Electronic Supplementary Information for:

A near-infrared fluorescence probe for imaging of pantetheinase in cells and mice in vivo

Yuantao Yang,‡[ab] Yiming Hu,‡[a] Wen Shi*[ab] and Huimin Ma[ab]

[a] *Beijing National Laboratory for Molecular Sciences, Key Laboratory of Analytical Chemistry for Living Biosystems, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China.*

[b] *University of Chinese Academy of Sciences, Beijing 100049, China.*

E-mail: shiwen@iccas.ac.cn

‡ *These authors contributed equally to this work.*

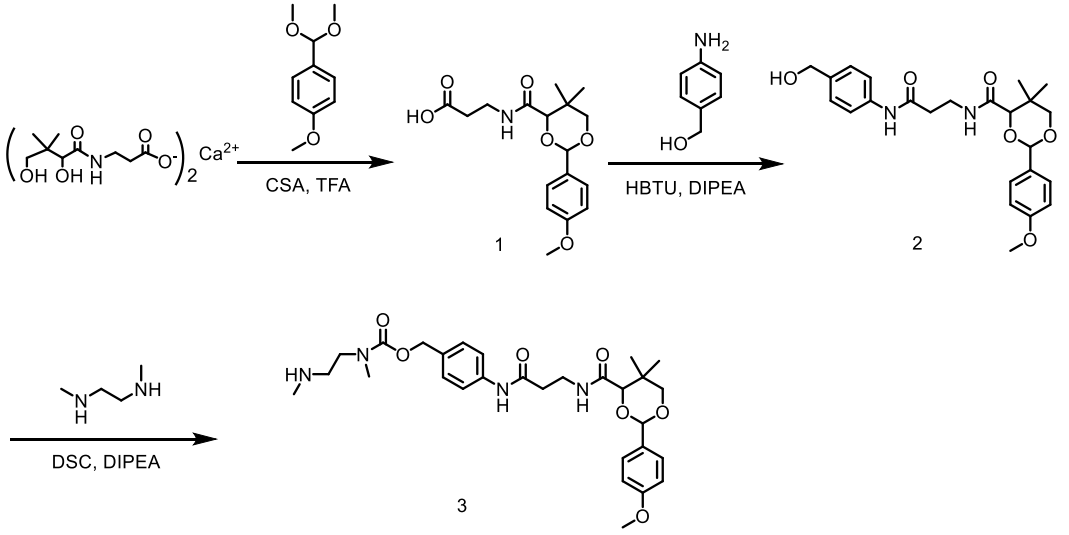
Table of contents

1. Apparatus and reagents

Fluorescence spectra were recorded on an F-4600 spectrophotometer (Hitachi, Japan). ^{1}H NMR and ^{13}C NMR spectra were obtained on Bruker Fourier-400 spectrometer. Electrospray ionization mass spectra (ESI-MS) were measured with a LC-MS 2010A instrument (Shimadzu, Japan). High-resolution electrospray ionization mass spectra (HR-ESI-MS) were measured on an APEX IVFTMS instrument (Bruker, Daltonics). Confocal fluorescence images were recorded on an FV 1200-IX83 confocal laser scanning microscope (Olympus, Japan). MTT analyses were made on a SpectraMax i3 microplate reader (Molecular Devices, USA). In-vivo imaging was carried out on In-vivo Master (Grand-imaging Technology, China).

(1S)-(+)-10-Camphorsulfonic acid (CSA), calcium D-pantothenate, trifluoroacetic acid (TFA), anisaldehyde dimethyl acetal, N,N-diisopropylethylamine (DIPEA), O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), N,N'-disuccinimidyl carbonate (DSC), N,N'-dimethylethylenediamine, triphosgene, IR-780 iodide, resorcinol, 4-aminobenzyl alcohol, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and dextran sulfate sodium (M_w = 40000) were obtained from Sigma. Lipopolysaccharides (LPS), and RR6 were obtained from MedChemExpress. Cell line (HK-2) and Dulbecco's modified Eagle's medium/Ham 's nutrient mixture F12 (DMEM/F12) were purchased from KeyGEN BioTECH Co., Ltd., Nanjing, China. Fetal bovine serum (FBS) was obtained from Thermofisher, and recombinant human vanin-1 from BioTechne China Co., Ltd. Ultrapure water (over 18 MΩ·cm) was produced from a Milli-Q reference system (Millipore). The stock solution (1 μg/mL) of pantetheinase was prepared in ultrapure water and stored in small aliquots at -70 °C to avoid repeated freeze-thaw cycles.

2. Syntheses and characterizations



Scheme S1 Syntheses of compounds **1**-**3**.

Synthesis of compound 1. CSA (93.5 mg) and calcium D-pantothenate (950 mg) were mixed in a 100 mL round-bottom flask, and then 15 mL of TFA was added dropwise with stirring at 0 °C. After dissolving all the solids, TFA was evaporated under reduced pressure to give a colorless oil. Next, 4-methoxybenzaldehyde dimethyl acetal (2.25 g) was added to the oil in CH_2Cl_2 (20 mL) and the mixture was stirred at room temperature for 8 h. After the reaction, 1 M Na_2CO_3 (30 mL) was added and the precipitate was filtered. The filtrate was then acidified to pH 4.5 with acetic acid, and the product in the solution was extracted with CH_2Cl_2. After the separated CH_2Cl_2 phase was dried over anhydrous Na_2SO_4, the solvent was removed through evaporation to give crude compound **1** as white solid (1.3 g, 48%), which was used in the next step without further purification.

Synthesis of compound 2. HBTU (1.14 g) and DIPEA (1.04 ml) were added to anhydrous THF containing compound **1** (1.01 g) under N_2 atmosphere, and the mixture was stirred for 20 min. Then p-aminobenzyl alcohol (443 mg) dissolved in THF was added via a syringe, followed by stirring for 4 h at room temperature. The solvent was removed by evaporation under reduced pressure and the residue was purified by silica gel chromatography eluted with CH_2Cl_2/CH_3OH (v/v, 5:1), affording compound **2** as light yellow solid (546 mg, 41%). ^{1}H NMR of compound **2** (300 MHz, CD_3OD; Fig. S1) δ 7.50 (d, J = 8.1 Hz, 2H), 7.42 (d, J =8.4 Hz, 2H), 7.29 (d, J = 8.1 Hz, 2H), 6.87 (d, J = 8.4 Hz, 2H), 5.51 (s, 1H), 4.56 (s, 2H), 4.14 (s, 1H), 3.78 (s, 3H), 3.78-3.67 (m, 2H), 3.57-3.55 (m, 2H), 2.59 (t, J = 6.3 Hz, 2H), 1.08 (s, 3H), 1.03 (s, 3H). ^{13}C NMR of compound **2** (75 MHz, CD_3OD; Fig. S2) δ 170.6, 170.2, 160.3, 137.5, 137.2, 130.4, 127.4, 127.2, 119.8, 113.1, 101.1, 83.6, 77.9, 63.5, 54.4, 35.7, 34.7, 32.6, 20.8, 18.3. ESI-MS: $[M+H]^+$ *m/z* 443.2.

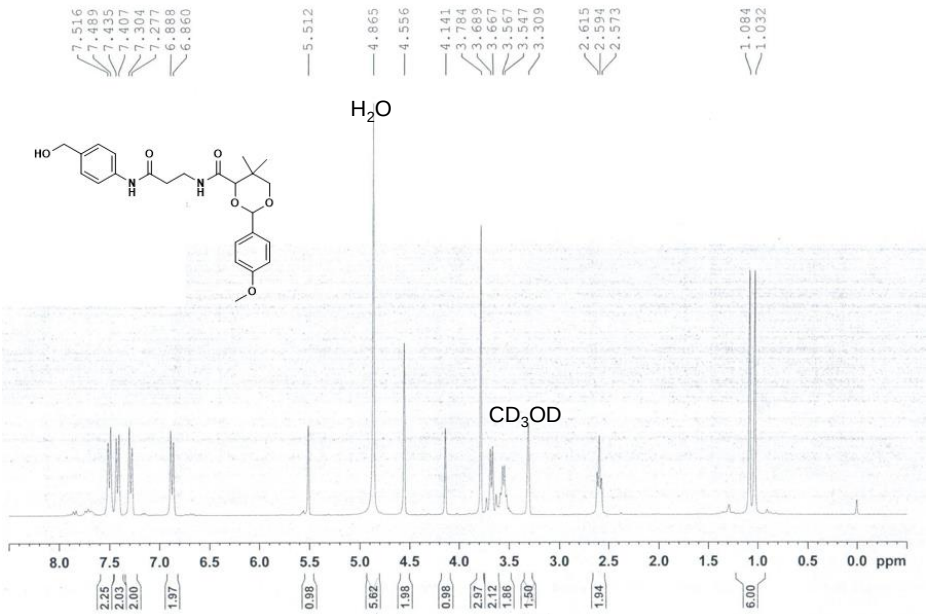


Fig. S1 ^{1}H NMR spectrum of compound **2** (300 MHz, CD_3OD, 298 K).

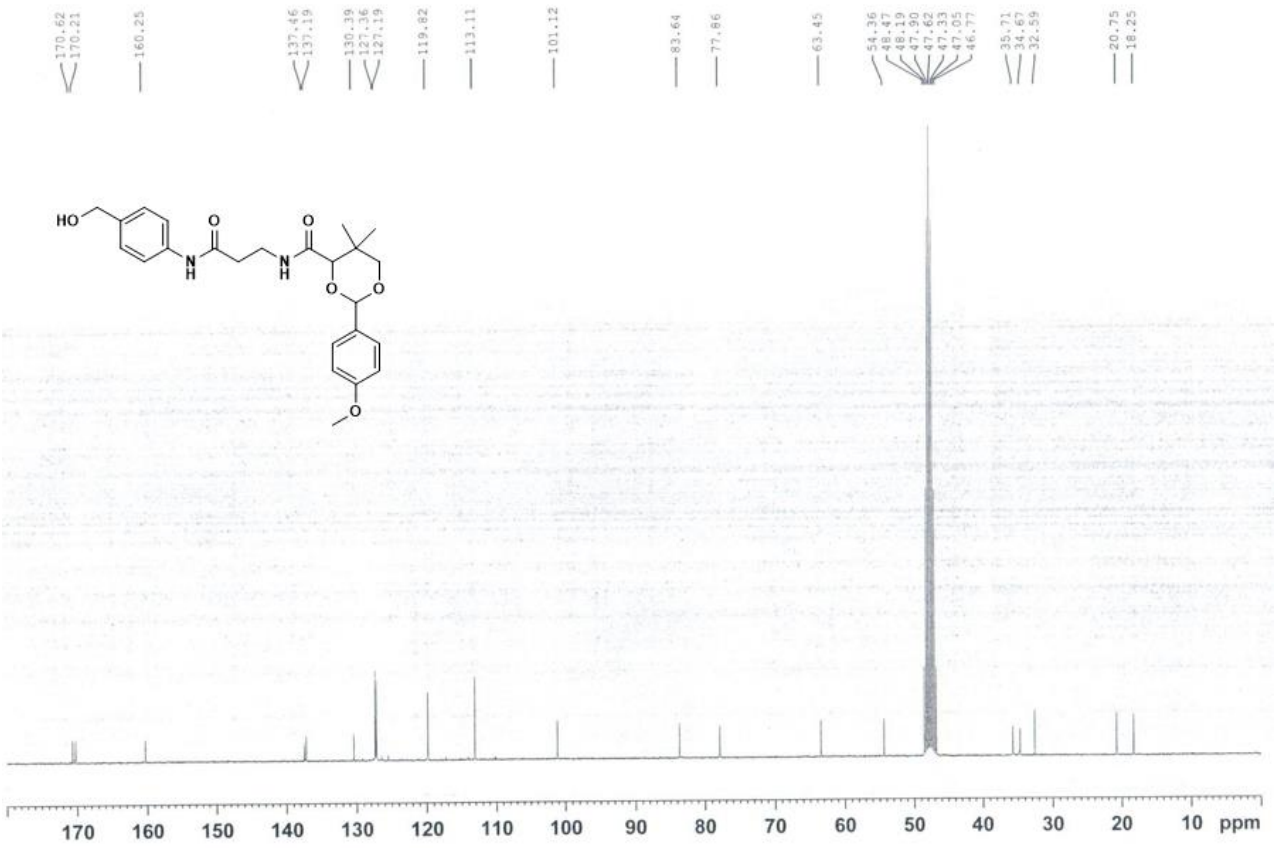


Fig. S2 ^{13}C NMR spectrum of compound **2** (75 MHz, CD_3OD, 298 K).

Synthesis of compound 3. Compound **2** (265 mg) and DIPEA (0.32 mL) were added to anhydrous CH_3CN containing DSC (384 mg). The reaction mixture was stirred at room temperature under N_2 atmosphere overnight. Then, N, N′-dimethylethylenediamine (528 mg) and DIPEA (1.04 ml) dissolved in anhydrous CH_3CN solution were added to the reaction solution, and stirred at room temperature under N_2 atmosphere for 8 h. After that, the solvent was removed under reduced pressure and the residue was dissolved in EtOAc. The organic phase was washed with 10% NH_4Cl solution and water, and dried over Na_2SO_4. After the solvent removal by evaporation, the residue was purified by silica gel chromatography eluted with CH_2Cl_2/CH_3OH (v/v, 5:1), affording compound **3** as white solid (174 mg, yield 52%). ^{1}H NMR of compound **3** (300 MHz, CD_3OD; Fig. S3) δ 7.54 (d, J = 8.1 Hz, 2H), 7.42 (d, J = 8.4 Hz, 2H), 7.33(d, J =8.1 Hz, 2H), 6.88 (d, J = 8.4 Hz, 2H), 5.52 (s, 1H), 5.07 (s, 2H), 4.15 (s, 1H), 3.79 (s, 3H), 3.73-3.63 (m, 2H), 3.57-3.55 (m, 2H), 3.42 (s, 2H), 2.94 (s, 3H), 2.77-2.70 (m, 2H), 2.60 (t, J = 6.3 Hz, 2H), 2.42-2.32 (m, 3H), 1.08 (s, 3H), 1.03 (s, 3H). ^{13}C NMR of compound **3** (75 MHz, CD_3OD; Fig. S4) δ 170.7, 170.2, 160.2, 138.3, 132.3, 130.4, 128.6, 128.3, 127.4, 119.7, 113.1, 101.1, 83.6, 77.8, 66.7, 54.4, 35.7, 34.6, 34.3, 33.9, 33.5, 32.6, 20.8, 18.2. ESI-MS: $[M+H]^+$ *m/z* 557.1.

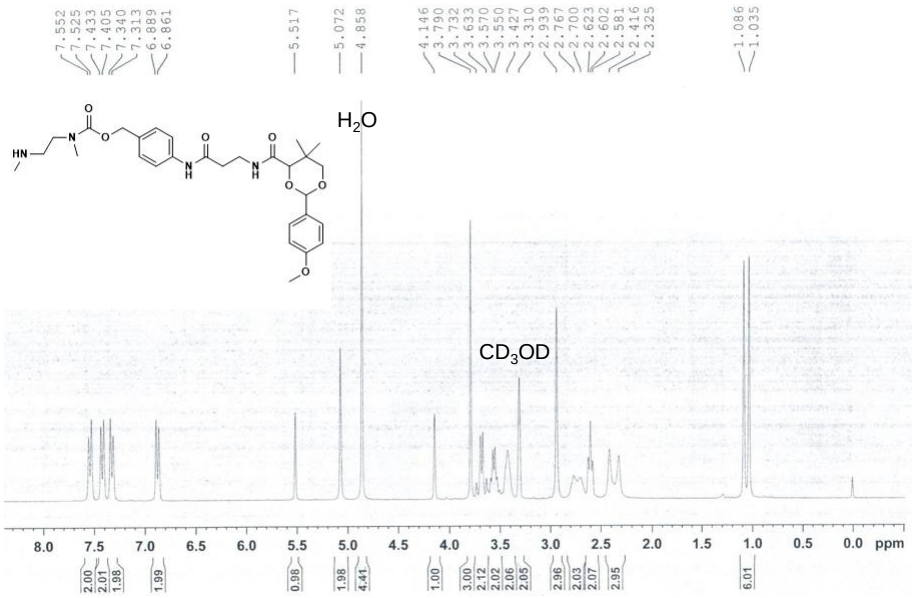


Fig. S3 ^{1}H NMR spectrum of compound **3** (300 MHz, CD_3OD, 298 K).

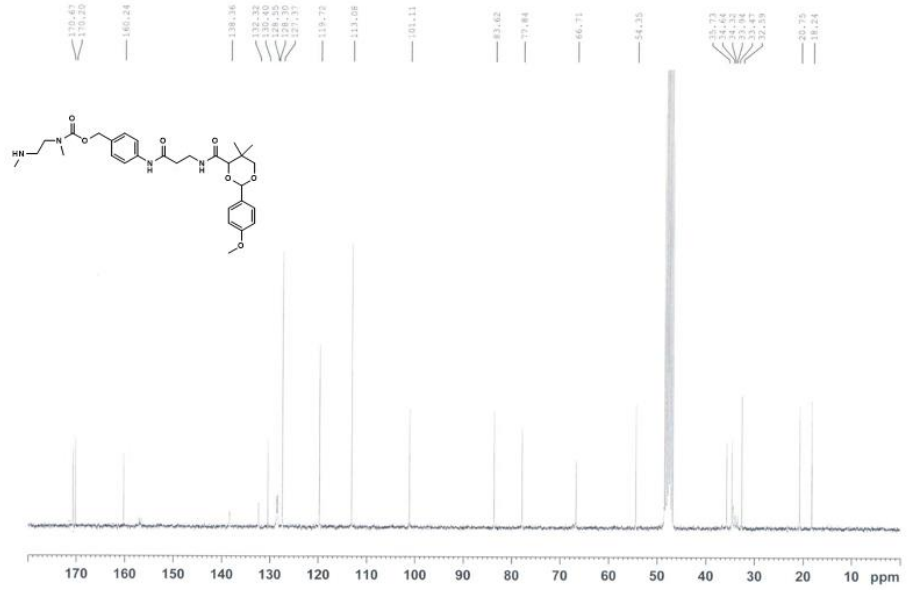
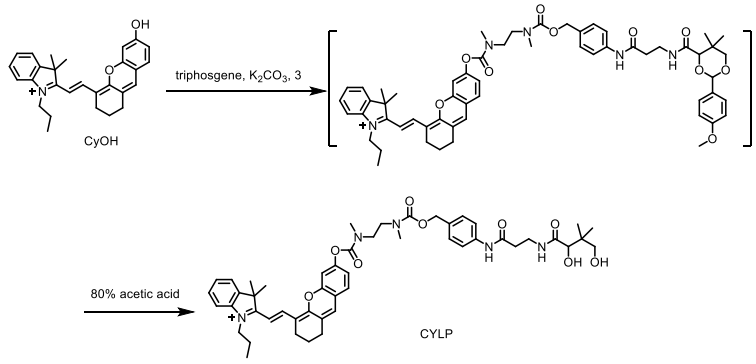


Fig. S4 ^{13}C NMR spectrum of compound **3** (75 MHz, CD_3OD, 298 K).



Scheme S2 Syntheses of CYLP.

Syntheses of CYLP: Hemicyanine (CyOH) was synthesized according to the previous literature (Li et al, *Anal. Chem.* **2014**, *86*, 6115). CyOH (82 mg) and K_2CO_3 (176 mg) dissolved in anhydrous CH_2Cl_2 were added dropwise to the solution of triphosgene (30 mg) in CH_2Cl_2 at 0 ºC under N_2 atmosphere. After reaction at room temperature for 1 h, compound **3** (89 mg) in anhydrous CH_2Cl_2 was added and the reaction mixture was stirred for 5 h at room temperature under N_2 atmosphere. The solvent was removed by evaporation under reduced pressure to obtain raw compound **4**. Then, 80% acetic acid was added to treat the compound **4** for 2.5 h to obtain CYLP. The product CYLP (67 mg, 47%) was purified by silica gel chromatography eluted with CH_2Cl_2/CH_3OH (v/v, 5:1). ^{1}H NMR of CYLP (400 MHz, CD_3OD; Fig. S5) δ 8.79-8.61 (m, 1H), 7.62-7.60 (m, 2H), 7.59-7.50 (m, 1H), 7.48-7.42 (m, 4H), 7.30-7.24 (m, 3H), 7.18-7.11 (m, 2H), 6.94 (m, 1H), 6.63-6.59 (m, 1H), 5.10-4.95 (m, 2H), 4.39 (t, J = 6.0 Hz, 2H), 3.90 (s, 1H), 3.70-3.30 (m, 8H), 3.13-2.96 (m, 6H), 2.75(d, J =28 Hz, 4H), 2.59 (m, 2H), 1.95 (t, J =8.0 Hz, 4H), 1.81 (s, 3H), 1.76 (s, 3H), 1.08 (t. J = 8.0 Hz, 3H), 0.90 (s, 6H). ^{13}C NMR of CYLP (100 MHz, CD_3OD; Fig. S6) δ 179.0, 174.7, 170.4, 160.4, 156.8, 154.4, 152.9, 146.4, 146.3, 142.5, 142.3, 141.5, 131.6, 129.6, 128.9, 128.7, 128.2, 127.9, 127.6, 122.4, 119.4, 119.3, 114.5, 113.0, 109.5, 109.1, 104.9, 75.9, 69.0, 53.4, 51.0, 50.9, 39.0, 36.0, 34.9, 34.0, 28.9, 26.9, 26.8, 21.0, 19.9, 19.5, 10.2. HR-ESI-MS: *m/z* calcd for $[C_{50}H_{62}N_5O_9]^+$, 876.4539; found, 876.4539 (Fig. S7).

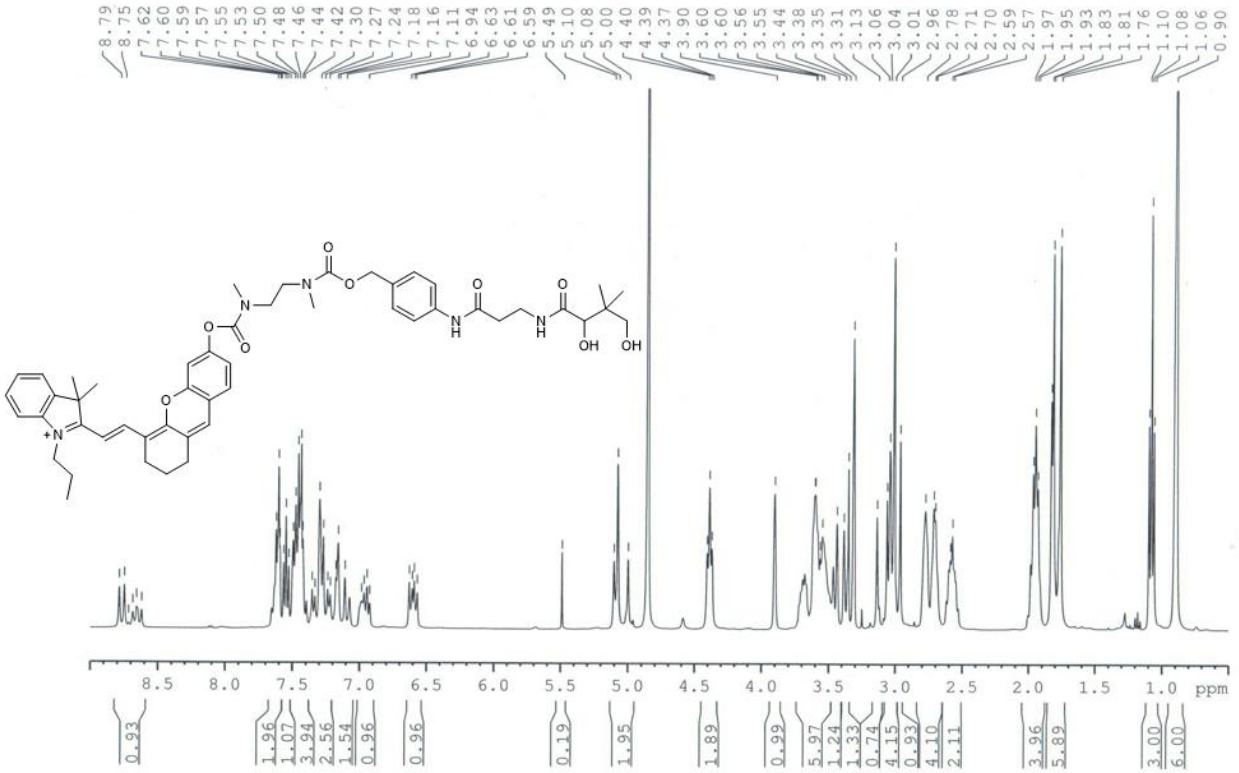


Fig. S5 ^{1}H NMR spectrum of CYLP (400 MHz, CD_3OD, 298 K).

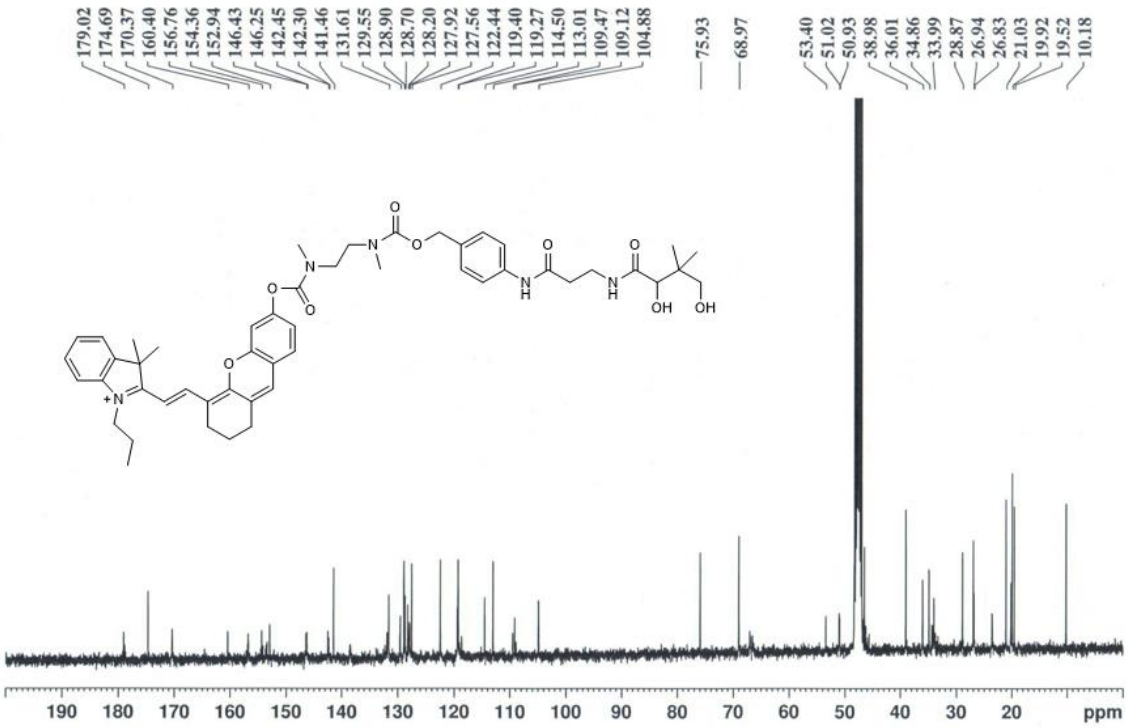


Fig. S6 ^{13}C NMR spectrum of CYLP (100 MHz, CD_3OD, 298 K).

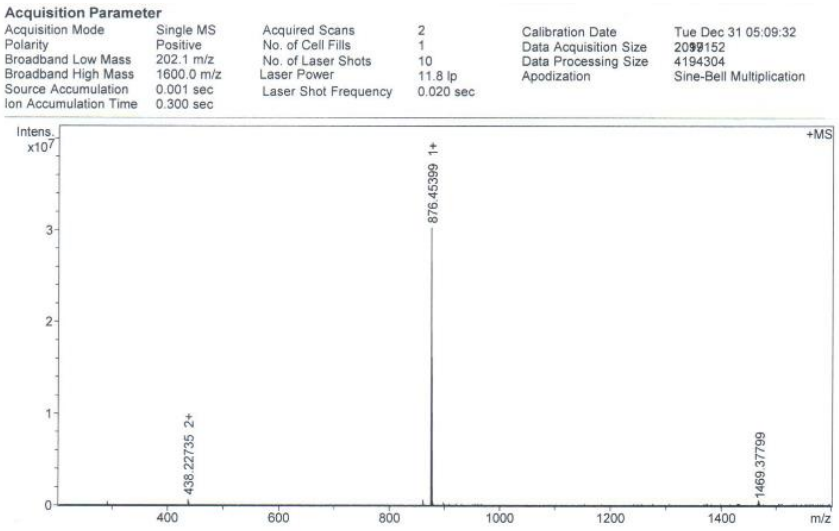
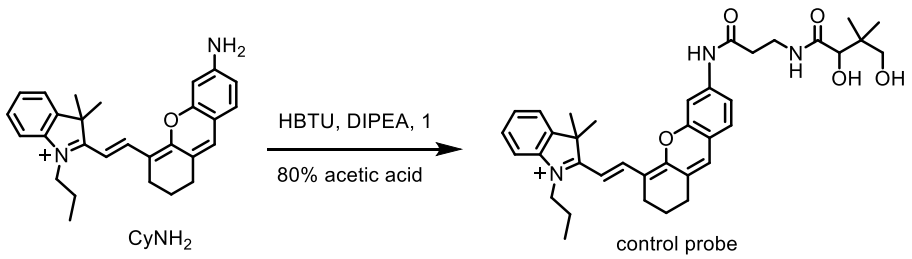


Fig. S7 HR-ESI-MS of CYLP.



Scheme S3 Synthesis of the control probe.

Syntheses of control probe: The control probe was synthesized in a similar manner. HBTU (380 mg) and DIPEA (400 μL) were added to 5 mL anhydrous CH_2Cl_2 containing compound **1** (337 g), and the mixture was stirred at 0 ºC for 20 min. Then $CyNH_2$ (411 mg, synthesized according to He et al, *Chem. Sci.* **2017**, *8*, 3479) dissolving in 5 mL CH_2Cl_2 was added via a syringe, followed by stirring for 8 h at room temperature. The solvent was removed by evaporation under reduced pressure to obtain crude protected product. Then, 80% acetic acid was added to the residue, and the solution was stirred for 1.5 h. The control probe (263 mg, 43%) was then obtained by silica gel chromatography eluted with CH_2Cl_2/CH_3OH (v/v, 20:1). ^{1}H NMR of control probe (300 MHz, CD_3OD; Fig. S9) δ 8.77 (d, *J* = 15.0 Hz, 1H), 8.05 (d, *J* = 1.5 Hz, 1H), 7.67 (d, *J* = 6.9 Hz, 1H), 7.56 - 7.43 (m, 4H), 7.32 - 7.29 (m, 2H), 6.56 (d, *J* = 15.0 Hz, 1H), 4.35 (t, *J* = 9.0 Hz, 2H), 3.92 (s, 1H), 3.67-3.58 (m, 2H), 3.49-3.38 (m, 2H), 2.76 (t, *J* = 6.9 Hz, 2H), 2.72 - 2.67 (m, 4H), 2.00- 1.92 (m, 4H), 1.84 (s, 6H), 1.09 (t. *J* = 7.5 Hz, 3H), 0.93 (s, 6H). ^{13}C NMR of control probe (75 MHz, CD_3OD; Fig. S9) δ 179.6, 176.2, 172.5, 162.6, 154.9, 147.3, 143.5, 143.0, 134.2, 130.3, 129.9, 129.3, 128.6, 123.8, 119.3, 117.9, 115.8, 114.1, 107.0, 105.4, 77.3, 70.3, 52.1, 40.4, 37.6, 36.1, 30.1, 28.3, 25.0, 22.3, 21.6, 21.3, 20.8, 11.6. HR-ESI-MS: *m/z* calcd for $[C_{37}H_{46}N_3O_5]^+$, 612.3432; found, 612.3434 (Fig. S10).

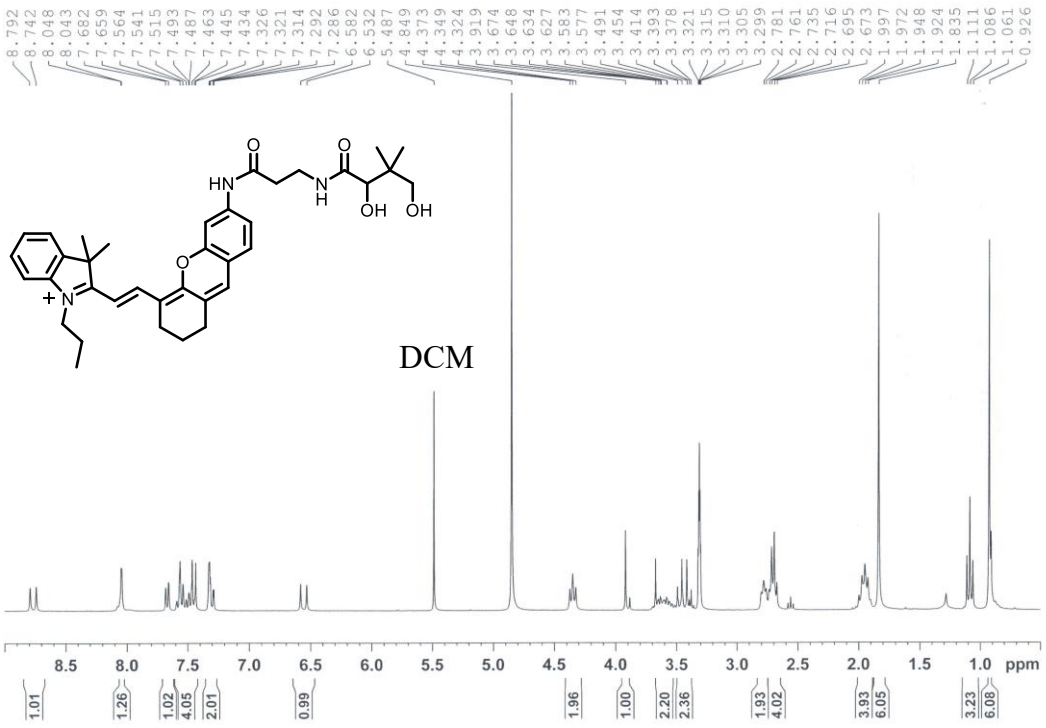


Fig. S8 ^{1}H NMR spectrum of control probe (300 MHz, CD_3OD, 298 K).

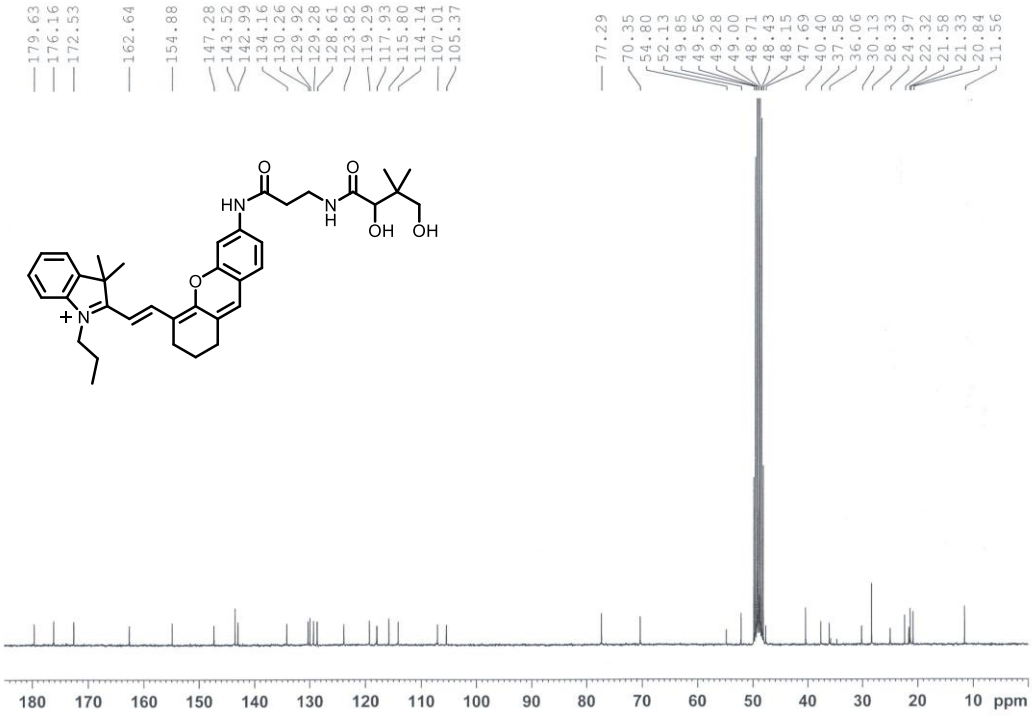


Fig. S9 ^{13}C NMR spectrum of control probe (75 MHz, CD_3OD, 298 K).

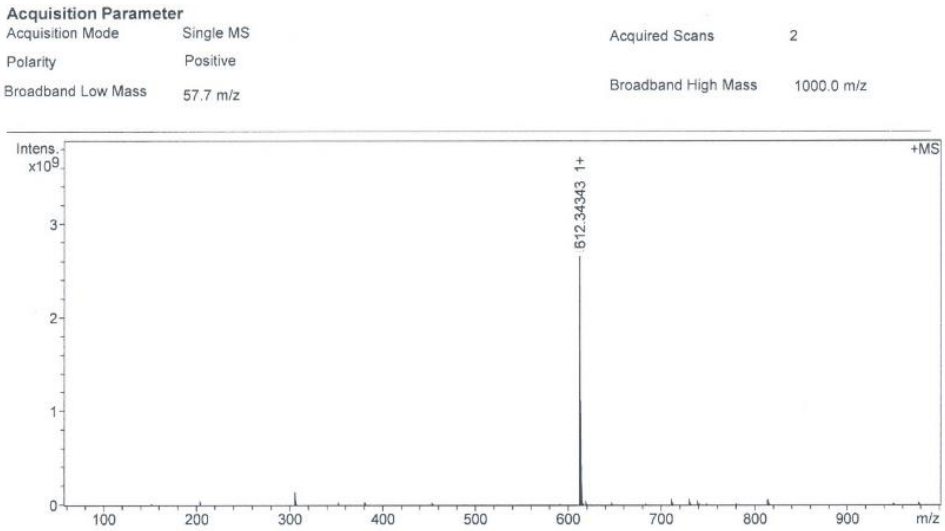


Fig. S10 HR-ESI-MS of control probe.

3. Supplementary methods

General procedure for pantetheinase detection

Unless otherwise stated, all spectral measurements were performed in phosphate buffer saline (PBS, pH 7.4) as follows. The probe CYLP was dissolved in DMSO and stored at −20 ºC for future use. In a 7 mL test tube, 4 mL of PBS was mixed with 50 μL of 1 mM probe. Then, the appropriate amount of pantetheinase was added, and the final volume was adjusted to 5 mL with PBS. After incubation at 37 °C for 1 h in a thermostat, the reaction solution was transferred to a 1-cm optical length quartz cell

to measure absorption spectra and fluorescence spectra. Meanwhile, pantetheinase-free solution as control was prepared and measured under the same conditions.

Detection limit

The detection limit (DL) was determined based on the following formula:

$$DL = 3SD/slope$$

in which SD is the standard deviation of eleven blank measurements (n = 11) and the slope is the slope of the linear curve from fluorescence titration.

Cells culture and imaging

HK-2 cells were cultured in DMEM/F12 supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin at 37 °C in a humidified 5% CO_2 incubator. And B16, HepG2 and HeLa cells were cultured in DMEM with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin in similar manner.

Cells were seeded in glass-bottom dishes for 24 h to adhere before experiments. Then, the cells were washed with FBS-free media for three times. For imaging, the cells were incubated with 10 μM CYLP in incubator for 1 h and subjected to FV 1200-IX83 confocal laser scanning microscope with a 40 × 0.95 NA objective lens.

Cell viability assay

The cytotoxicity of CYLP was evaluated by the MTT assay. Briefly, Cells were seeded in 96-well plates at a density of 7000 cells/well, and incubated with CYLP at varied concentrations at 37 ℃ for 12 h. Then, the culture media were discarded, and 0.1 mL of the MTT solution (0.5 mg/mL in DMEM) was added to each well, followed by incubation at 37 ℃ for 4 h. The supernatant was abandoned, and 110 μL of DMSO was added to each well to dissolve the formed formazan. After shaking the plates for 10 min, the absorbance of each well was read with a microplate reader at 490 nm. The cell viability was calculated according to the equation: $CR = A/A_0 \times 100\%$, where A is the absorbance of the experimental group (i.e., the cells were treated by CYLP) and A_0 is the absorbance of the control group (i.e., the cells were untreated by CYLP). The cell survival rate from the control group was considered to be 100%. The cytotoxicity of RR6 was tested by the similar procedure except that the RR6 was incubated with cell for 24 h.

In vivo imaging of mice

All animal care and experimental protocols complied with the Animal Management Rules of the Ministry of Health of the People's Republic of China and were approved by the Institute of Process

Engineering, Chinese Academy of Sciences. Excitation light was provided by a 680 nm diode laser. The emission light was filtered by a 730 nm bandpass filter.

Kunming mice were divided into two groups. They were intraperitoneally injected with RR6 in saline (30 mg/kg) or equal amount of vehicle (control), respectively. After 1 h, all mice were intraperitoneally injected with CYLP (400 μM × 200 μL). The fluorescent images of each group were measured at the different time interval within 50 min.

For inflammation Kunming mice, 200 μL of 1 mg/mL LPS was injected in the right leg of the mouse, and an equal amount of saline was injected in the left leg (control). After 14 h, CYLP (400 μM × 20 μL) was injected into both legs of the mouse by subcutaneous injection. Fluorescent images of mice were captured at different time interval within 50 min. For negative control, 30 min prior to the administration of CYLP, the inhibitor RR6 (100 μM × 50 μL) was injected.

For inflammatory bowel disease model, the mice were fed with the dextran sulfate sodium (2% in water) for 8 days. Then the mice were administrated with CYLP (400 μM × 100 μL) through the anus into the colon. For control group, the mice were fed with water without dextran sulfate sodium for 8 days and then administrated with the probe. The images were taken 1 h after the administration of the probe.

4. Supplementary figures

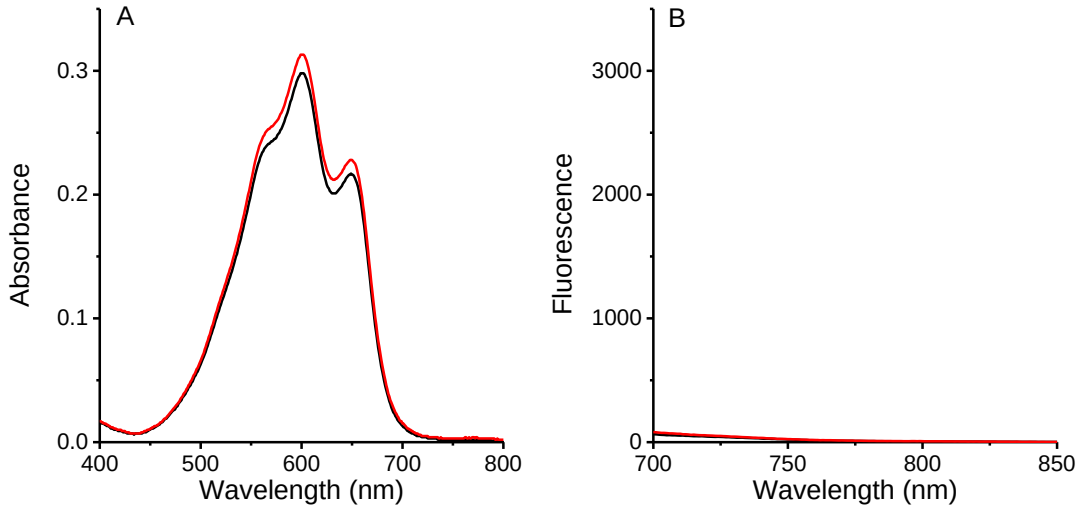


Fig. S11 (A) Absorption and (B) fluorescence spectra of the control probe (10 μM) incubated with (red curves) and without (black curves) pantetheinase (100 ng/mL) at 37 °C for 1 h. λ_{ex} = 680 nm.

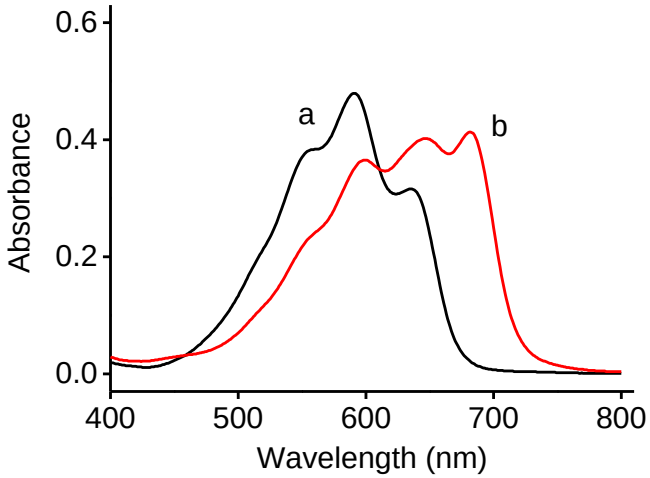


Fig. S12 Absorption spectra of CYLP (10 μM) reacting without (a) and with (b) pantetheinase (50 ng/mL) at 37 ºC for 1 h.

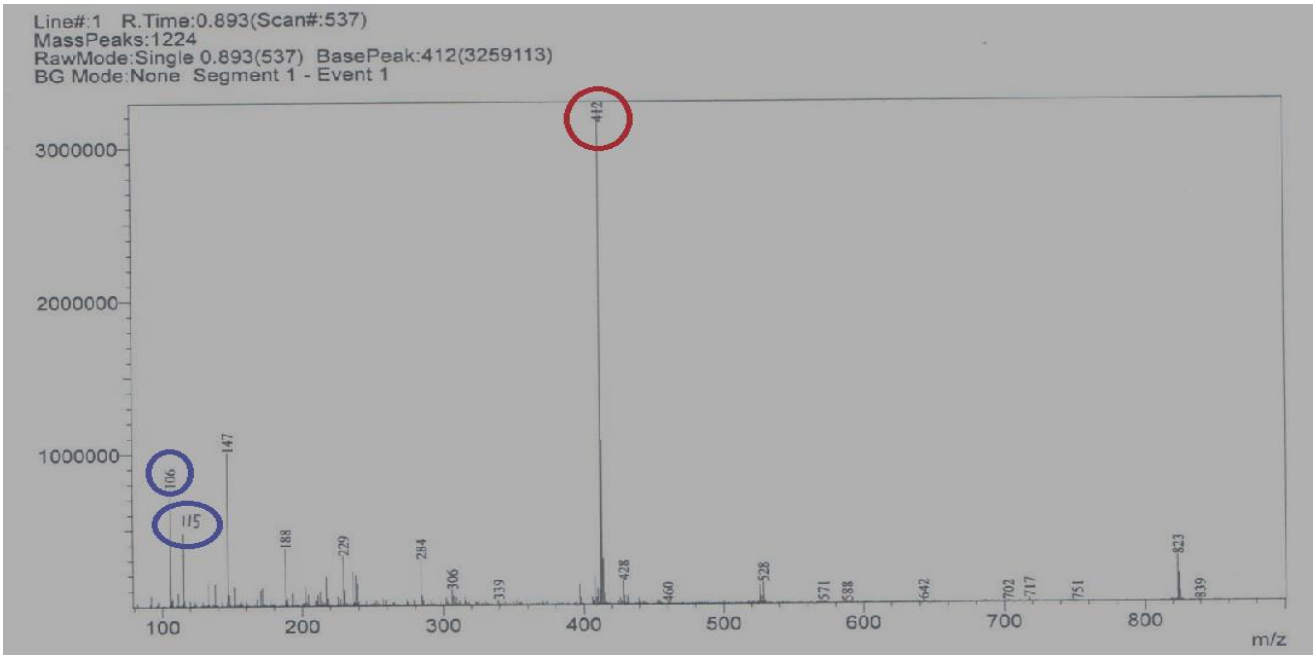


Fig. S13 ESI-MS of the reaction system. The peak at m/z = 412 indicates $[CyOH]^+$; the peaks at m/z = 106 and m/z = 115 indicate the other two fragments of the self-immolative linker.

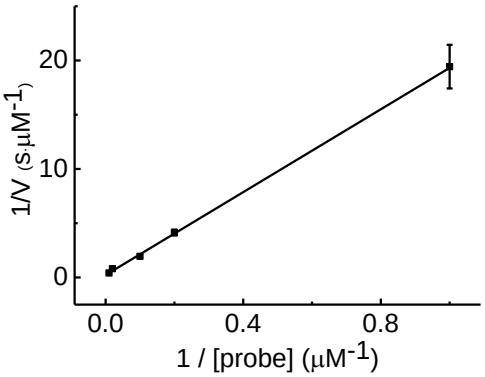


Fig. S14 Lineweaver-Burk plot of CYLP (1-100 μM) catalyzed by pantetheinase (10 ng/mL) in PBS (pH 7.4). $\lambda_{ex/em}$ = 680/710 nm.

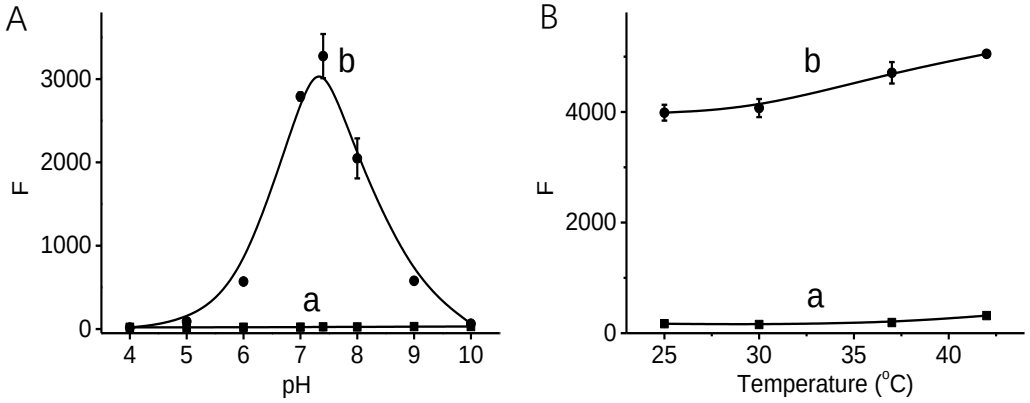


Fig. S15 Effects of pH (A) and temperature (B) on the fluorescence of CYLP (10 μM) reacting with (a) 0 and (b) 50 ng/mL pantetheinase for 1 h. $\lambda_{ex/em}$ = 680/710 nm.

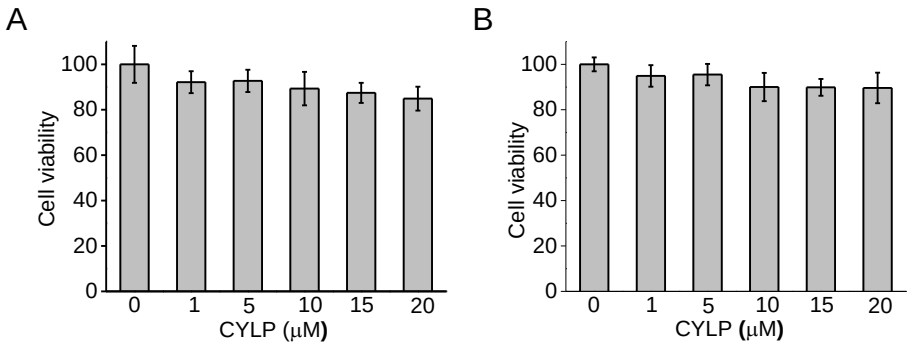


Fig. S16 Viability of (A) HK-2 and (B) HepG2 cells treated with CYLP at varied concentrations. The results are the mean ± standard deviation of five separate measurements.

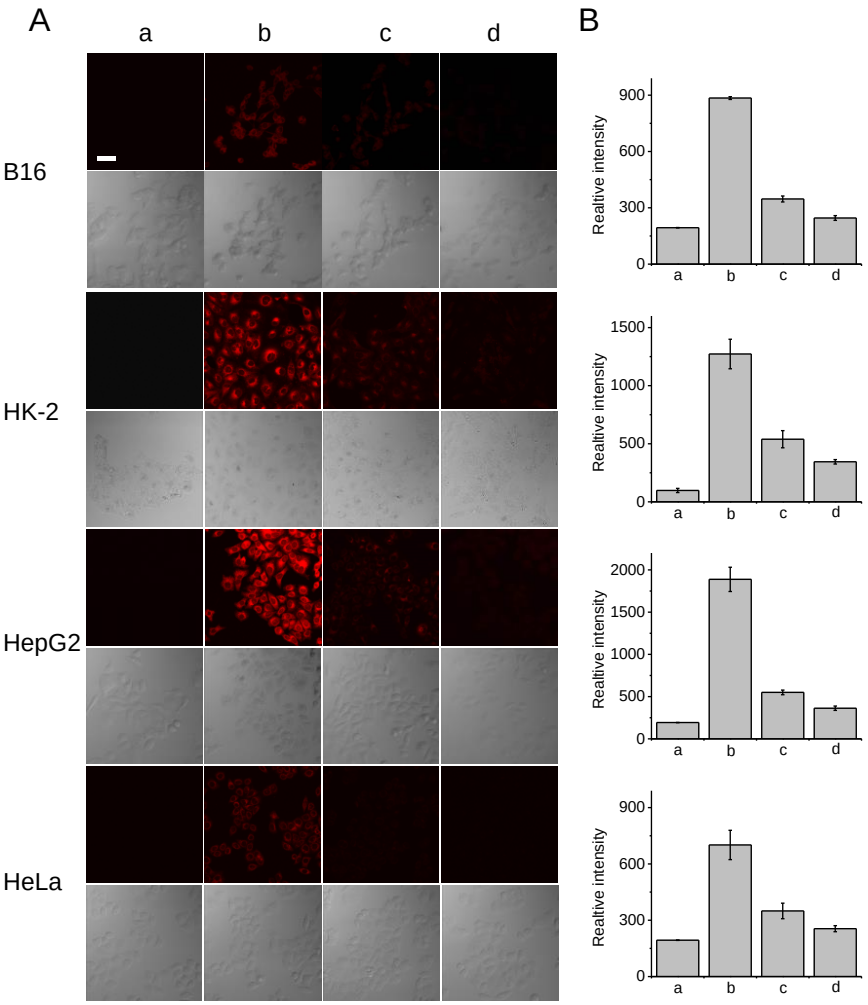


Fig. S17 (A) Confocal fluorescence images and corresponding differential interference contrast (DIC) images of different cells. Column a: cells themselves (control); column b: the cells incubated with CYLP (10 μM) for 1h; column c: the cells pretreated with RR6 (10 μM) for 1 h, followed by incubation with CYLP (10 μM) for 1 h; column d: the cells pretreated with RR6 (50 μM) for 1 h, followed by incubation with CYLP (10 μM) for 1 h. Scale bar, 50 μm. (B) The relative fluorescence intensity of the corresponding fluorescence images in panel A.

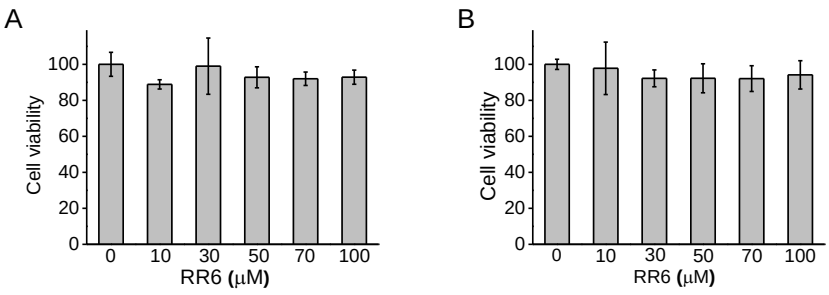


Fig. S18 Viability of (A) HK-2 and (B) HepG2 cells treated with RR6 at varied concentrations. The results are the mean ± standard deviation of five separate measurements.

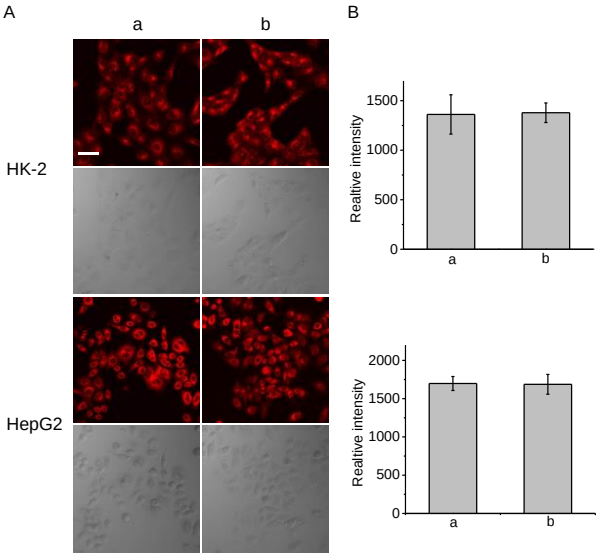


Fig. S19 (A) Confocal fluorescence images and corresponding DIC images of HK-2 and HepG2 cells. Column a: cells pretreated with CYLP (10 μM) for 1 h, followed by washing and incubation in DMEM for 1 h.; (b) cells pretreated with CYLP (10 μM) for 1 h, followed by washing and incubation with RR6 (50 μM) in DMEM for 1 h. Scale bar, 50 μm. (B) The relative fluorescence intensity of the corresponding fluorescence images in panel A.

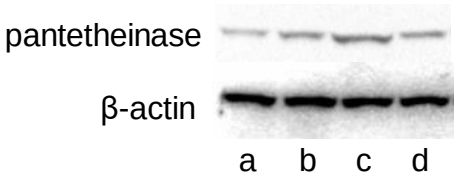


Fig. S20 Western blot analyses of pantetheinase in different cells. a: B16 cells; b: HK-2 cells; c: HepG2 cells; d: HeLa cells. β-Actin was used as a protein standard.

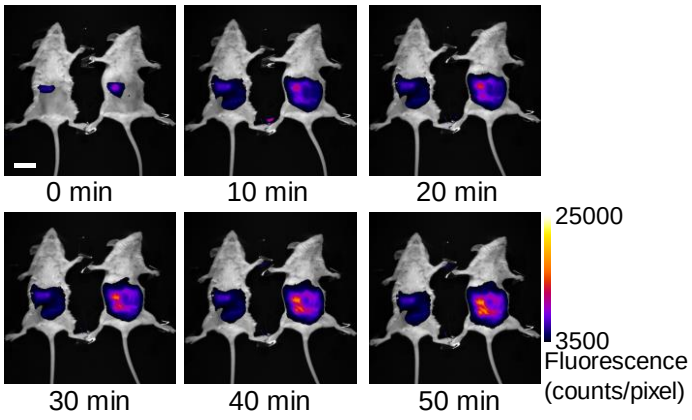


Fig. S21 Time-dependent fluorescence images of the RR6 group (left) and the experimental group (right) after administration of CYLP. Scale bar: 2 cm.

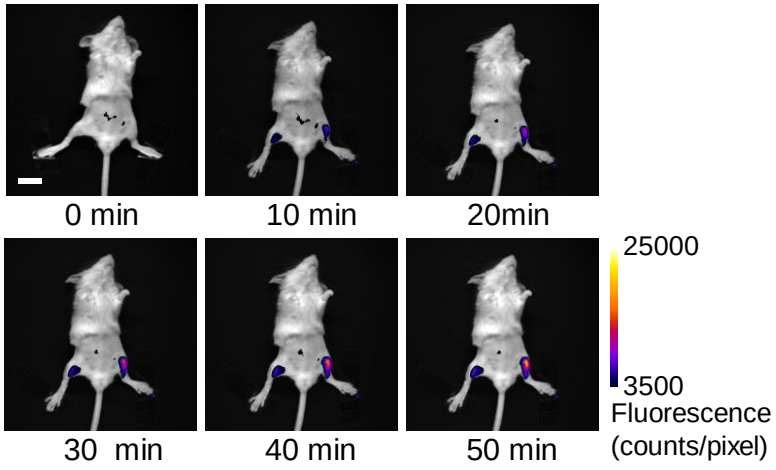


Fig. S22 Time-dependent fluorescence images of the control leg (left) and the inflammatory leg (right) of the mouse after administration of CYLP. Scale bar: 2 cm.

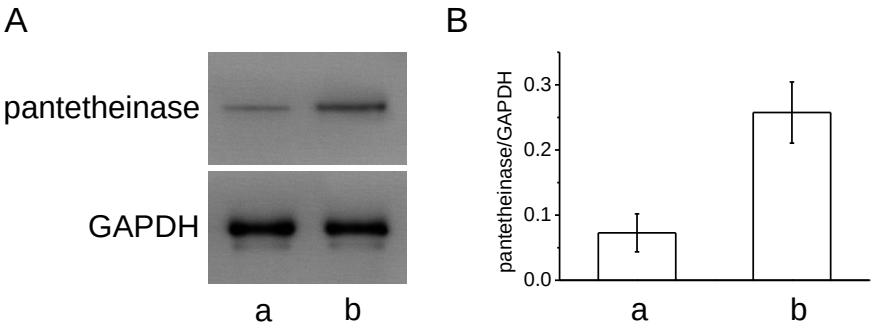


Fig. S23 Western blot analyses of pantetheinase in the tissue homogenate of the same part from (a) the control leg and (b) the inflammation leg. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein standard. The results are the mean±SD (n=3).

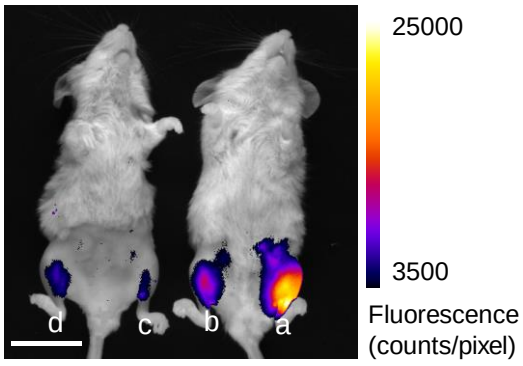


Fig. S24 Comparison of the fluorescence images of the inflammatory legs with and without inhibitor RR6. (a) Inflammatory leg (treated with LPS); (c) the inflammatory leg was pre-treated with RR6 for 30 min prior to the administration of CYLP; (b and d) non-inflammatory legs (treated with saline). Scale bar: 2 cm.

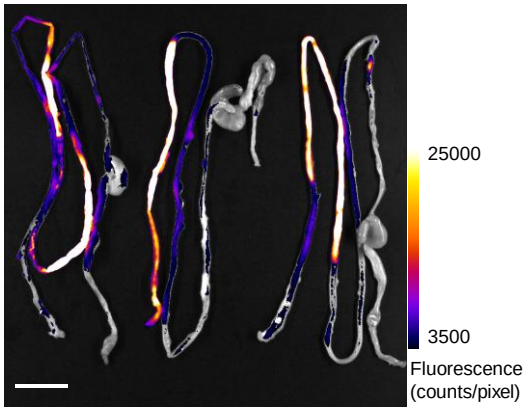


Fig. S25 Fluorescence image of intestines of the IBD mice. The intestines were obtained via dissection after 2 h injection of the probe. Scale bar: 2 cm.