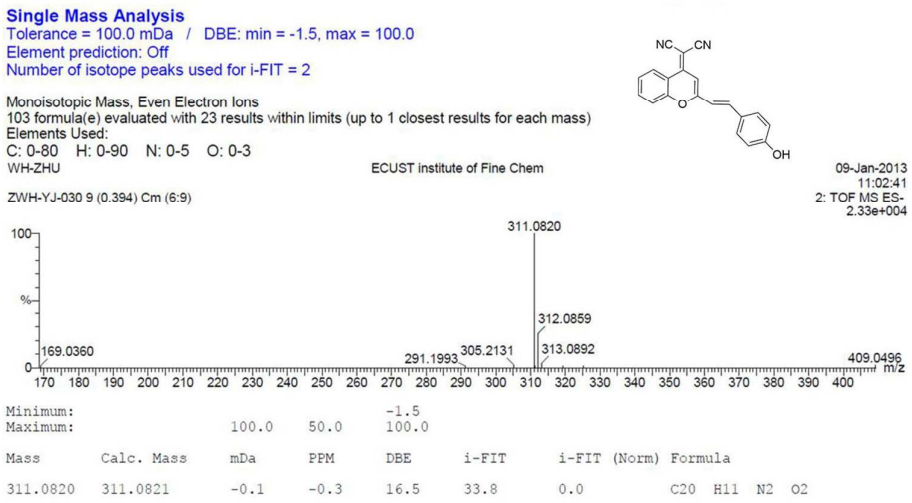


Figure S15. HRMS spectrum of DCM-OH

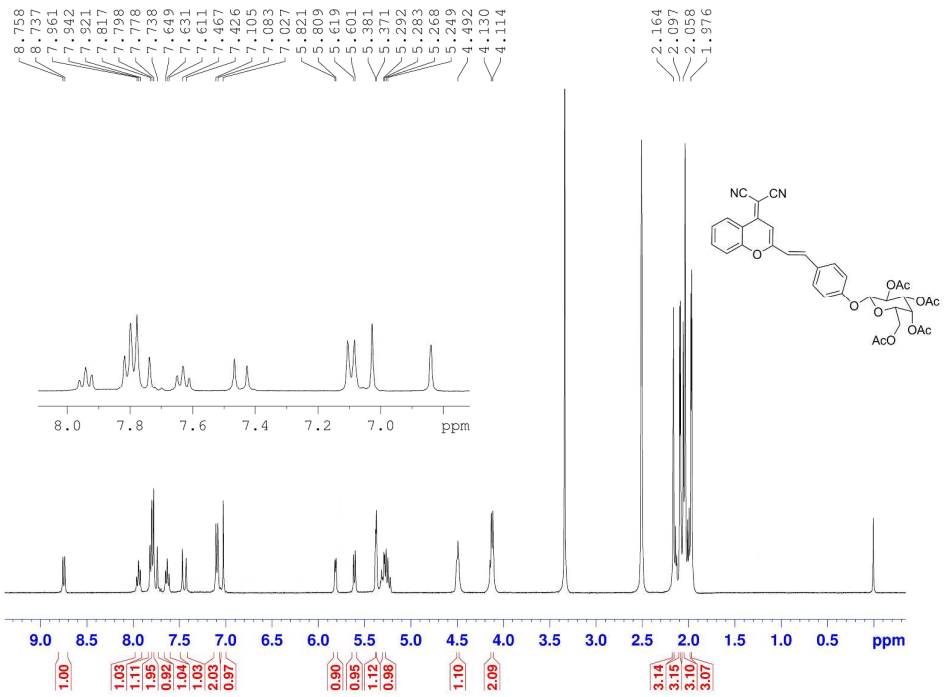


Figure S16. ^{1}H NMR spectrum of DCM-βgalAc in DMSO-d_6

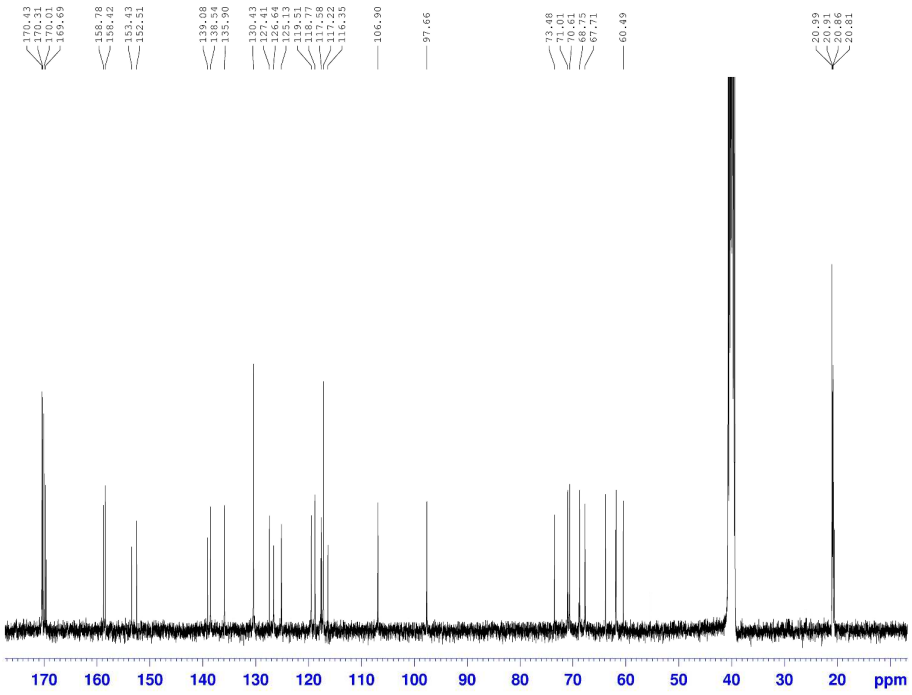
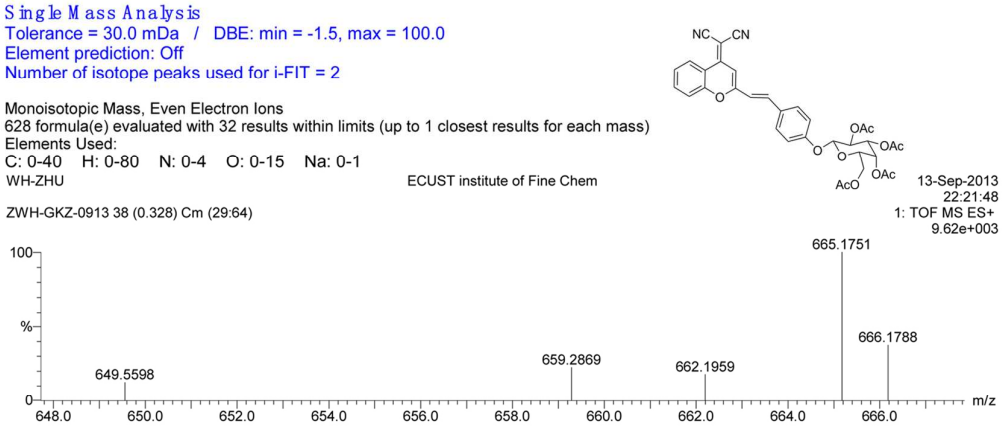


Figure S17. ^{13}C NMR spectrum of DCM-βgalAc in DMSO-d_6



Minimum:				-1.5			
Maximum:		30.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
665.1751	665.1747	0.4	0.6	20.5	23.0	0.0	C34 H30 N2 O11 Na

Figure S18. HRMS spectrum of DCM-βgalAc

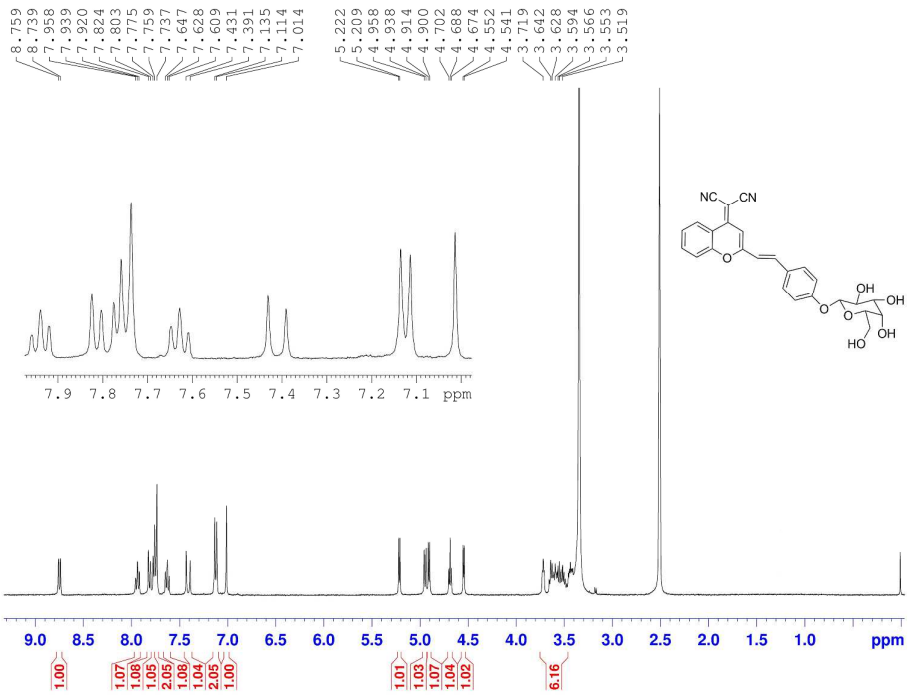


Figure S19. ^{1}H NMR spectrum of DCM-βgal in DMSO-d_6

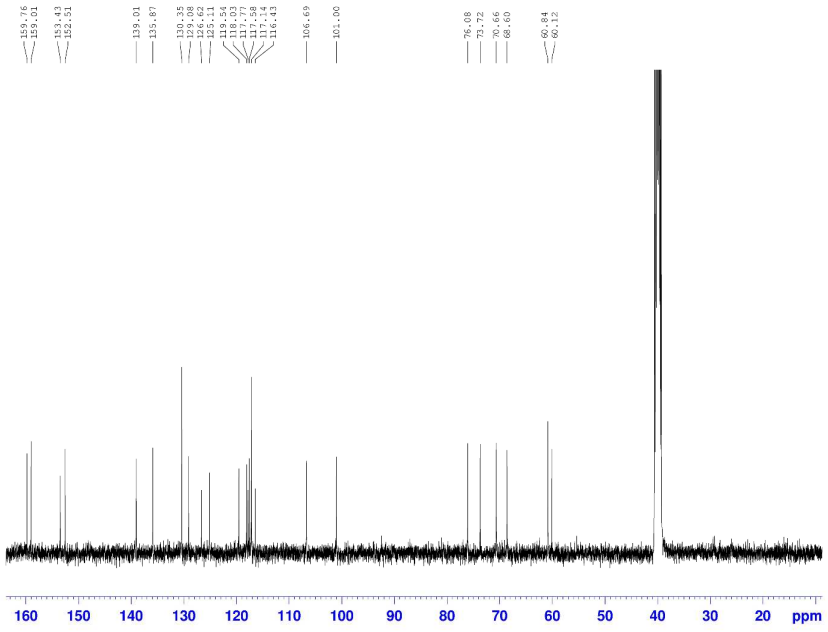
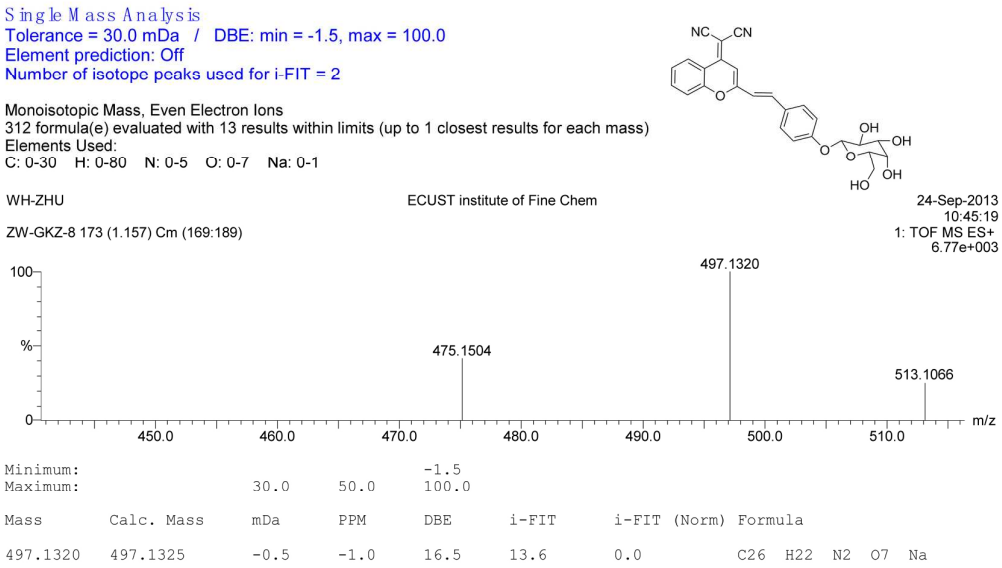


Figure S20. ^{13}C NMR spectrum of DCM-βgal in DMSO-d_6



Minimum:				-1.5			
Maximum:		30.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
497.1320	497.1325	-0.5	-1.0	16.5	13.6	0.0	C26 H22 N2 O7 Na

Figure S21. HRMS spectrum of DCM-*β*gal