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A diketopyrrolopyrrole-based ratiometric fluorescent probe for endogenous leucine aminopeptidase detecting and imaging with specific phototoxicity in cancer cells

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Table of Contents

Experimental reagents and instruments

All reagents used in the reaction were purchased from Shanghai Titan Scientific Co., Ltd., if not otherwise stated, they were used directly. 4-Aminobenzyl alcohol and Boc-Leu-Osu (1) were acquired commercially from Energy Chemical, Shanghai. The reaction was monitored by TLC and the silica gel used for column chromatography was 200-300 mesh. ^{1}H and ^{13}C NMR spectra were all measured by a Bruker AM-400 MHz spectrometer with $CDCl_3$ or MeOH-d_4 as the solvent and tetramethylsilane (TMS) as an internal reference. Absorption spectra and fluorescence spectra of the solution were recorded on a Varian Cary 500 ultraviolet-visible (UV-vis) spectrophotometer and an F97pro fluorescence spectrophotometer, respectively. The cell survival rate was obtained by a multifunctional microplate reader (Synergy H1, BioTek Instruments, America). Confocal images of cells were carried out on a confocal laser scanning microscope (CLSM, Leica Microsystems, TCSSP5 II, Germany).

Sample preparation

The target product **DPP-Leu** was dissolved in ethanol (EtOH, analytical reagent) to prepare a mother liquor with a concentration of 1.0 mM in the *in vitro* test. All UV-vis absorption spectra and fluorescence spectra were obtained at a final concentration of 10^{-5} M after shaking for 20 minutes at a constant temperature of 37 ℃ in EtOH/PBS buffer solution (2:8, v/v, pH = 7.4).

In selective experiments, Na_2CO_3, Na_2SO_4, KCl, NaClO, H_2O_2 (100 μM); homocysteine (Hcy), cysteine (Cys), glutathione (GSH), adenosine triphosphate (ATP), adenosine diphosphate (ADP), human serum albumin (HSA), bovine serum albumin (BSA) (50 μM); alpha-fetoprotein (AFP), esterase (10.0 ng/mL) were separately added into the probe solution (1.0 mL) with a concentration of 10^{-5} M, oscillated at 37 ℃ for 20 min, and then tested with a fluorescence

spectrophotometer. Finally, LAP (18 U/L) was added to each reaction solution and oscillated for another 20 min to obtain fluorescence spectra.

ROS study of DPP-Leu

1,3-Diphenylisobenzofuran (DPBF, ROS indicator) was dissolved in *N*,*N*-dimethylformamide (DMF) to prepare a mother liquor with a concentration of 10 mM and then added into the corresponding test solution. Absorption spectra were immediately acquired by an UV-vis spectrophotometer after illuminating by a 530 nm laser (20 mW/cm^2) for different times.

Cell culture and CLSM imaging

Human breast cancer cells (MCF-7), human liver cancer cells (HepG2), human embryonic kidney cells (293T) and human normal thyroid cells (Nthy) were all cultured 37 ℃ under 5% CO_2 atmosphere and incubated with RPMI1640 medium containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Calsbad, CA, USA) and 1% penicillin-streptomycin (10000 U/mL penicillin and 10 mg/mL streptomycin).

The above-mentioned cells were cultured in 96-well plates at a density of 3×10^4 cells per well for 24 h and then co-incubated with **DPP-Leu** (10 μM) for 60 min at 37 ℃. The culture media were aspirated, washed three times with PBS buffer solution and immediately used for confocal fluorescence imaging. The fluorescence signals of cells were collected at 520-570 nm and 620-670 nm respectively with 490 nm laser as excitation light.

Cytotoxicity assay by CCK-8

The MCF-7, HepG2, 293T and Nthy cells were seeded in a 96-well plate at a density of 1 × 10^4 cells per well and cultured in 0.2 mL of DMEM medium containing 10% FBS for 24 h. The various cells were then incubated with different concentrations of **DPP-Leu** for 24 h, subsequently co-incubated with tetrazolium salt (WST-8) for another 3 h, which was conducted to test the cytotoxicity of **DPP-Leu**. Finally, the culture medium was replaced with DMSO and absorbance at 450 nm was measured on a multifunctional microplate reader. The relative cell viability (%) is calculated by the formula: cell viability = $OD_{treated}/OD_{control}$ × 100%.

The phototoxicity of **DPP-Leu** (20 μM) to various cells was tested by 530 nm laser irradiation for different times. The cells were incubated for 24h after light irradiation and WST-8 was then added into the culture medium to calculate the relative cell viability according to the above method.

Cell fluorescence imaging after light irradiation

The cells were seeded in a culture dish with a density of 5 × 10^4 cells per well and then co-incubated with **DPP-Leu** (20 μM) for 60 min. The cells continued to be cultured for 24 hours after 530 nm laser irradiation for different times and then co-incubated with calcein AM and propidium iodide (PI) for staining. Finally, the culture media were removed after 30 minutes and washed three times with PBS buffer solution for fluorescence imaging.

Synthetic steps of DPP-Leu

Synthesis of compound **2**. (4-Aminophenyl)methanol (862.05 mg, 7.0 mmol) and compound **1** (2.3 g, 7.0 mmol) were added into a two-necked round-bottom flask

containing dichloromethane (25 mL) and *N*,*N*-dimethylformamide (5 mL), and the flask was deaerated with argon. Then the solution was stirred at room temperature overnight. After evaporating the organic solvent, the residue was further purified by column chromatography (silica gel, dichloromethane/ethyl acetate 10:1) to obtain the compound **2** as a white solid (1.49 g): yield 63.24%; ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 9.44 (s, 1H), 7.36 (d, J = 8.0 Hz, 2H), 7.04 (d, J = 8.0 Hz, 2H), 5.77 (d, J = 8.0 Hz, 1H), 4.52 (s, 2H), 4.49 – 4.43 (m, 1H), 1.82-1.75 (m, 1H), 1.73 – 1.62 (m, 2H), 1.41 (s, 9H), 0.96 (dd, J = 10.3, 6.5 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 172.32, 156.65, 137.27, 136.47, 127.47, 119.93, 80.19, 64.40, 53.98, 41.42, 28.38, 24.78, 23.12, 21.65. HRMS (ESI, m/z): $[M+Na]^+$ calcd for $C_{18}H_{28}N_2O_4Na^+$: 359.1947, found: 359.1929.

Synthesis of compound **3**. Compound **2** (672.86 mg, 2.0 mmol), carbon tetrabromide (663.26 mg, 2.0 mmol) and triphenyl phosphine (524.58 mg, 2.0 mmol) were added to a 50 mL two-necked round-bottom flask containing 20 mL ultra-dry dichloromethane as the reaction solvent under argon atmosphere. The solution was stirred at room temperature for 8 h and then quenched with 5 mL saturated sodium bicarbonate solution. The crude product was extracted with CH_2Cl_2 for 3 times and purified by the column chromatographic (silica gel, dichloromethane/ethyl acetate 30:1) to afford a white solid. Compound **3** was immediately put into the next reaction.

Synthesis of compound **DPP-Boc-Leu**. Compound **3** (119.80 mg, 0.3 mmol) and compound **4** (91.53 mg, 0.2 mmol) were added to a 50 mL two-necked flask, and the flask was deaerated with argon. After the addition of acetonitrile (20 mL) by a syringe, the solution was stirred for 12 h at 80 °C. After evaporating the solvent, the resulting crude product was purified by column chromatography (silica gel, ethanol/dichloromethane 1:20) to obtain the product as a purple solid (50.23 mg): yield 32.25%; ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 9.66 (d, J = 5.7 Hz, 2H), 9.60 (s, 1H), 8.43 (d, J = 6.8 Hz, 2H), 7.83 (d, J = 5.8 Hz, 2H), 7.59 – 7.51 (m, 7H), 6.50 (d, J = 13.5 Hz, 1H), 6.38 (s, 1H), 5.67 (d, J = 8.3 Hz, 1H), 4.45 (q, J = 7.3 Hz, 1H), 3.88 – 3.82 (m, 2H), 3.79 – 3.74 (m, 2H), 1.77 (s, 8H), 1.66 (d, J = 5.6 Hz,

1H), 1.56 (d, J = 37.4 Hz, 5H), 1.43 (s, 9H), 1.29 – 1.23 (m, 8H), 0.96 (s, 6H), 0.81 (t, J = 6.3 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 162.26, 161.40, 154.91, 145.24, 142.40, 139.82, 138.36, 132.75, 130.41, 129.17, 129.05, 126.96, 125.68, 120.65, 116.26, 110.19, 80.37, 31.24, 31.07, 29.91, 29.15, 28.44, 26.41, 26.25, 24.81, 23.13, 22.46, 22.38, 21.62, 13.97, 13.91. HRMS (ESI, m/z): $[M]^+$ calcd. for $C_{47}H_{62}N_5O_5^+$: 776.4751, found 776.4761.

Synthesis of compound **DPP-Leu**. Compound **DPP-Boc-Leu** (130.0 mg, 0.18 mmol) was dissolved in 20 mL dichloromethane under argon atmosphere and then 2 drops of trifluoroacetic acid were added into the flask. The reaction was carried out at room temperature for 3 hours. The organic solvent was then evaporated under reduced pressure and the obtained crude product was further purification by column chromatography (silica gel, ethanol/dichloromethane 1:10) to obtain a purple solid product **DPP-Leu** (101.13 mg) : yield 32.25%; ^{1}H NMR (400 MHz, MeOH-d_4), δ (ppm): 9.16 (d, J = 6.8 Hz, 2H), 8.54 (d, J = 6.8 Hz, 2H), 7.89 (d, J = 7.0 Hz, 2H), 7.81 – 7.75 (m, 4H), 7.65 – 7.57 (m, 6H), 5.86 (s, 2H), 4.08 – 4.04 (m, 1H), 3.87 (dt, J = 7.4, 7.4 Hz, 4H), 1.81 – 1.78 (m, 3H), 1.26 – 1.22 (m, 8H), 1.21 – 1.16 (m, 8H), 0.84 (dd, J = 11.5, 6.3 Hz, 12H).^{13}C NMR (100 MHz, MeOH-d_4), δ (ppm): 169.40, 156.56, 146.24, 140.91, 131.47, 130.33, 130.29, 128.48, 127.65, 121.99, 111.18, 65.14, 53.79, 41.69, 32.33, 32.21, 30.48, 29.91, 27.34, 27.22, 23.53, 23.44, 23.29, 22.11, 14.33, 14.27. HRMS (ESI, m/z): $[M]^+$ calcd. for $C_{42}H_{54}N_5O_3^+$ 676.4227, found 676.4230.

Original spectral copy of new compounds

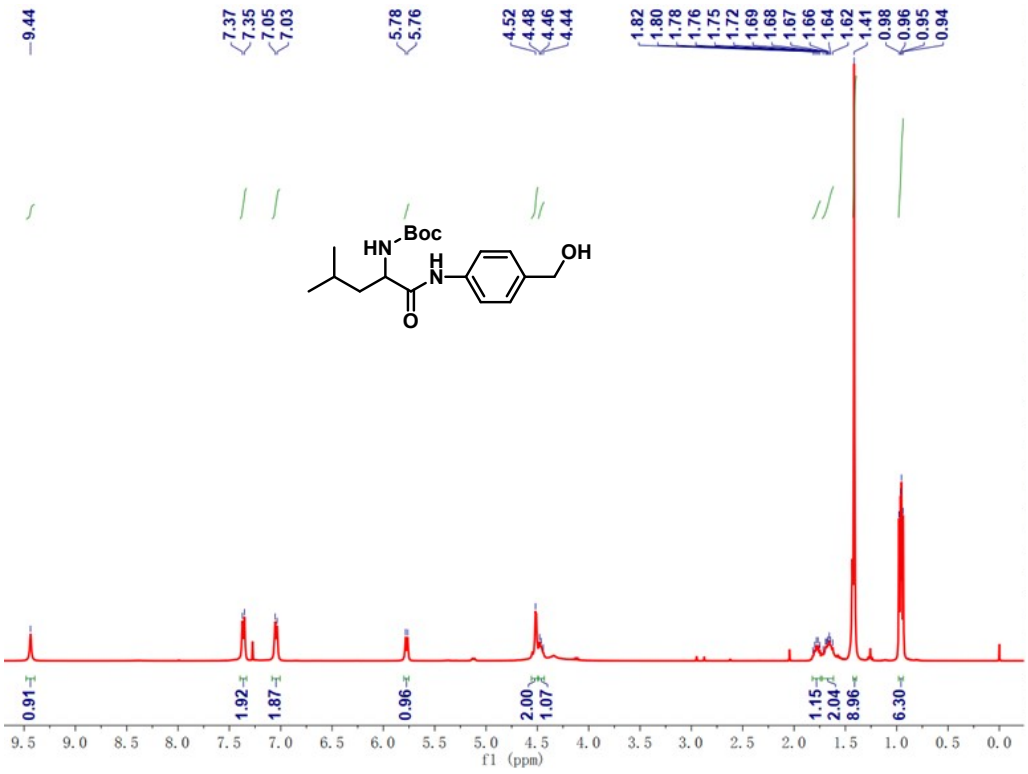

Fig. S1. ^{1}H NMR of compound **2** was conducted in $CDCl_3$.

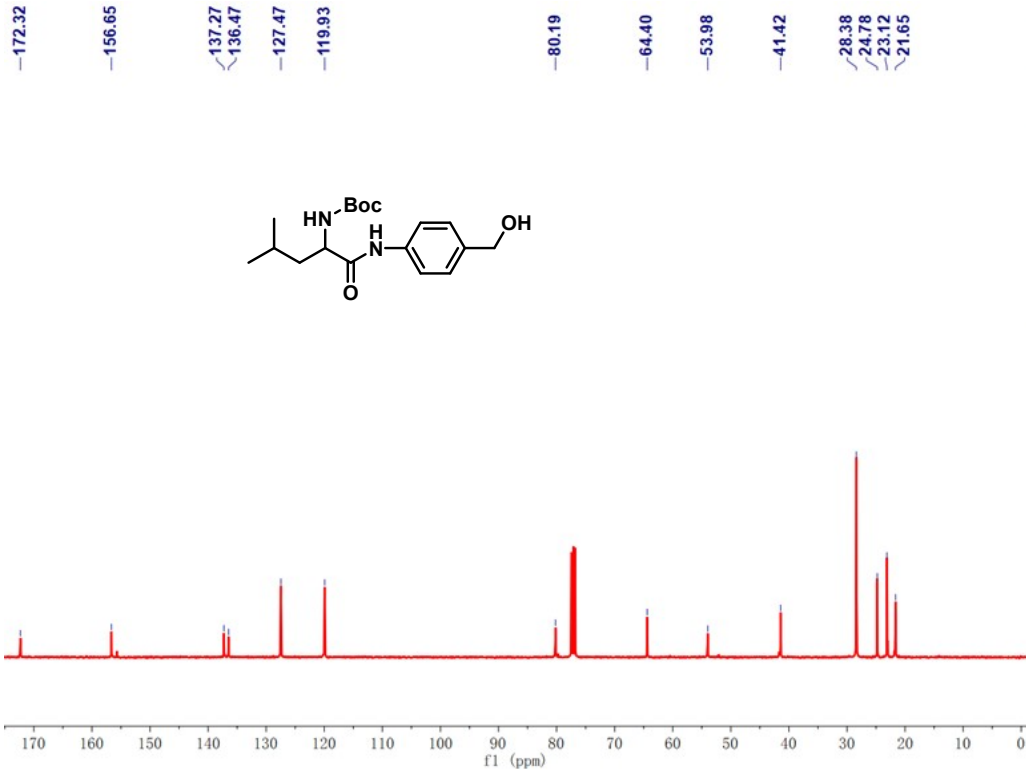

Fig. S2. ^{13}C NMR of compound **2** was conducted in $CDCl_3$.

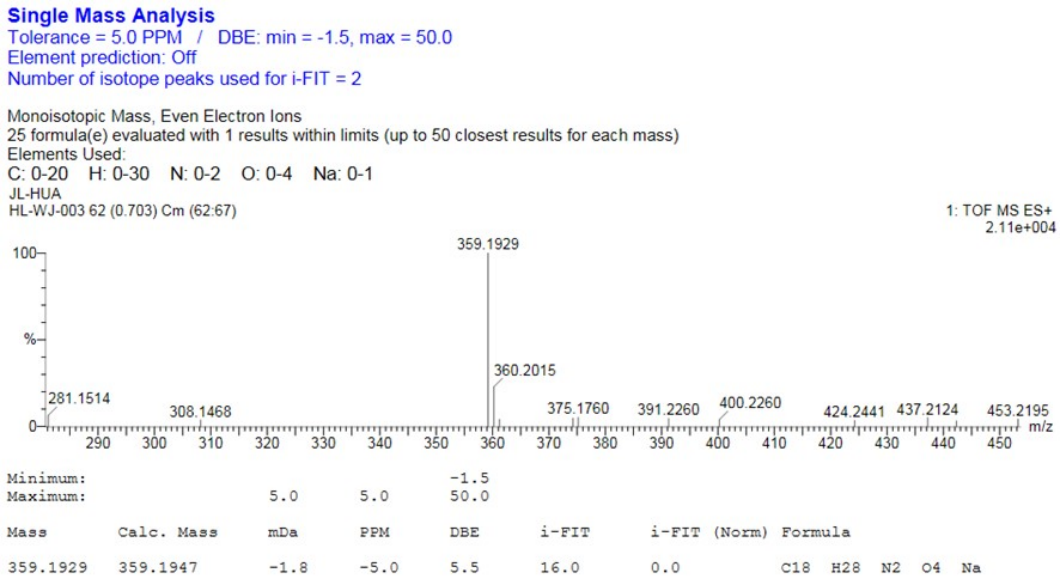

Fig. S3. High-Res ESI-TOF mass spectrum of compound **2**.

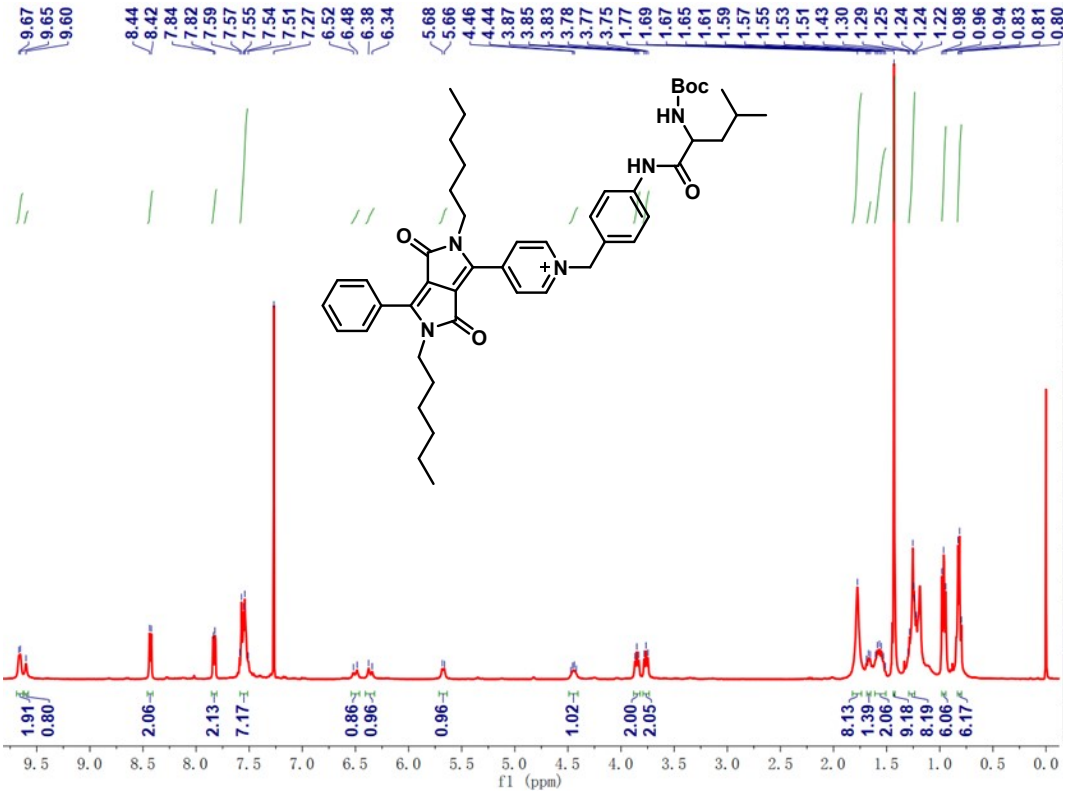

Fig. S4. ^{1}H NMR of compound **DPP-Boc-Leu** was conducted in $CDCl_3$.

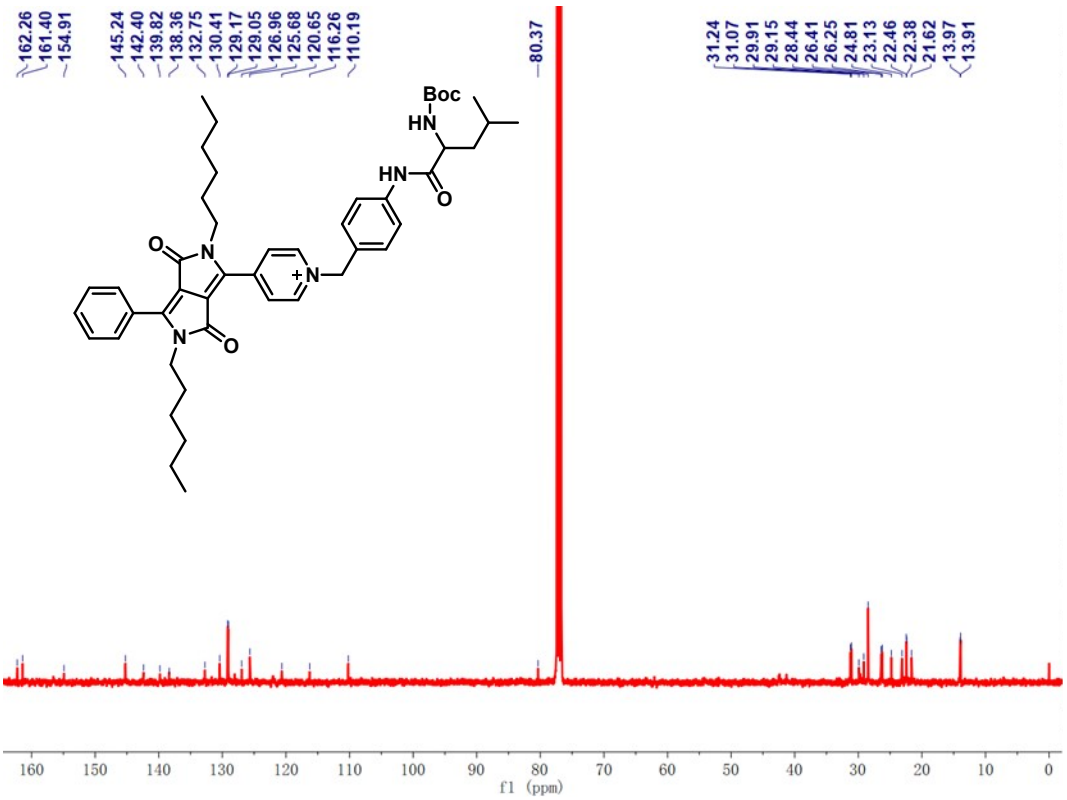

Fig. S5. ^{13}C NMR of compound **DPP-Boc-Leu** was conducted in $CDCl_3$.

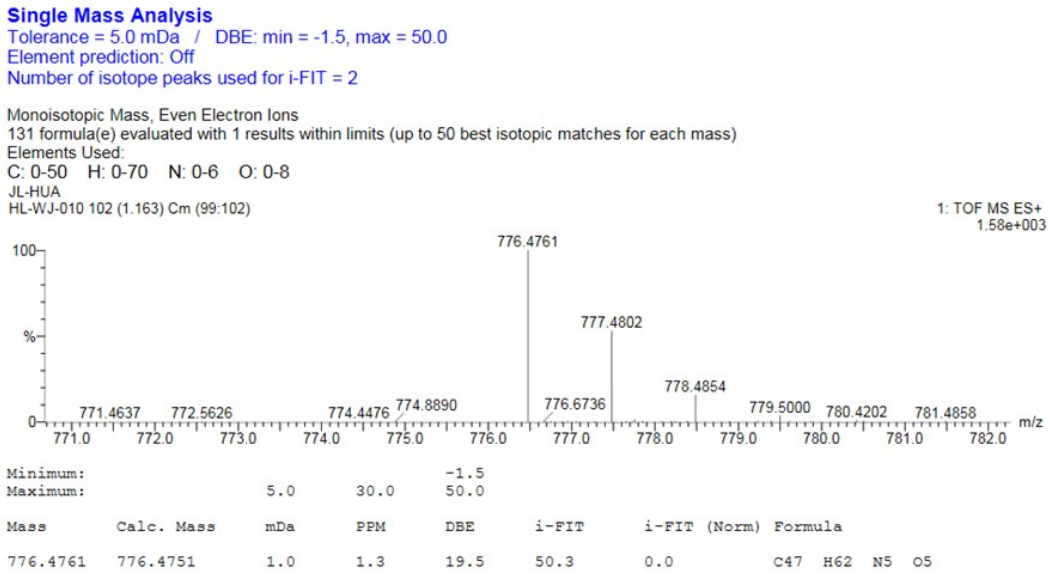

Fig. S6. High-Res ESI-TOF mass spectrum of compound **DPP-Boc-Leu**.

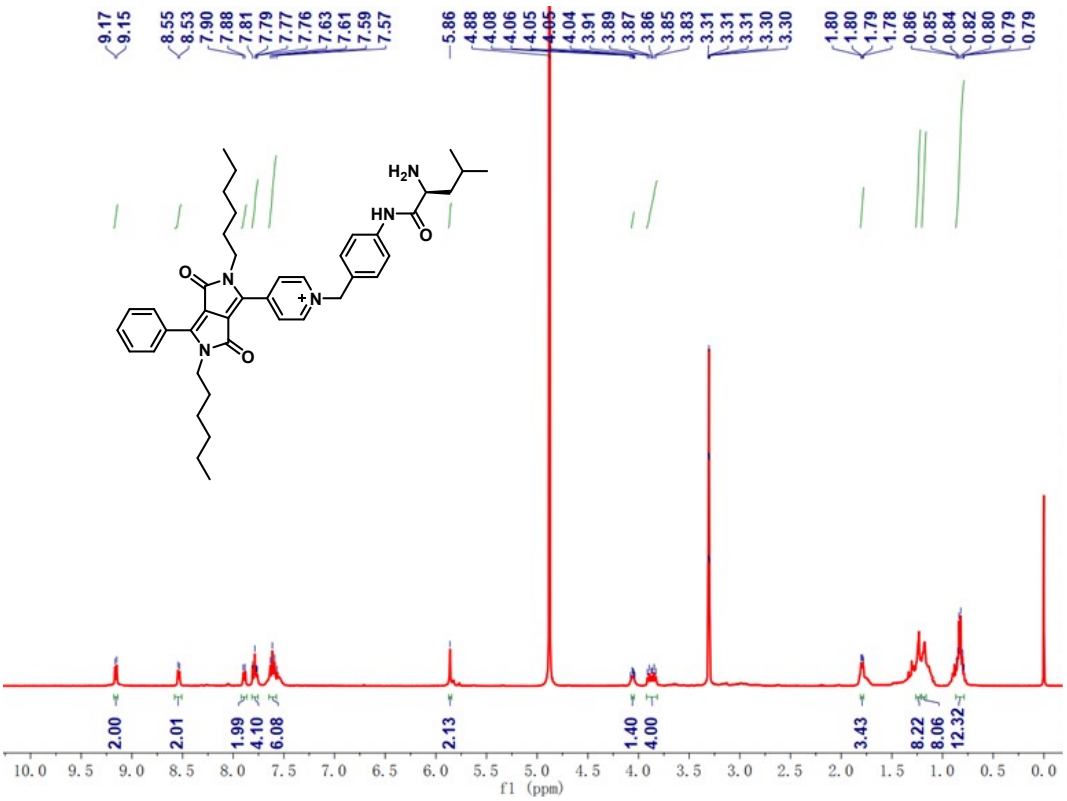

Fig. S7. [1]H NMR of compound **DPP-Leu** was conducted in MeOH-d_4.

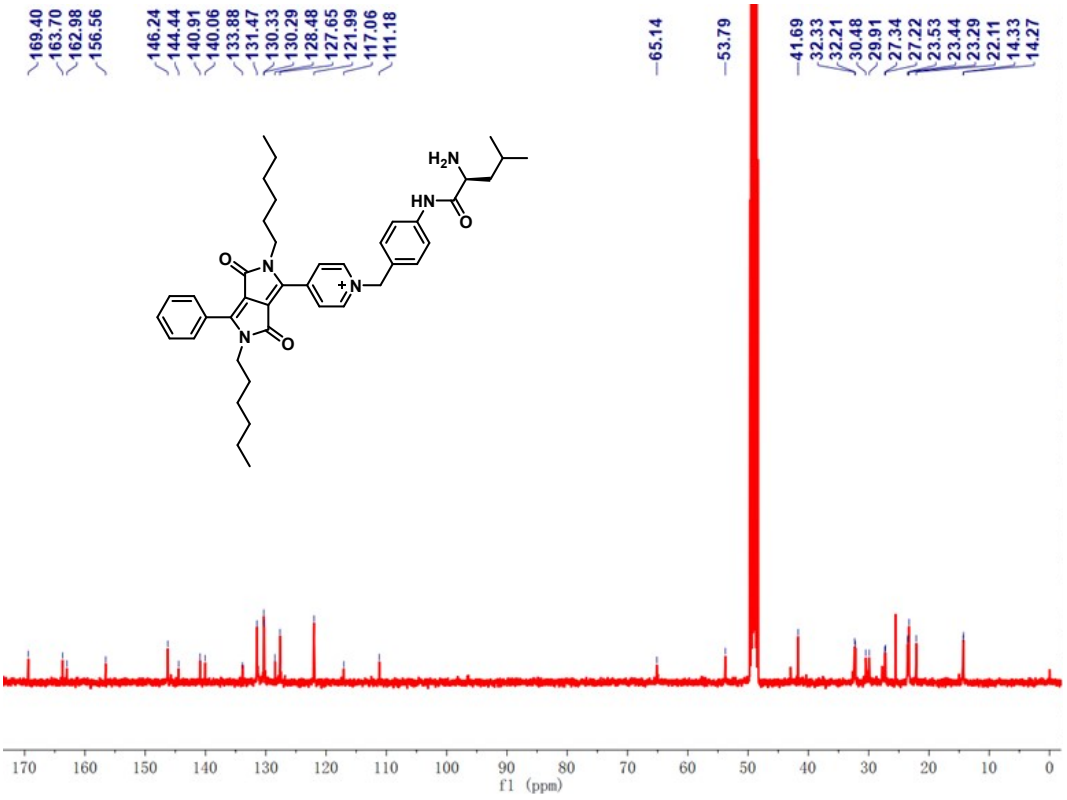

Fig. S8. [13]C NMR of compound **DPP-Leu** was conducted in MeOH-d_4.

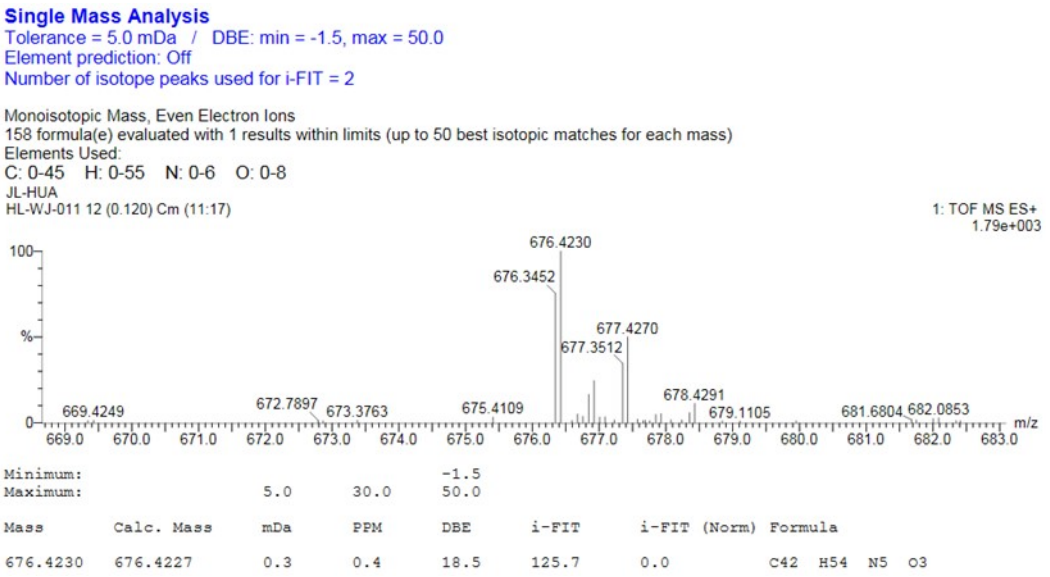

Fig. S9. High-Res ESI-TOF mass spectrum of compound **DPP-Leu**.

Normalized absorbance and fluorescence spectra of compound 4 and the reaction mixture of DPP-Leu and LAP

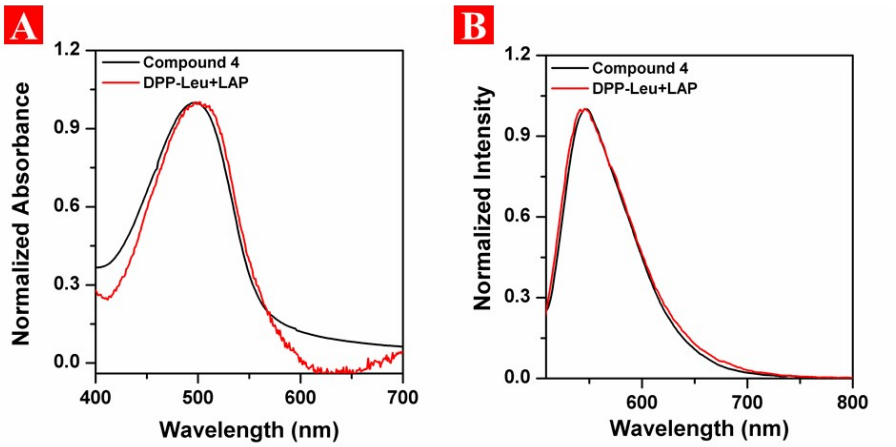

Fig. S10. (A) Normalized UV-vis absorption spectra of compound **4** and the reaction mixture of **DPP-Leu** and LAP (18 U/L); (B) Normalized fluorescence spectra of compound **4** and the reaction mixture of **DPP-Leu** and LAP (18 U/L)

Michaelis-Menten analysis of the Michaelis constant of probe toward LAP

The *Michaelis constant* was determined according to:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[c]} + \frac{1}{V_{max}}$$

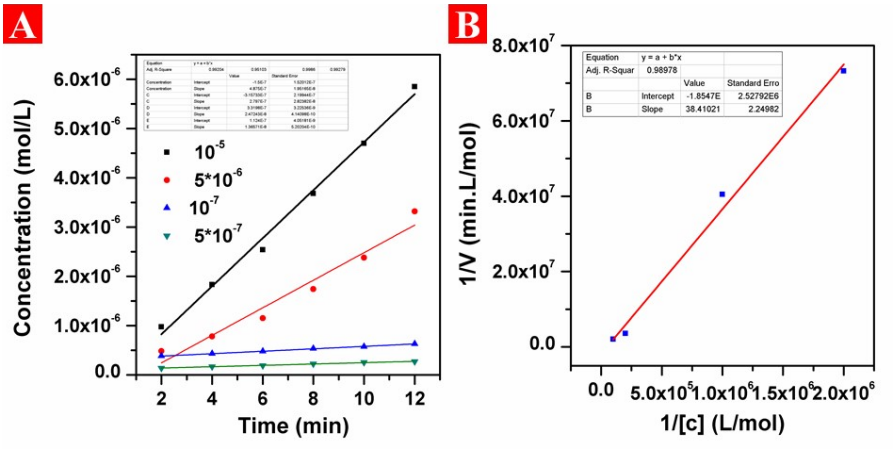

Fig. S11. Linear relationship between (A) the concentration of compound **4** and the reaction time and (B) the 1/V and 1/[c]. LAP = 18 U/L.

Time-dependent imaging of DPP-Leu towards endogenous LAP

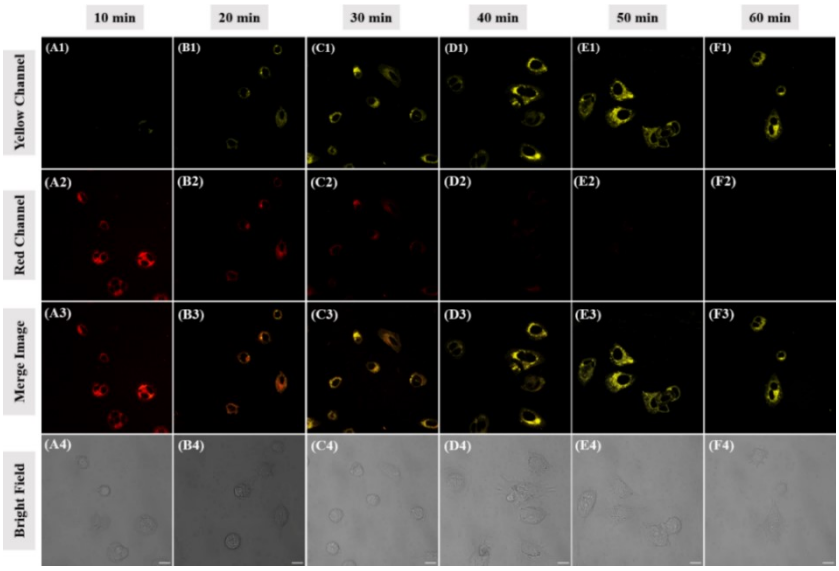

Fig. S12. Fluorescent imaging of MCF-7 cells stained with 10 μM of **DPP-Leu** with different incubation times. Incubation time: (A1-A4) 10 min; (B1-B4) 20 min; (C1-C4) 30 min; (D1-D4) 40 min; (E1-E4) 50 min; (F1-F4) 60 min. (A1-F1) Yellow channel (520 nm-570 nm); (A2-F2) Red channel (620 nm-670 nm); (A3-F3) Merge image; (A4-F4) Bright field. λex = 490 nm. Scale bar = 25 μm.

Cell toxicity of DPP-Leu towards different cells in darkness

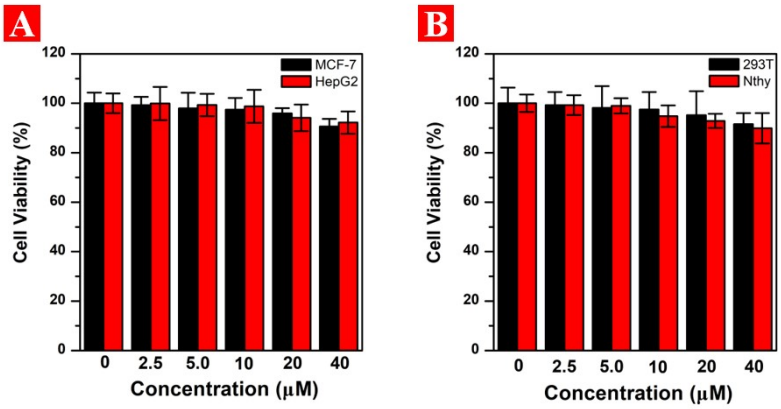

Fig. S13. The cell viability of (A) MCF-7 and HepG2 cells (B) 293T and Nthy cells incubated with 0-40 μM of **DPP-Leu** for 24h in the dark.

Live/dead staining of 293T cells after irradiation

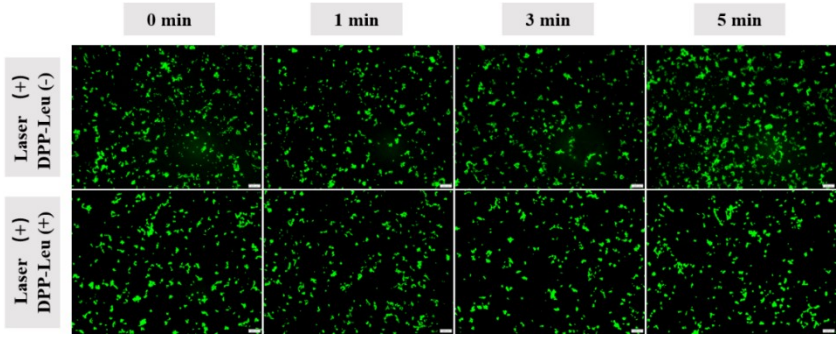

Fig. S14. Live/dead staining of 293T cells without (first row) and with (second row) **DPP-Leu** (20 μM) treated with 530 nm light (20 mW/cm^2) irradiation for 0 min, 1 min, 3 min and 5 min. The live cells were stained by calcein AM (green) and dead cells were stained by PI (red). Bar = 200 μm.