Supplementary Information

Highly Selective and Wash-free Visualization of Resistant Bacteria With a Relebactam-derived Fluorogenic Probe

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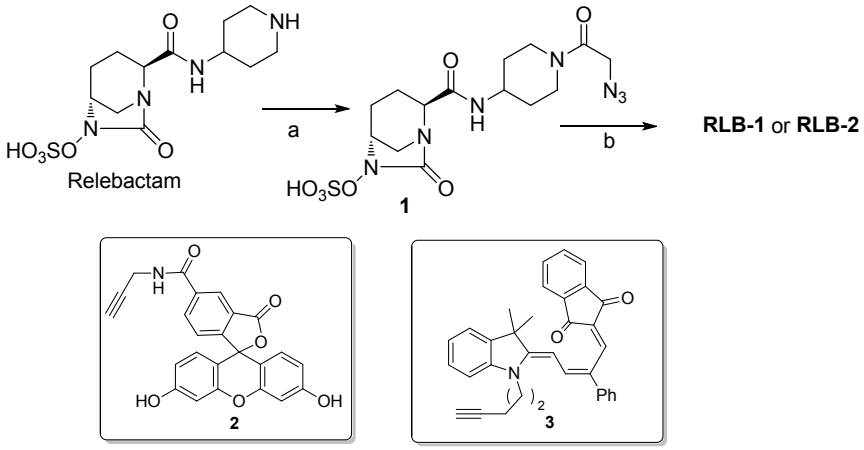


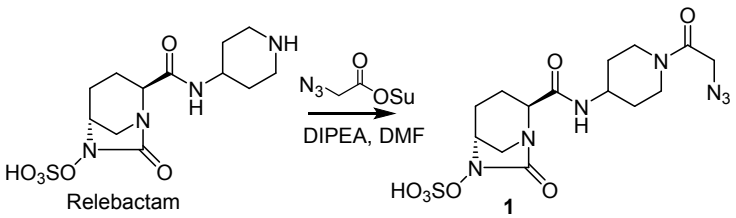
Scheme S1. Preparation of RLB-1 or RLB-2.

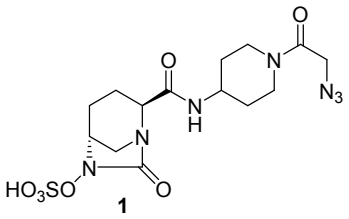
a) Azidoacetic acid NHS ester, DIPEA, DMF, rt, 1 h ; b) **2** or **3**, $CuSO_4$, Vc, THPTA, DMSO:H_2O = 1:1, rt, 0.5 h. THPTA: Tris(3-hydroxypropyltriazolylmethyl)amine.

General Information

Unless otherwise noted, all chemicals were purchased from commercial sources (e.g. Adamas-Beta, Energy Chemical and TCI) and used without further purification. Relebactam was purchased from Shanghai Biopharmaleader Co.,Ltd. Analytical thin layer chromatography was performed on 0.20 mm Qingdao Haiyang silica gel plates and visualized by ultraviolet light. A number of synthesized chemicals were purified using a SepaBean machine equipped with Sepaflash columns produced by Santai Technologies Inc. in China. HPLC was performed on a Shimadzu HPLC System equipped with a LC-20AT gradient pump and an inline diode array UV-Vis detector. A reversed-phase C18 (Inertsil ODS-SP, 5 μm, 4.6 x 250 mm or phenomenex, 5 μm, 21.2 x 250 mm) column was used with a MeCN/H_2O gradient mobile phase containing 0.1% trifluoroacetic acid at a flow of 1 or 12 mL/min for the analysis or purification. The ^{1}H and ^{13}C NMR spectra were taken on Bruker nuclear magnetic resonance spectrometer (^{1}H, 400 MHz or 600 MHZ; ^{13}C, 150 MHz). 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 Waters Xevo G2-XS time of flight mass spectrometer with electrospray ionization. Mass spectrometry (ESI) of protein was recorded on a MS SCIEX TripleTOF 5600+ System. Fluorescence spectra were carried out on a wavelength-calibrated FluoroMax-3 fluorometer (Horiba Jobin Yvon). Kinetic experiments were conducted in a microplate reader (Molecular Devices, SpectraMax i3). Fluorescence imagings were taken using Gel Documentation and Image Analysis System (SAGECREATION, China) or Infrared Imaging Systems ODYSSEY Sa (LICOR). Microscope imaging of resistant bacteria was carried out on a TCS SP8 confocal microscopy (Leica). Image processing was made on image J software (National Institutes of Health, USA).

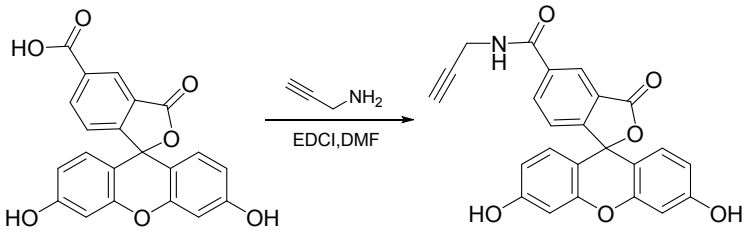
Synthesis and characterization

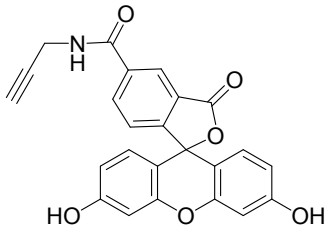




(2*S*,5*R*)-2-((1-(2-azidoacetyl)piperidin-4-yl)carbamoyl)-7-oxo-1,6-diazabicyclo[3.2.1]octan-6-yl hydrogen sulfate (1)

To a solution of relebactam (50.0 mg, 0.144 mmol) in DMF (300 μL) were added azidoacetic acid NHS ester (34.1 mg, 0.172 mmol) and *N*,*N*-diisopropylethylamine (DIPEA, 59 μL, 0.359 mmol). The resulting mixture were stirred at room temperature for 1 h. RP-HPLC purification on a C18 column afforded title compound as white powder (38.0 mg, 61%). ^{1}H NMR (400 MHz, D_2O) δ 4.41 (d, *J* = 13.2 Hz, 1H), 4.36-4.22 (m, 3H), 4.07 (t, *J* = 11.2 Hz, 2H), 3.81 (d, *J* = 14.0 Hz, 1H), 3.38 (d, *J* = 11.7 Hz, 1H), 3.28 (t, *J* = 12.9 Hz, 1H), 3.13 (d, *J* = 12.2 Hz, 1H), 2.99 (t, *J* = 12.6 Hz, 1H), 2.26 (dd, *J* = 15.0, 5.1 Hz, 1H), 2.14 (dd, *J* = 14.5, 1.8 Hz, 1H), 2.09-1.93 (m, 3H), 1.93-1.81 (m, 1H), 1.72-1.45 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 168.78, 166.14, 165.64, 59.39, 57.61, 49.72, 46.47, 45.97, 45.95, 43.03, 40.60, 40.56, 31.53, 31.27, 30.80, 30.63, 20.50, 18.40. HRMS (ESI) m/z calcd for $C_{14}H_{21}N_7O_7S$ $[M-H]^-$ 430.1145, found 430.1146.

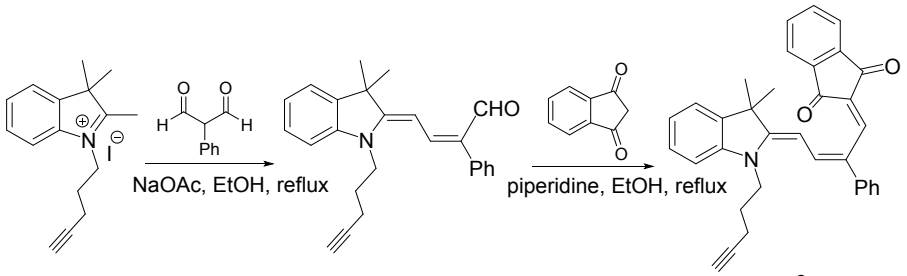




2

3',6'-dihydroxy-3-oxo-N-(prop-2-yn-1-yl)-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamide (2)

To a solution of 5-carboxyfluorescein (50.0 mg, 0.133 mmol) in DMF (500 μL) were added 2-propynylamine (11 μL, 0.173 mmol) and EDCI (38.2 mg, 0.199 mmol), the resulting solution were stirred at room temperature for 3 h. RP-HPLC purification on a C18 column afforded title compound as orange powder (35.2 mg, 64%), whose ^{1}H NMR spectrum is consistent with reported data.[1]



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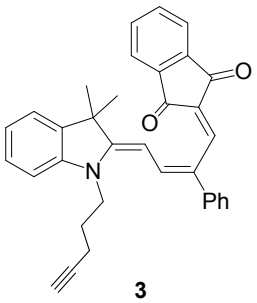
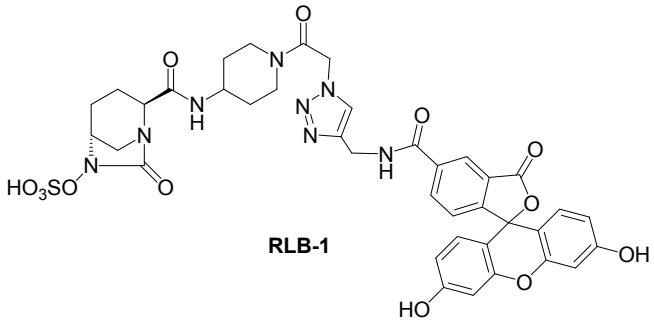


3

2-((2E,4Z)-4-(3,3-dimethyl-1-(pent-4-yn-1-yl)indolin-2-ylidene)-2-phenylbut-2-en-1-ylidene)-1H-indene-1,3(2H)-dione (3)

The synthesis of title compound followed reported procedure[2] with minor modifications. In brief, a mixture of sodium acetate (139.3 mg, 1.698 mmol), **4** (500.0 mg, 1.415 mmol), and 2-phenylmalondialdehyde (314.5 mg, 2.123 mmol) in ethanol (5 mL) were heated to reflux for 0.5 h. The solvent was then removed *via* Rota-Vap and the residue was purified by flash column chromatography to afford compound **5** as crude product (230.0 mg), which was used in next step without further purification. A mixture of **5** (230.0 mg), 1,3-indandione (113.7 mg, 0.776 mmol), and piperidine (30 μL) in ethanol (5 mL) were heated to reflux for 3 h. The solvent was removed *via*

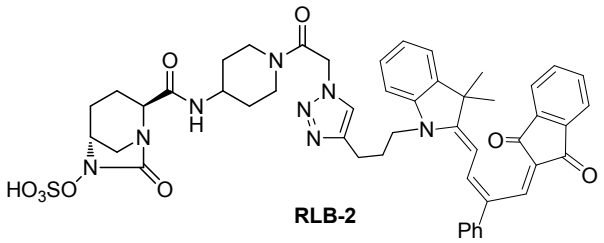
Rota-Vap and the residue was purified by flash column chromatography to afford title compound as blue solid (43%, 136.0 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86-7.78 (m, 2H), 7.66-7.60 (m, 2H), 7.57 (s, 1H), 7.47-7.26 (m, 8H), 7.12 (t, *J* = 7.4 Hz, 1H), 6.94 (d, *J* = 7.9 Hz, 1H), 5.77 (d, *J* = 13.6 Hz, 1H), 3.76 (t, *J* = 7.1 Hz, 2H), 2.15 (td, *J* = 6.6, 2.5 Hz, 2H), 1.89 (s, 6H), 1.86-1.83 (m, 1H), 1.83-1.75 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 191.47, 170.07, 149.28, 148.27, 142.88, 141.26, 141.08, 140.91, 133.61, 130.74, 129.92, 128.45, 128.28, 127.05, 123.64, 122.36, 122.01, 120.11, 108.95, 99.22, 82.29, 70.29, 48.66, 42.18, 27.52, 25.74, 16.07.



RLB-1

(2S,5R)-2-((1-(2-(4-((3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)methyl)-1H-1,2,3-triazol-1-yl)acetyl)piperidin-4-yl)carbamoyl)-7-oxo-1,6-diazabicyclo[3.2.1]octan-6-yl hydrogen sulfate (RLB-1)

The synthesis of title compound was according to reported procedure[3] with minor modification. In brief, a mixture of **1** (5.0 mg, 0.012 mmol), **2** (5.3 mg, 0.013 mmol), $CuSO_4$ (0.2 mg, 0.001 mmol), L-(+)-Ascorbic acid (8.5 mg, 0.048 mmol) and THPTA (0.4 mg, 0.001 mmol) in DMSO: H_2O = 1:1 (100 μL) were stirred at room temperature for 0.5 h. RP-HPLC purification on a C18 column afforded compound **RLB-1** as orange powder (4.3 mg). The purity of the title compound was confirmed by HPLC analysis. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.43 (t, *J* = 5.7 Hz, 1H), 8.50 (s, 1H), 8.28 (d, *J* = 8.1 Hz, 1H), 8.04 (d, *J* = 5.8 Hz, 1H), 7.90 (s, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 6.68 (d, *J* = 2.3 Hz, 2H), 6.61-6.51 (m, 4H), 5.53-5.35 (m, 2H), 4.58 (d, *J* = 5.6 Hz, 2H), 4.21 (d, *J* = 12.7 Hz, 1H), 3.99 (s, 1H), 3.87 (d, *J* = 12.4 Hz, 2H), 3.72 (d, *J* = 6.4 Hz, 1H), 3.17 (t, *J* = 12.7 Hz, 1H), 3.03-2.93 (m, 2H), 2.83-2.70 (m, 1H), 2.09-1.98 (m, 1H), 1.91-1.61 (m, 5H), 1.60-1.48 (m, 1H), 1.45-1.32 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 168.90, 168.20, 166.24, 164.65, 164.12, 159.70, 154.79, 151.89, 144.51, 135.97, 134.77, 129.24, 126.61, 124.75, 124.36, 123.52, 112.77, 109.13, 102.32, 59.44, 57.67, 50.62, 46.50, 46.04, 46.01, 43.23, 40.74, 40.69, 35.08, 31.59, 31.34, 30.80, 30.64, 20.54, 18.45. HRMS (ESI) m/z calcd for $C_{38}H_{35}N_8O_{13}S$ $[M-H]^-$ 843.2044, found 843.2043.



(2S,5R)-2-((1-(2-(4-(3-((Z)-2-((E)-4-(1,3-dioxo-1H-inden-2(3H)-ylidene)-3-phenylbut-2-en-1-ylidene)-3,3-dimethylindolin-1-yl)propyl)-1H-1,2,3-triazol-1-yl)acetyl)piperidin-4-yl)carbamoyl)-7-oxo-1,6-diazabicyclo[3.2.1]octan-6-yl hydrogen sulfate (RLB-2)

A mixture of **1** (5.0 mg, 0.012 mmol), **3** (6.2 mg, 0.013 mmol), $CuSO_4$ (0.2 mg, 0.001 mmol), L-(+)-Ascorbic acid (8.5 mg, 0.048 mmol) and THPTA (0.4 mg, 0.001 mmol) in DMSO: H_2O = 1:1 (100 μL) were stirred at room temperature for 0.5 h. RP-HPLC purification on a C18 column afforded compound **RLB-2** as blue powder (3.7 mg). The purity of the title compound was confirmed by HPLC analysis. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.04 (dd, J = 7.8, 2.7 Hz, 1H), 7.79-7.66 (m, 4H), 7.59 (d, J = 7.4 Hz, 1H), 7.45 (t, J = 7.5 Hz, 2H), 7.39-7.24 (m, 5H), 7.22-7.15 (m, 2H), 7.08 (s, 1H), 7.00 (s, 1H), 5.81 (d, J = 14.0 Hz, 1H), 5.50-5.33 (m, 2H), 4.22 (d, J = 12.7 Hz, 1H), 3.99 (s, 1H), 3.94-3.78 (m, 4H), 3.72 (d, J = 6.3 Hz, 1H), 3.17 (t, J = 12.9 Hz, 1H), 3.02-2.92 (m, 2H), 2.82-2.72 (m, 1H), 2.60-2.53 (m, 2H), 2.11-1.57 (m, 14H), 1.57-1.49 (m, 1H), 1.43-1.34 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 190.94, 174.36, 172.00, 168.86, 166.19, 164.13, 148.82, 145.88, 145.20, 142.28, 141.10, 141.04, 133.67, 129.68, 129.32, 128.59, 128.42, 128.36, 126.99, 124.25, 123.74, 122.46, 121.25, 117.49, 110.50, 99.73, 59.40, 57.63, 50.59, 48.72, 46.48, 46.01, 45.98, 43.20, 42.89, 40.72, 40.67, 39.52, 31.60, 31.36, 30.78, 30.62, 26.56, 26.25, 22.43, 20.52, 18.42. HRMS (ESI) m/z calcd for $C_{48}H_{50}N_8O_9S$ $[M-H]^-$ 913.3343, found 913.3345.

Plasmid construction

Plasmid or genomic DNAs from AmpC *P. Aeruginosa* (PAO1) and OXA-1 *Klebsiella pneumoniae* (ATCC BAA 2146) were used as template for PCR. Following manufacturer's protocol (TransGen), the PCRs were conducted with Pfu DNA polymerase, and template DNA, as well as F (Forward) and R (Reverse) primers (Table S1) . The purified PCR product was inserted into SalI and NcoI sites of *p*BAD/*Myc*-His vector with Ezmax One-step Cloning kit (Tolo Biotech) following manufacturer's protocol. The resulting plasmid DNAs were transformed into Top10 or LMG194 *E. coli*.

Table S1. Primers for cloning of β-lactamases.

Primer	Sequence
AmpC F	5'-taacaggaggaattaaccatgggcatgcgcgataccagattcccctgc-3'
AmpC R	5'-atgatgatgatgatgatggtcgacgcgcttcagcggcaccttgccctg-3'
OXA-1 F	5'-acaggaggaattaaccatgggcttgttagccgttaaaattaagcc-3'
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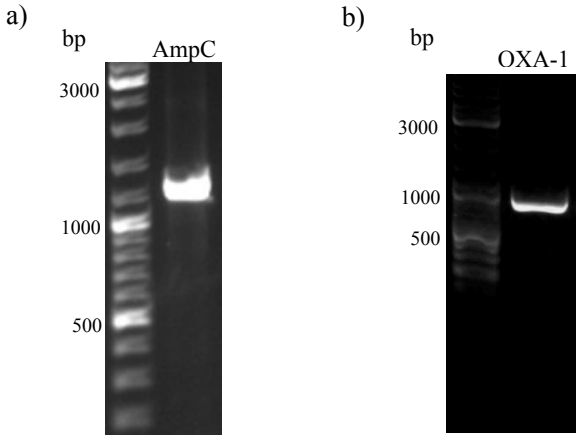


Figure S1. Agarose gel electrophoresis of the PCR product. (a) AmpC. (b) OXA-1.

Protein expression and purification

The TEM-1[4], KPC-2[4], VIM-27[4], NDM-1[4], IMP-1[4] and CphA[5] was overexpressed and purified as previously described.

The AmpC and OXA-1 blas was overexpressed and purified as previously described[5]. In brief, the plasmid was transformed into LMG194 *E. coli* competent cells and the transformed *E. coli* was cultured in Luria-Bertani (LB) media containing 100 μg/mL ampicillin at 37 ^{o}C overnight. Overexpression of protein was induced by the addition of 0.5 mM – (+) Arabinose at 30 ^{o}C for 7 h. The cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 400 mM

NaCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.2% BME). The cells suspension was lysed by Ultrasonic cell crusher and the cell debris was removed by centrifugation at 4 ^{o}C. The resulting supernatant was incubated with Ni-NTA agarose bead and washed with native wash buffer with 5 mM imidazole, 20 mM imidazole and 250 mM imidazole,subsequently. The imidazole was removed by PD-10 column. The protein concentration was determined by the BCA protein assay.

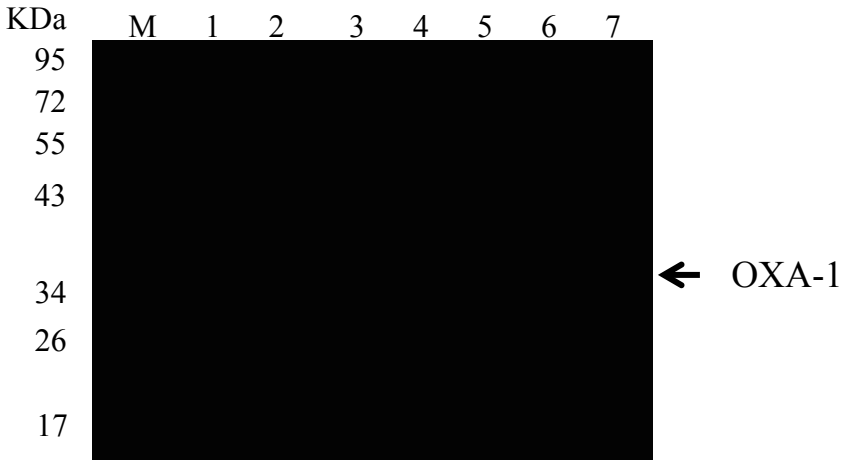


Figure S2. The SDS-PAGE assay of OXA-1 bla.
M: protein standard; 1: lysate of *E. coli* before induction by Arabinose; 2: lysate of *E. coli* after induction by Arabinose; 3: Supernatent; 4: flow-through after Ni-NTA binding; 5: washing with 5 mM imidazole; 6: washing with 20 mM imidazole; 7: eluted by 250 mM imidazole.

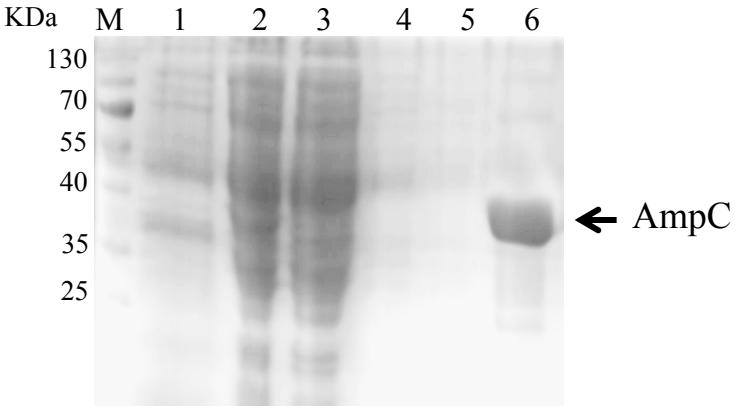


Figure S3. The SDS-PAGE assay of the purified AmpC bla.
M: protein marker; 1: lysate of *E. coli* before induction by Arabinose; 2: lysate of *E. coli* after induction by Arabinose; 3: flow-through after Ni-NTA binding; 4: washing with 5 mM imidazole; 5: washing with 20 mM imidazole; 6: eluted by 250 mM imidazole.

Inhibition test assay

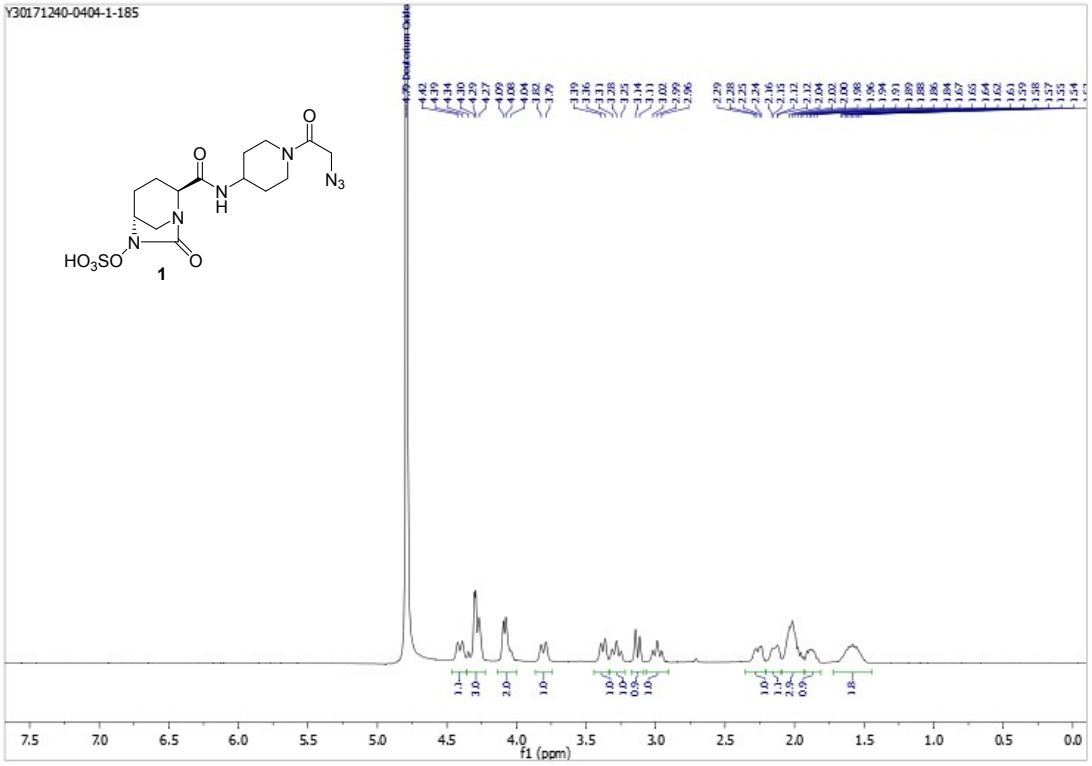
To a 96-well plate (black and flat bottom) was added TEM-1 bla (500 pM) in PBS (containing 0.1% CHAPS, pH 7.4) with or without RLB-1 (1 μM). The total volume was adjusted to 100 μL/each well. Upon incubation at room temperature for 10 minutes, fluorogenic substrate CDC-1 was added to have the final concentration at 10 μM. These samples were placed to a Microplate reader at room temperature immediately and the fluorescent intensity at 460 nm was recorded over a period of 10 minutes under excitation at 365 nm. All of these experiments were triplicated.
CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulfonate

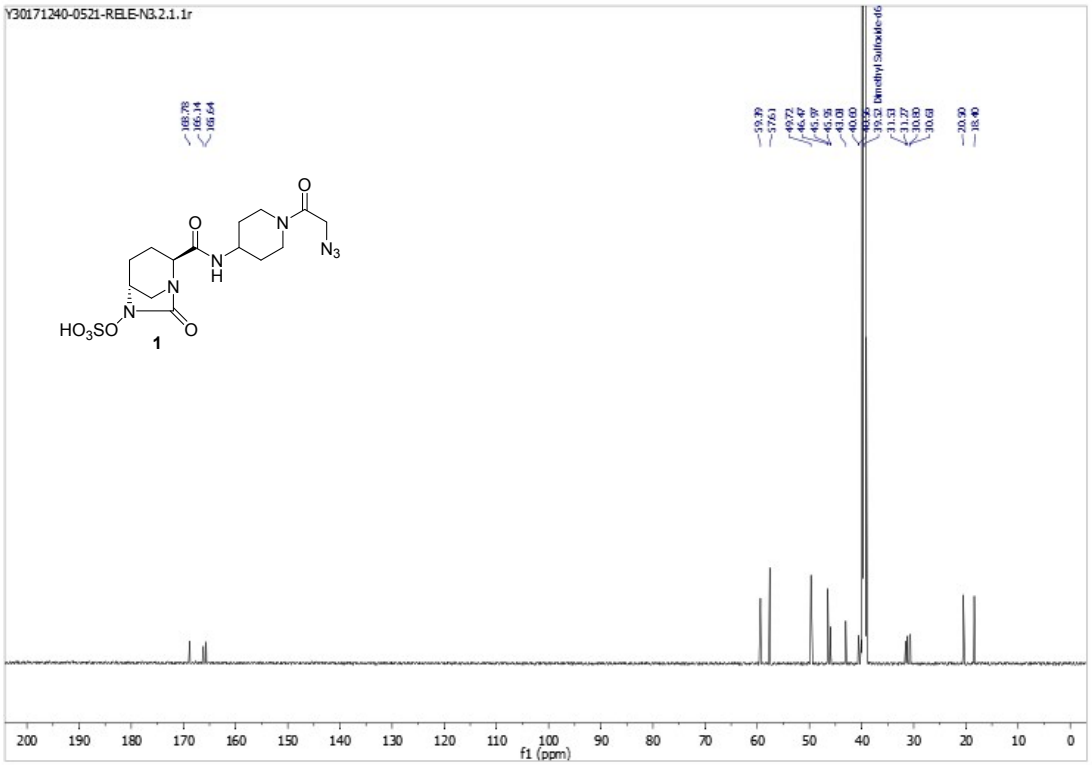
References.

1. M. A. Brun, K. T. Tan, E. Nakata, M. J. Hinner and K. Johnsson, *J. Am. Chem. Soc.*, 2009, **131**, 5873-5884.
2. H. J. Chen, C. Y. Chew, E. H. Chang, Y. W. Tu, L. Y. Wei, B. H. Wu, C. H. Chen, Y. T. Yang, S. C. Huang, J. K. Chen, I. C. Chen and K. T. Tan, *J. Am. Chem. Soc.*, 2018, **140**, 5224-5234.
3. X. Liu, A. Thakur, and D. Wang. *Biomacromolecules.*, 2007, **8**, 2653-2658.
4. W. Mao, L. Xia and H. Xie, *Angew. Chem. Int. Ed.*, 2017, **56**, 4468-4472.
5. X. Qian, S. Zhang, S. Xue, W. Mao, M. Xu, W. Xu and H. Xie, *Bioorg. Med. Chem. Lett.*, 2019, **29**, 322-325.

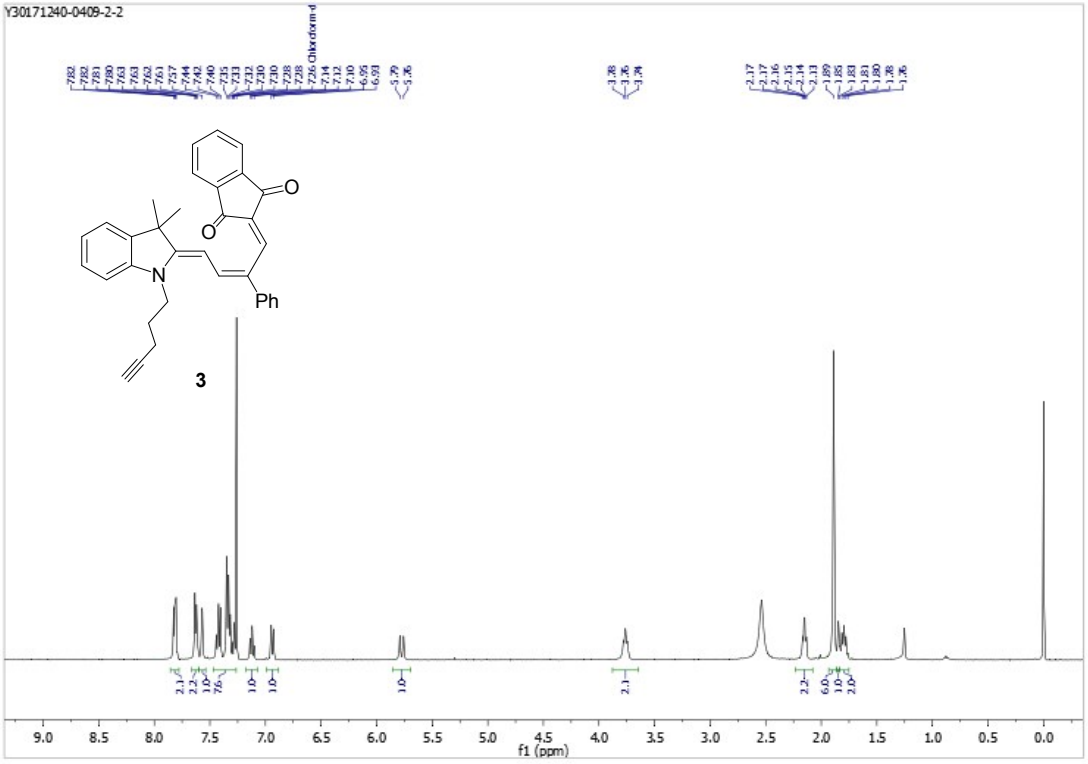
^{1}H and ^{13}C NMR spectra.

Compound 1





Compound 3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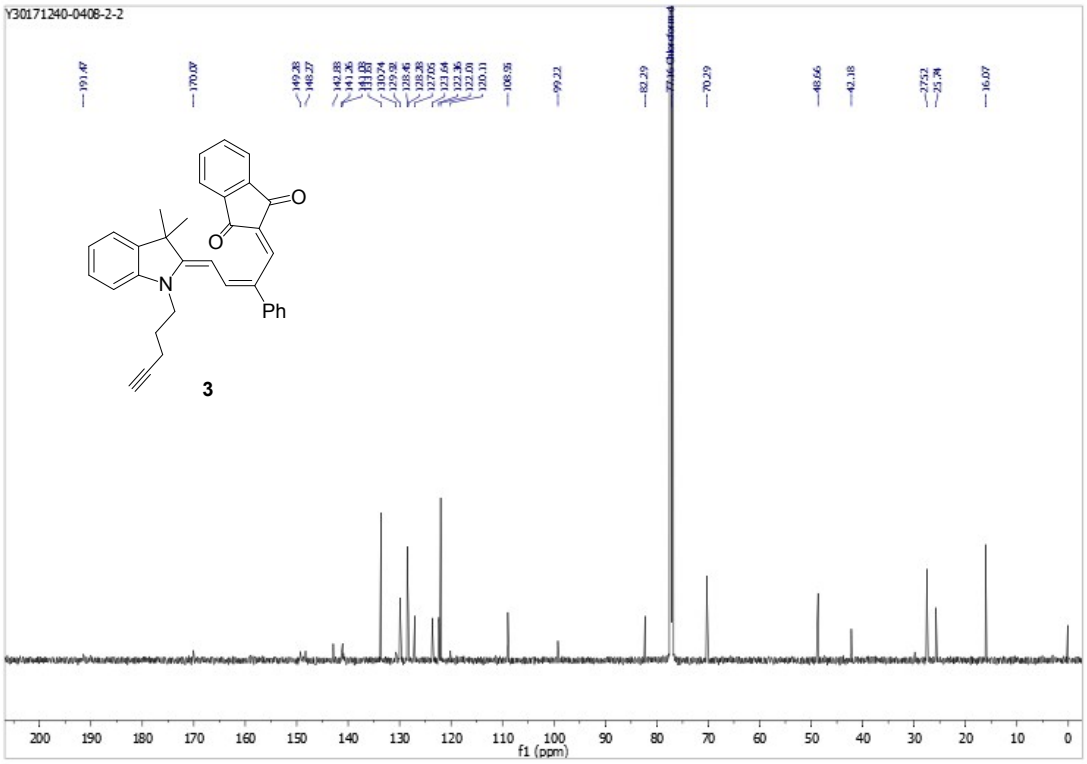
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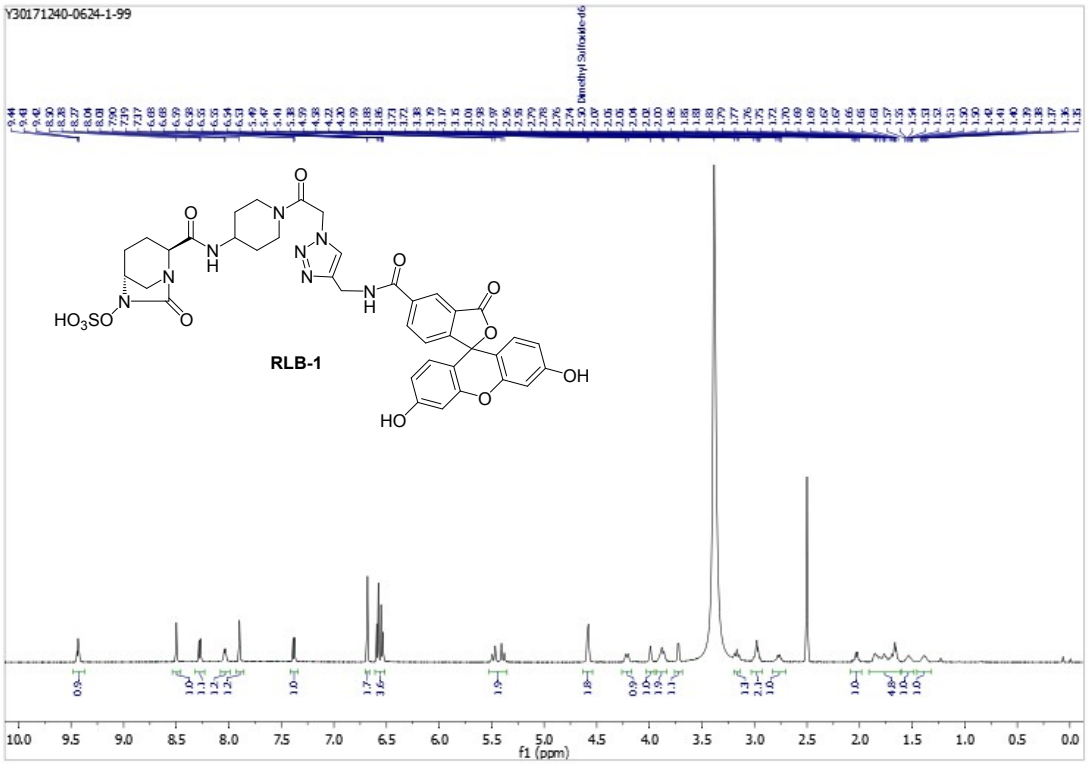
RLB-1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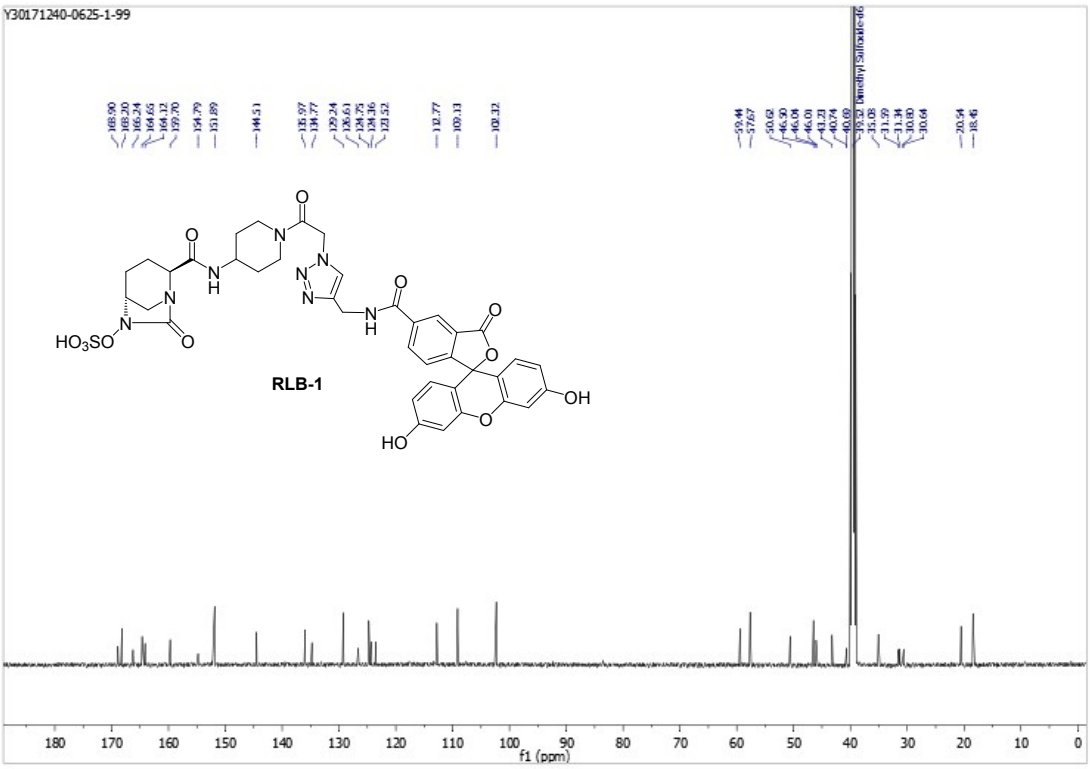
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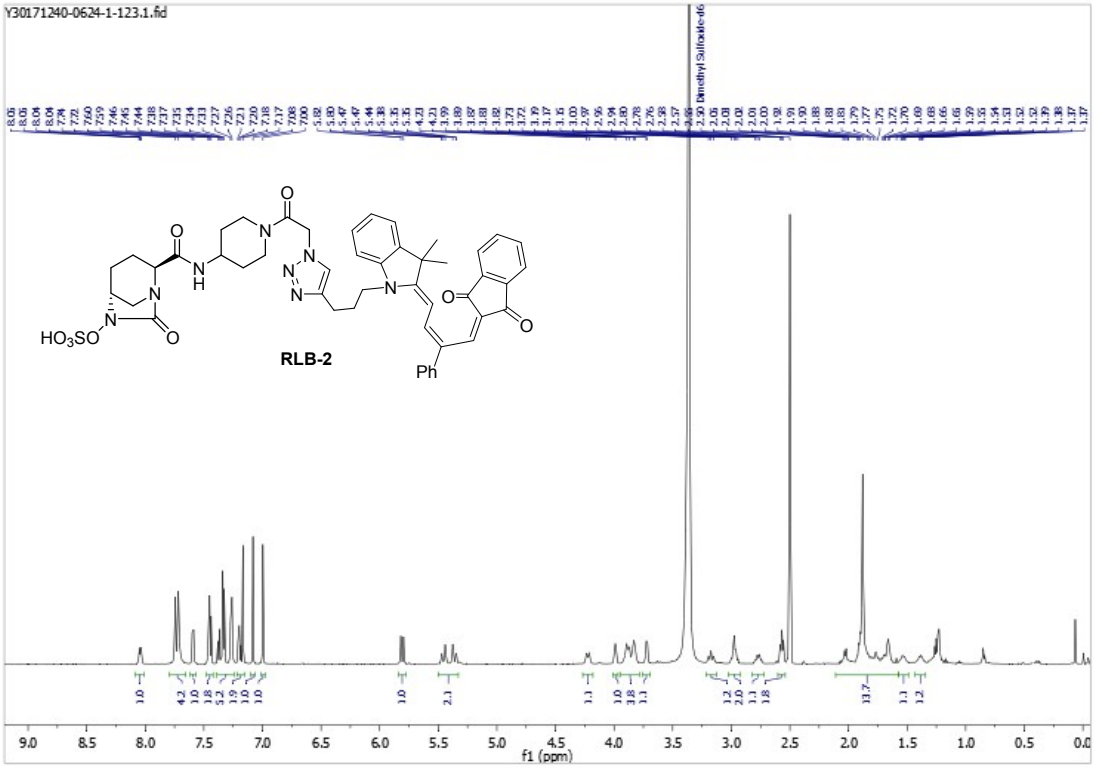
RLB-2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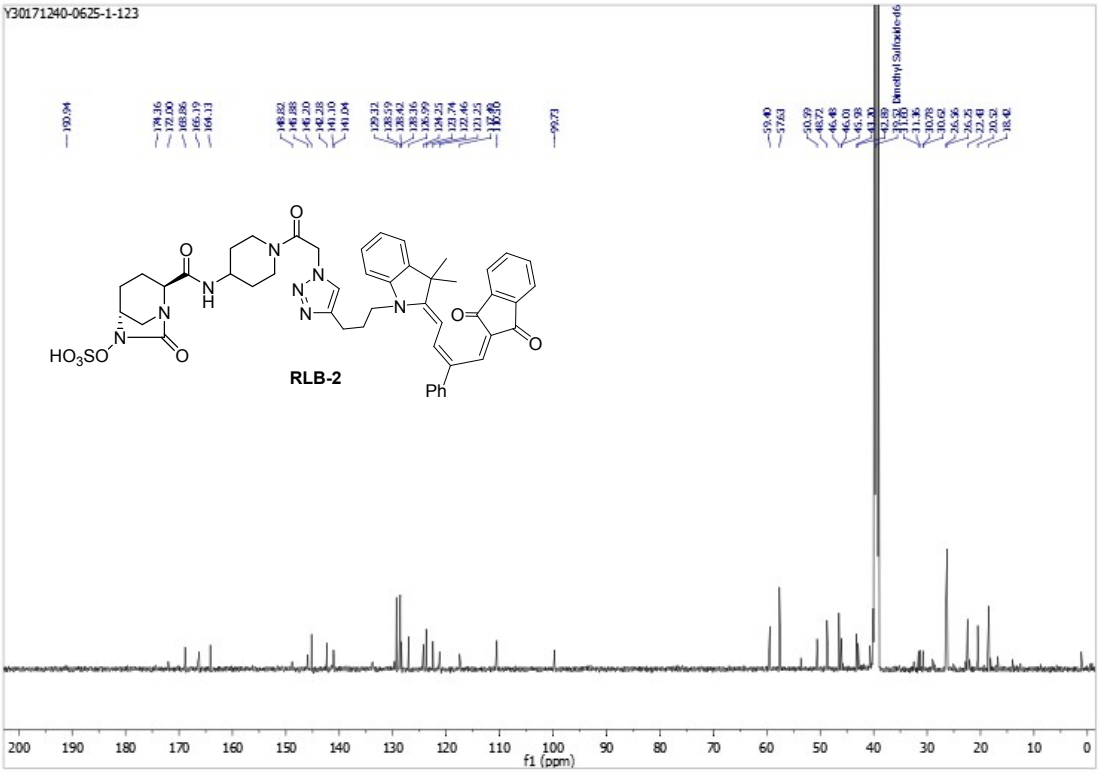
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Dimethyl Sulfoxide-d6
RLB-2
f1 (ppm)

HPLC traces of RLB-1 and RLB-2.