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Far-red imaging of β-galactosidase through a phospha-fluorescein

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1. General Methods.

Materials. General chemicals were of the best grade available, supplied by Adamas-beta, Shanghai Chemical Reagent Co., Tokyo Chemical Industries (TCI), J&K chemical LTD., and Acros Organics. Unless otherwise stated, all commercial reagents were used without additional purification. All solvents were freshly distilled according to standard procedures prior to use.

Apparatus. All reactions were monitored by thin-layer chromatography (TLC) on gel F254 plates. Flash chromatography was carried out on silica gel (200-300 mesh; Qingdao Ocean Chemicals). The condensation reactions were performed in sealable pressure tubes (Beijing Synthware Glass) behind a blast shield. NMR spectra were recorded on a Bruker AC-300P spectrometer at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR or on a Bruker AC-600P spectrometer at 600 MHz for ^{1}H NMR and at 150 MHz for ^{13}C NMR. δ values are given in ppm relative to tetramethylsilane. Mass spectra (MS) were measured with an API-3000 MS spectrometer using electrospray ionization (ESI). High-resolution mass spectra (HRMS) were recorded on an Aglilent Technologies 6538 UHD Accurate-Mass Q-TOF MS spectrometer using ESI. UV-visible spectra were obtained on a Analytikjena Specord 210 PLUS UV–vis spectrophotometer. Fluorescence spectroscopic studies were performed on a Hitachi F-7000. HPLC analysis was performed on an Diamonsil C18 (4.6 × 250 mm) column (Dikma technologies) using an HPLC system composed of a pump (LC-20AD, Shimadzu) and a detector (SPD-M20A, Shimadzu).

Synthesis of DiMe-PF and DiMe-PF-Gal.

Compound 1. Compound **1** was synthesized according to reported procedures.

DiMe-PF. To a 50 mL well-dried flask flushed with argon, 2-bromo-1,3-dimethylbenzene (0.28 g, 1.5 mmol) and anhydrous THF (5 mL) were added. The solution was cooled to -78 °C, *s*-BuLi (1.0 M in *n*-hexane, 2.0 mL, 2.0 mmol) was added and the reaction mixture was stirred at -78 °C for 30 min. Compound **1** (0.56 g, 1.0 mmol) dissolved in anhydrous THF (5 mL) was added dropwise, and the reaction mixture was slowly warmed to room temperature, then stirred for 1 h. The reaction was quenched by addition of 2 N HCl, then neutralized with $NaHCO_3$ and extracted with DCM. The organic layers were combined, washed with brine and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent under reduced pressure, the residue was dissolved in CH_2Cl_2 (10 mL). To this solution, *p*-toluenesulfonic acid monohydrate (0.17 g, 1.0 mmol) was added and the resulting mixture was stirred at room temperature for 1h. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel to afford orange solid **DiMe-PF** (0.18 g, 43% yield). ^{1}H NMR (600MHz, $CDCl_3$): δ 7.58-7.49 (m, 3H), 7.39 (td, J = 7.9, 3.2 Hz, 2H), 7.32 (t, J = 7.6 Hz, 1H), 7.19 (dd, J = 12.1, 7.7 Hz, 2H), 6.90 (dd, J = 9.4, 6.8 Hz, 2H), 2.03 (s, 3H), 2.00 (s, 3H); ^{13}C NMR (125MHz, $CDCl_3$): δ 152.2, 152.2, 138.6, 136.1, 135.2, 135.0, 134.1, 132.3, 132.3, 131.5, 129.7, 129.6, 128.8, 128.7, 128.5, 127.4, 127.4, 121.1, 119.8, 19.4, 19.4; ^{31}P NMR (240MHz, $CDCl_3$): δ 10.75 (s). HRMS (ESI) calcd. for $C_{27}H_{21}O_3P$ $[M+H]^+$: 425.1301, found: 425.1350.

DiMe-PF-Gal. A mixture of **DiMe-PF** (82 mg, 0.2 mmol), 2,3,4,6-tetra-O-acetyl-α-galactopyranosyl bromide (0.25 g, 0.6 mmol) and Cs_2CO_3 (0.20 g, 0.6 mmol) in MeCN (5 mL) was stirred at room temperature under argon overnight. The inorganic precipitate was filtered off, and the filtrate was evaporated to dryness. The resulting residue was dissolved with dry MeOH (5 mL) and the solution was cooled to 0 °C.

Then 30 μL of 28 % NaOMe in MeOH was slowly added, and the mixture was stirred for 1 h. The reaction was quenched by addition of 2 N HCl, then neutralized with $NaHCO_3$ and extracted with DCM. The organic layers were combined, washed with brine and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel to afford **DiMe-PF-Gal** (48 mg, 41% yield). ^{1}H NMR (600MHz, $CDCl_3$): δ 7.71-7.65 (m, 1H), 7.57-7.46 (m, 2H), 7.37-7.26 (m, 4H), 7.21-7.11 (m, 3H), 6.98-6.77 (m, 3H), 6.32-6.20 (m, 1H), 4.99-4.86 (m, 1H), 4.11-3.86 (m, 2H), 3.67-3.49 (m, 4H), 1.93-1.90 (m, 6H); ^{13}C NMR (125MHz, $CDCl_3$): δ 183.4, 183.3, 159.3, 159.2, 159.1, 151.0, 138.4, 136.0, 135.8, 135.0, 134.9, 132.3, 131.5, 129.9, 129.8, 129.6, 128.8, 128.7, 128.6, 127.5, 125.0, 121.2, 100.3, 99.9, 74.8, 74.5, 73.0, 72.9, 70.2, 68.0, 60.5, 60.3, 19.4, 19.3, 19.3; ^{31}P NMR (240MHz, $CDCl_3$): δ 9.52 (s), 9.42 (s). HRMS (ESI) calcd. for $C_{33}H_{31}O_8P$ $[M+H]^+$: 587.1829, found: 187.1842.

Cellular culture

HEK 293T cell (purchased from National Infrastructure of Cell Line Resource in Shanghai) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic (AA) at 37 °C in a 5% CO2/95% air incubator. Ovcar-3 cell (purchased from National Infrastructure of Cell Line Resource in Shanghai) were cultured in RPMI 1640 containing 20% FBS and 1% Antibiotic-Antimycotic (AA) at 37 °C in a 5% CO2/95% air incubator.

MTT assay.

The cytotoxicity of **DiMe-PF-Gal** were evaluated using MTT assay. HEK 293T cell lines and Ovcar-3 cells were cultivated in 96-well plates by mixture of 90% (V/V) Dulbecco's Modified Eagle's Medium (DMEM), 10% (V/V) FBS containing probes of various concentrations: 1 μM, 2 μM, 5 μM, 10 μM and 20 μM in a 5% CO_2/95% air incubator at 37 ℃. After incubation for 24 hours, 20 μL of MTT reagent, 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL in PBS) was added into each well and incubated for another 4 hours in the 5% CO_2/95% air incubator at 37 ℃. Then 80 μL DMSO were added into each well to dissolve the formazan crystals. Each experiment was performed for three times. Absorbance of each well was analysed at 565 nm with a SynergyTM H1 multimode microplate reader (BioTek).

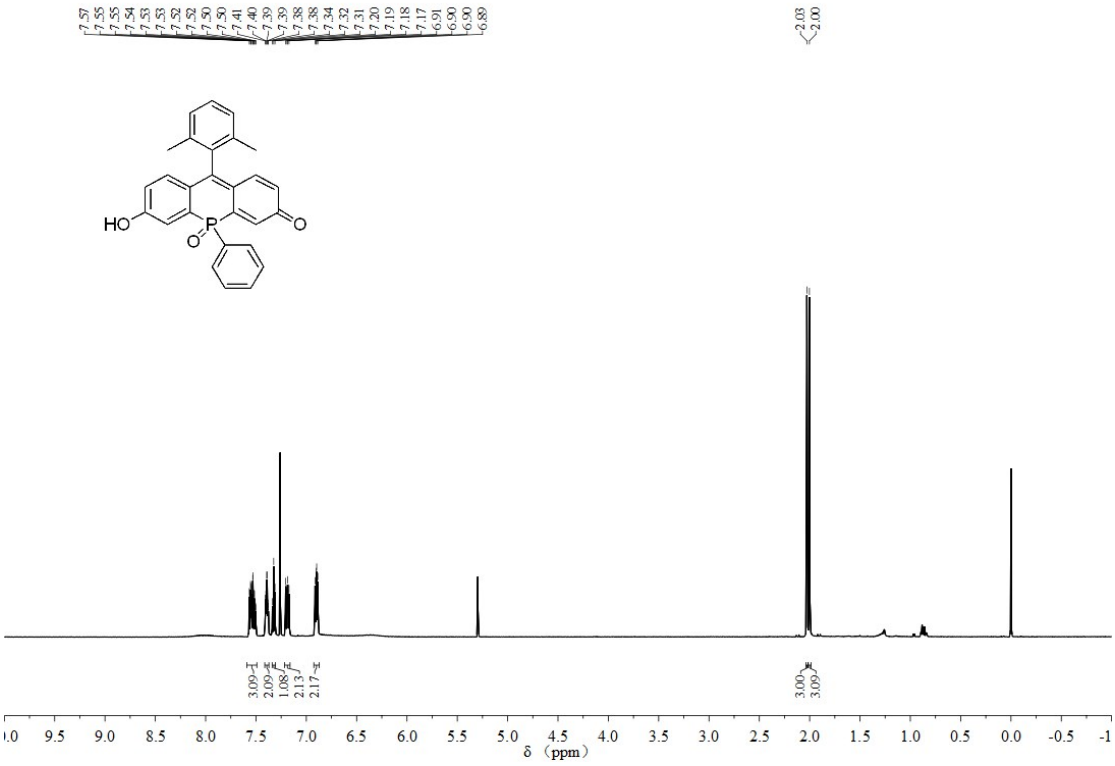
***LacZ* gene transient transfection**

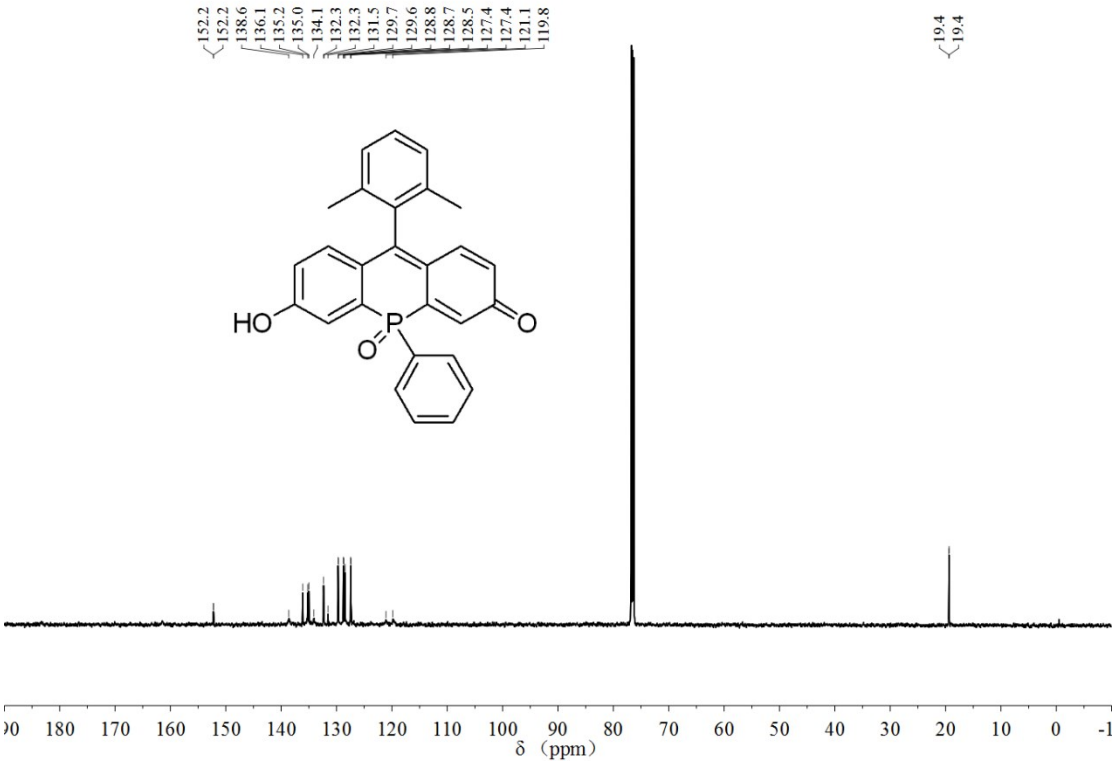
HEK 293T cells were centrifuged and resuspended in fresh serum-free DMEM at a density of 1.0×10^6 cells mL^{-1} before transfection. 500 μL cell suspension was distributed per well in a 12-well plate. DNA was diluted in fresh serum-free DMEM (in a volume equivalent to one-tenth of the culture to be transfected), 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.

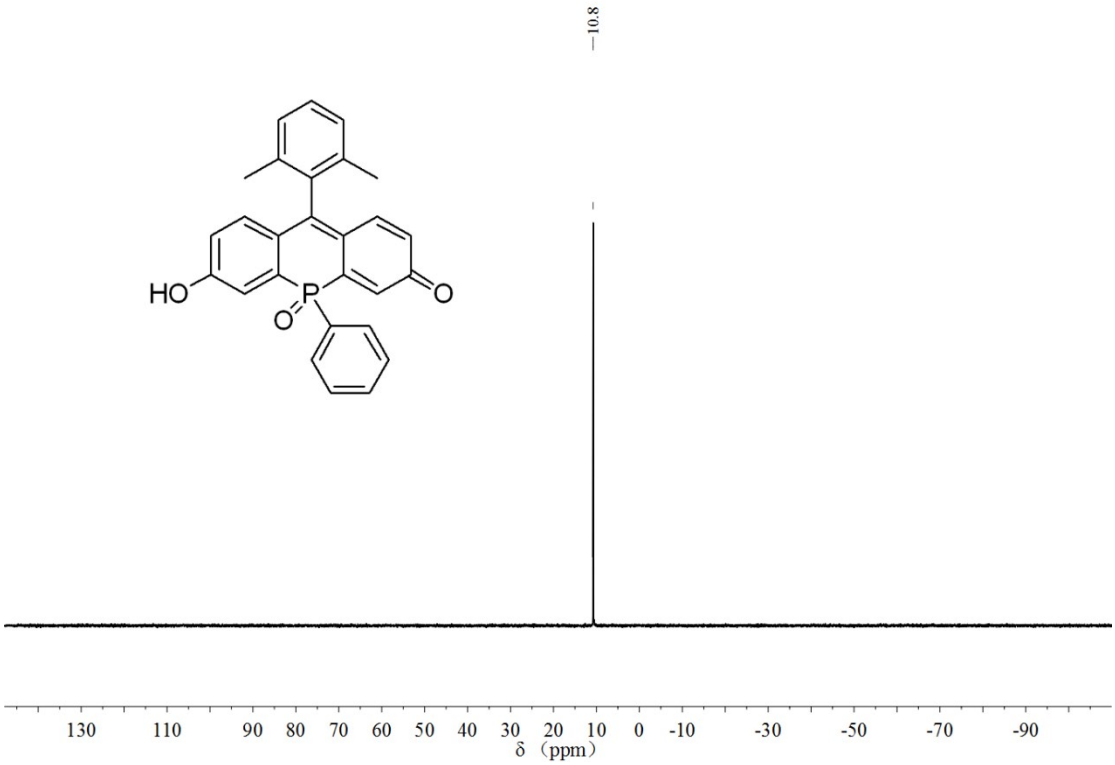
Confocal imaging.

HEK 293T cell and Ovcar-3 cell were incubated with **DiMe-PF-Gal** (5.0 μM) for 30 min and washed by PBS for three times. Fluorescent imaging was performed using Leica TCS SP5 confocal laser scanning microscopy with a 63× objective.

^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra



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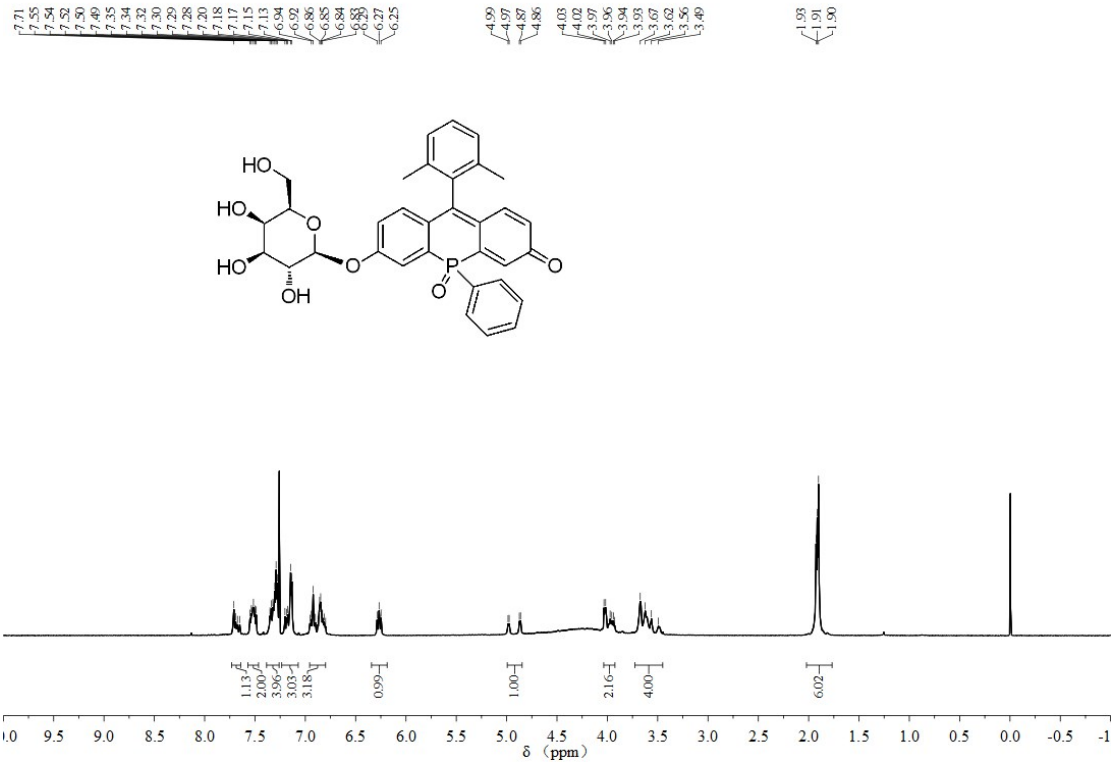
δ（ppm）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1.91
1.90
HO
HO
HO
OH
O
O
P
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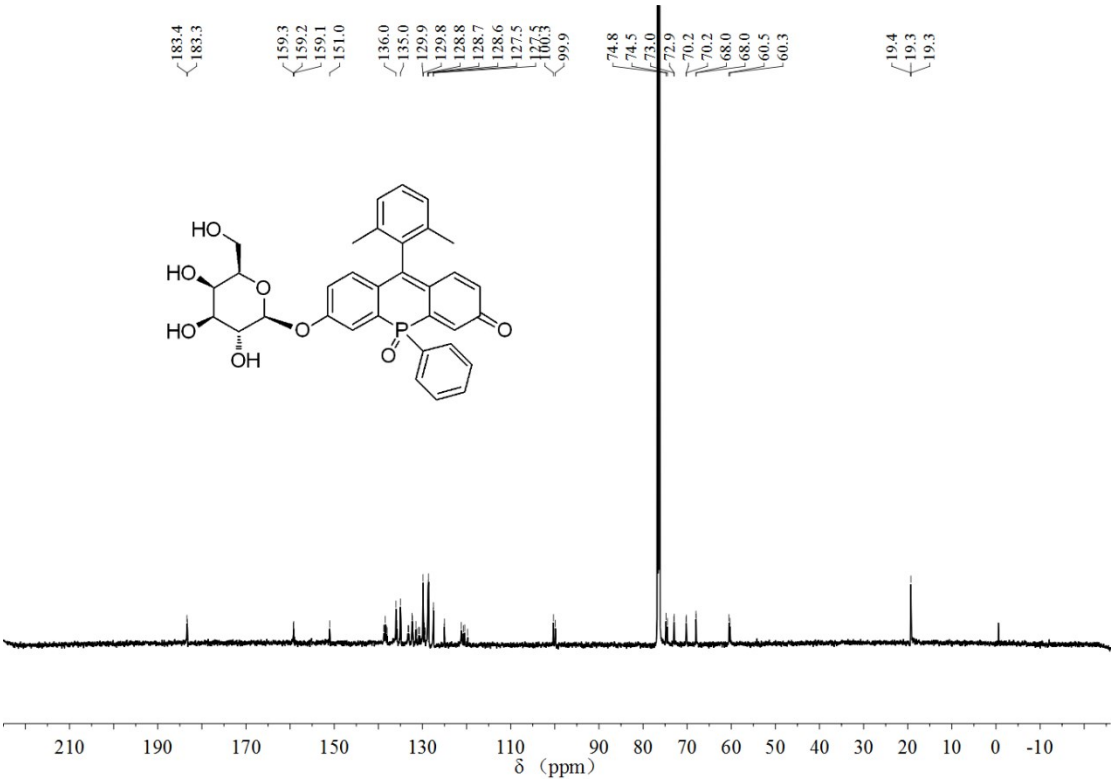
δ (ppm)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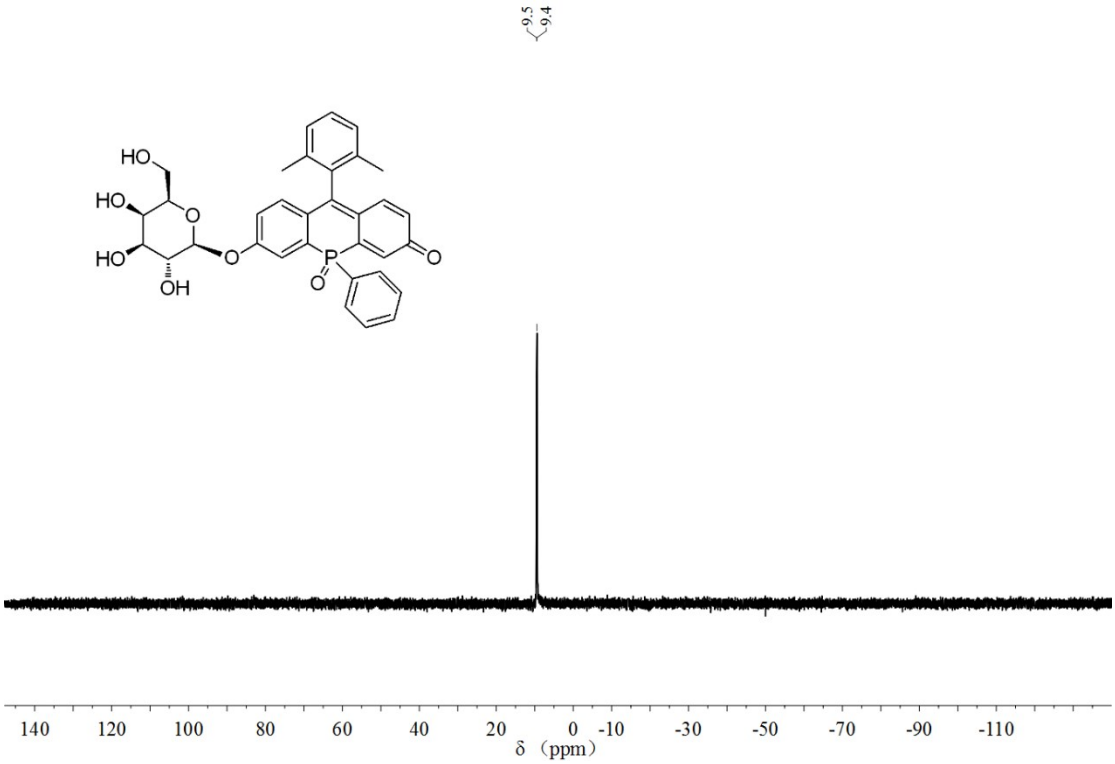
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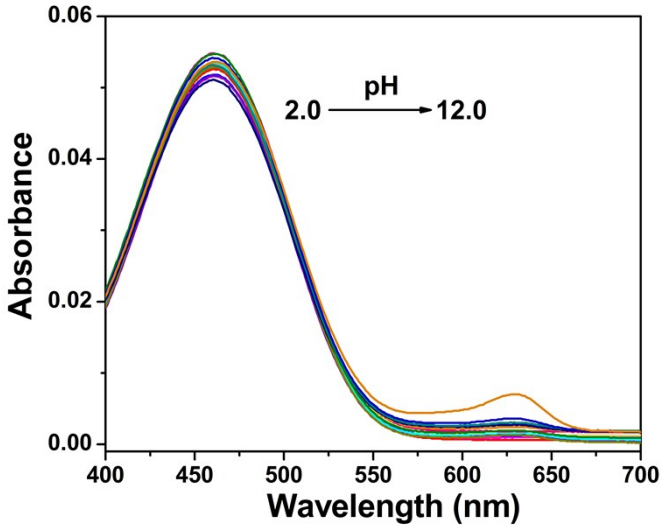

Fig. S1 The absorption spectra of **DiMe-PF-Gal** in varied pH (2.5 μM).

Table S1 The excited states and corresponding oscillator strength (*f*) of **DiMe-PF**.

Excited State	*f*	Excited State	*f*	Excited State	*f*
1	0.0002	11	0.0854	21	0.0390
2	0.3885	12	0.0545	22	0.0190
3	0.0009	13	0.1193	23	0.0175
4	0.0004	14	0.0434	24	0.0070
5	0.0475	15	0.0059	25	0.0042
6	0.0003	16	0.0049	26	0.0054
7	0.0009	17	0.0276	27	0.0019
8	0.054	18	0.0012	28	0.0033
9	0.0228	19	0.0038	29	0.0247
10	0.0132	20	0.0001	30	0.0910

```
Excited State   2:      Singlet-A      2.9571 eV  419.28 nm  f=0.3885  <S**2>=0.000
    107 -> 112        0.13292
    111 -> 112        0.67675
```

Table S2 The excited states and corresponding oscillator strength (*f*) of **DiMe-PF-Gal.**

Excited State	*f*	Excited State	*f*	Excited State	*f*
1	0.0003	11	0.0161	21	0.0050
2	0.4925	12	0.0875	22	0.0001
3	0.0013	13	0.0799	23	0.0016
4	0.0006	14	0.0013	24	0.0803
5	0.0533	15	0.0182	25	0.0008
6	0.0002	16	0.1068	26	0.0107
7	0.0008	17	0.0060	27	0.0008
8	0.0071	18	0.0030	28	0.0039
9	0.0251	19	0.0068	29	0.0060
10	0.0069	20	0.0254	30	0.0025

```
Excited State   2:      Singlet-A      2.9608 eV  418.75 nm  f=0.4925  <S**2>=0.000
    150 -> 155       -0.12310
    154 -> 155        0.67701
```

Table S3 The excited states and corresponding oscillator strength (*f*) of deprotonated **PF**.

Excited State	*f*	Excited State	*f*	Excited State	*f*
1	0.000	11	0.0088	21	0.0024
2	0.0020	12	0.1968	22	0.0002
3	0.0033	13	0.0754	23	0.0031
4	0.0000	14	0.0022	24	0.0314
5	0.0040	15	0.0028	25	0.0140
6	0.0000	16	0.0105	26	0.0015
7	0.0001	17	0.0000	27	0.0027
8	0.0054	18	0.0636	28	0.0233
9	0.2302	19	0.0018	29	0.0012
10	0.0000	20	0.0009	30	0.0328

```
Excited State   9:      Singlet-A      2.4662 eV  502.74 nm  f=0.2302  <S**2>=0.000
    101 -> 111        0.43452
    104 -> 111        0.53457
    109 -> 112        0.13943
    104 <- 111       -0.11102
```

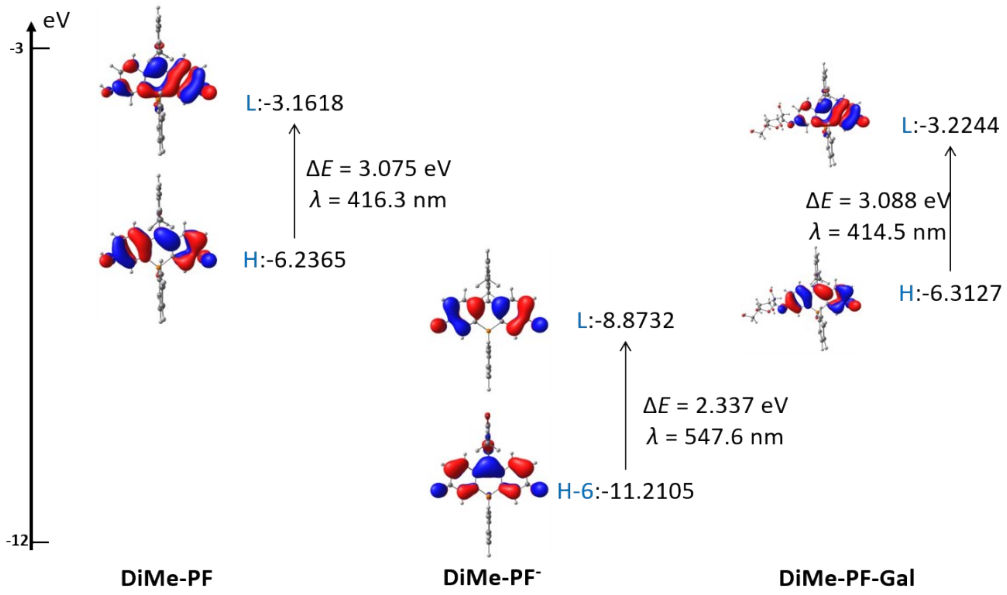

Fig. S2 The dominated excited states of **DiMe-PF**, deprotonated **DiMe-PF** and **DiMe-PF-Gal**.

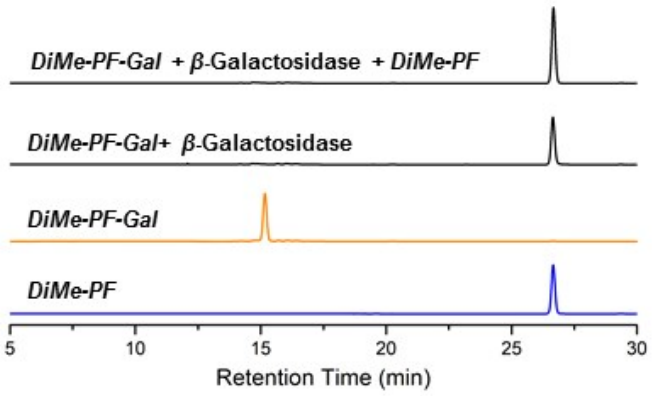

Fig. S3 HPLC analysis of probe **DiMe-PF-Gal** reacted with β-galactosidase.

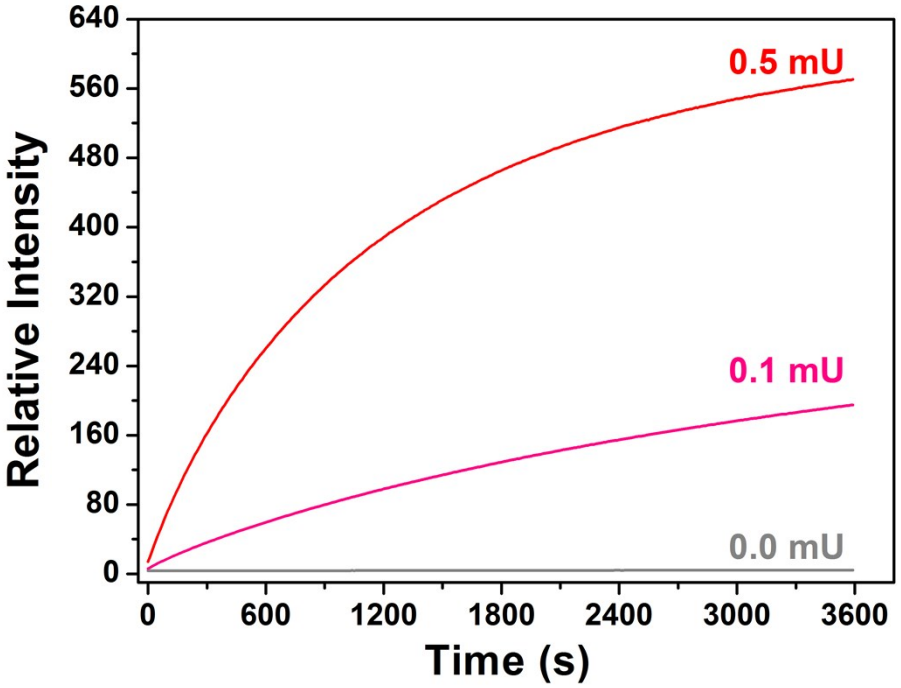

Fig. S4 Time-dependent fluorescence intensity changes of probe **DiMe-PF-Gal** (2.5 μM) upon addition of various concentrations *β*-galactosidase.

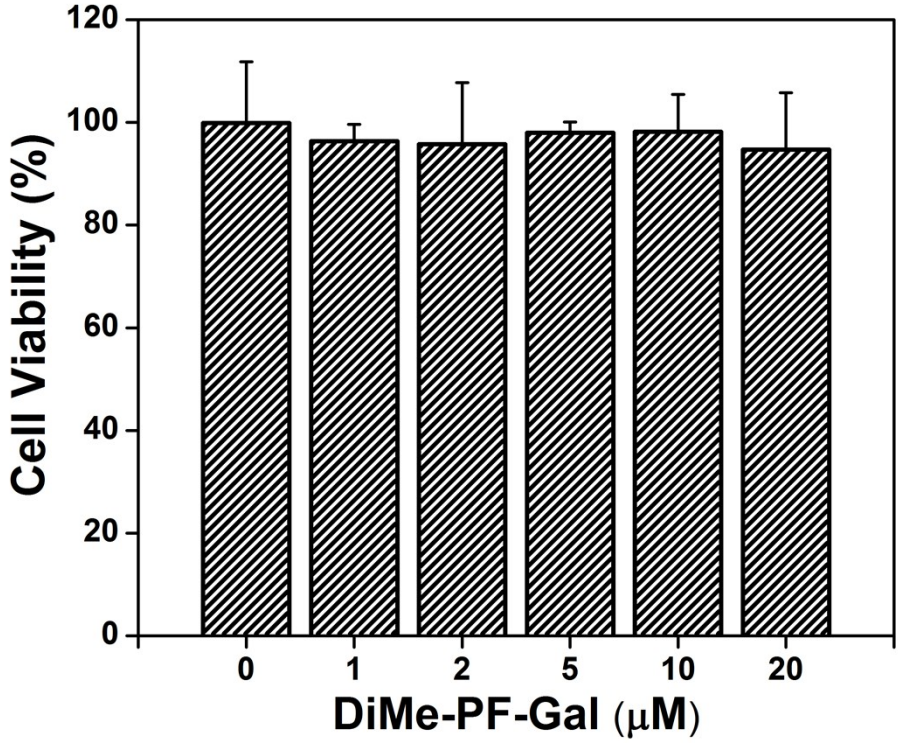

Fig. S5 Cytotoxic effects of probe **DiMe-PF-Gal** on HEK 293T cells.

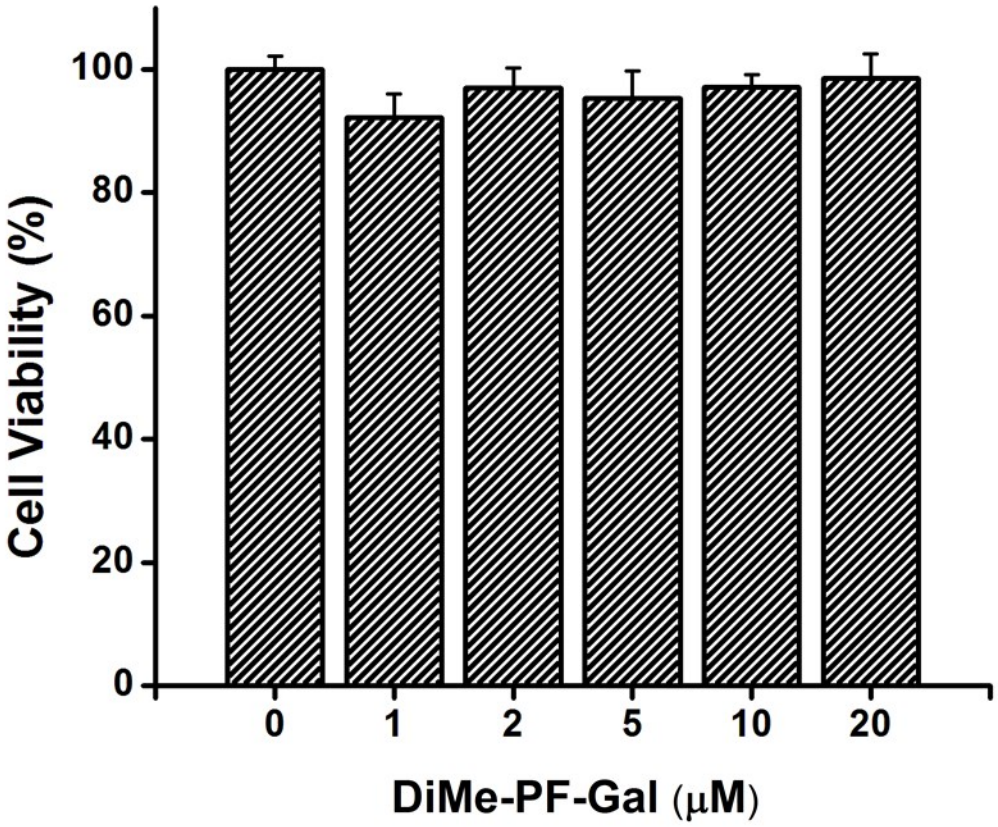

Fig. S6 Cytotoxic effects of probe **DiMe-PF-Gal** on Ovcar-3 cells.

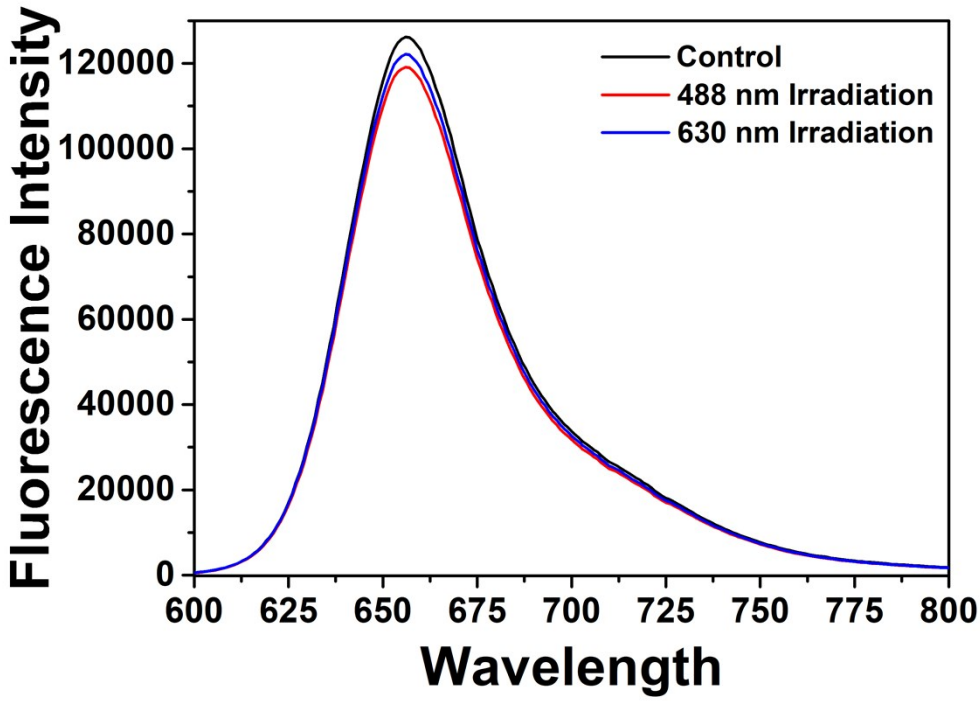

Fig. S7 Intensity of fluorescence for **DiMe-PF-Gal** irradiated with light at 488 nm or 630 nm for 1 hour.

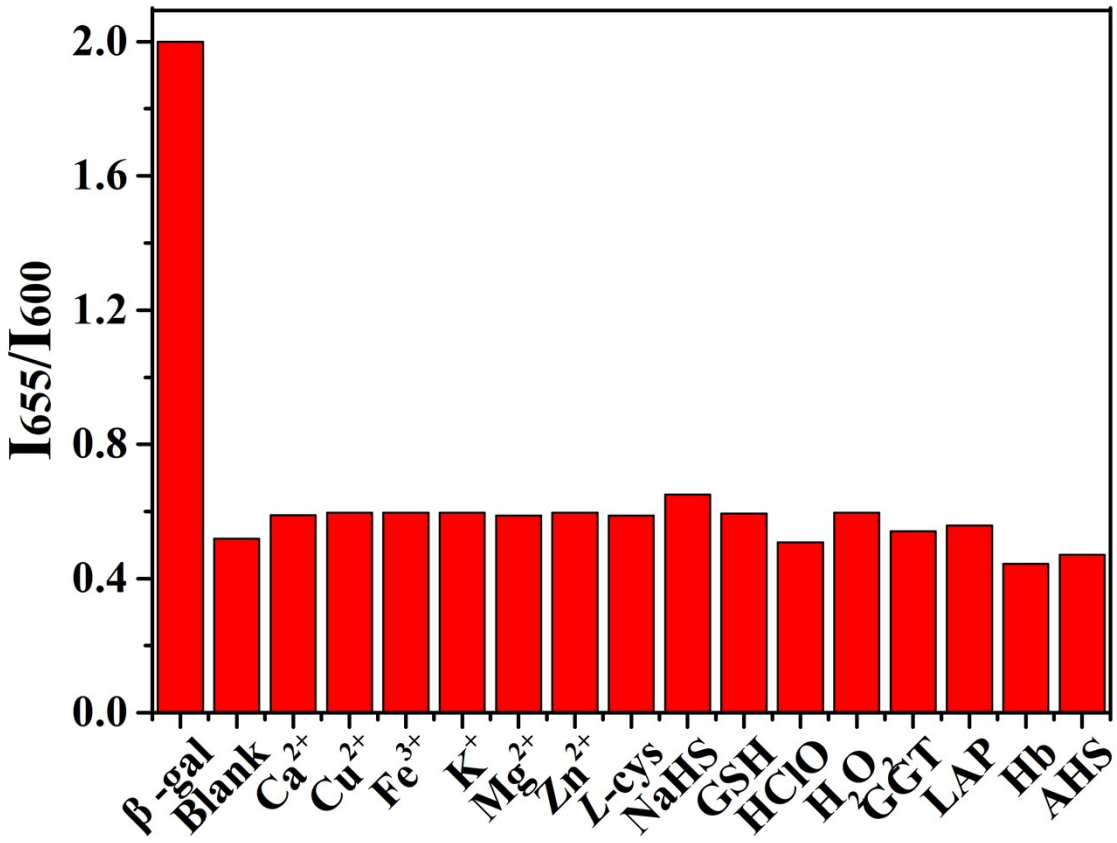

Fig. 8 Fluorescence intensity of **DiMe-PF-Gal** in the presence of other species in PBS buffer solution at 7.4 (500 μM for metal ions, L-cys, NaHS, GSH, HClO and H_2O_2, 200 μU for *β*-Gal, GGT and LAP, 200 μM for Hb and AHS).