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# A sensitive fluorescent probe for β-galactosidase activity detection and application in ovarian tumors imaging

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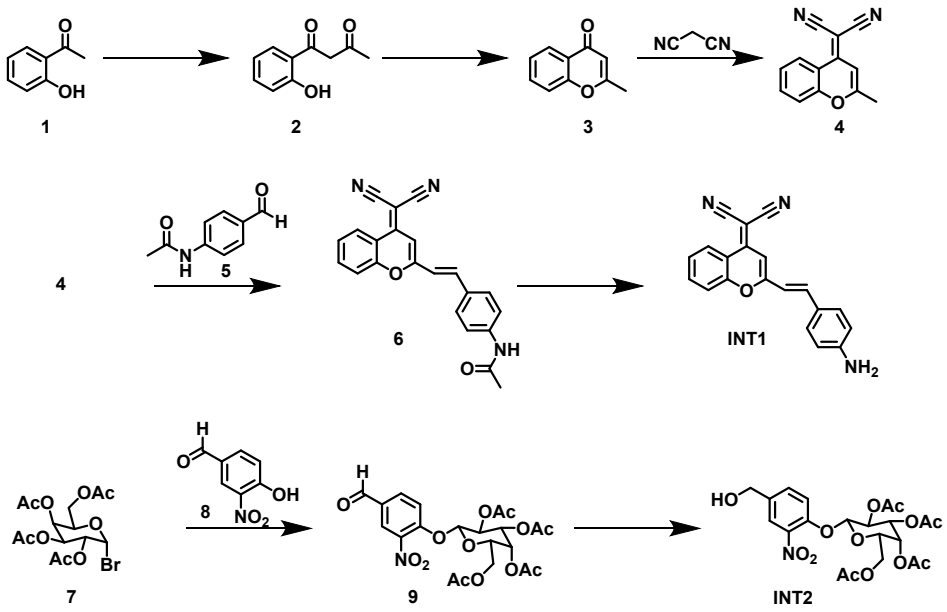
## 1□ Synthesis of DCMCA-βgal and other intermediates

Scheme S1. Synthesized processes of intermediates.




**Synthesis of compound 3.**

Sodium hydride (60% dispersion in mineral oil, 11.75 g, 293.8 mmol) was rinsed three times with hexanes, then suspended in THF (30 mL). A mixture of compound 1 (10 g, 73.45 mmol) and EtOAc (16.18 g, 183.62 mmol) in THF (2.5 mL) was added dropwise to the above suspension at room temperature. A vigorous reaction was observed, and the temperature rose to reflux. After complete addition, the reaction mixture was stirred for a further 5 min, then quenched by pouring onto ice, then acidified to pH 6 with 6 M aq HCl. The resulting precipitate was collected via filtration, washed with water, and dried under high vacuum to afford the title compound 3 as a crystalline white solid (12.2 g, 93.2%). $^1$H NMR (500 MHz, DMSO-$d_6$): δ 7.99-8.01 (m, 1H), 7.75-7.78 (m, 1H), 7.57-7.59 (d, $J$ = 8.2 Hz, 1H), 7.44-7.47 (m, 1H), 6.24 (s, 1H), 2.39 (s, 3H).

**Synthesis of compound 4.**

The solution of malononitrile (4 g, 62.46 mmol) and compound 3 (6.9 g, 43.08 mmol) in acetic anhydride (40 mL) was refluxed for 12 h under argon protection. Then acetic anhydride was removed by under reduce pressure. Then water (50 mL) was added to and refluxed for 30 min. The resulting mixture was filtered and the filter cake was dissolved in DCM (150 mL). Compound 4 was obtained by chromatography on silica gel with DCM, an orange-yellow solid (3.4 g, 37.9%). $^1$H NMR (500 MHz,

DMSO-$d_6$): δ 8.73-8.75 (m, 1H), 7.89-7.93 (m, 1H), 7.71-7.73 (d, *J* = 8.2 Hz, 1H), 7.61-7.64 (m, 1H), 6.82 (s, 1H), 2.51 (s, 3H).

**Synthesis of compound INT1.**

Compound 4 (500 mg, 2.4 mmol) and 4-acetamidobenzaldehyde (470.2 mg, 2.88 mmol) were dissolved in toluene (15 mL) along with acetic acid (0.25 mL) and piperidine (0.5 mL), the system was under argon protection and then refluxed for 10 h. Toluene was removed by evaporation to give the crude compound 6 (850 mg) and used directly next step without further purification. Then the crude was dissolved in EtOH-conc. HCl (10 mL: 20 mL) and refluxed for overnight. Then mixture was cooled and saturated NaHCO3 was added to adjust pH to about 7. The resulting mixture was extracted with DCM (3×50 mL), the organic layer was combined, and dried over Na2SO4 and concentrated by reduced pressure. The residue was purified by silica gel chromatography with DCM to get the desired compound INT1, a dark orange solid (330 mg, 44.2% yield). $^1$H NMR (500 MHz, DMSO-$d_6$): δ 8.72-8.74 (d, *J* = 8.2 Hz, 1H), 7.88-7.91 (m, 1H), 7.77-7.78 (d, *J* = 7.9 Hz, 1H), 7.63-7.66 (d, *J* = 15.6 Hz, 1H), 7.57-7.61(t, *J* = 7.6 Hz, 1H), 7.48-7.50 (d, *J* = 8.5 Hz, 2H), 7.08-7.11 (d, *J* = 15.9 Hz, 1H), 6.87 (s, 1H), 6.61-6.63 (d, *J* = 8.5 Hz, 2H), 6.02 (s, 2H).

**Synthesis of compound 9.**

Silver oxide (1.63 g, 9.73 mmol) and 4-hydroxy-3-nitro benzaldehyde (1.24g, 5.35 mmol) were added to a solution of compound 8(2.0 g, 4.86 mmol) in acetonitrile (50 mL). The mixture was stirred at room temperature for overnight. Then the reaction mixture was filtered on celite and the pad washed with EtOAc. The filtrate was concentrated under vacuum and purified by chromatography on silica gel with Hexane/EtOAc (10%-50%) as the eluent to afford compound 9 as white solid (2.01 g, 83.1 % yield). $^1$H NMR (500 MHz, $CDCl_3$): δ 10.0 (s, 1H), 8.32-8.33 (d, *J* = 2.1 Hz, 1H), 8.08-8.10 (dd, $J_1$ = 8.5 Hz, $J_2$ = 1.8 Hz, 1H), 7.50-7.52 (d, *J* = 8.9 Hz, 1H), 5.59-5.63 (m, 1H), 5.51-5.52 (d, *J* = 3.0 Hz, 1H), 5.22-5.24 (d, *J* = 7.6 Hz, 1H), 5.14-5.17 (dd, $J_1$ = 10.4 Hz, $J_2$ = 3.4 Hz, 1H), 4.27-4.30 (m, 1H), 4.12-4.22 (m, 2H), 2.22 (s, 3H), 2.15 (s, 3H), 2.11 (s, 3H), 2.05 (s, 3H).

**Synthesis of compound INT2.**

Sodium borohydride (167.3 mg, 4.42 mmol) was added portion-wise to a cooled solution of compound 9 (1.0 g, 2.01 mmol) in $CHCl_3$-iPrOH (25 mL:8 mL) at 0 °C. The solution was allowed to reach room temperature and stirred for 6 h. An aqueous solution of citric acid was added dropwise in ice-bath. After washing with NaHCO3 solution (10% (w/w), 3×15 mL) and water (15 mL), the organic layer was dried over Na2SO4. The residue was evaporated under vacuum and purified by chromatography on silica gel (Hexane-EtOAc 1:1) to give compound INT2c as white solid (800 mg, 80% yield). $^1$H NMR (500 MHz, $CDCl_3$): δ 7.83 (d, $J$ = 1.2 Hz, 1H), 7.54-7.56 (m, 1H), 7.37-7.39 (d, $J$ = 8.6 Hz, 1H), 5.55-5.58 (m, 1H), 5.49 (d, $J$ = 3.1 Hz, 1H), 5.11-5.14 (dd, $J_1$ = 10.4 Hz, $J_2$ = 3.4 Hz, 1H), 5.07-5.09 (d, $J$ = 7.9 Hz, 1H), 4.76 (s, 2H), 4.26-4.30 (m, 1H), 4.07-4.21 (m, 2H), 2.22 (s, 3H), 2.16(s, 3H), 2.10 (s, 3H), 2.04 (s, 3H).

**DCMCA-βgal synthesis**

First, compound INT3 was synthesized by dropwise addition of the INT1 solution (90 mg, 0.29 mmol) and DIEA (112.08 mg, 0.87 mmol) in anhydrous THF (7 mL) to a triphosgene solution (43 mg, 0.14 mmol) in anhydrous THF (5 mL) in an ice-bath. The mixture was stirred at 50 °C for 30 min and the INT2 solution (158.8 mg, 0.32 mmol) and DIEA (80 mg, 0.60 mmol) were then added at 0 °C. Next, the reaction mixture was stirred at 50 °C for 16 h, concentrated, dissolved in DCM (60.0 mL), washed with water (3 × 30 mL), and the organic layer was then dried over $Na_2SO_4$. Compound INT3 was obtained by thin layer chromatography (pre-TLC) (DCM/Hexane/EtOAc = 1:1:1, retention factor [Rf] = 0.45) as a yellow solid (70 mg, 28.9% yield). $^1$H NMR (500 MHz, $CDCl_3$): δ 8.94 – 8.96 (d, $J$ = 7.6 Hz, 1H), 7.90 (d, $J$ = 1.8 Hz, 1H), 7.75 – 7.78 (m, 1H), 7.57 – 7.63 (m, 5H), 7.47 – 7.51 (m, 3H), 7.39 – 7.41 (d, $J$ = 8.5 Hz, 1H), 6.89 (s, 1H), 6.85 (s, 1H), 6.76 – 6.79 (d, $J$ = 15.9 Hz, 1H), 5.56 – 5.59 (dd, $J_1$ = 10.4 Hz, $J_2$ = 7.9 Hz, 1H), 5.49 – 5.50 (d, $J$ = 3.1 Hz, 1H), 5.24 (s, 2H), 5.09 – 5.14 (m, 2H), 4.27

– 4.30 (m, 1H), 4.08 – 4.20 (m, 2H), 2.22 (s, 3H), 2.15 (s, 3H), 2.10 (s, 3H), 2.04 (s, 3H). HR-MS $[M+Na]^+$: m/z Calcd 859.2069, Found 859.2062.

Next, compound INT3 (50 mg, 0.06 mmol) was dissolved in dry methanol (4.5 mL) and MeONa (27 mg, 0.5 mmol) in dry methanol (1 mL) was added dropwise to the solution; the solution was stirred for 6 h at 30 °C. Once the reaction was completed, the mixture was neutralized by the addition of Amberlite IR-120 plus (H+) until the pH value reached approximately 7. Purification by pre-TLC (DCM/MeOH = 10:1) provided a pure product probe, a dark red solid (30 mg, 78.9% yield). $^1$H NMR (500 MHz, DMSO-$d_6$): δ 10.13 (s, 1H), 8.74 – 8.76 (d, *J* = 8.2 Hz, 1H), 7.97 (d, *J* = 1.8 Hz, 1H), 7.92 – 7.95 (m, 1H), 7.81 – 7.82 (d, *J* = 8.2 Hz, 1H), 7.71 – 7.74 (m, 4H), 7.61 – 7.64 (m, 1H), 7.57 – 7.59 (d, *J* = 8.9 Hz, 2H), 7.46 – 7.48 (d, *J* = 8.9 Hz, 1H), 7.38 – 7.41 (d, *J* = 16.2 Hz, 1H), 7.01 (s, 1H), 5.17-5.19 (m, 3H), 5.06 – 5.07 (d, *J* = 7.6 Hz, 1H), 4.90 (br, 1H), 4.66 (br, 1H), 4.61 (br, 1H), 3.71 (br, 1H), 3.63 – 3.66 (m, 1H), 3.49 – 3.58 (m, 3H), 3.40 – 3.42 (m, 1H). HR-MS $[M+Na]^+$: m/z Calcd 691.1647, Found 691.1632.

**2、$^1$H NMR spectra of Compound 4 and INT1 (Figure S1-S2)**

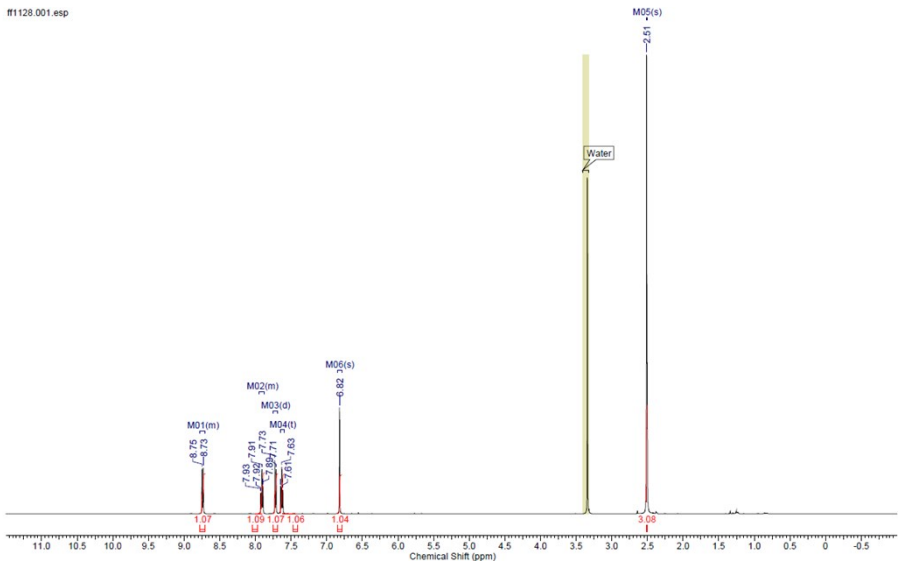



Figure S1. $^{1}$H NMR spectrum of compound 4.

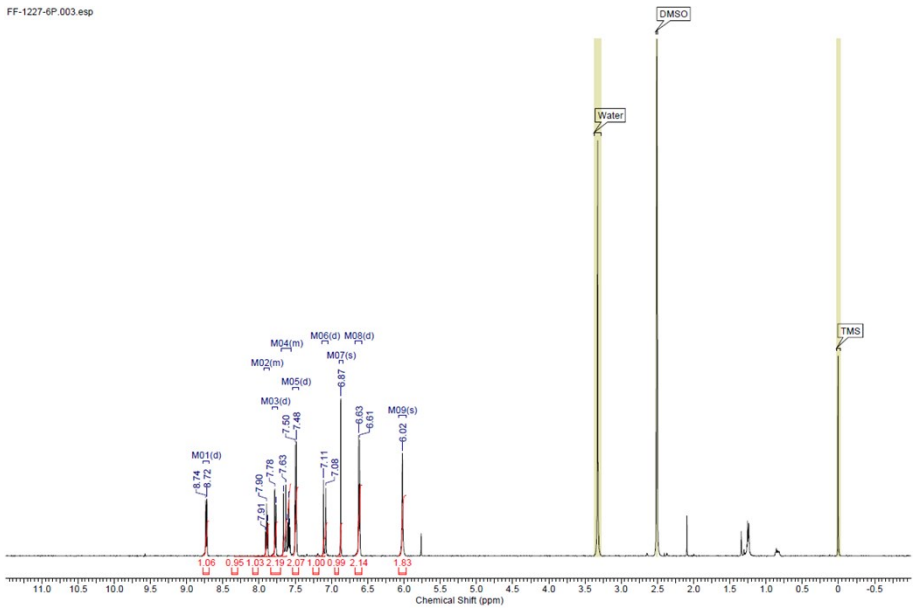



Figure S2. $^{1}$H NMR spectrum of INT1.

## 3、$^{1}$H NMR spectra of Compound 9 and INT2 (Figure S3-S4)

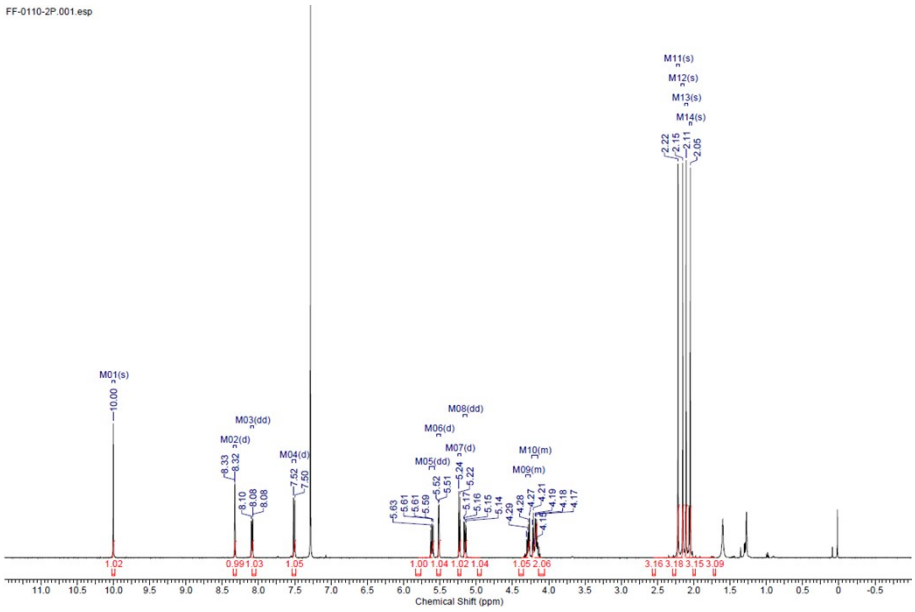



Figure S3. $^{1}$H NMR spectrum of compound 9.

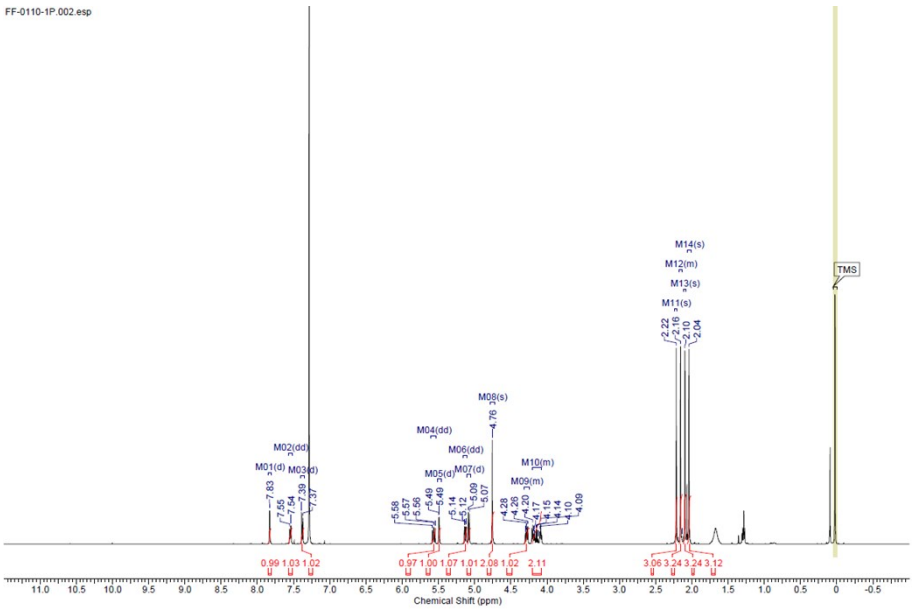



Figure S4. $^{1}$H NMR spectrum of INT2.

**4、$^{1}$H NMR and HR-MS spectra of Compound INT3 and DCMCA-βgal (Figure S5 -S8)**

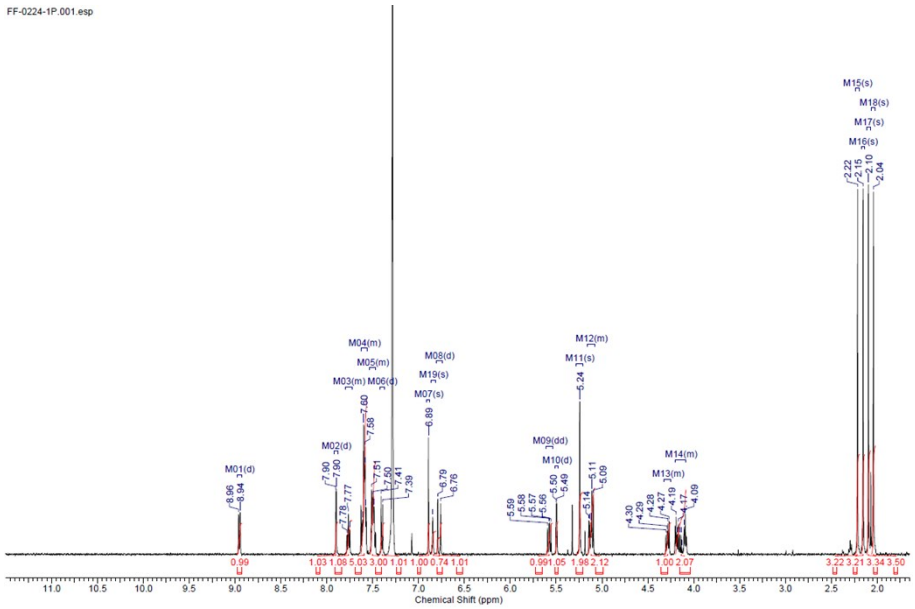



Figure S5. $^{1}$H NMR spectrum of INT3.

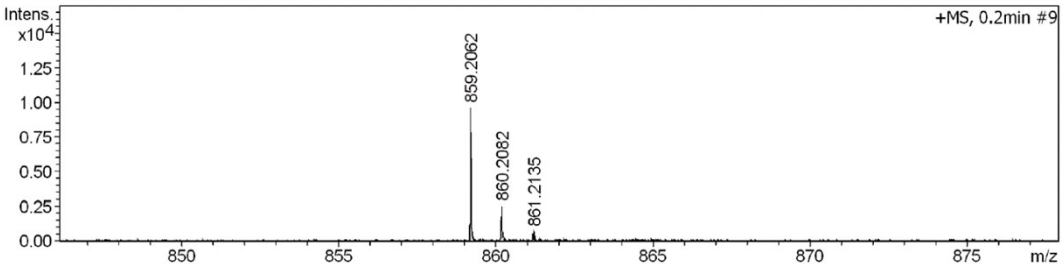



Figure S6. HR-MS spectrum of INT3.

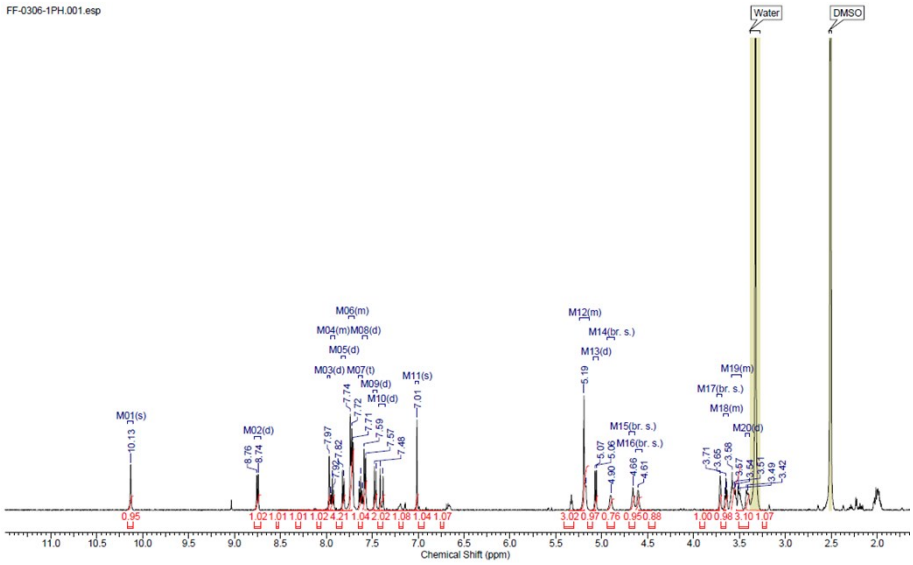



Figure S7. $^{1}$H NMR spectrum of DCMCA-βgal.

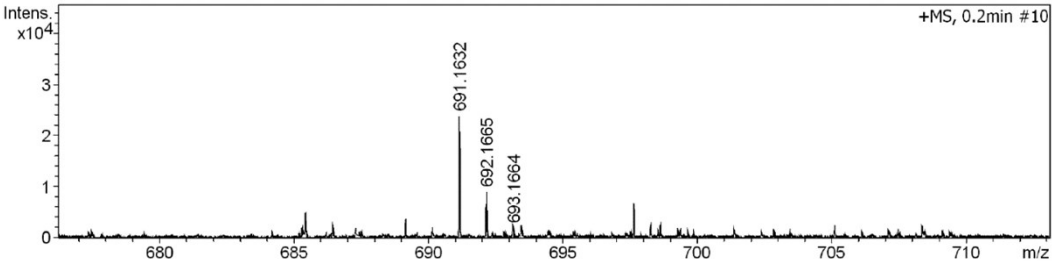



Figure S8. HR-MS spectrum of DCMCA-βgal.

## 5、Optimization of DCMCA-βgal concentration (Figure S9)

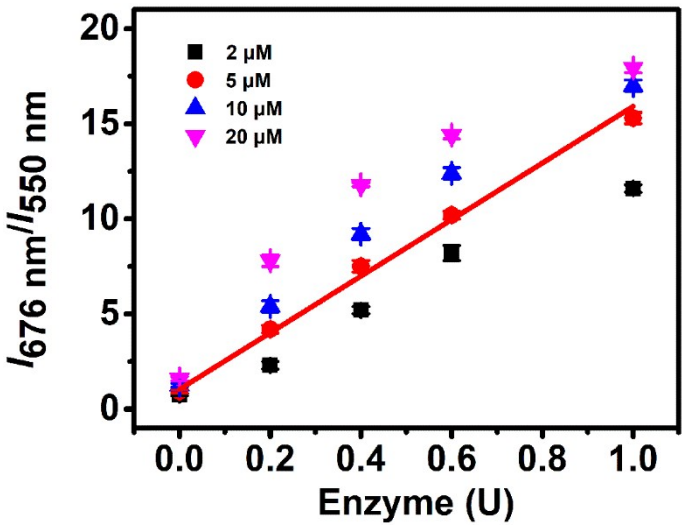



Figure S9. The concentration of DCMCA-βgal was optimized for determination of β-gal. The curves represent the concentration of DCMCA-βgal at 2, 5, 10 and 20 μM. Fluorescence measurements are obtained at λex = 464 nm.

**6、pH independence of DCMCA-βgal (Figure S10)**

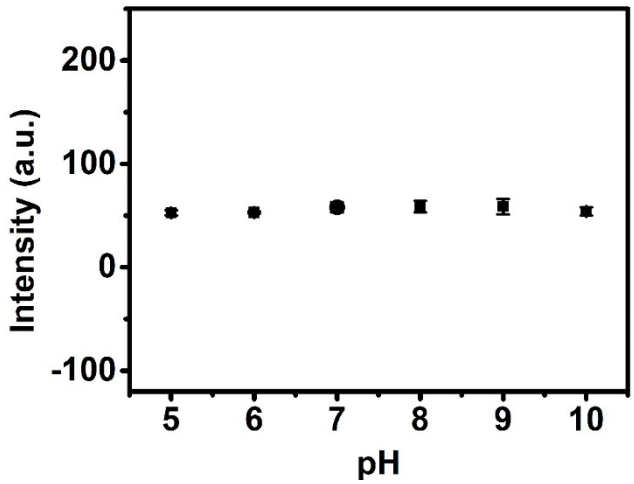



Figure S10. Fluorescence intensity ($I_{676\ nm}$) of DCMCA-βgal (5 μM) against pH, λex = 464 nm.

**7、Cytotoxicity of DCMCA-βgal (Figure S11)**

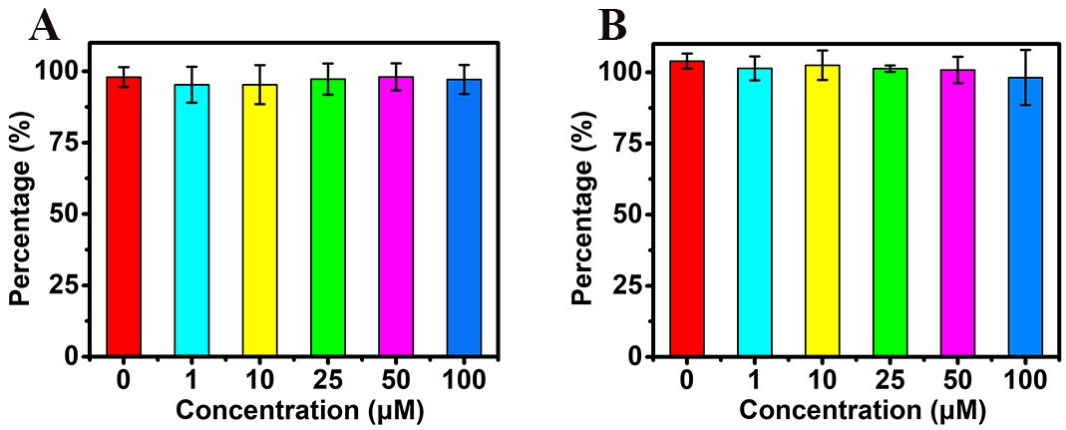



Figure S11. Cell viability of 293T cells (a) and SK-OV-3 cells (b) cultured in the presence of DCMCA-βgal at various concentrations (0-100 μM) for 24 h.

**8、Comparison of fluorescent intensity of DCMCA-βgal (5 μM) in 293T and 293T/lacZ cells (Figure S12)**

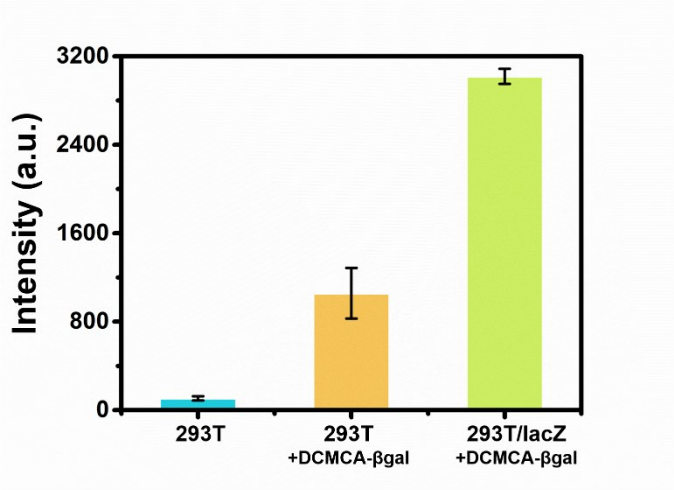



Figure S12. Comparison of fluorescent intensity of DCMCA-βgal (5 μM) in 293T and 293T/lacZ cells. Error bars represent standard deviation (±S.D.) with n=3.

**9、Comparison of fluorescent intensity of DCMCA-βgal (5 μM) in different SK-OV-3 cells (Figure S13)**

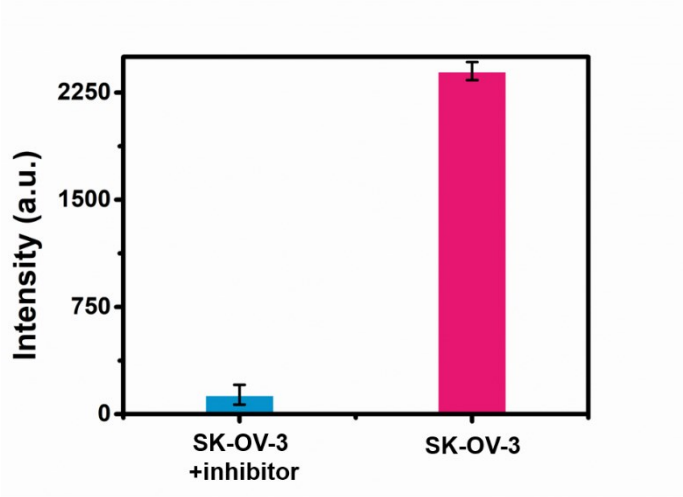



Figure S13. Comparison of fluorescent intensity of DCMCA-βgal (5 μM) in inhibitor-treated and untreated SK-OV-3 cells. Error bars represent standard deviation (±S.D.) with n=3.

**10、Time-dependence imagaing of tumor-bearing mice with DCMCA-βgal tumor injection (Figure S14)**

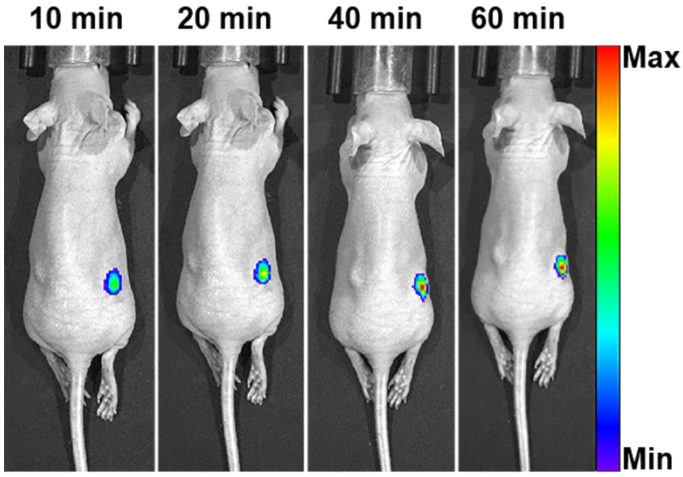



Figure S14. Time-dependence imaging of tumor-bearing mice with DCMCA-βgal tumor injection.