Minor structure modification serendipitously leads to a highly carbapenemase-specific fluorogenic probe

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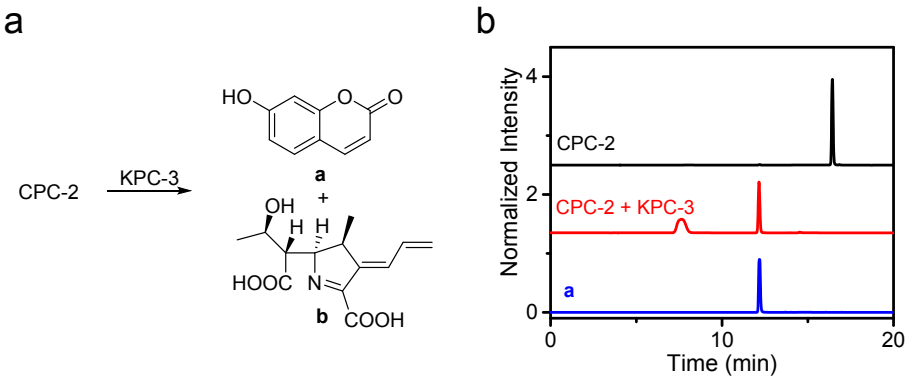


Figure S1. (a) Hydrolysis of CPC-2 mediated by KPC-3. (b) HPLC traces of CPC-2 before and after hydrolysis by KPC-3. CPC-2 (50 μM) in PBS (pH 7.4) was incubated with KPC-3 (44 nM) at room temperature for 30 minutes before HPLC analysis.

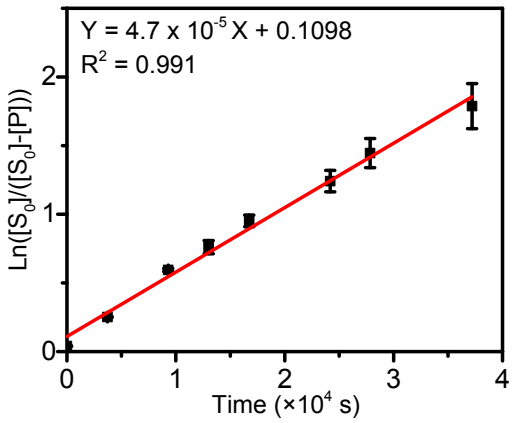


Figure S2. Determination of spontaneous hydrolysis rate of CPC-2 in PBS (pH 7.4) at room temperature. Error bars are ± SD.

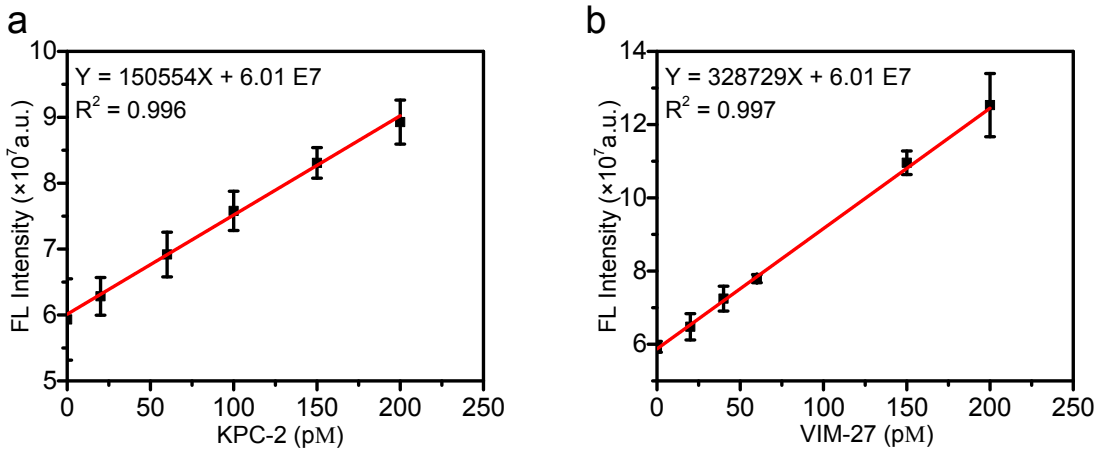
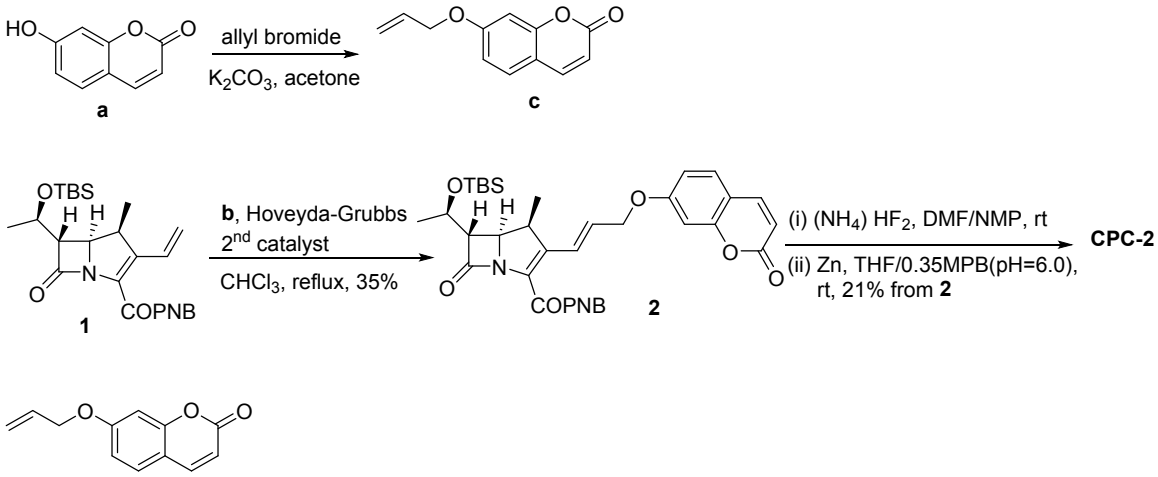


Figure S3. Linear correlation between fluorescent intensity of CPC-2 and concentration of KPC-2 (a) and VIM-27 (b). CPC-2 (10 μM) was incubated with a serial of concentrations of indicated β-lactamases at 37 °C for 30 min, fluorescence intensity was measured with microplate reader. $\lambda_{ex/em}$ = 365/460 nm. All experiments were triplicated and error bars are ± SD.

General Information

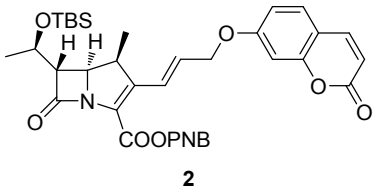
All chemicals were purchased from commercial sources (e.g. Adamas-Beta, Energy Chemical and TCI China) and used without further purification. Phosphate-buffered saline (PBS, pH 7.4) were obtained from Invitrogen Corporation. Analytical thin layer chromatography was performed with 0.20 mm silica gel 60F plates with fluorescent indicator (254 nm), and visualized by ultraviolet light. HPLC was performed on a Shimadzu HPLC System equipped with a LC-20AT gradient pump and an inline diode array UV-Vis detector. An analytic or semi-preparative reversed-phase C18 column (Phenomenex, 5 μm) was used with a MeCN/H_2O gradient mobile phase containing 0.1% or 0.01% or no trifluoroacetic acid at a flow of 1 or 3 mL/min for the analysis or purification. The ^{1}H and ^{13}C NMR spectra were taken on Bruker nuclear magnetic resonance spectrometer (400 MHz for ^{1}H NMR; 150 MHz for ^{13}C NMR). Data for ^{1}H NMR spectra are reported as follows: chemical shifts are reported as δ in units of parts per million (ppm) relative to tetramethylsilane (δ = 0, s); multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), or br (broadened); coupling constants are reported as a *J* value in Hertz (Hz); the number of protons (n) for a given resonance is indicated nH, and based on the spectral integration values. High-resolution mass spectra (HRMS) were recorded on a Bruker micro-TOF-QII time of flight mass spectrometer with electrospray ionization. The PBS used in this study contains 0.1% 3-[(3-cholamidopropyl)dimethylammonium]-1-propane sulfonate (CHAPS) as surfactant. Fluorescence spectra were obtained on a wavelength-calibrated FluoroMax-3 fluorometer (Horiba Jobin Yvon). Kinetic experiments were conducted in a microplate reader (Molecular Devices, SpectraMax *i*3). β-Lactamase-expressing bacteria was obtained from Microbiologics. TEM-1, KPC-2, KPC-3, CTX-M-9, NDM-1, VIM-27, CphA, L1, AmpC β-lactamases were obtained as previously described.[1]

Synthesis of fluorogenic probe CPC-2



7-allyloxycoumarin (c)

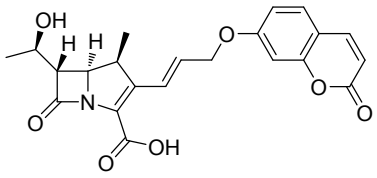
The title compound was synthesized according to previously reported procedure[2] with minor modification. A solution of umbelliferone **a** (1.04 g, 6.4 mmol), allyl bromide (0.6 mL, 6.9 mmol) and potassium carbonate (1.06 g, 7.7 mmol) in acetone (64 mL) were heated to refluxed overnight. Then volatile reagent or solvent was removed via Rota-Vap and the residue was dissolved with ethyl acetate (50 mL). The organic phase was washed with H_2O (50 mL x 2) and brine (60 mL x 1), dried over Na_2SO_4 and concentrated. Purification by chromatography on silica gel column afforded title compound as a white solid (1.26 g, 97%), whose ^1H NMR is consistent with reported data.[2]



(4S, 5R, 6S)-6-((R)-1-((tert-butyldimethylsilyl) oxy)ethyl)-4-methyl-7-oxo-3-((*E*) -3-((2-oxo-2H-chromen-7-yl) oxy) prop-1-en-1-yl)-1- azabicycl [3.2.0] hept-2-ene -2-carboxylic 4-nitrobenzoic anhydride (2).

Under N_2 atmosphere, to a solution of **1** (408.8 mg, 0.84 mmol) and 7-allyloxycoumarin **c** (340.0 mg, 1.68 mmol) in $CHCl_3$ (2.8 mL) was added Hoveyda-Grubbs 2nd catalyst (157.9 mg, 0.252 mmol). The resulting mixture were heated to reflux for 11 h (monitored by TLC). Purification by chromatography on silica gel column afforded title compound (194.3 mg, 35%). ^1H NMR (400 MHz, $CDCl_3$) δ 8.22 (d, *J* = 8.8 Hz, 2H), 7.67 (d, *J* = 8.8 Hz, 2H), 7.64 (d, *J* = 9.6 Hz, 1H), 7.47 (d, *J* = 16.4 Hz, 1H), 7.38 (d, *J* = 8.8 Hz, 1H), 6.85 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.82 (d, *J* = 2.4 Hz, 1H), 6.27 (d, *J* = 9.6 Hz, 1H), 6.24-6.17 (m, 1H), 5.45 (d, *J* = 13.9 Hz, 1H), 5.28 (d, *J* = 13.9 Hz, 1H), 4.75 (d, *J* = 5.7 Hz, 2H), 4.28 – 4.22 (m, 2H), 3.44 – 3.36 (m, 1H), 3.25 (dd, *J* = 5.4, 2.4 Hz, 1H), 1.27 (d, *J* = 6.0 Hz, 3H), 1.24 (d, *J* = 7.2 Hz, 3H),

0.86 (s, 9H), 0.09 (s, 3H), 0.07(s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.81, 161.53, 161.52, 161.21, 160.84, 155.88, 147.71, 147.41, 143.47, 142.91, 131.30, 129.01, 128.20, 126.49, 125.67, 123.84, 113.50, 113.09, 112.98, 101.86, 68.90, 65.96, 65.45, 59.37, 56.29, 39.38, 25.77, 22.53, 18.04, 16.92, -4.10, -4.90. HRMS (ESI) m/z calcd for $C_{35}H_{40}N_2O_9Si$ $(M+Na)^+$ 683.2401, found 683.2399.



CPC-2

(4S, 5R, 6S)-6-((R)-1-hydroxyethyl)-4-methyl-7-oxo-3-((E)-3-((2-oxo-2H-chromen -7-yl) oxy) prop-1-en-1-yl)-1-azabicyclo [3.2.0] hept-2-ene-2-carboxylic acid (CPC-2).

To a solution of **2** (23.7 mg, 0.036 mmol) in NMP/DMF (1:3, 0.3 mL) at room temperature was added ammonium hydrogen difluoride (20.5 mg, 0.36 mmol). The reaction mixture were stirred for 40 h at room temperature, and then diluted with ethyl acetate (40 mL). The solution was washed with water (15 mL x 3) and brine (15 mL x 1), subsequently. After being dried over Na_2SO_4 and concentrated *in vacuo*, the residues were dissolved in THF/PB (0.35M, pH 6.0) (1.2mL, 1:1) before stirred with activated zinc powder at room temperature for 25min. Then the mixture were passed through a 0.22 μM PTFE syringe filter to remove precipitate, and the filtrate was purified with preparative RP-HPLC with CH_3CN-H_2O as mobile phase to afford title compound as a white solid (3.1 mg, 21% from **2**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.88 (d, *J* = 9.6 Hz, 1H), 7.53 (d, *J* = 8.4 Hz, 1H), 7.48 (d, *J* = 16.3 Hz, 1H), 6.98 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.94 (d, *J* = 2.4 Hz, 1H), 6.24 (d, *J* = 9.2 Hz, 1H), 6.04 (dt, *J* = 16.2, 6.0 Hz, 1H), 4.75 (d, *J* = 5.2 Hz, 2H), 4.11 – 4.05 (m, 2H), 3.16 (dd, *J* = 7.2Hz, 2.4Hz, 1H), 3.14 – 3.12 (m, 1H), 1.29 (d, *J* = 6.3 Hz, 3H), 1.18 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (151 MHz, CD_3OD) δ 174.61, 168.70, 163.70, 163.42, 157.14, 145.82, 140.40, 136.02, 130.51, 128.67, 127.72, 114.37, 114.18, 113.44, 102.86, 70.62, 67.18, 59.25, 58.38, 40.20, 21.89, 16.95. HRMS (ESI) m/z calcd for $C_{22}H_{27}NO_7$ $(M+Na)^+$ 434.1216, found 434.1217.

Characterization of Probe

Determination of limit of detection (LOD): CPC-2 (10 μM) and a series of concentrations (ranging from 20 to 300 pM) of VIM-27, KPC-2, and KPC-3 in PBS (pH 7.4, total volume: 100 μL) were added to a 96-well black plate. After incubation at 37 °C for 30 min, the fluorescence intensity of these samples were measured on a microplate reader (Molecular Devices, SpectraMax *i*3, $\lambda_{ex/em}$ = 365/460 nm). The slope (*k*) was obtained from linear fitting between fluorescent intensity and

concentration of enzymes. The LOD was calculated as LOD = 3N/*k* (N is the standard deviation of a blank sample). All experiments were performed in triplicate.

Inhibition assay: β-lactamases (0.5 nM) and inhibitors (PBA: 2 mM; EDTA: (400 μM) were pre-mixed and incubated in a 96-well black plate for 20 min at room temperature before CPC-2 (10 μM in PBS，total volume: 100 μL) was added and incubated at 37 ^{o}C for 30 min. Fluorescence intensity was measured using a microplate reader (Molecular Devices, SpectraMax *i*3, $\lambda_{ex/em}$ = 365/460 nm). All experiments were performed in triplicate.

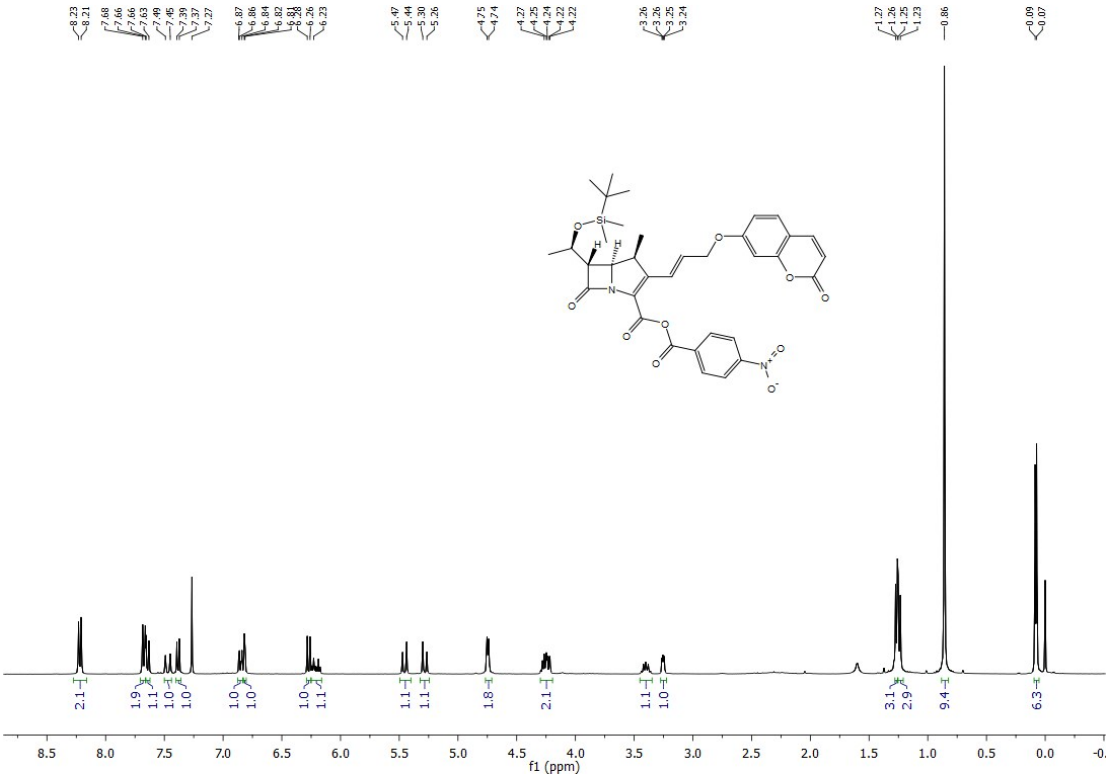
Fluorescence response of CPC-2 to live pathogenic bacteria: CPC-2 (15 μM in PBS, total volume: 20 μL) and a series of pathogenic bacteria (ATCC 25922, ATCC 35218, ATCC BAA 2340, and NCTC 13440, 4×10^5 CFU) in a 384-well black plate were incubated at room temperature for 1 hour, the fluorescence intensity of these samples were measured on a microplate reader (Molecular Devices, SpectraMax *i*3, $\lambda_{ex/em}$ = 365/460 nm).

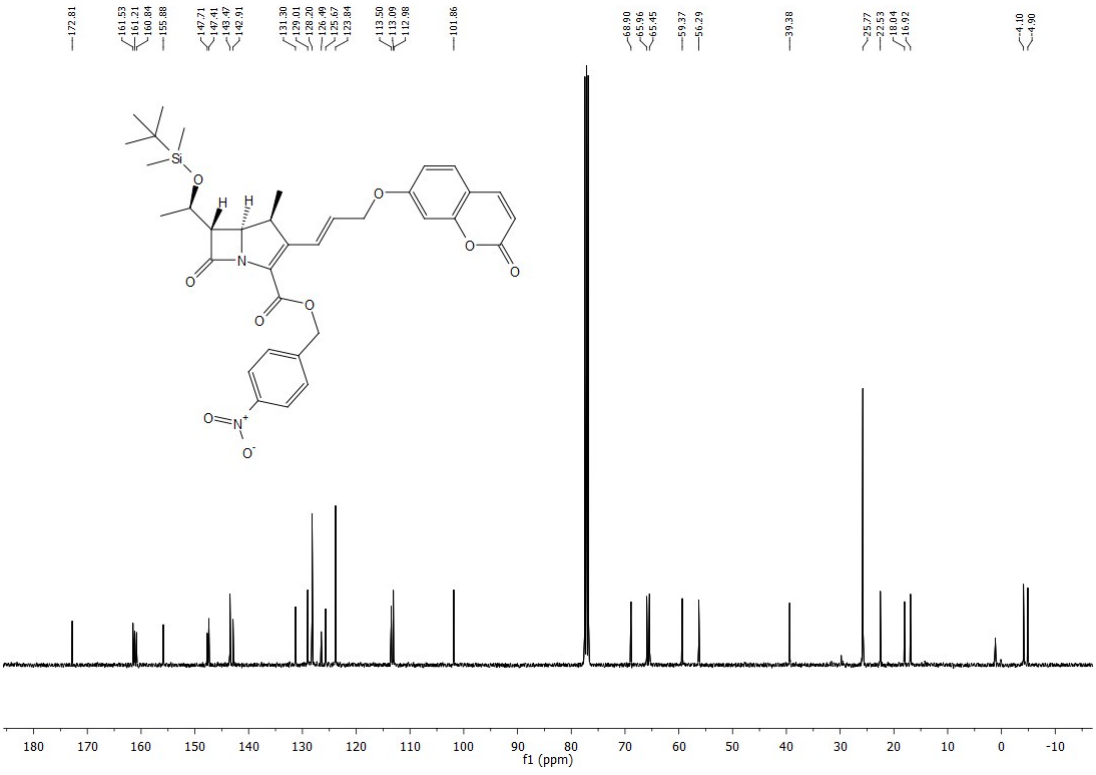
References

1. (a) W. Mao, L. Xia and H. Xie, *Angew. Chem. Int. Ed.*, 2017, **56**, 4468; (b) W. Mao, Y. Wang, X. Qian, L. Xia and H. Xie, *Chembiochem*, 2019, **20**, 511; (c) X. Qian, S. Zhang, S. Xue, W. Mao, M. Xu, W. Xu and H. Xie, *Bioorg. Med. Chem. Lett.*, 2019, **29**, 322; (d) W. Mao, L. Xia, Y. Wang and H. Xie, *Chem. Asian J.*, 2016, **11**, 3493; (e) W. Mao, X. Qian, J. Zhang, L. Xia and H. Xie, *Chembiochem*, 2017, **18**, 1990;
2. Jacob Kofoed, Tamis Darbre and Jean-Louis Reymond, *Org. Biomol. Chem.*, 2006, 4, 3268-3281.

^{1}H, ^{13}C NMR spectra

Compound **2**





Compound CPC-2