Supplementary Information for

Detecting *Mycobacterium Tuberculosis* by nitrofuranyl calanolide-trehalose probe based on nitroreductase Rv2466c

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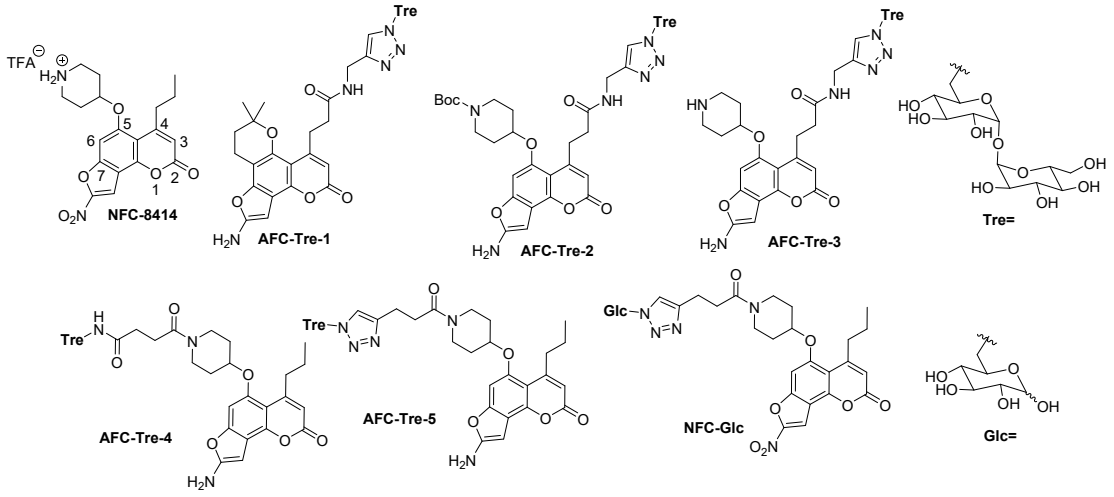
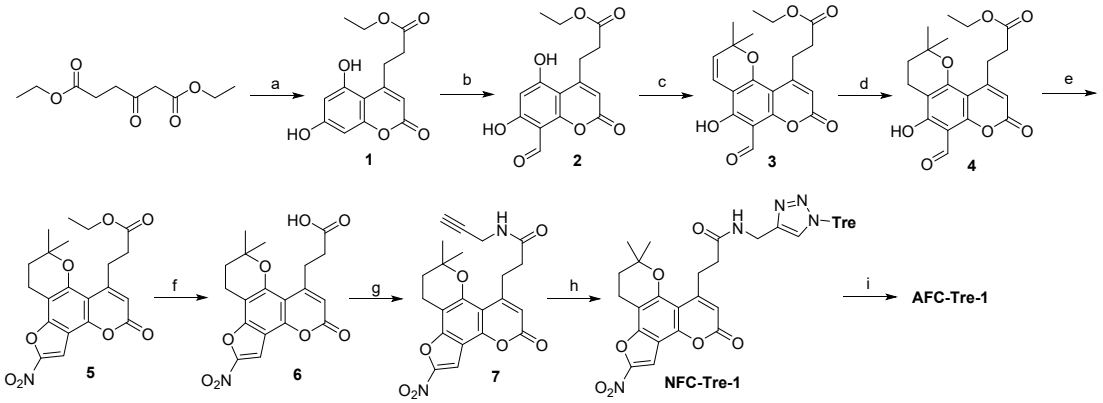
1. General Chemical Methods

Details about the synthesis of all chemical entities described in this article are in the Supporting Information. All chemicals were purchased as reagent grade and used without further purification unless otherwise noted. Analytical thin-layer chromatography (TLC) plates, preparative TLC plates, and silica gel for column chromatography were purchased from Qingdao Haiyang Chemical and Special Silica Gel Co., Ltd. Automatic liquid chromatography−mass spectrometry (LC−MS) analysis was performed on a Waters SQ Advantage mass spectrometer equipped with an ultraperformance LC (UPLC) system and an eluent splitter (5% eluent was split into the MS system). High-resolution LC−MS was conducted using an Agilent LC/mass selective detector (MSD) time of flight (TOF) with an Agilent ZORBAX SB-C18 (rapid resolution, 3.5 μm, 2.1 mm × 30 mm) column at a flow rate of 0.40 mL/min. The solvent was MeOH/water (75:25 (v/v)) containing 5 mmol/L ammonium formate. The ion source was electrospray ionization (ESI). Proton nuclear magnetic resonance (^{1}H NMR) and carbon nuclear magnetic resonance (^{13}C NMR) spectroscopy were performed on Bruker Advance 400M NMR and 800M NMR spectrometers.



Scheme S1. Structures of **NFC-8414, AFC-Tre** and **NFC-Glc**.



Scheme S2. Synthesis of Compounds NFC-Tre-1 and AFC-Tre-1. Reagents and conditions:(a) ethanol, phloroglucinol, $BF_3{\cdot}C_2H_5OC_2H_5$, reflux, overnight, 90%; (b) DCM, DMF, $POCl_3$, rt, overnight, 55%; (c) toluene, 1,1-Diethoxy-3-methyl-2-butene, pyridine, reflux, 3h, 35%; (d) (I) Ac_2O, IR-120, rt, overnight (II) methanol, H_2, Pd/C, 50℃, 1h (III) methanol，K_2CO_3, rt, 2h, 80%;(e) (I) acetone, $BrCH_2NO_2$, K_2CO_3, 3h (II) acetic anhydride, reflux, overnight, 60%; (f) 6N HCl, reflux, 1h, 50%;(g) DCM, propargulamine, EDCI, HOBt, DIPEA, rt, 2h, 55-65%;(h) DMF, Tre-N_3, Vc-Na, $CuSO_4$, H_2O, rt, 30min, 50%; (i) Fe, NH_4Cl, EtOH/H_2O, rt, overnight, 10%.

Synthetic procedure of intermediate Ethyl 3-(5,7-dihydroxy-2-oxo-2H-chromen-4-yl)propanoate (1). Boron trifluoride ether (21.3 g, 150 mmol) was added to a solution of phloroglucinol (12.6 g, 100 mmol) and diethyl 3-oxoadipate (23.8 g, 110 mmol) in 300 ml ethanol at 0°C, and the reaction mixture was stirred at reflux overnight and monitored by LCMS until all the starting material was consumed completely. Water (500 mL) was added and the residue was washed with 5% sodium bicarbonate aqueous solution. The dried crude product can be used in the next step without purification.

Synthetic procedure of intermediate Ethyl 3-(8-formyl-5,7-dihydroxy-2-oxo-2H-chromen-4-yl)propanoate (2). Phosphorus trichloride (12.2 mL, 15 mmol) was added to DMF (10.5 mL, 15 mmol) in an ice bath and stirred for 30 min. Then a solution of intermediate **1** (20.8 g, 7.5 mmol) in dichloromethane was added to DMF-$POCl_3$ mixture and stirred overnight at room temperature. After the reaction was completed, dichloromethane was removed in vacuo and 400 mL ice water was slowly added for stirring for 30 min. The residue was washed with water and ethyl acetate to obtained intermediate **2** (12.5 g, 55%). ^{1}H NMR (400 MHz, DMSO) δ 12.25 (s, 1H), 10.16 (s, 1H), 6.27 (s, 1H), 6.07 (s, 1H), 4.15 – 3.98 (m, 2H), 3.17 (t, J = 7.5

Hz, 2H), 2.63 (t, J = 7.6 Hz, 2H), 1.18 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 190.7, 172.32, 165.6, 164.7, 159.2, 158.8, 157.0, 110.7, 103.7, 102.3, 99.1, 60.5, 33.6, 31.1, 14.6.

Synthetic procedure of intermediate Ethyl3-(6-formyl-5-hydroxy-2,2-dimethyl-8-oxo-2H,8H-pyrano[2,3-f]chromen-10-yl)propanoate (3). To a solution of intermediate **2** (8 g, 26 mmol) in 100 mL toluene was added 1,1-diethoxy-3-methylbut-2-ene (6.2 g, 39 mmol) and pyridine (320 μL, 4 mmol). The mixture was stirred under reflux for 3 hours. After the reaction wased complete, the reaction mixture was concentrated in vacuum and the residue was purified by column chromatography on silica gel (petroleum: ethyl acetate=4:1) to obtained intermediate **3** (3.4g, 35%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.82 (s, 1H), 10.39 (s, 1H), 6.68 (d, J = 10.1 Hz, 1H), 6.07 (s, 1H), 5.61 (d, J = 10.1 Hz, 1H), 4.31 – 4.06 (m, 2H), 3.25 (dd, J = 9.5, 5.9 Hz, 2H), 2.73 – 2.52 (m, 2H), 1.55 (s, 6H), 1.29 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 192.1, 172.0, 161.5, 158.7, 158.2, 157.9, 155.7, 126.8, 115.0, 111.8, 105.5, 104.0, 102.3, 80.6, 60.8, 34.0, 31.7, 28.2, 14.3.

Synthetic procedure of intermediate Ethyl3-(6-formyl-5-hydroxy-2,2-dimethyl-8-oxo-3,4-dihydro-2H,8H-pyrano[2,3-f]chromen-10-yl)propanoate (4). To a solution of intermediate **3** (3 g, 8 mmol) in 30 mL of acetic anhydride was added 3 g Amberlite IR-120 and stirred for overnight at room temperature. The reaction was monitored by LCMS until all the starting material was consumed completely, then the acetic anhydride was evaporated under vacuum. The residue was dissolved in 60 mL methanol and added 1g 10% Pd/C, the mixture was stirred under atmosphere of H_2 at 50°C for 1 hours. The reaction was monitored by LCMS until all the starting material was consumed completely, then the reaction mixture was filtered. Then add potassium carbonate (5.6 g, 40 mmol) to the solution and stir for 2 hours at room temperature. The reaction monitored by LCMS until all the starting material was consumed completely. The reaction mixture was filtered and concentrated in vacuum. The residue was purified by column chromatography on silica gel (petroleum: ethyl acetate=4:1) to obtained intermediate **4** (2.4 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.96 (s, 1H), 10.41 (s, 1H), 6.07 (s, 1H), 4.28 – 4.10 (m, 2H), 3.34 – 3.14 (m, 2H), 2.73 (t, J = 6.8 Hz, 2H), 2.67 – 2.55 (m, 2H), 1.88 (t, J = 6.8 Hz, 2H), 1.46 (s, 6H), 1.30 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 192.0, 172.0, 164.5, 159.2, 158.8, 157.2, 156.2, 111.6, 105.1, 103.4, 102.4, 78.7, 60.8, 34.3, 32.0, 31.0, 26.6, 15.9, 14.3.

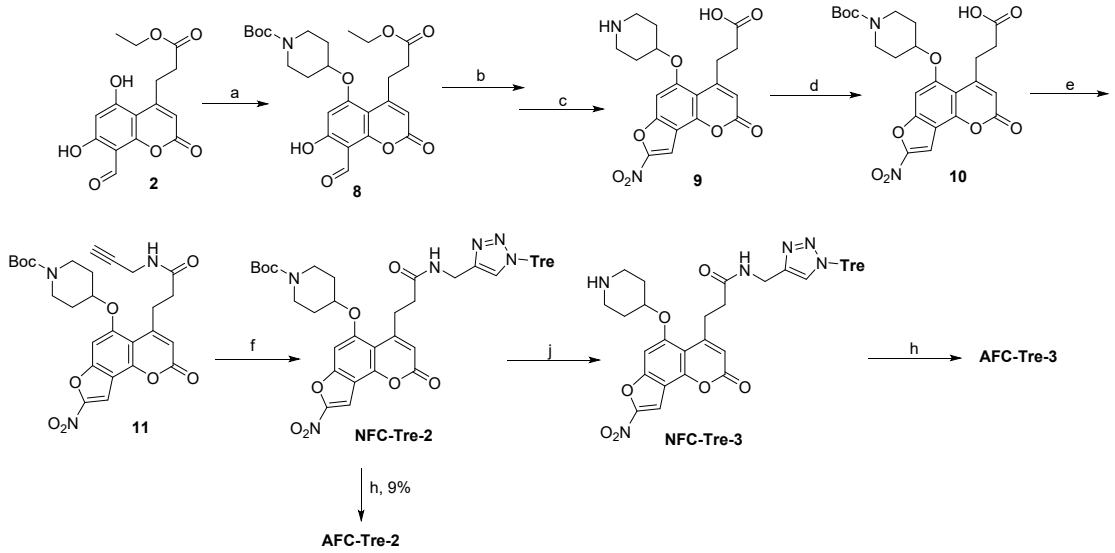
Synthetic procedure of intermediate Ethyl3-(9,9-dimethyl-2-nitro-5-oxo-10,11-dihydro-5H,9H-furo[2,3-f]pyrano[2,3-h]chromen-7-yl)propanoate (5). To a solution of the intermediate **4** (2.4 g, 6.4 mmol) in 20 mL acetone was added K_2CO_3 (2.7 g, 19.2 mmol) and $BrCH_2NO_2$ (672 μL, 9.6 mmol) at room temperature and the reaction mixture was stirred for 1.5 hours. The reaction mixture was filtered and concentrated in vacuum and the residuewas stirred in 30 mL acetic anhydride for overnight at 140 °C. Then the reaction mixture was cooled and concentrated in vacuum. The residue was purified by column chromatography on silica gel (petroleum: ethyl acetate=4:1) to obtained intermediate **5** (2 g, 60%).^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (s, 1H), 6.24 (s, 1H), 4.30 – 4.14 (m, 2H), 3.43 – 3.27 (m, 2H), 3.06 (t, *J* = 6.8 Hz, 2H), 2.76 – 2.61 (m, 2H), 1.98 (t, *J* = 6.8 Hz, 2H), 1.50 (s, 6H), 1.31 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.0, 159.2, 156.4, 153.9, 153.7, 152.6, 149.5, 114.1, 108.8, 107.7, 106.1, 102.1, 78.3, 60.8, 34.3, 32.1, 30.6, 26.5, 16.5, 14.3.

Synthetic procedure of intermediate 3-(9,9-dimethyl-2-nitro-5-oxo-10,11-dihydro-5H,9H-furo[2,3-f]pyrano[2,3-h]chromen-7-yl)propanoic acid (6). The intermediate **5** was dissolved in 30mL 6N hydrochloric acid to (1.3 g, 3 mmol) and stirred overnight at 100℃. The reaction monitored by LCMS until all the starting material was consumed completely, then the reaction mixture was filtered and washed with methanol to obtain intermediate **6** (0.93 g 50%). ^{1}H NMR (400 MHz, DMSO) δ 8.31 (s, 1H), 6.30 (s, 1H), 3.32 (s, 2H), 2.93 (t, *J* = 6.7 Hz, 2H), 2.59 – 2.51 (m, 2H), 1.92 (t, *J* = 6.7 Hz, 2H), 1.41 (s, 6H). ^{13}C NMR (101 MHz, DMSO) δ 173.7, 158.8, 157.0, 154.0, 153.6, 152.6, 149.3, 114.2, 108.7, 107.6, 107.4, 102.4, 78.7, 49.1, 34.4, 31.9, 30.2, 26.4, 16.4.

Synthetic procedure of intermediate 3-(9,9-dimethyl-2-nitro-5-oxo-10,11-dihydro-5H,9H-furo[2,3-f]pyrano[2,3-h]chromen-7-yl)-N-(prop-2-yn-1-yl)propenamide (7). To a solution of EDCI (385 mg, 2 mmol) and HOBt (270 mg, 2 mmol) in 20 ml anhydrous dichloromethane was added the intermediate **6** (0.6 g, 1.5 mmol) and stirred for half an hour, then propargylamine (110 mg, 2 mmol) and DIPEA (774 mg, 6 mmol) were added, continue stirring for 2 hours. The reaction monitored by LCMS until all the starting material was consumed completely, then the reaction mixture was concentrated in vacuum. The residue was purified by column chromatography on silica gel (petroleum: ethyl acetate=4:1) to obtain intermediate **7** (41 mg 65%).^{1}H NMR (400 MHz, DMSO) δ 8.34 (s, 1H), 8.32 (s, 1H), 6.25 (s, 1H), 3.89 (dd, *J* = 5.3,

2.3 Hz, 2H), 3.18 (dd, *J* = 10.3, 4.8 Hz, 2H), 3.10 (t, *J* = 2.3 Hz, 1H), 2.94 (t, *J* = 6.6 Hz, 2H), 2.45 (t, *J* = 7.6 Hz, 2H), 1.93 (t, *J* = 6.6 Hz, 2H), 1.43 (s, 6H). ^{13}C NMR (101 MHz, DMSO) δ 170.8, 158.9, 157.6, 154.2, 153.7, 152.7, 149.4, 114.1, 108.6, 107.7, 107.5, 102.4, 81.6, 78.8, 73.4, 35.4, 32.2, 30.3, 28.3, 26.5, 16.4.

Synthetic procedure of compound NFC-Tre-1. To a solution of terminal alkyne (0.23 mmol) and Tre-N_3 (90 mg, 0.23 mmol, the intermediate synthesized according to reported literature[1]) in 3 ml of DMF was stirred for 10 minutes, then Vc-Na (20 mg, 0.1 mmol) and copper sulfate (37.5 mg, 0.15 mmol) were added. The reaction monitored by LCMS until all the starting material was consumed completely, then the reaction mixture was concentrated in vacuum. The residue was purified by HPLC to obtain compound **NFC-Tre-1** (81 mg, 45%). ^{1}H NMR (400 MHz, DMSO) δ 8.39 (t, *J* = 5.4 Hz, 1H), 8.33 (s, 1H), 7.81 (s, 1H), 6.25 (s, 1H), 5.29 (d, *J* = 5.3 Hz, 1H), 4.95 (d, *J* = 4.9 Hz, 1H), 4.84 (d, *J* = 3.4 Hz, 1H), 4.81 – 4.73 (m, 3H), 4.69 (d, *J* = 6.1 Hz, 1H), 4.62 – 4.53 (m, 2H), 4.43 (dd, *J* = 14.3, 7.5 Hz, 1H), 4.32 (dd, *J* = 11.6, 5.6 Hz, 3H), 4.12 (t, *J* = 7.7 Hz, 1H), 3.66 – 3.48 (m, 4H), 3.46 – 3.39 (m, 1H), 3.20 (dd, *J* = 8.8, 5.1 Hz, 4H), 3.09 (td, *J* = 9.3, 5.2 Hz, 1H), 3.01 – 2.90 (m, 3H), 2.48 – 2.43 (m, 2H), 1.91 (t, *J* = 6.5 Hz, 2H), 1.39 (s, 6H). ^{13}C NMR (101 MHz, DMSO) δ 170.7, 158.4, 157.3, 153.8, 153.2, 152.1, 148.8, 144.6, 123.3, 113.5, 108.1, 107.2, 107.0, 101.9, 93.4, 78.4, 72.9, 72.6, 71.5, 71.4, 71.3, 70.1, 69.8, 60.8, 56.1, 50.7, 35.0, 34.2, 31.8, 29.8, 26.0, 18.6, 15.9. HRMS (ESI): m/z (M+H$^+$) calcd for $C_{34}H_{42}O_{17}N_5$, 792.2570, found: 792.2551.



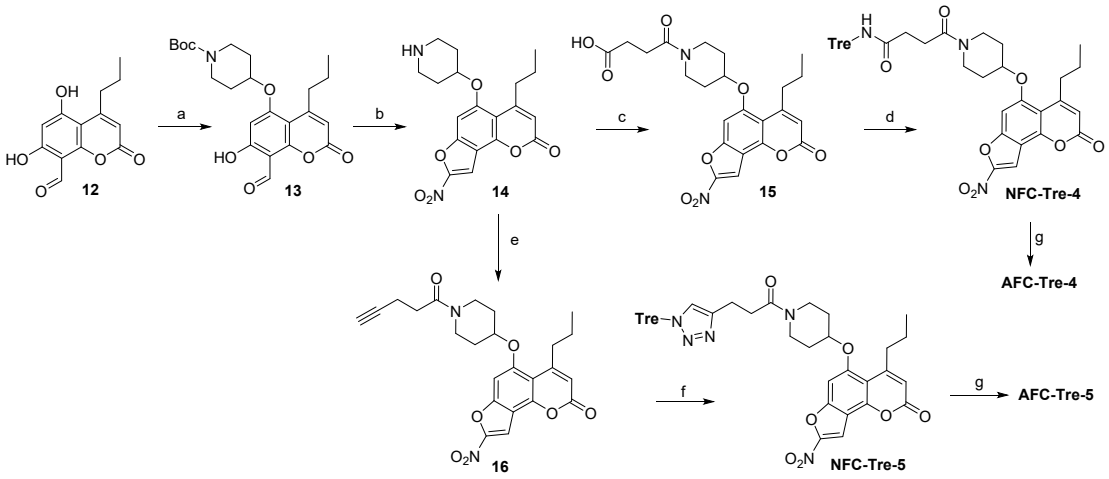
Scheme S3. Synthesis of Compounds NFC-Tre-2, **NFC-Tre-3**, **AFC-Tre-2** and **AFC-Tre-**

3. Reagents and conditions:(a) THF, PPh_3, DIAD, rt, overnight, 30%; (b) acetone, $BrCH_2NO_2$, K_2CO_3, rt, 3h; (c) 6N HCl, reflux, overnight, 50%; (d) DCM, Boc_2O, DIPEA, rt, 3h, 65%; (e) DCM, propargulamine, EDCI, HOBt, DIPEA, rt, 2h, 55-65%; (f) DMF, Tre-N_3,Vc-Na, $CuSO_4$, H_2O, rt, 30min, 50%; (g) DCM, TFA, rt, 1h, 80-90%; (h) Fe, NH_4Cl, EtOH/H2O, rt, overnight, 9%-15%.

Synthetic procedure of compounds NFC-Tre-2 and NFC-Tre-3. The compounds **NFC-Tre-2** and **NFC-Tre-3** were synthesized according to the synthetic procedure of compounds **NFC-Tre-1.**

NFC-Tre-2 ^{1}H NMR (400 MHz, DMSO) δ 8.44 – 8.32 (m, 2H), 7.79 (s, 1H), 7.65 (s, 1H), 6.26 (s, 1H), 5.28 (d, *J* = 5.1 Hz, 1H), 4.94 (d, *J* = 4.5 Hz, 1H), 4.88 – 4.81 (m, 2H), 4.78 – 4.73 (m, 2H), 4.67 (d, *J* = 6.1 Hz, 1H), 4.62 – 4.52 (m, 2H), 4.43 (dd, *J* = 14.4, 7.7 Hz, 1H), 4.33– 4.27 (m, 2H), 4.11 (t, *J* = 8.2 Hz, 1H), 3.81 (d, *J* = 13.3 Hz, 2H), 3.66 – 3.48 (m, 4H), 3.46 – 3.39 (m, 1H), 3.25 – 3.17 (m, 4H), 3.15 – 3.04 (m, 3H), 3.02 – 2.92 (m, 1H), 2.46 (d, *J* = 7.5 Hz, 2H), 2.08 (d, *J* = 10.3 Hz, 2H), 1.65 (d, *J* = 9.2 Hz, 2H), 1.41 (s, 9H). ^{13}C NMR (101 MHz, DMSO) δ 170.6, 158.2, 157.8, 157.2, 155.1, 153.9, 152.3, 150.6, 144.6, 123.2, 113.4, 109.1, 107.6, 106.6, 93.3, 93.1, 78.9, 75.3, 72.8, 72.6, 71.5, 71.4, 71.3, 70.1, 69.8, 60.8, 50.7, 34.58, 34.2, 31.9, 29.9, 28.1. HRMS (ESI): m/z ($M+H^+$) calcd for $C_{39}H_{51}O_{19}N_6$, 907.3203, found: 907.3186.

NFC-Tre-3 ^{1}H NMR (400 MHz, DMSO) δ 4.87 – 4.77 (m, 2H), 7.83 (s, 1H), 7.66 (s, 1H), 6.29 (s, 1H), 5.32 (d, *J* = 4.7 Hz, 1H), 5.01 (d, *J* = 4.0 Hz, 1H), 4.93 (s, 1H), 4.87 – 4.77 (m, 4H), 4.71 (d, *J* = 5.6 Hz, 1H), 4.62 – 4.54 (m, 2H), 4.43 (dd, *J* = 14.6, 7.8 Hz, 1H), 4.37 – 4.30 (m, 2H), 4.14 – 4.07 (m, 1H), 3.66 – 3.47 (m, 5H), 3.46 – 3.40 (m, 1H), 3.26 – 3.17 (m, 4H), 3.10 (d, *J* = 8.4 Hz, 3H), 3.01 – 2.93 (m, 1H), 2.30 – 2.20 (m, 2H), 2.02 – 1.92 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 170.7, 158.2, 157.5, 157.1, 155.0, 152.4, 150.6, 123.4, 113.3, 109.4, 107.6, 106.5, 93.3, 93.2, 79.3, 79.0, 78.7, 72.9, 72.6, 72.3, 71.5, 71.4, 71.3, 70.1, 69.8, 60.7, 56.1, 50.8, 41.2, 34.5, 34.2, 32.0, 26.9, 18.6. HRMS (ESI): m/z ($M+H^+$) calcd for $C_{34}H_{43}O_{17}N_6$, 807.2679, found: 807.2668.



Scheme S4. **Synthesis of NFC-Tre-4 and NFC-Tre-5.** Reagents and conditions:(a) THF, 1-Boc-4-piperidinol, PPh_3, DIAD, rt, overnight, 68%; (b) i, NO_2CH_2Br, K_2CO_3, acetone, rt, 3 h; ii, TFA, 80°C, 1 h, 50%; (c) Toluene, Succinic anhydride, DIPEA, 110°C, 3 h, 82%; (d) DMF, Tre-NH_2, EDCI, HOBt, DIPEA, rt, 55%.; (e) DMF, Pentynoic acid, EDCI, HOBt, DIPEA, rt, 87%; (f) DMF, Tre-N_3,Vc-Na, $CuSO_4$, H_2O, rt, 30min, 89%; (g) Fe, NH_4Cl, EtOH/H2O, rt, overnight, 8%-10%.

Synthetic procedure of intermediate tert-butyl 4-((8-formyl-7-hydroxy-2-oxo-4-propyl-2H-chromen-5-yl)oxy)piperidine-1-carboxylate (13). Intermediate **12** was synthesized according to our previous report[2]. To a solution of Intermediate **12** (6.45 g, 26 mmol) and the 1-Boc-4-piperidinol (5.2 g, 26 mmol) in 150 mL of THF was added PPh_3 (10.2 g, 39 mmol) and diisopropyl azodiformate (8.9 g, 44 mmol) at 0°C. The reaction mixture was stirred for overnight, then concentrated in vacuum. The residue was purified by column chromatography on silica gel (petroleum: ethyl acetate=4:1) to obtain intermediate **13** (7.6 g, 68%).

Synthetic procedure of intermediate 8-nitro-5-(piperidin-4-yloxy)-4-propyl-2H-furo[2,3-h]chromen-2-one (14). To a solution of intermediate **13** (3.4 g, 8 mmol) in 40 mL acetone was added K_2CO_3 (4.5 g, 32 mmol) and $BrCH_2NO_2$ (840 μL, 12 mmol) at room temperature and the reaction mixture was stirred for 1.5 hours. The reaction mixture was filtered and concentrated in vacuum, then the residue was treated with 30 mL CF_3COOH and was stirred for 1 hour at 80 °C. Then the reaction mixture was cooled, then the residue was recrystallization in methanol to obtain the crude product **14** that can be used in the next step without purification.

Synthetic procedure of intermediate 3-(5-((1-(tert-butoxycarbonyl)piperidin-4-yl)oxy)-8-nitro-2-oxo-2H-furo[2,3-h]chromen-4-yl)propanoic acid (15). To a solution of intermediate

14 (1.4 g, 3.7 mmol) in 20 mL toluene was added succinic anhydride (0.55 g, 5.5 mmol) and DIPEA (900 mg, 7 mmol). The reaction mixture was stirred for 3h at 110℃, then concentrated in vacuum. The residue was recrystallization in ether to obtain intermediate **15** (1.5 g, 82%).

Synthetic procedure of compound NFC-Tre-4. To a solution of intermediate **15** (94 mg, 0.2 mmol) in 20 ml anhydrous dichloromethane was added EDCI (39 mg, 0.2 mmol) and HOBt (27 mg, 0.2 mmol), stirred for half an hour, then added Tre-NH_2 (68 mg, 0.2 mmol, the intermediate synthesized according to reported literature[1]) and DIPEA (77 mg, 0.6 mmol), continue stirring for 2 hours. Upon completion as shown by LC-MS, the reaction mixture was concentrated in vacuum. The residue was purified by HPLC to obtain intermediate **NFC-Tre-4** (87 mg, 55%). ^{1}H NMR (400 MHz, DMSO) δ 8.33 (s, 1H), 7.77 (s, 1H), 7.67 (s, 1H), 6.30 (s, 1H), 4.95 – 4.89 (m, 3H), 4.85 (d, *J* = 12.0 Hz, 2H), 4.79 – 4.75 (m, 2H), 4.71 – 4.61 (m, 3H), 4.37 – 4.31 (m, 1H), 4.14 (d, *J* = 13.3 Hz, 1H), 3.86 (d, *J* = 11.5 Hz, 1H), 3.76 – 3.70 (m, 1H), 3.67 – 3.62 (m, 1H), 3.58 – 3.44 (m, 5H), 3.30 – 3.21 (m, 3H), 3.17 – 3.04 (m, 3H), 3.99 – 3.89 (m, 3H), 2.63 – 2.55 (m, 2H), 2.42 – 2.31 (m, 2H), 2.16 (dd, *J* = 25.8, 11.4 Hz, 2H), 1.69 (d, *J* = 9.1 Hz, 1H), 1.64 – 1.53 (m, 3H), 0.96 (t, *J* = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 172.2, 172.1, 170.0, 158.3, 158.0, 157.8, 155.0, 152.3, 150.7, 113.3, 109.2, 107.7, 106.6, 93.4, 93.3, 93.1, 75.2, 72.7, 72.5, 71.7, 71.6, 71.4, 70.5, 70.1, 60.8, 42.5, 38.1, 30.6, 30.4, 30.0, 27.9, 22.5, 13.6. HRMS (ESI): m/z ($M+H^+$) calcd for $C_{35}H_{46}O_{18}N_3$, 796.2771, found: 796.2756.

Synthetic procedure of compound NFC-Tre-5. Compound **NFC-Tre-5** was synthesized according to **NFC-Tre-1.** ^{1}H NMR (400 MHz, DMSO) δ 8.28 (s, 1H), 7.78 (s, 1H), 7.62 (s, 1H), 6.27 (s, 1H), 5.31 (d, *J* = 5.2 Hz, 1H), 4.97 (d, *J* = 4.6 Hz, 1H), 4.90 (s, 1H), 4.83 (d, *J* = 3.5 Hz, 1H), 4.81 – 4.77 (m, 3H), 4.59 – 4.52 (m, 2H), 4.42 – 4.34 (m, 2H), 4.19 – 4.08 (m, 2H), 3.85 (d, *J* = 13.1 Hz, 1H), 3.64 – 4.52 (m, 5H), 3.26 – 3.16 (m, 3H), 3.12 – 3.06 (m, 2H), 3.03 – 2.96 (m, 1H), 2.95 – 2.89 (m, 2H), 2.87 – 2.82 (m, 2H), 2.71 (d, *J* = 7.4 Hz, 2H), 2.22 – 2.09 (m, 2H), 1.66 (d, *J* = 7.1 Hz, 1H), 1.58 (dt, *J* = 14.8, 7.4 Hz, 4H), 0.95 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 170.2, 158.8, 158.4, 158.3, 155.5, 152.7, 151.1, 146.5, 122.8, 113.8, 109.6, 108.1, 107.1, 93.7, 93.5, 75.6, 73.3, 73.1, 72.0, 71.9, 71.7, 70.5, 70.3, 70.2, 61.1, 51.2, 42.9, 38.5, 32.5, 31.1, 30.4, 22.9, 21.5, 14.0. HRMS (ESI): m/z ($M+H^+$) calcd for $C_{36}H_{46}O_{18}N_5$, 820.2883, found: 820.2870.

The general route of the NFC-Tre reduction products

NFC-Tre (1eq) was dissolved in ethanol and reduced iron powder (10eq) was added. Then ammonium chloride (10eq) dissolved in water was added and stirred overnight. After the reaction was completed, the iron mud was removed by filtration, and the filtrate was separated and purified by HPLC.

AFC-Tre-1

^{1}H NMR (800 MHz, DMSO) δ 10.21 (s, 1H), 8.36 (s, 1H), 7.80 (s, 1H), 5.95 (s, 1H), 5.28 (s, 1H), 4.95 – 4.88 (m, 1H), 4.84 (s, 1H), 4.81 – 4.66 (m, 4H), 4.60 – 4.5 (m, 2H), 4.43 (dd, J = 14.0, 7.4 Hz, 1H), 4.29 (d, J = 4.4 Hz, 2H), 4.13 – 4.10 (m, 1H), 3.84 (s, 2H), 3.64 – 3.61 (m, 1H), 3.59 – 3.56 (m, 1H), 3.54 – 3.50 (m, 2H), 3.45 – 3.42 (m, 2H), 3.21 (d, J = 7.3 Hz, 2H), 3.14 – 3.08 (m, 4H), 2.98 (t, J = 8.8 Hz, 1H), 2.64 – 2.61 (m, 2H), 2.44 – 2.41 (m, 2H), 1.80 – 1.76 (m, 2H), 1.30 (s, 6H).^{13}C NMR (201 MHz, DMSO) δ 170.8, 159.3, 157.6, 156.5, 152.2, 152.0, 144.7, 144.7, 123.2, 118.5, 109.9, 105.6, 102.5, 97.5, 93.3, 93.2, 75.9, 72.8, 72.6, 71.5, 71.3, 71.3, 70.0, 69.7, 60.7, 50.7, 35.1, 34.2, 31.9, 30.6, 25.9, 17.4, 11.5. HRMS (ESI): m/z ($M+H^+$) calcd for $C_{34}H_{44}O_{15}N_5$, 762.2828, found: 762.2820.

AFC-Tre-2

^{1}H NMR (400 MHz, DMSO) δ 8.37 (s, 1H), 7.80 (s, 1H), 6.55 (s, 1H), 5.94 (s, 1H), 5.29 (s, 1H), 4.95 (s, 1H), 4.84 (s, 1H), 4.81 – 4.73 (m, 3H), 4.71 – 4.66 (m, 1H), 4.62 – 4.55 (m, 3H), 4.48 – 4.39 (m, 1H), 4.31 – 4.25 (m, 2H), 4.16 – 4.00 (m, 2H), 3.77 (s, 2H), 3.73 – 3.67 (m, 2H), 3.66 – 3.49 (m, 6H), 3.46 – 3.43 (m, 1H), 3.23 – 3.19 (m, 2H), 3.15 – 3.11 (m, 3H), 3.01 – 2.05 (m, 1H), 2.45 – 2.40 (m, 2H), 2.02 – 1.94 (m, 2H), 1.68 – 1.58 (m, 2H), 1.41 (s, 9H).^{13}C NMR (201 MHz, DMSO) δ 170.8, 159.1, 157.4, 155.5, 154.4, 153.8, 144.6, 123.2, 118.3, 109.2, 105.4, 102.4, 97.5, 96.6, 93.3, 91.3, 87.9, 78.9, 73.8, 72.8, 72.6, 72.6, 71.5, 71.3, 71.3, 70.1, 69.7, 60.8, 50.7, 40.0, 34.7, 34.2, 31.7, 29.8, 28.1, 11.0. HRMS (ESI): m/z ($M+H^+$) calcd for $C_{39}H_{53}O_{17}N_6$, 877.3462, found: 877.3448.

AFC-Tre-3

^{1}H NMR (400 MHz, DMSO) δ 8.41 (s, 1H), 7.82 (s, 1H), 6.67 (s, 1H), 5.79 (s, 1H), 4.83 (s, 1H), 4.68 – 4.49 (m, 3H), 4.48 – 4.38 (m, 1H), 4.30 (s, 2H), 4.12 (t, *J* = 9.1 Hz, 1H), 3.72 (s, 2H), 3.65 – 3.40 (m, 7H), 3.22 (d, *J* = 6.4 Hz, 3H), 3.14 – 3.03 (m, 4H), 3.01 – 2.91 (m, 2H), 2.44 – 2.24 (m, 4H), 2.16 – 1.97 (m, 2H), 1.79 – 1.62 (m, 2H).^{13}C NMR (201 MHz, DMSO) δ 170.9, 160.0, 159.9, 159.9, 158.1, 157.9, 157.7, 155.4, 155.4, 144.7, 123.2, 93.3, 93.2, 72.8,

72.6, 72.5, 71.5, 71.4, 71.3, 70.0, 69.7, 60.7, 56.7, 56.0, 55.9, 50.7, 45.1, 35.1, 34.2, 31.8, 15.7, 11.0. HRMS (ESI): m/z ($M+H^+$) calcd for $C_{34}H_{45}O_{15}N_6$, 777.2937, found: 777.2927.

AFC-Tre-4

^1H NMR (400 MHz, DMSO) δ 7.72 (d, *J* = 4.4 Hz, 1H), 6.51 (s, 1H), 5.91 (s, 1H), 4.91 (d, *J* = 3.4 Hz, 1H), 4.87 – 4.85 (m, 1H), 4.76 (dd, *J* = 19.7, 3.4 Hz, 2H), 4.68 – 4.63 (m, 1H), 4.62 – 4.58 (m, 1H), 4.03 – 3.91 (m, 1H), 3.82 – 3.70 (m, 3H), 3.69 – 3.61 (m, 1H), 3.60 – 3.42 (m, 4H), 3.42 – 3.37 (m, 1H), 3.18 – 3.09 (m, 3H), 3.00 – 2.93 (m, 1H), 2.90 – 2.82 (m, 2H), 2.59 – 2.54 (m, 1H), 2.41 – 2.31 (m, 3H), 2.11 – 2.00 (m, 2H), 1.71 – 1.52 (m, 4H), 0.94 (t, *J* = 7.3 Hz, 3H). HRMS (ESI): m/z ($M+H^+$) calcd for $C_{35}H_{48}O_{16}N_3$, 766.3029, found: 766.3022.

AFC-Tre-5

^1H NMR (400 MHz, DMSO) δ 8.41 – 8.39 (m, 1H), 7.77 (s, 1H), 6.26 (s, 1H), 5.67 (s, 1H), 5.29 (s, 1H), 4.86 – 4.74 (m, 3H), 4.63 – 4.50 (m, 3H), 4.43 – 4.34 (m, 1H), 4.16 – 4.08 (m, 1H), 4.02 – 3.91 (m, 1H), 3.75 – 3.66 (m, 3H), 3.64 – 3.56 (m, 2H), 3.56 – 3.48 (m, 3H), 3.15 – 3.06 (m, 3H), 3.03 – 2.92 (m, 2H), 2.87 – 2.77 (m, 4H), 2.66 (dd, *J* = 10.5, 4.6 Hz, 2H), 2.07 – 1.93 (m, 2H), 1.61 – 1.46 (m, 4H), 0.93 (t, *J* = 6.2 Hz, 3H).^{13}C NMR (101 MHz, DMSO) δ 169.7, 156.0, 158.6, 155.5, 155.2, 146.1, 122.4, 119.3, 99.6, 98.6, 98.2, 98.1, 98.0, 93.6, 93.3, 78.6, 72.9, 72.7, 72.6, 71.6, 71.5, 71.3, 70.1, 69.8, 60.8, 50.7, 42.3, 38.1, 32.1, 30.8, 30.1, 22.9, 21.0, 13.8, 11.1. HRMS (ESI): m/z ($M+H^+$) calcd for $C_{36}H_{48}O_{15}N_5$, 790.3141, found: 790.3126.

NFC-Glc

Synthetic procedure of compound NFC-Glc. Compound **NFC-Glc** was synthesized according to **NFC-Tre-5.** ^1H NMR (400 MHz, DMSO) δ 8.33 (s, 1H), 7.81 (s, 1H), 7.67 (s, 1H), 6.30 (s, 1H), 5.46 – 5.32 (m, 1H), 5.03 – 4.89 (m, 2H), 4.87 – 4.81 (m, 1H), 4.71 – 4.62 (m, 1H), 4.49 (s, 1H), 4.36 – 4.25 (m, 1H), 4.21 – 4.10 (m, 1H), 3.93 – 3.77 (m, 1H), 3.66 (t, *J* = 9.2 Hz, 1H), 3.23 – 3.18 (m, 1H), 3.06 (d, *J* = 10.6 Hz, 1H), 3.02 – 2.97 (m, 4H), 2.96 – 2.91 (m, 2H), 2.89 – 2.81 (m, 2H), 2.76 – 2.68 (m, 2H), 2.24 – 2.08 (m, 2H), 1.70 – 1.53 (m, 4H), 0.96 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 170.1, 158.8, 158.4, 158.3, 155.5, 152.7, 151.1, 146.5, 123.4, 113.8, 109.6, 108.1, 107.0, 100.2, 93.5, 75.6, 73.5, 72.2, 72.1, 71.6, 71.0, 54.7, 51.2, 42.9, 32.3, 31.1, 30.4, 22.9, 21.4, 14.0.

2. Bacterial Growth Conditions and Strains

Mtb H37Rv, BCG and M. *smeg* were grown in Middlebrook 7H9 supplemented with 0.2% glycerol, 0.05% Tween-80, and 10% oleate-albumin-dextrose-catalase (OADC) enrichment. *Escherichia coli*, *Enterococcus faecalis*, *Listeria monocytogenes* and *Bacillus subtilis* were grown in LB medium, and *Staphylococcus aureus* was grown in THY medium. All these strains were grown in a shaking incubator at 37 °C and 200 rpm except for *Staphylococcus aureus*, which was grown in a static state. All experiments concerning *Mtb* were conducted in accordance with biosafety level III laboratory operating standards.

The bacteria were cultured to the log phase and incubated with 100 μM **NFC-Tre-1**, **NFC-Tre-5**, and **DMN-Tre** at 37 °C for 1 h, respectively. **Dil** stained M. *smeg* according to the manufacturer's protocol. A blank control was obtained in cells treated under the same conditions but without fluorescent probes. For *Mtb*, labeled cells were harvested by centrifugation (3000×g for 10 min) and then fixed in an equal volume of 4% paraformaldehyde at room temperature for 30 min.

3. Animals and Cell Culture

Male C57BL/6 mice (6−8 weeks old) were purchased from Vital River Experimental Animal Co., Ltd. (Beijing, China). Mice were housed in specific pathogen-free conditions with a 12 h light/dark cycle and free access to food and water in the animal research center laboratory of Tsinghua University. All animal protocols were conducted in compliance with the Institute of Animal Care and Use Committee (IACUC) of Tsinghua University approved based on institutional and national guidelines.

Mouse RAW 264.7 macrophages were cultured in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, CA, USA) with 10% (v/v) fetal bovine serum (FBS) (Gibco, Australia). Bone marrow-derived macrophages were prepared based on the general cultural method. All the cells were grown at 37 °C in a 5% CO_2 humidified incubator and guaranteed to be mycoplasma free.

4. The Fluorescence Intensity of Bacteria Was Analyzed by Flow Cytometry

Bacteria were labeled and analyzed by flow cytometry with a BD LSRFortessa equipped with a four-laser, 14-color analysis from Tsinghua University Instrument Sharing Platform. The pacific blue channel with excitation at 405 nm and filtered at 450/50 nm was selected to detect **NFC-Tre** fluorescence. The PE with excitation at 561 nm and filtered at 586/15 nm was selected to detect **Dil** fluorescence. Flow cytometry data analysis was performed using FlowJo v10 software. A paired sample t-test was also performed for fluorescence intensity to determine significant differences between each experimental group. Significance was determined at $P \leq 0.05$. Figures were prepared by GraphPad Prism v8.0.

5. Microscope for Fluorescence Imaging

In general, all the bacteria were labeled and then imaged. A DeltaVision wide-field fluorescence microscope was used in selective evaluation and nonreplication labeling experiments. An Olympus FV1000 confocal microscope was used in the *Mtb*-labeling experiments. An Andor Dragonfly Spinning Disk Confocal Microscope was used for the detection of intracellular mycobacteria. A Super-resolution Nikon SIM Microscope was used for fluorescence distribution imaging of M. *smeg*.

6. Visualizing Mycobacteria in Macrophages with NFC-Tre-5

Macrophages (2.5×10^5) were incubated with freshly cultured BCG strain and M. *smeg* at a multiplicity of infection (MOI) of 5 for 4 h in confocal microscopy dishes 15mm. Then, the cells were washed with PBS (1 mL×3), and a solution of **NFC-Tre-5** (100 μM) in DMEM was added and incubated for 15 min. The cells were washed and fixed in 4% paraformaldehyde for 30 min. Then, the nucleus and cell membrane were stained with NucRed™ Live 647 ReadyProbes™ dye and CellMask™ orange plasma dye, respectively, according to the manufacturer's protocol.

Mice were treated with $1x10^6$ CFU of mycobacteria by nasal infection, and alveolar lavage fluid was collected at 24 h. Alveolar macrophages were collected according to published literature.[3] Visualization of mycobacteria in host cells was accomplished with an Andor

Dragonfly confocal microscope, setting excitation at 405 nm and emission at 445/46 nm for cyan fluorescence, excitation at 561 nm and emission at 594/43 nm for red fluorescence, and excitation at 637 nm and emission at 698/77 nm for deep red fluorescence.

7. Figures

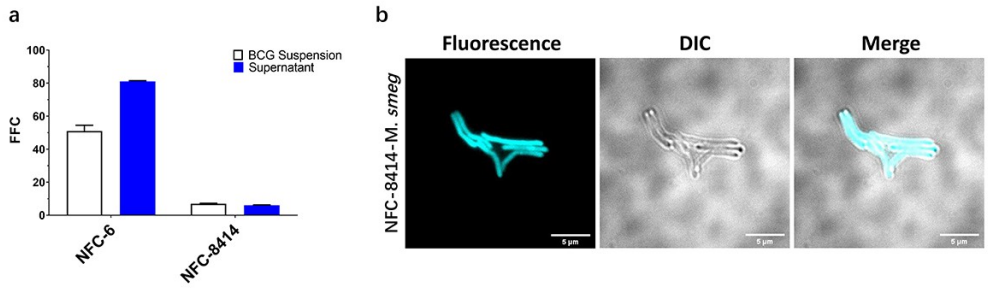


Fig. S1 NFC-8414 label single Mycobacteria. (**a**) Compound **NFC-6** and **NFC-8414** (5 μM) were incubated with BCG (OD=0.1) that were grown at 37 °C in Middlebrook 7H9 medium for 24h. The reactions were monitored in Ex (390 nm)/Em (470 nm) by EnVision® Multi-mode Plate Reader. **NFC-6** was described in previous work. (**b**) M. *smeg* were treated with 100 μM **NFC-8414** for 15min and then imaged.

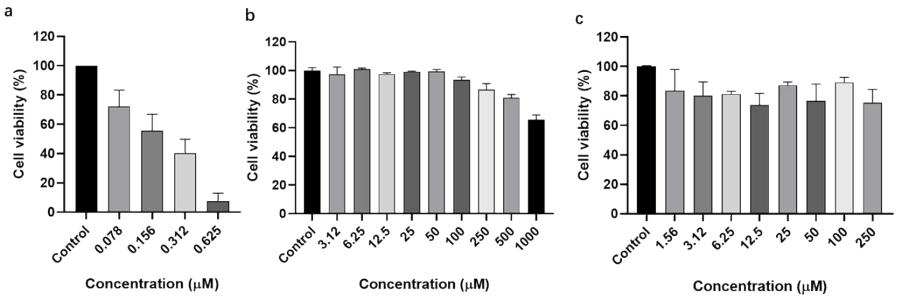


Fig. S2 Cell viability with MTT assay. The cell viability of RAW 264.7 macrophages with the treatment of **NFC-8414** (**a**), **NFC-Tre-5** (**b**) and **NFC-Glc** (**c**) for 24 h. The MTT assay is performed according to the reported literature[4]. Error bars represent standard deviation of three independent measurements.

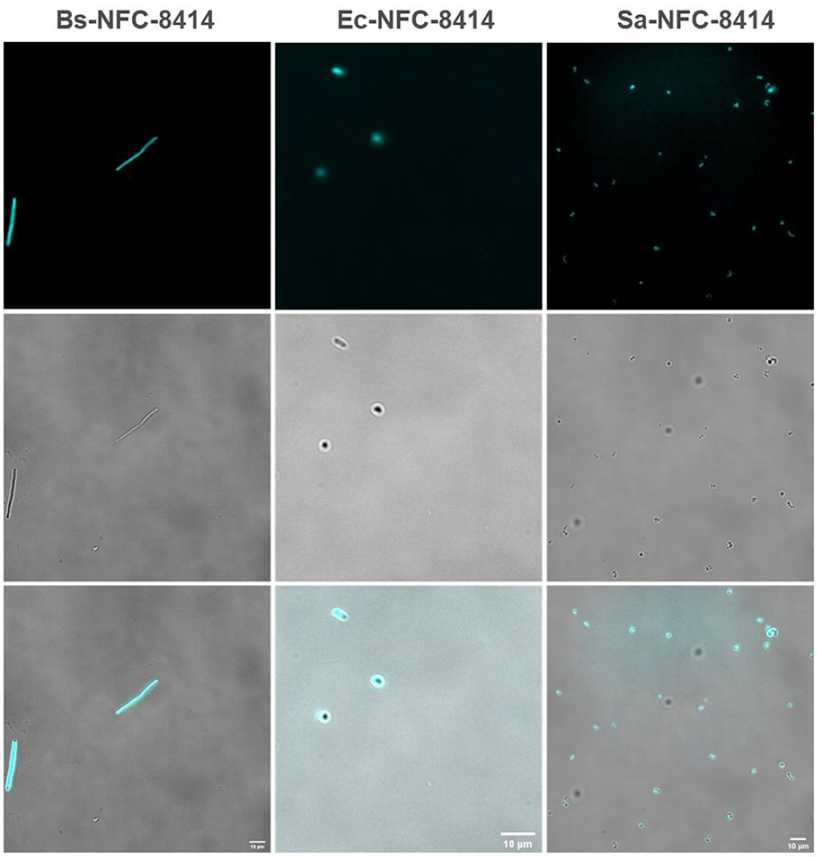


Fig. S3 Non-specific label bacteria with NFC-8414. Imaging of *Bacillus subtilis* (Bs), *Escherichia coli* (Ec) and *Staphylococcus aureus* (Sa) cells incubated with 100 μM **NFC-8414** for 1 h.

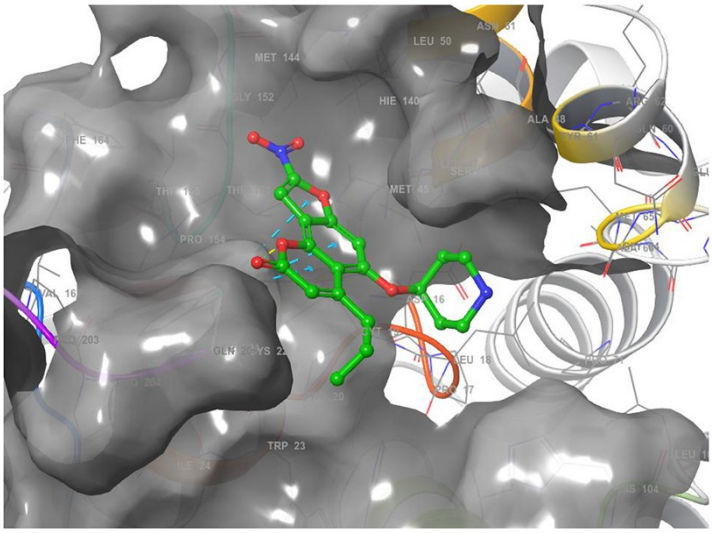
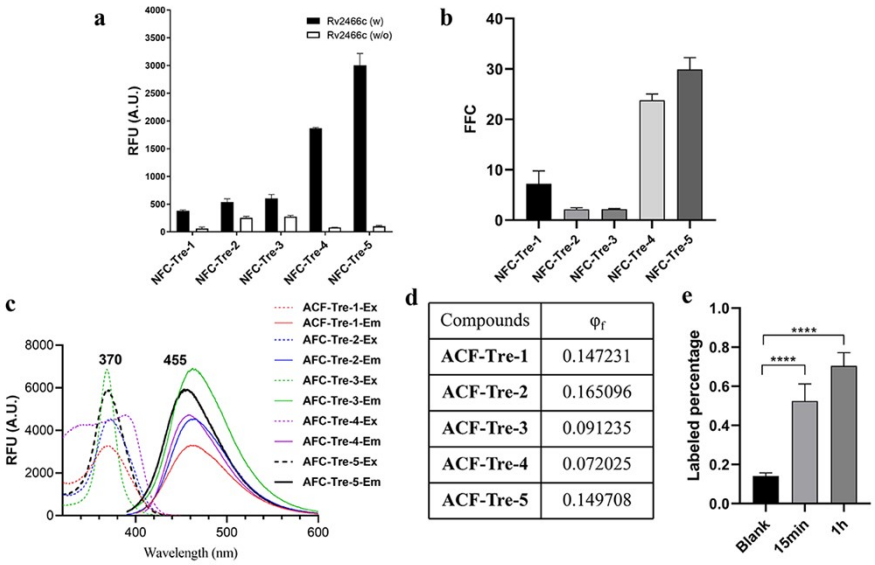


Fig. S4 Predicted binding mode of compound NFC-8414 with Rv2466c. NFC-8414 and the WT Rv2466c crystal structure (PDB ID: 4NXI)[5] was prepared using LigPrep and Protein Preparation Wizard,[6] respectively, in Maestro v11.7 (Schrodinger, Inc.: Maestro v11.7, 2018)

and docked using Glide with standard settings. Compound **NFC-8414** (green, sticks), Rv2466c (white, ribbon).



Compounds	φ_f
ACF-Tre-1	0.147231
ACF-Tre-2	0.165096
ACF-Tre-3	0.091235
ACF-Tre-4	0.072025
ACF-Tre-5	0.149708

Fig. S5 NFC-Tres are reduced by Rv2466c to produce fluorescence. (**a**) **NFC-Tre** are reduced by Rv2466c. Fluorescence data were collected with 200 μg/mL Rv2466c and 5 μM **NFC-Tre**. (**b**) The FFC with and without Rv2466c. (**c**) The spectra of compound **AFC-Tre-5**, λ_{ex} = 370 nm and λ_{em} = 455 nm. (**d**) Relative fluorescence quantum yield of compounds **AFC-Tre-1** to **AFC-Tre-5**. The φf of NFC-Tre was measured by means of a comparative method. **NFC-Tre** were dissolved in dd H_2O and quinine sulfate in 0.1 M H_2SO_4 was used as a reference.[7] (**e**) The ratio of the number of **NFC-Tre-5** labeled cells to mCherry (*Mycobacterium smegmatis*) M. *smeg* cells was analyzed after mCherry M. *smeg* cells were incubated with 100 μM **NFC-Tre-5** for 15 or 1h.

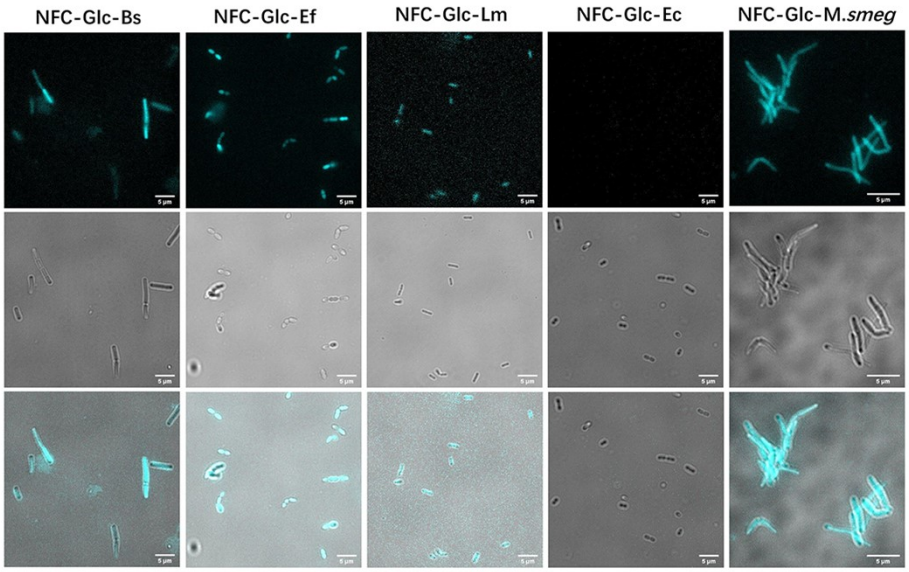


Fig. S6 Non-specific label bacteria with NFC-Glc. Imaging of Bacillus subtilis (Bs), Escherichia coli (Ec), Enterococcus faecalis (Ef), Listeria monocytogenes (Lm) and *Mycobacterium smegmatis* (M. *smeg*) cells incubated with 100 μM **NFC-Glc** for 1 h.

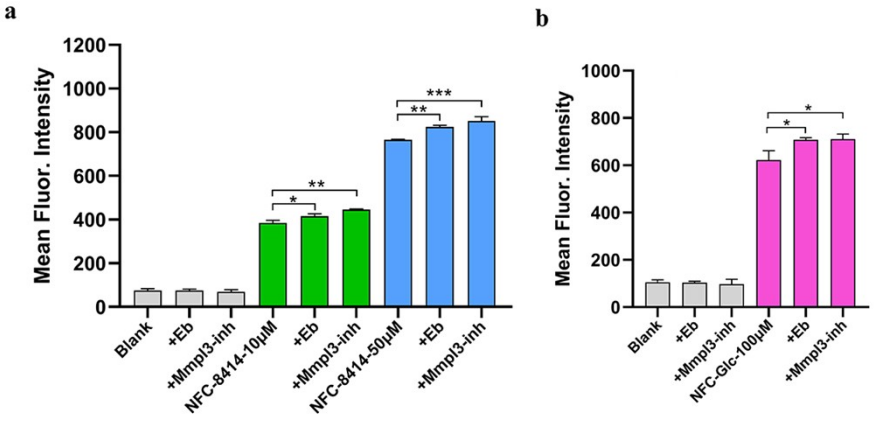


Fig. S7 (a) Flow cytometry analysis of the mean fluorescence intensity of M. *smeg* treated with or without 10μM NFC-8414 and 100 μM NFC-8414; Mmpl3 inhibitor 100 μM (Mmpl3-inh); preincubated ebselen 50 μg/mL (Eb) for 1h, respectively. **(b)** Flow cytometry analysis of the mean fluorescence intensity of M. *smeg* treated with or without 100μM NFC-Glc; Mmpl3 inhibitor 100 μM (Mmpl3-inh); ebselen 50 μg/mL (Eb) for 1h.

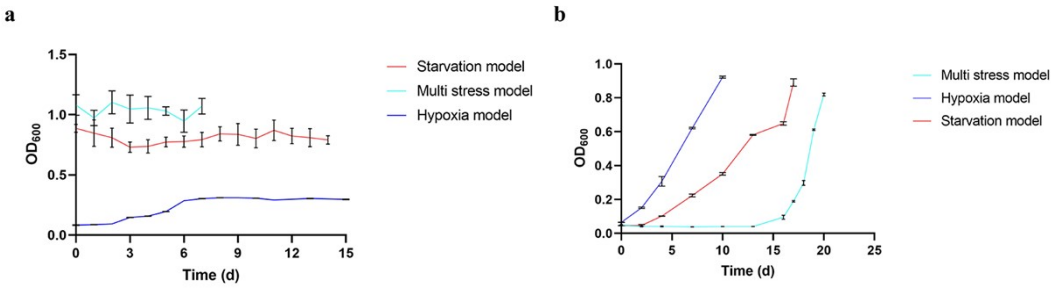


Fig. S8 Growth of BCG under different stress conditions. (**a**) For starvation culture model, BCG was grown to log-phase, harvested and resuspended in PBS plus 0.05% w/v tyloxapol to an OD_{600} of 0.9 and incubated standing for 2 weeks. For multiple stress culture model, BCG was grown to log-phase, and resuspended in PCB solution to an OD_{600} of 1.0 and incubated for 7 days. PCB solution was prepared by combining 448 mL of 0.2 M dibasic sodium phosphate with 552 mL of 0.1 M citric acid. The pH was adjusted to pH 4.5 with 0.2 M dibasic sodium phosphate or 0.1 M citric acid solution at room temperature. Tyloxapol was added to a final concentration of 0.05% v/v. BCG was grown to log-phase, then were diluted 10-fold in 7H9 medium and transferred to sealed tubes. The cultures were grown at 37 ℃ for 15 days. (**b**) Non-replicating BCG produced by different stress conditions harvested and resuspended in 7H9 medium, The cultures were grown under normal conditions for 10-20 days.

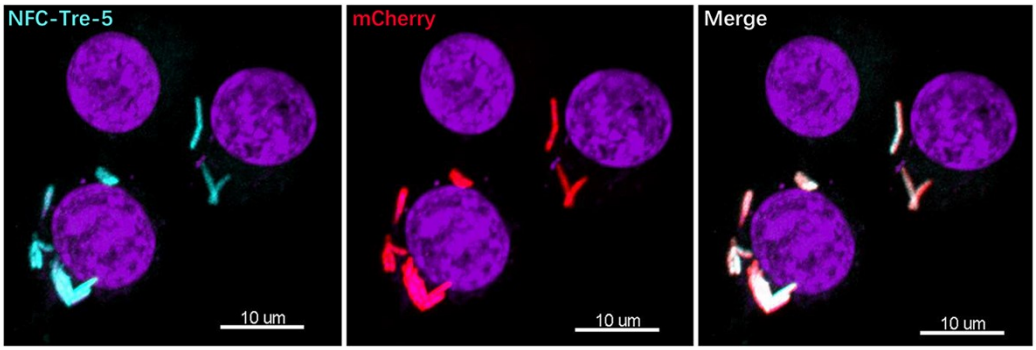


Fig. S9 Detecting live mycobacteria in macrophages with NFC-Tre-5. Prepared Raw 264.7 cells were incubated with freshly cultured mCherry M. *smeg* strain at a 1-to-5 ratio for 4 h and washed three times with PBS, then **NFC-Tre-5** (100 μM) was added and incubated for 15 min. Raw 264.7 cells were imaged with Nuclear dye (NucRed) according to the manufacturer's protocol.

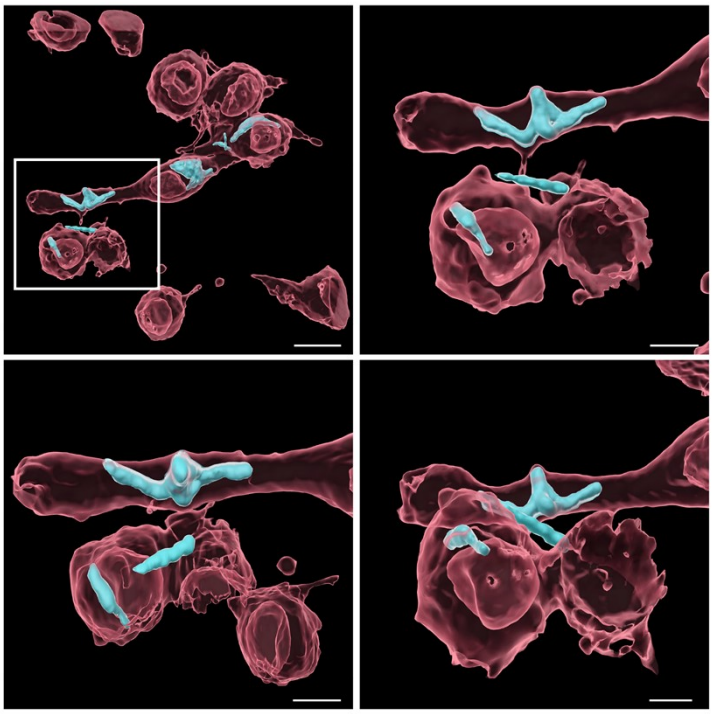
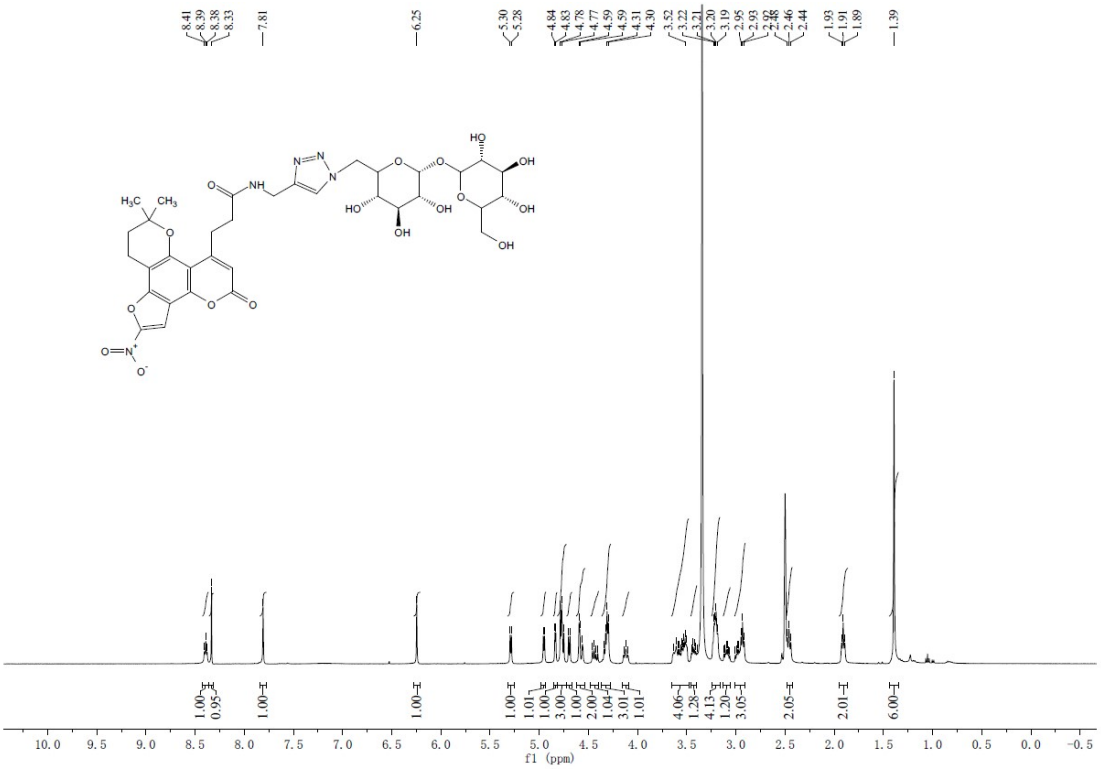


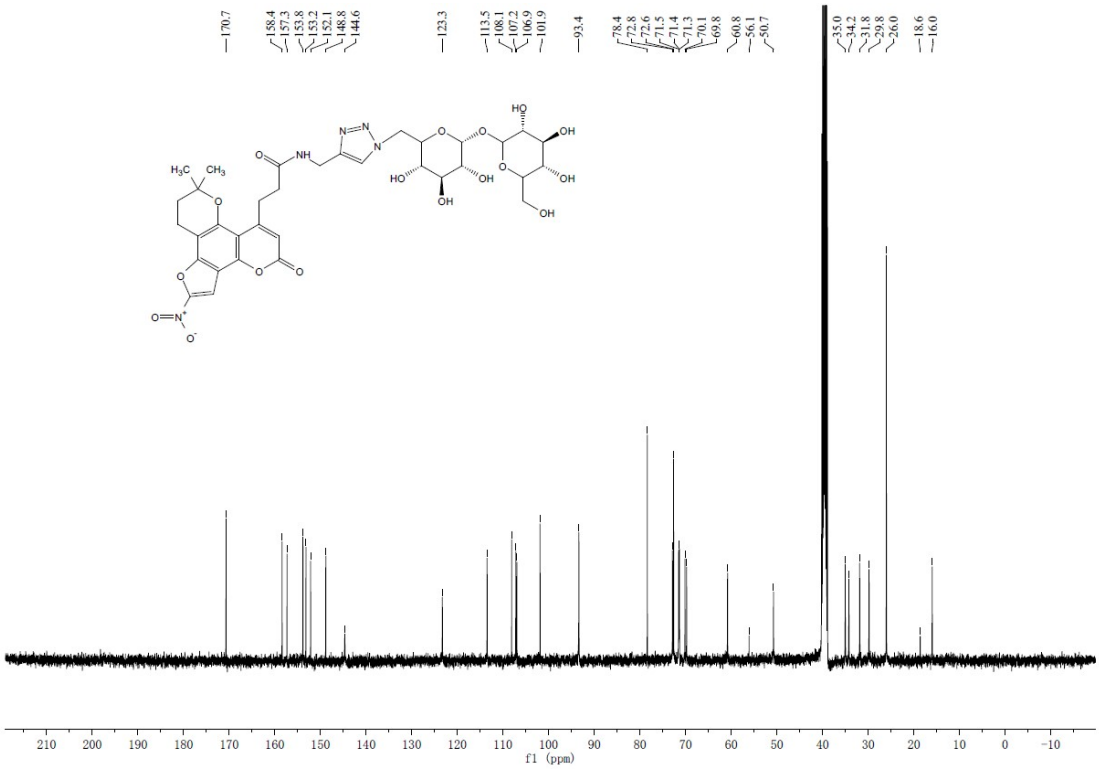
Fig. S10 Reconstructing the virtual surface of M. *smeg*-infected RAW 264.7 cells in Figure 5. Transparent red represents the cell membrane; solid cyan represents M. *smeg*.

9. NMR Spectra

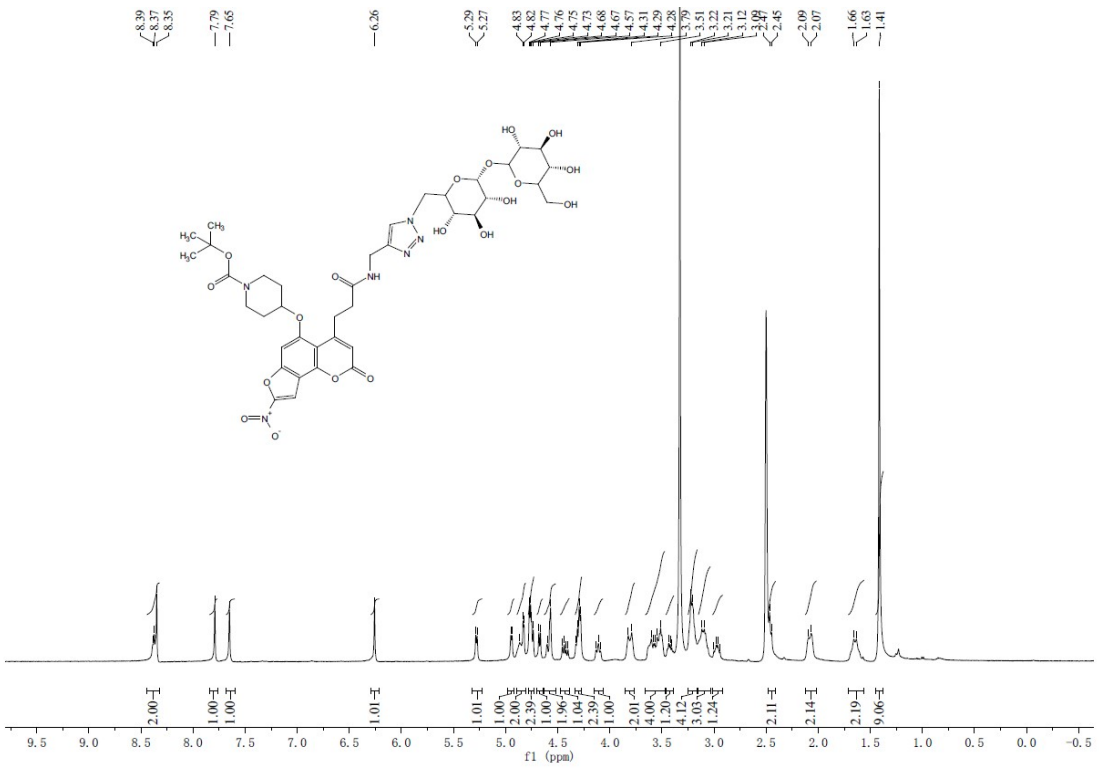
^{1}H NMR spectra of **NFC-Tre-1**.



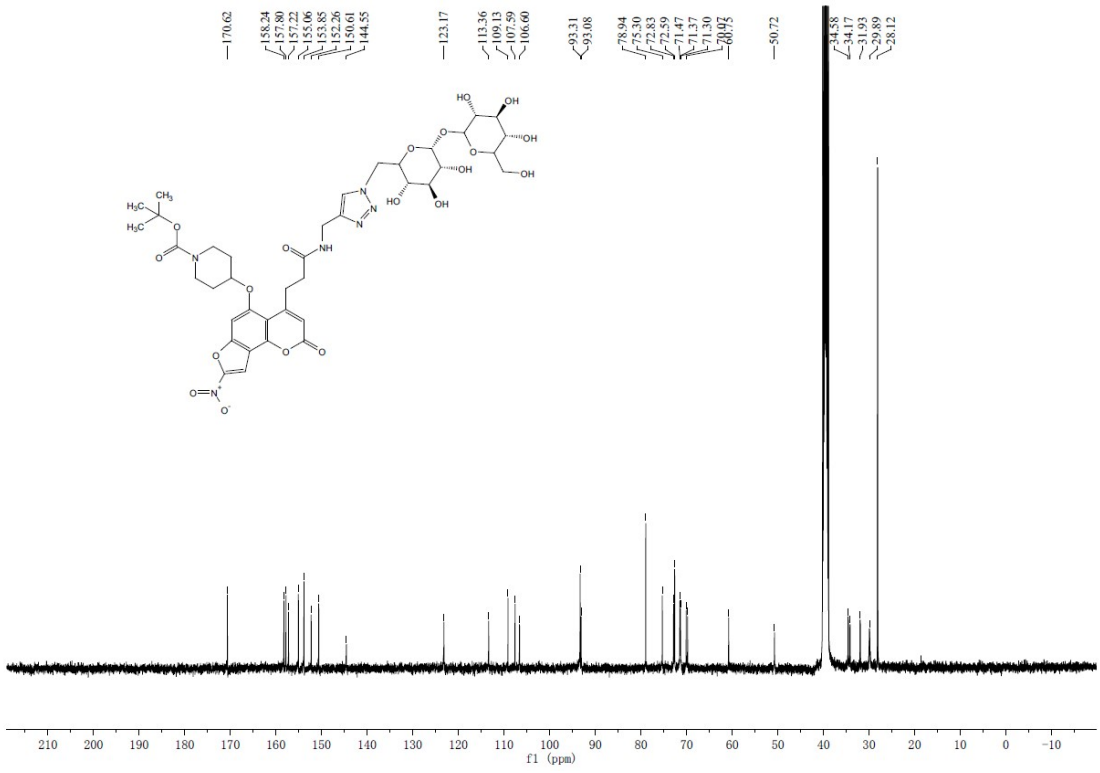
^{13}C NMR spectra of **NFC-Tre-1**



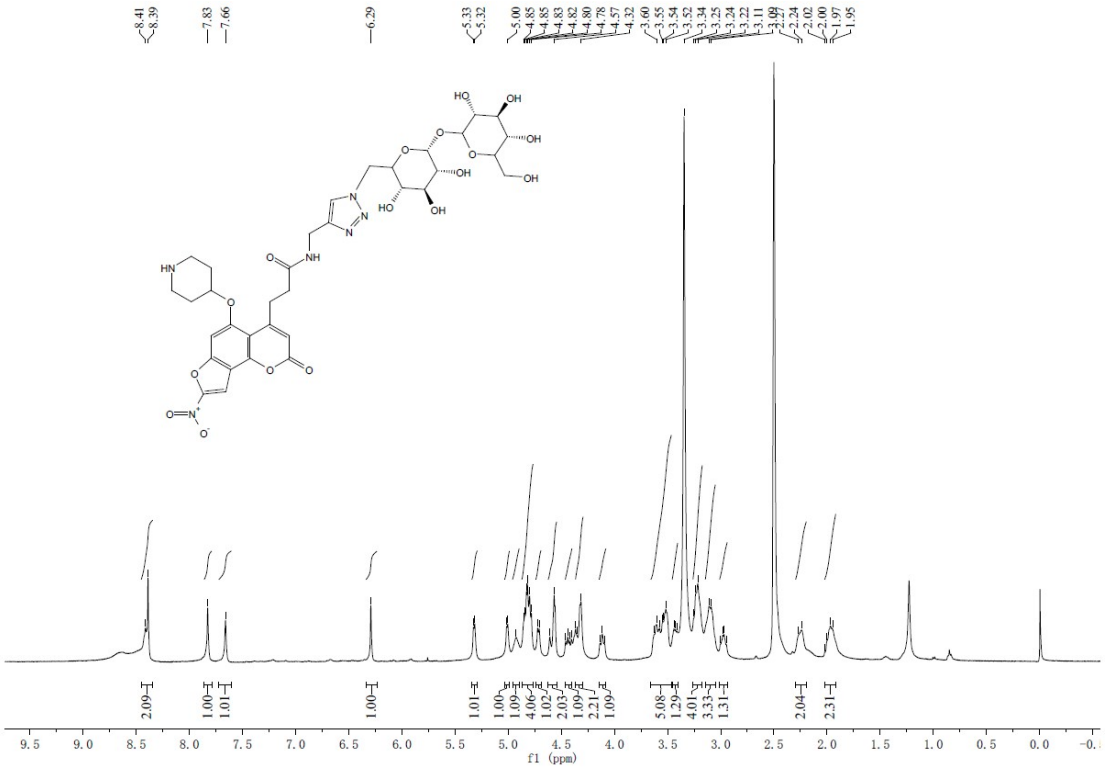
^{1}H NMR spectra of **NFC-Tre-2**



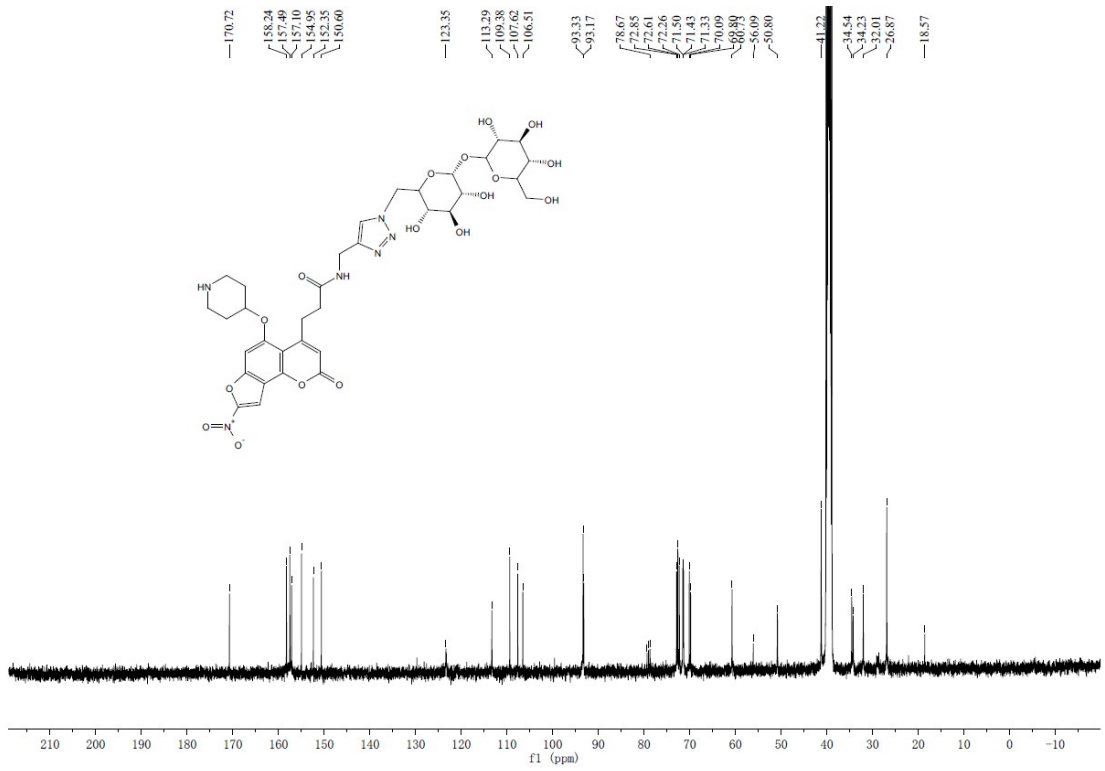
^{13}C NMR spectra of **NFC-Tre-2**



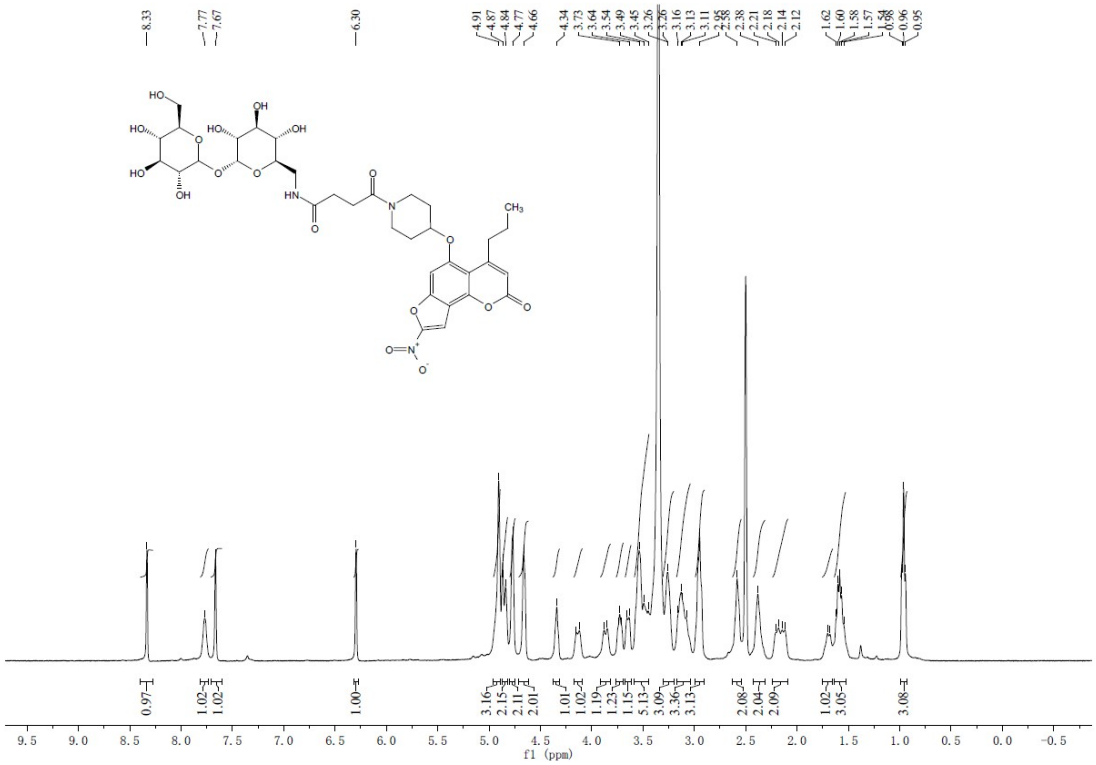
^{1}H NMR spectra of **NFC-Tre-3**



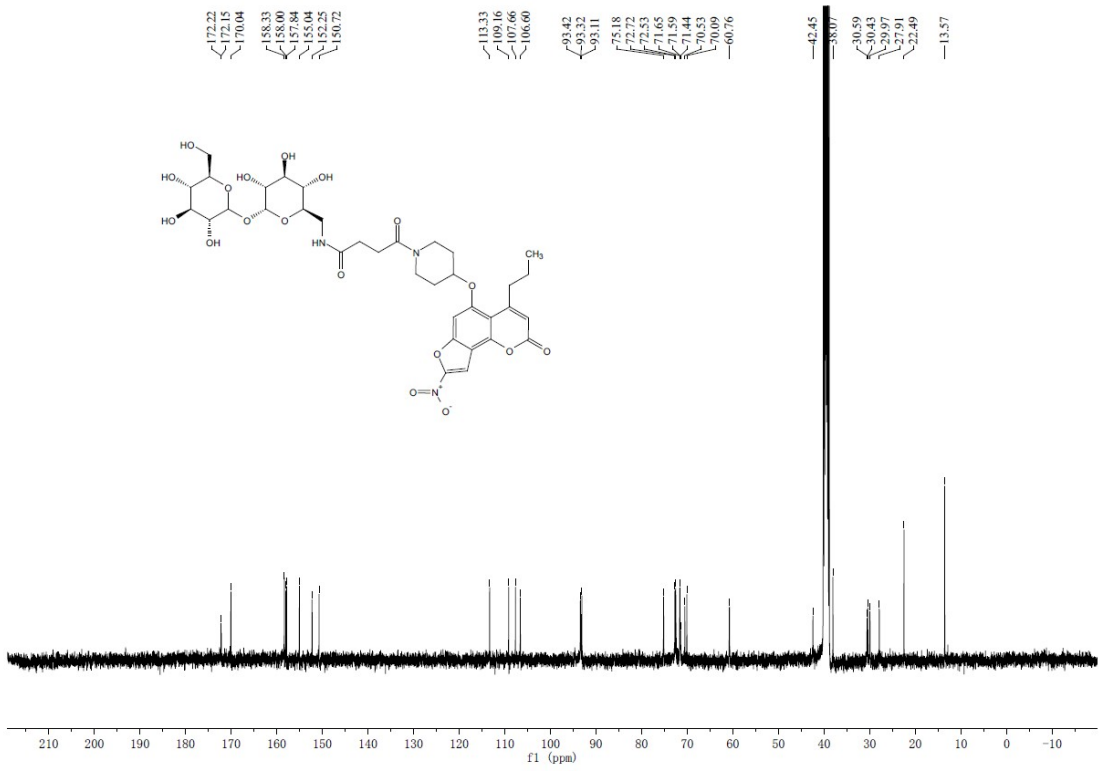
^{13}C NMR spectra of **NFC-Tre-3**



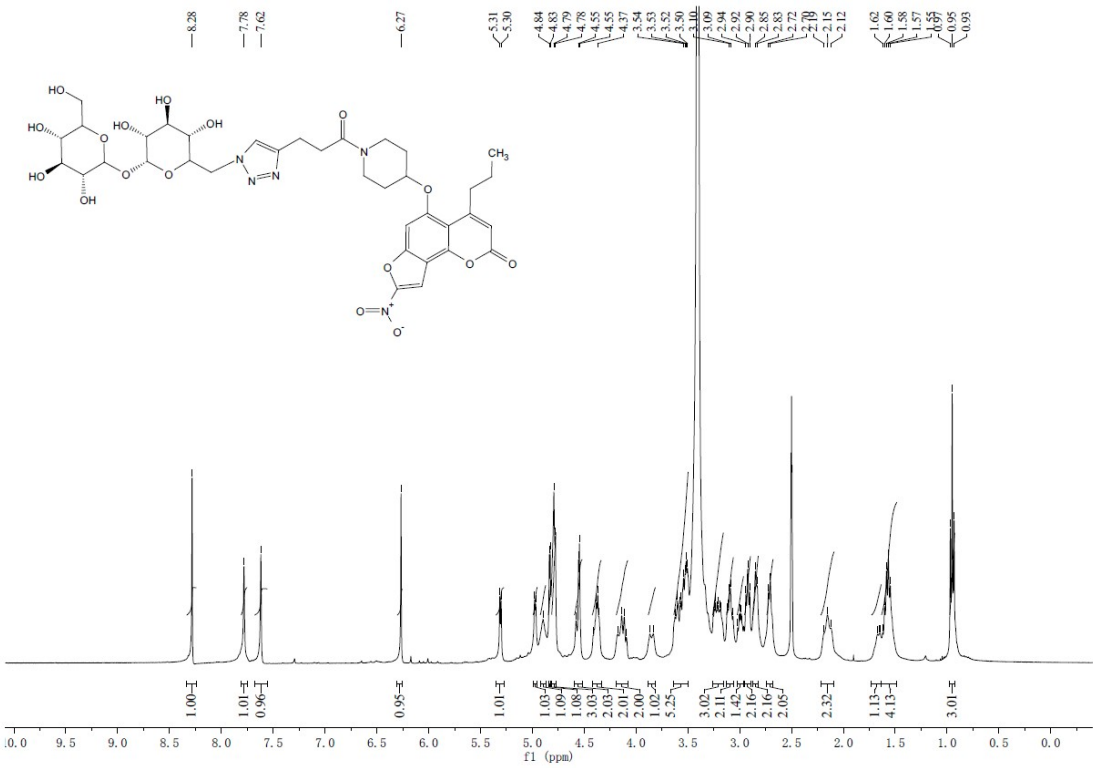
^{1}H NMR spectra of **NFC-Tre-4**



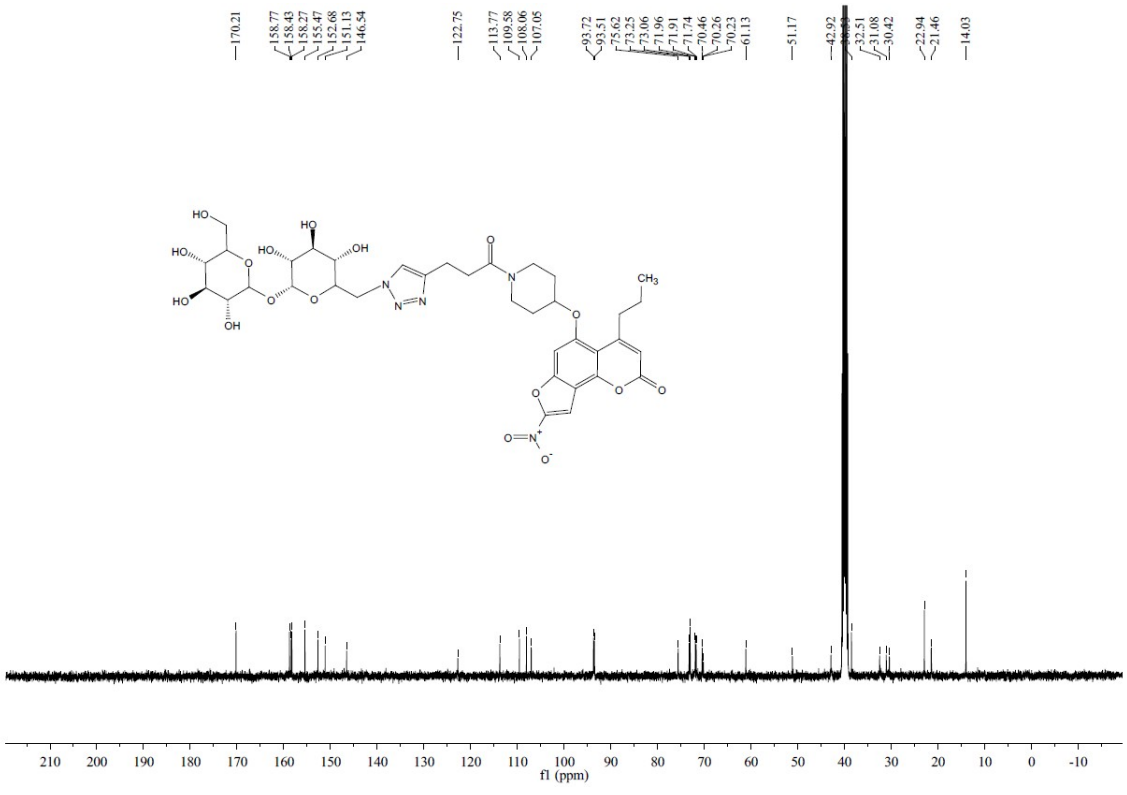
^{13}C NMR spectra of **NFC-Tre-4**



^{1}H NMR spectra of **NFC-Tre-5**



^{13}C NMR spectra of **NFC-Tre-5**



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11. Acknowledgment

We gratefully appreciate Professors Babak Javid and Jingren Zhang at Tsinghua University for their kind provision of bacteria. We would like to sincerely acknowledge the assistance of the Imaging Core Facility, Tsinghua Center for Protein Science, Tsinghua University, for assistance with the use of DeltaVision, Andor Dragonfly Spinning Disk and Nikon SIM microscopes, especially Yanli Zhang for her enthusiastic and professional guidance in cell imaging technology. We thank Bin Yu (Center of Biomedical Analysis, Tsinghua University) for her kind help with the flow cytometry analysis. We gratefully acknowledge the funding support of grants from the National Natural Science Foundation of China (grants 81773575 and 81573289). This work was supported by funds from the Beijing Advanced Innovation Center for Structural Biology, Tsinghua University, and Beijing Municipal Natural Science

Foundation (21JG0034).