A fluorogenic trehalose probe for tracking phagocytosed *Mycobacterium tuberculosis*

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Materials and Methods

1. Chemistry

1.1 General Information

All chemicals were purchased from commercial sources as specified below. Kinetic experiments were performed in a M1000 microplate reader (TECAN, research triangle park, NC). Analytical TLC was performed with 0.25 mm silica gel 60F plates with fluorescent indicator (254 nm). The ^{1}H and ^{13}C NMR spectra were taken on Varian 500 MHz or 600 MHz magnetic resonance spectrometer. 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 chloroform-d (δ 7.26, 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. HPLC was performed on a Dionex HPLC System (Dionex Corporation) equipped with a GP50 gradient pump and an inline diode array UV-Vis detector. A reversed-phase C18 (Phenomenax, 5 μm, 10 x 250 mm or Dionex, 5 μm, 4.6 x 250 mm) column was used with a MeCN (B) / H_2O (A) gradient mobile phase containing 0.1% trifluoroacetic acid at a flow of 1 or 3 mL/min for the analysis.

1.2 Probe syntheses and characterizations.

Preparation of compound N-(2-(4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylphenoxy)ethyl)hept-6-ynamide **(2)**. To a flask charged with tetrakis(triphenylphosphine)palladium(0) (26 mg, 0.0226 mmole), 1,3-dimethylbarbituric acid (1,3-DMBA) (106 mg, 0.68 mmol) and **1** [1] (100 mg, 0.226 mmol) was added in DCM (1.5 mL) under N_2 atmosphere. The resulting mixture was then reacted at 40 ºC for 3 hr. DCM (50 mL) and saturated sodium bicarbonate (20 mL) were added, and the separated organic layer was further washed with brine (20 mL) and dried over $MgSO_4$. After removing the solvent, the residue was re-dissolved in DMF (1 mL) and used in the next step without further purification. N, N-Diisopropylethylamine (118 μL, 0.68 mmol) was added to adjust pH > 7 followed by a pre-mixed solution of heptynoic acid (29 mg 0.226 mmol) and N,N,N′,N′-Tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (TSTU) (68 mg, 0.226 mmol). The resulting mixture was then stirred at room temperature overnight. After solvent concentration and ethyl acetate extraction, the resulted residue was further washed with water, brine and dried over $MgSO_4$. Silicon column

purification afforded pure compound **2** (65 mg, 62% for 2 steps). ^{1}H NMR (600 MHz, CD_3OD) δ 7.27 – 7.21 (m, 2H), 7.18 (d, *J* = 8.3 Hz, 1H), 7.10 (d, *J* = 2.5 Hz, 1H), 7.05 (dd, *J* = 8.4, 2.5 Hz, 1H), 6.90 – 6.78 (m, 4H), 4.19 (t, *J* = 5.4 Hz, 2H), 3.65 (t, *J* = 5.4 Hz, 2H), 2.28 (t, *J* = 7.4 Hz, 2H), 2.23 – 2.19 (m, 3H), 2.05 (s, 3H), 1.77 (p, *J* = 7.5 Hz, 2H), 1.61 – 1.51 (m, 2H). ^{13}C NMR (151 MHz, CD_3OD) δ 174.86, 174.65, 160.10, 158.18, 156.86, 137.69, 131.46, 131.38, 130.25, 130.13, 124.27, 121.16, 120.96, 116.35, 116.19, 115.69, 111.92, 102.86, 102.66, 66.27, 57.06, 47.98, 47.92, 47.84, 47.75, 47.69, 47.55, 47.41, 47.27, 47.13, 38.71, 38.57, 38.43, 35.02, 27.88, 27.72, 27.56, 24.66, 24.32, 18.68, 18.42, 17.35. MS (ESI): m/z calculated for $C_{29}H_{27}NO_5^+$ ($[M+H]^+$): 470.19; found: 470.3.

Synthesis of compounds TG-Tre: 4-(1-((2S,3S,4S,5R,6R)-4,5-dihydroxy-2-(hydroxymethyl)-6-(((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy) tetrahydro-2H-pyran-3-yl)-1H-1,2,3-triazol-4-yl)-N-(2-(4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylphenoxy)ethyl) butanamide (4-TG-Tre) and N-(2-(4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylphenoxy)ethyl)-5-(1-(((2R,3S,4S,5R,6R)-3,4,5-trihydroxy-6-(((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy) tetrahydro-2H-pyran-2-yl)methyl)-1H-1,2,3-triazol-4-yl)pentanamide (6-TG-Tre) were obtained following the general procedure of click reaction. Compound **2** (1 eq) was dissolved in DMSO/H_2O (1:2), Tre-Az (1.2 eq) was added to the solution, followed by $CuSO_4$ (3 eq), Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) (3 eq) and sodium ascorbate (50 eq). The resulting mixture was then stirred at room temperature for 1 hr. HPLC purification and lypholization afforded the respective compound. 4-TreAz [2] (**3**) from Prof. Bertozzi's lab was used to obtain 4-TG-Tre, and 6-TreAz [2] (**4**) was synthesized as previously described[1] and used to obtain 6-TG-Tre.

^{1}H NMR (600 MHz, CD_3OD) for 4-TG-Tre: δ 7.89 (s, 1H), 7.53 (d, *J* = 9.2 Hz, 2H), 7.26 – 7.19 (m, 3H), 7.17 – 7.08 (m, 3H), 7.05 (dd, *J* = 8.4, 2.5 Hz, 1H), 5.25 (d, *J* = 3.7 Hz, 1H), 5.10 (d, *J* = 3.7 Hz, 1H), 4.50 (t, *J* = 10.4 Hz, 1H), 4.44 – 4.34 (m, 2H), 4.16 (t, *J* = 5.5 Hz, 2H), 3.91 – 3.76 (m, 3H), 3.71 – 3.59 (m, 4H), 3.47 (dd, *J* = 9.8, 3.7 Hz, 1H), 3.40 (dd, *J* = 12.5, 2.1 Hz, 1H), 3.37 – 3.31 (m, 1H), 3.08 (dd, *J* = 12.4, 4.1 Hz, 1H), 2.72 (t, *J* = 6.8 Hz, 2H), 2.26 (t, *J* = 7.0 Hz, 2H), 2.01 (s, 3H), 1.75 – 1.61 (m, 4H). HRMS: calculated for $C_{41}H_{48}N_4O_{15}^+$ ($[M+H]^+$): 837.31; found: 837.3093.

^{1}H NMR (600 MHz, d_6-DMSO) for 6-TG-Tre δ 8.09 (t, J = 5.6 Hz, 1H), 7.73 (s, 1H), 7.19 (d, J =

8.4 Hz, 1H), 7.10 – 7.04 (m, 3H), 7.01 (dd, J = 8.4, 2.6 Hz, 1H), 6.78 (d, J = 7.4 Hz, 4H), 4.83 (d, J = 3.6 Hz, 1H), 4.55 (dd, J = 12.1, 3.1 Hz, 2H), 4.38 (dd, J = 14.3, 8.3 Hz, 1H), 4.15 – 4.05 (m, 3H), 3.66 – 3.42 (m, 7H), 3.22 (dd, J = 9.6, 3.7 Hz, 1H), 3.17 (dd, J = 9.6, 3.7 Hz, 1H), 3.11 (dd, J = 9.9, 8.8 Hz, 1H), 2.97 (dd, J = 9.9, 8.7 Hz, 1H), 2.62 – 2.57 (m, 2H), 2.14 (t, J = 6.9 Hz, 2H), 1.56 (td, J = 11.0, 9.9, 5.7 Hz, 4H). HRMS: calculated for $C_{41}H_{48}N_4O_{15}^+$ ([M+H]$^+$): 837.31; found: 837.3096.

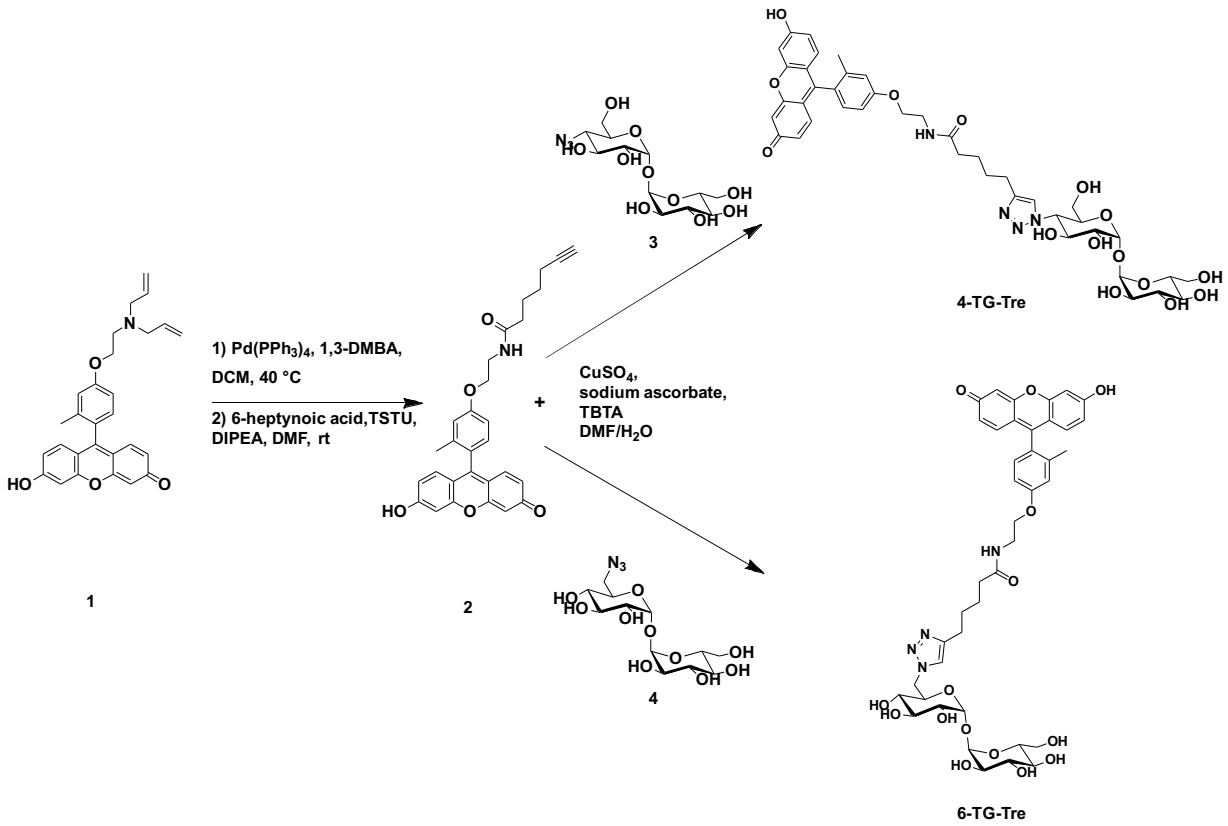


Fig. S1. Synthesis of TG-Tre.

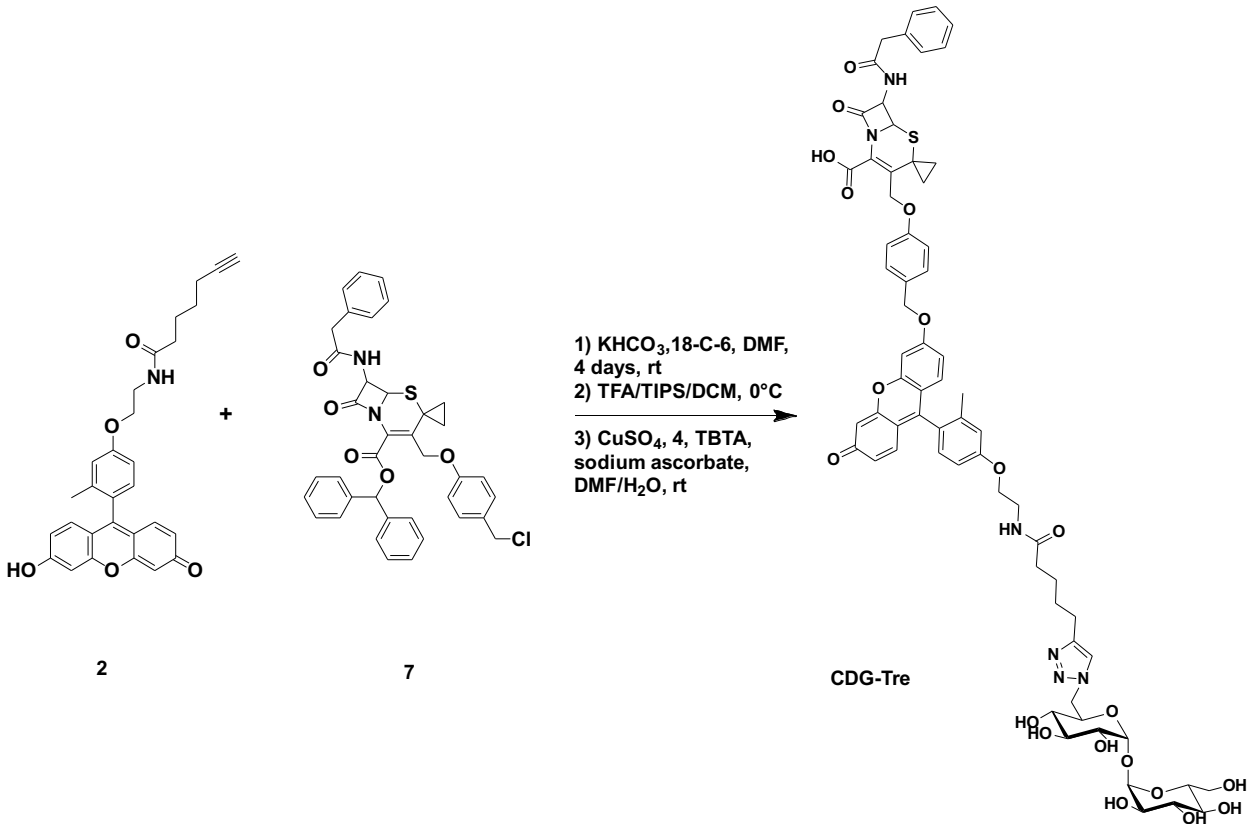


Fig. S2. Synthesis of CDG-Tre.

Synthesis of compound CDG-Tre: 4-((4-(((9-(2-methyl-4-(2-(5-(1-(((2*R*,3*S*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(((2*R*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl) oxy)tetrahydro-2*H*-pyran-2-yl)methyl)-1*H*-1,2,3-triazol-4-yl)pentanamido)ethoxy) phenyl)-3-oxo-3*H*-xanthen-6-yl)oxy)methyl)phenoxy)methyl)-7-oxo-8-(2-phenylacetamido)-2-thia-6-azaspiro[bicyclo[4.2.0]octane-3,1'-cyclopropan]-4-ene-5-carboxylic acid.

To a mixture of **2 (**24.3 mg, 0.03 mmol) , potassium bicarbonate (9.6 mg, 0.06 mmol) and 18-crown-6 (7.9 mg, 0.03 mmol) in anhydrous DMF (1 mL) were stirred at room temperature for 5 min, then **7** [3] (23 mg, 0.03 mmol) was added and the resulting mixture was stirred in dark at room temperature for 72 h. Ethyl acetate (50 mL) and H_2O (10 mL) was added to the reaction solution, and the separated organic layer was further washed with H_2O (10 mL) twice and saturated brine (10 mL). After removal of the solvent under Rota-Vap, residue was dissolved in a pre-cold solution of DCM/TFA/TIPS (5 mL/1 mL/0.5 mL). The resulting mixture was then stirred at 0°C for 2 hours. After condensation and ether precipitation, the crude was used in the next step and reacts with 6-TreAz following the general procedure of click reaction. Following HPLC purification and

lyophilization afforded the titled compound CDG-Tre (5.5 mg, 11.5% over three steps) ^{1}H NMR (600 MHz, DMSO-d6) δ 9.11 (d, *J* = 8.2 Hz, 1H), 8.05 (t, *J* = 5.6 Hz, 1H), 7.70 (s, 1H), 7.42 – 6.88 (m, 18H), 6.50 (d, *J* = 9.8 Hz, 1H), 6.33 (s, 1H), 5.75 (dd, *J* = 8.3, 4.8 Hz, 1H), 5.29 – 5.14 (m, 3H), 4.80 (d, *J* = 3.6 Hz, 1H), 4.57 – 4.43 (m, 3H), 4.41 – 4.30 (m, 2H), 4.12 – 4.00 (m, 3H), 3.61 – 3.40 (m, 4H), 3.20 – 2.91 (m, 2H), 2.61 – 2.50 (m, 3H), 2.35 (p, *J* = 1.9 Hz, 1H), 2.11 (t, *J* = 6.9 Hz, 2H), 1.95 (s, 3H), 1.52 (dp, *J* = 12.6, 6.8, 6.4 Hz, 4H), 1.47 – 1.42 (m, 2H), 1.29 (dd, *J* = 10.1, 4.7 Hz, 1H), 1.20 (s, 1H), 0.90 (dt, *J* = 9.6, 2.7 Hz, 1H), 0.82 (s, 1H). MALDI-TOF: calculated for $C_{66}H_{70}N_6O_{20}S^+$ ([M+H]$^+$): 1300.37; Found: 1300.30.

2. Biology

2.1 General information

Plasmid pMRLB.16 containing gene Rv0129c for expressing recombinant Ag85c from Mtb, NR-13275, was obtained through BEI Resources (NIAID, NIH). The design and clone of BlaC from Mtb H37Rv were described previously[4]. TALON metal affinity resin for affinity purification was purchase from Clontech (Mountain view, CA). SimpleBlue SafeStain protein gel staining reagent was purchased from Invitrogen (Carlsbad, CA). Dulbecco's Modified Eagle Medium (DMEM) and premixed Penicillin-Streptomycin (100X, 5,000 U/ml) were purchased from Thermo Fisher Scientific (Waltham, MA). Hoechst was purchased from Sigma-Aldrich (St Louis, MO). The CellMask deep red plasma membrane stain reagent was purchased from Life Technologies (Carlsbad, CA). Western blots were imaged using a LI-COR Odyssey imaging system (Lincoln, NE).

2.2. Expression, purification and SDS-PAGE analysis of Mtb Ag85c and BlaC

The general expression and purification procedures of Ag85c and BlaC were described previously[5]. Briefly, a single colony of *E. coli* (TOP10) containing either pMRLB.16-Ag85c or pET28b-BlaC was inoculated into 100 ml of Lysogeny broth (LB broth) with 50 μg/ml of kanamycin (Ag85c) or 100 μg/ml ampicillin (BlaC), followed by incubation at 37°C, 205 rpm overnight. The overnight culture was added into 500 ml of fresh LB broth with antibiotic and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After incubation for up to 6 h at 37°C 205 rpm, bacteria were harvested, washed, and the pellet was frozen at -80°C. The pellets were later resuspended and lysed

in Novagen Bugbuster protein extraction reagent (EMD Millipore, Burlington, MA). Proteinase inhibitor cocktail (cOmplete, mini, EDTA-free, Roche) was added to the bacterial lysate before two rounds of metal-affinity purification using TALON metal affinity resin. The affinity purified fraction was eluted using lysis buffer containing up to 500 mM imidazole. Buffer was changed to PBS (supplemented with 10% glycerol) by centrifugation with centrifugal filter units (EMD Millipore, Burlington, MA) at regenerated size 30K. The concentration of purified proteins was determined with Pierce protein BCA assay reagents (Thermo Scientific, Rockford, IL). Protein samples were denatured in LDS loading buffer (Life Technologies, Carlsbad, CA) with heating and analyzed by SDS-PAGE (NuPAGE, Life Technologies, Carlsbad, CA). The gels were stained by SimpleBlue SafeStain protein gel staining reagent. The analysis was performed in duplicates.

2.3. Fungus and bacteria growth and staining

C. albicans (ATCC 10231) was provided by Dr. Niaz Banaei at Stanford Medical Center, *S. aureus* (ATCC 25923), *C. diphtheriae* (ATCC 13812), and BCG (ATCC 35734) were purchased from ATCC (American Type Culture Collection). *M. smegmatis* expressing mcherry fluorescent protein was previously reported and utilized in this study.

Generally, *C. albicans* was cultured in YPD (yeast extract-peptone-dextrose) medium. *M. smegmatis* and BCG were cultured in Middlebrook 7H9 broth supplemented with ADC enrichment. *E. coli* (TOP10) were grown in LB medium. *S. aureus* and *C. diphtheriae* were grown on tryptic soy agar plates supplemented with defibrinated sheep blood at 37°C. Colony forming units per milliliter (CFU/ml) was determined by measuring the UV absorbance at OD_{600}.

For 4-TG-Tre, 6-TG-Tre and CDG-Tre staining, 5 mM probes in pure DMSO were frozen at -80 °C as stock solutions. 50 μM working solution was prepared freshly by diluting stock solution in PBS (PH 7.05). Staining was done by resuspending fungus or bacteria in 50 μM working solution, incubated at 37 °C for 2 h, then centrifuged at 3000*g* and washed twice with PBS. A light-safe tube or foil wrap was used to prevent photobleaching during incubation. Bacteria or fungus were fixed in 10% formalin for 30 min before analyzed by flow cytometry and microscope.

2.4. Fungus and bacteria analysis using flow cytometry and microscope

Flow cytometry analysis was done with instrumentation and assistance provided by the Stanford Shared FACS Facility. Bacterial cell fluorescence was analyzed using a FACScan with a 4-laser, 12-color DxP12 Cytek upgrade (Becton Dickinson, Cytek Biosciences) and evaluated according to their granularity (SSC, side Scatter) property on a log scale, excited by 488 nm later, filtered by 560 nm, SP (Short Pass filter) and 525/50 nm, BP (Band Pass filter). Flow cytometry data were collected and analyzed using FlowJo V10 software. The MFI (mean fluorescence intensity) was plotted against the natural autofluorescence of bacteria in PBS.

The super-resolution images were acquired by OMX BLAZE 3D-structured illumination, super-resolution microscope (SIM) (Applied Precision) with simultaneous excitation at 405 nm for Hoechst (Emission filters 435/31 nm), 488 nm for TokyoGreen (Emission filters 528/48 nm), 568 nm for TAMRA (Emission filters 609/37 nm) and 642 nm for Deep Red (Emission filters 683/40 nm), according to the standard procedure in the manufacturer's instructions, and reconstructed with OMX softWoRx image-processing software. Images were further processed using ImageJ.

Confocal images were taken with a Zeiss LSM710 inverted confocal microscope using a 40x/oil or 63x/oil immersion objective. Fluorescence and bright-field images were gathered sequentially and stacked. Essential sequential Z sections of stained fungus, bacteria and cells were recorded for generation of stacked images. Multi-channel 3D projections of fluorescent images were constructed from sequential Z sections assembled in ImageJ.

2.5. Inhibitory study with *M. smegmatis*

One hundred microliter (100 µl) of freshly cultured *M. smegmatis* was pretreated with PBS, 2 mg/ml potassium clavulanate, 2 mg/ml avibactam, 200 µg/ml ebselen or 2.5, 5, 7.5 mg/ml spermidine at 37°C for 1 h. The samples were then incubated with 50 µM CDG-Tre in a 100 µl final volume for an additional 3h. Cells were washed with PBS twice before analysis by flow cytometry.

For inhibitory study with free trehalose, CDG-Tre (final concentration = 10 µM) was added to 200 µL aliquots of freshly cultured *M. smegmatis* at OD of 0.5. Free trehalose was also added to the sample at a ratio of CDG-Tre: Free trehalose = 0, 1:1, 1:10, 1:100, or 1:1000. These samples were then incubated for an hour with shaking at 37 ºC. The samples were fixed in 10% formalin and

washed twice with 0.05% PBS tween before fluorescence analysis by flow cytometry.

2.6. Visualization of BCG in macrophage

Mouse RAW 264.7 macrophages were grown on sterilized coverslips in a 6-well plate in DMEM containing 10% (v/v) fetal bovine serum (FBS), 4.5 g/L glucose, 2 mM L-glutamine, and 1% (v/v) penicillin/streptomycin (complete medium) at 5% CO_2, 37°C to ~60-70% confluence. Freshly cultured BCG were pre-labeled with TAMRA-Tre[6] (50 μM in 7H9 medium) overnight then washed and incubated with macrophage at 10 to 1 ratio in complete medium for 6 h. Macrophages were washed with HBSS (Hanks' balanced salt solution) for three times to remove free bacteria, then incubated with CDG-DNB3, 6-TG-Tre or CDG-Tre at 20 μM in serum-free medium for 4 h, or with 6-TG-Tre (10 and 100 μM) or CDG-Tre (2 and 10 μM) for 22 h then incubated in complete medium overnight. Cells were further stained with Hoechst and CellMask Deep Red cell plasma staining reagent according to manufacturer's protocol. Cells were washed and fixed in 10% formalin before mounted for imaging. Images of infected macrophages were obtained by either a confocal microscopy (Zeiss LSM710 inverted confocal microscope) or a structured illumination, super-resolution microscope (SR-SIM, OMX BLAZE 3D).

2.7. Stability of CDG-Tre

CDG-Tre (final concentration at 20 μM) was incubated in PBS, 7H9 with or without nutrition supplement (M0553 Middlebrook ADC Growth Supplement, sigma Aldrich), and DMEM for 22 h at 37 °C followed by analysis via HPLC (20 %ACN, 0.1% TFA, 0 min to 100 % ACN, 0.1% TFA, 30min). The peak area of CDG-Tre pre- and post- incubation in buffers were compared.

2.8. Measurement of cell proliferation of 6-TG-Tre and CDG-Tre treated macrophage

Mouse RAW 264.7 macrophages were grown in a 96-well plate to ~60-70% confluence, then incubated with serum free medium containing 0.5% PBS, 0.5% DMSO, 2, 5, 10, 20, 50, 100 or 200 μM TG-Tre and CDG-Tre in 0.5% DMSO for 22h. Cells were further analyzed by CellTiter 96 AQueous one solution cell proliferation assay (MTS assay). The average reading of PBS treated macrophages was arbitrarily set to 1, to normalize the relative viability of other conditions.

3. Statistical Analysis

GraphPad Prism 7 was used for plotting and statistical analysis. The significant difference was determined by performing one-way ANOVA (Figure 3c, 3e, and Figure S11) followed by Bonferroni's multiple comparison test to determine the statistical significance with 95% confidence intervals with *$p < 0.0332$; **$p < 0.0021$, ***$p < 0.0002$, ****$p < 0.0001$, ns: not significant. All error bars in figures represent ± SD, n = 3.

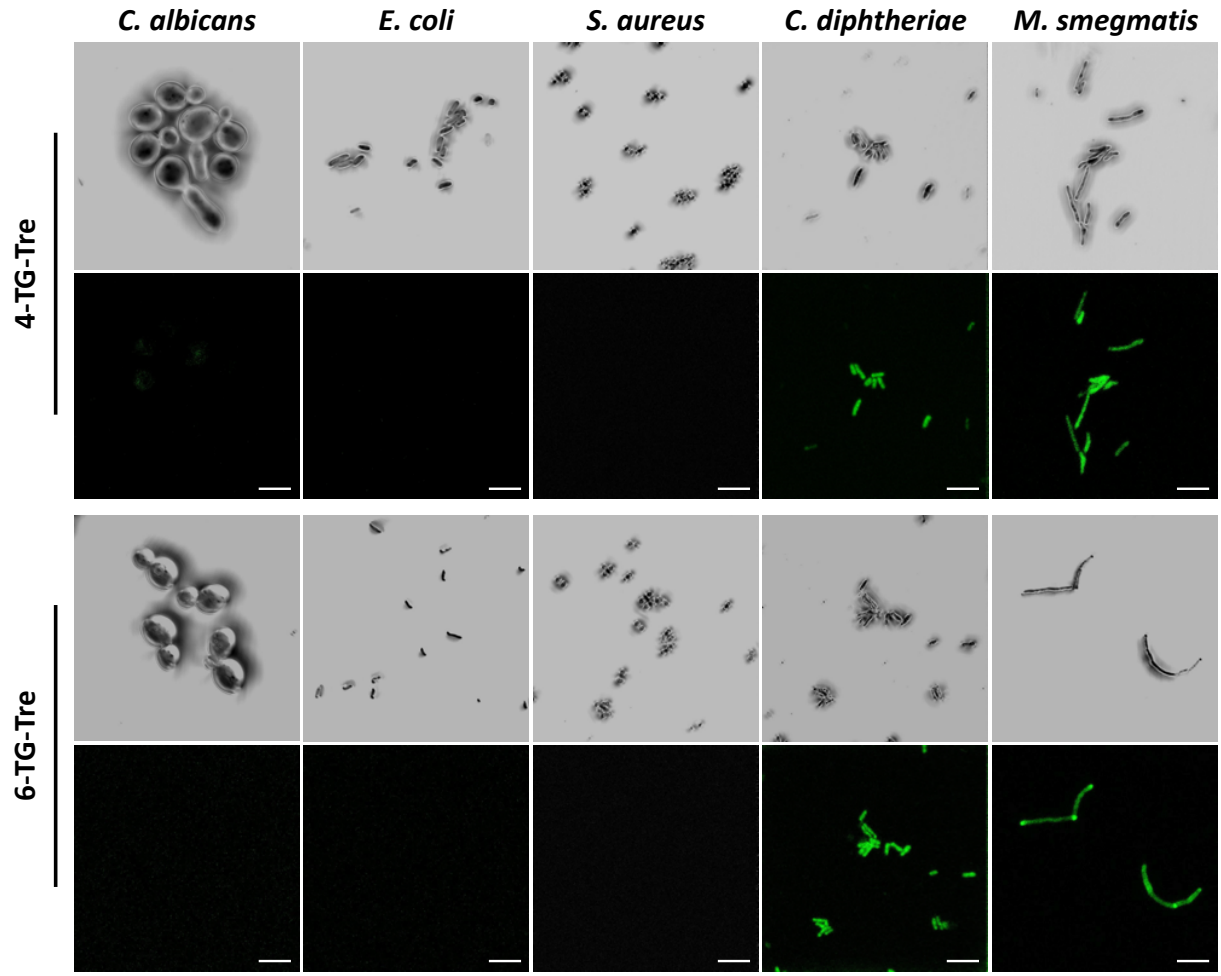


Fig. S3. **Fluorescence analysis of different species of bacteria and fungi labeled with 4-TG-Tre or 6-TG-Tre.** Confocal images of freshly cultured *C. albicans*, *E. coli* (TOP10), *S. aureus*, *C. diphtheriae* and *M. smegmatis* stained with 50 μM of 4-TG-Tre and 6-TG-Tre in PBS for 2 h at 37 °C. For each set of images, the top row = bright field; bottom row = green fluorescence, 63x/oil, Ex-490 nm/Em-520 nm. Scale bars indicate 5 μm.

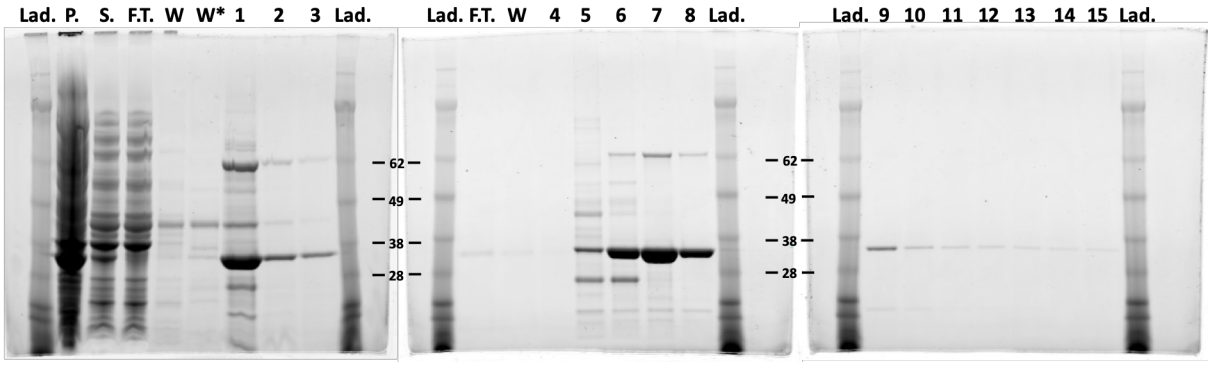
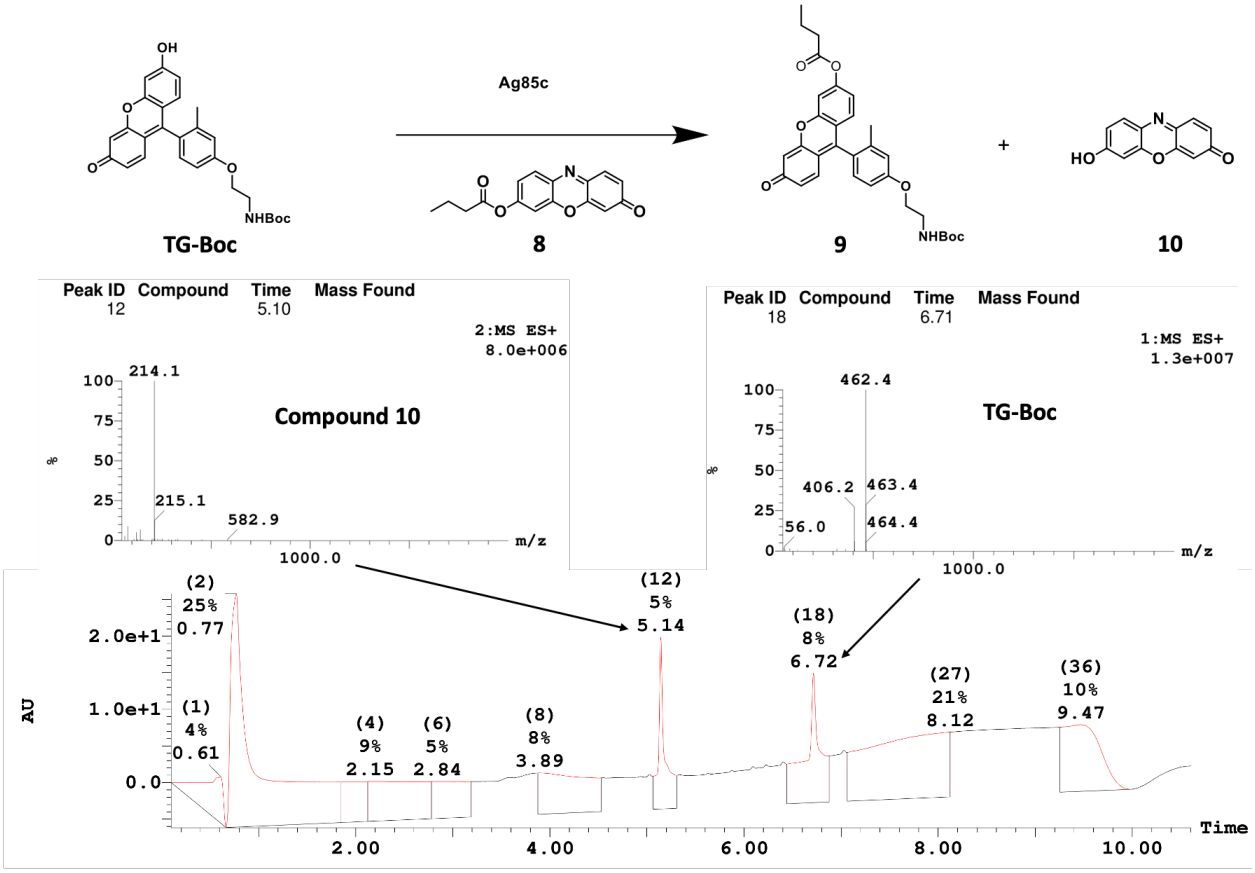
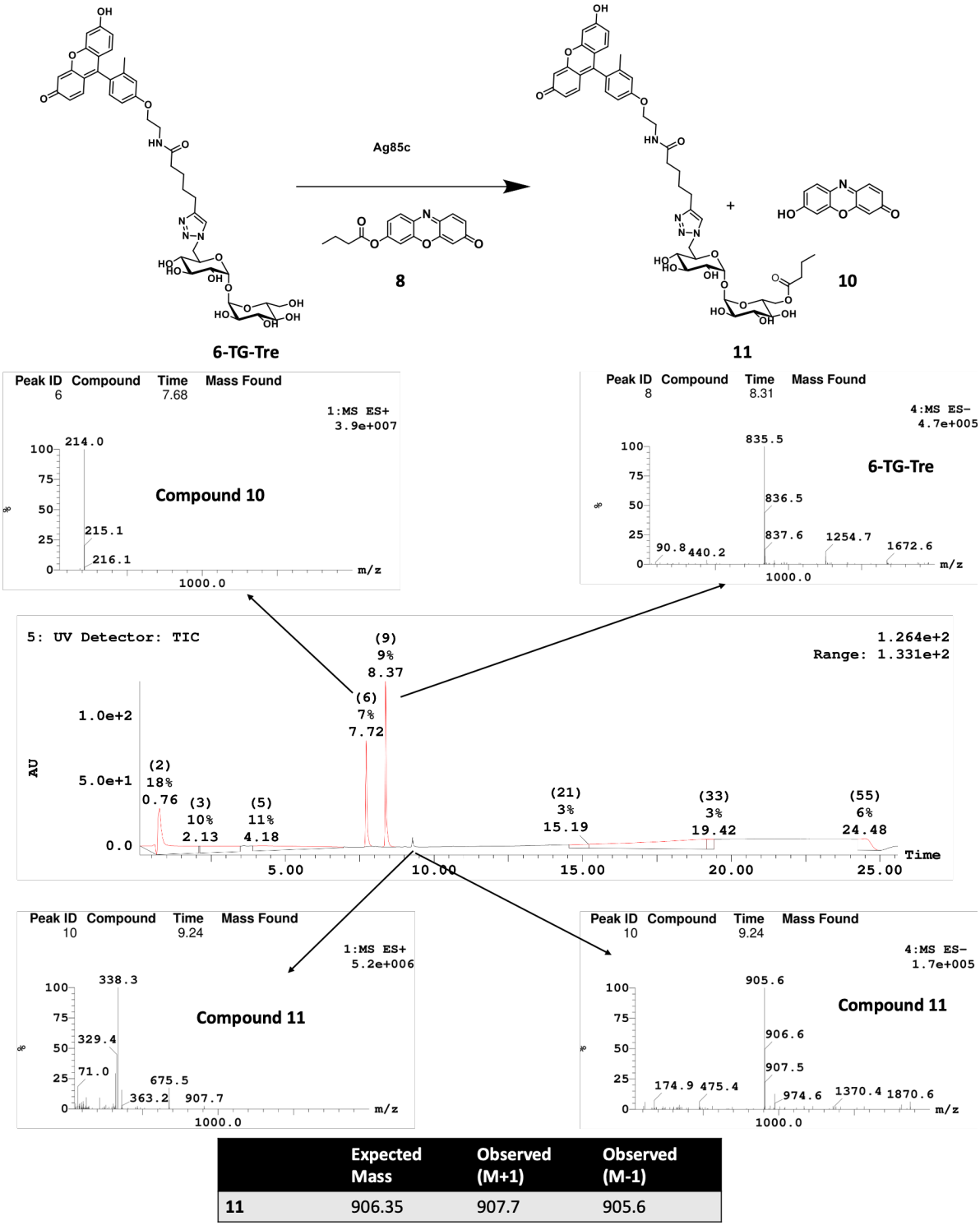


Fig. S4. SDS-PAGE analysis of purified *M. tuberculosis* Ag85c without signaling peptides in pET23b-Ag85c. Lad.: SeeBlue Plus 2 pre-stained protein standard; P: pellet of lysate; S: supernatant of lysate; F.T.: flow through fraction of supernatant from TALON resin column; W: wash by HEPES buffer; W*: wash by 10mM Imidazole; Lanes 1-3: elution from the first TALON resin column by 500mM Imidazole (fraction 1 contained purified Ag85c); Lanes 4-15: elution from the second TALON resin column with 10, 20, 40, 60, 80, 100, 120, 140,160, 180, 200 and 250 mM imidazole.



	Expected Mass	Observed (M+1)
9	531.6	N/A

Fig. S5. **LCMS analysis of Ag85c-mediated transformation using TG-Boc as an acyl group acceptor.** TG-Boc (50 μM) and resorufin butyrate **8** (100 μM) were incubated with 500 nM of Ag85c for 6 h at 37°C and analyzed by LCMS. No butylrated product **9** but resorufin (**10**) was observed, suggesting that the Ag85c was able to hydrolyze resorufin butyrate **8** but unable to transfer the butyrate group to the substrate. N/A: not detected.



	Expected Mass	Observed (M+1)	Observed (M-1)
11	906.35	907.7	905.6

Fig. S6. LCMS analysis of Ag85c-mediated transformation using 6-TG-Tre as an acyl group acceptor. 6-TG-Tre (50 μM) and resorufin butyrate **8** (100 μM) were incubated with 500 nM of Ag85c for 6 h at 37°C and analyzed by LCMS. The butyrated product **11** was observed, suggesting that the Ag85c was able to transfer the butyrate group to the substrate.

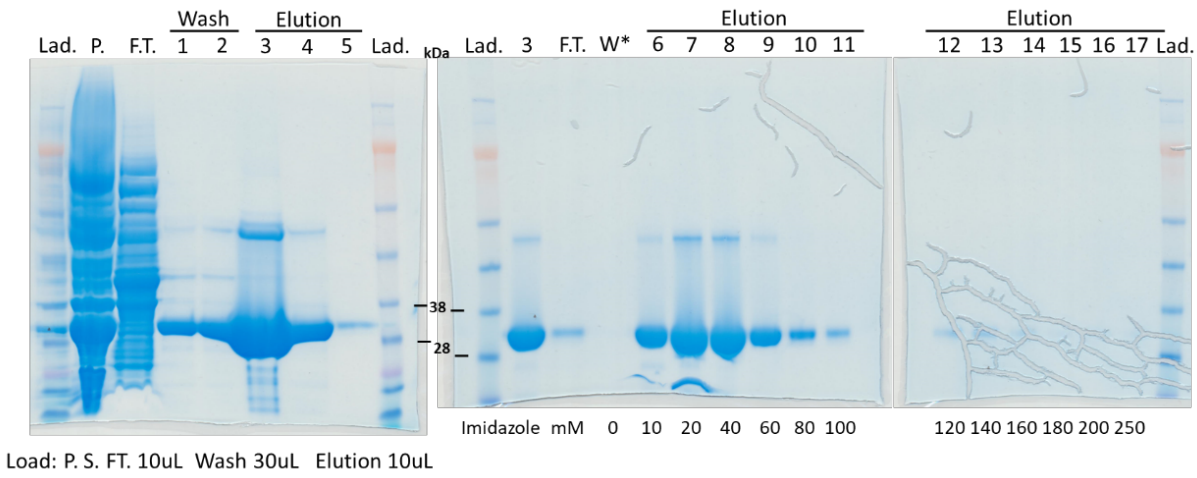


Fig. S7. SDS-PAGE analysis of purified *M. tuberculosis* BlaC. Lad: SeeBlue Plus 2 pre-stained protein standard; P: pellet of lysate; S: supernatant of lysate; F.T.: flow through fraction of supernatant from TALON resin column; Wash by HEPES buffer; W*: wash by 10mM Imidazole; Lanes 1-5: elution from the first TALON resin column by 500 mM Imidazole (fraction 1 contained purified Ag85c); Lanes 6-17: elution from the second TALON resin column with 10, 20, 40, 60, 80, 100, 120, 140,160, 180, 200 and 250 mM imidazole.

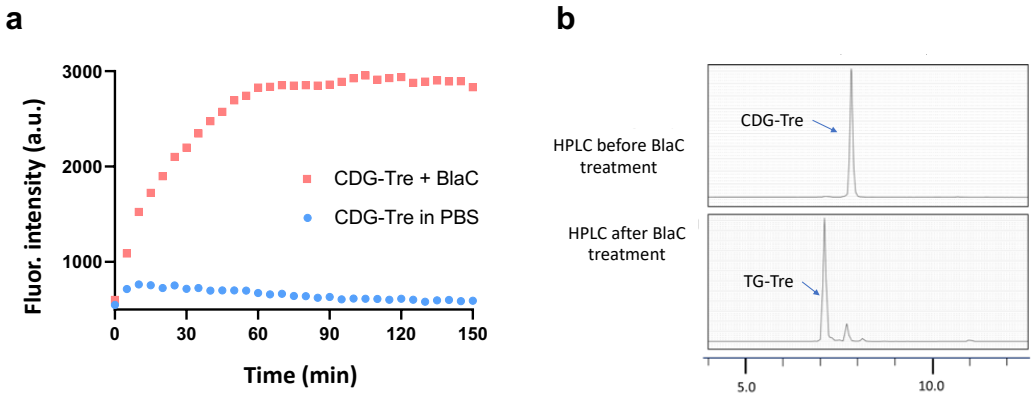
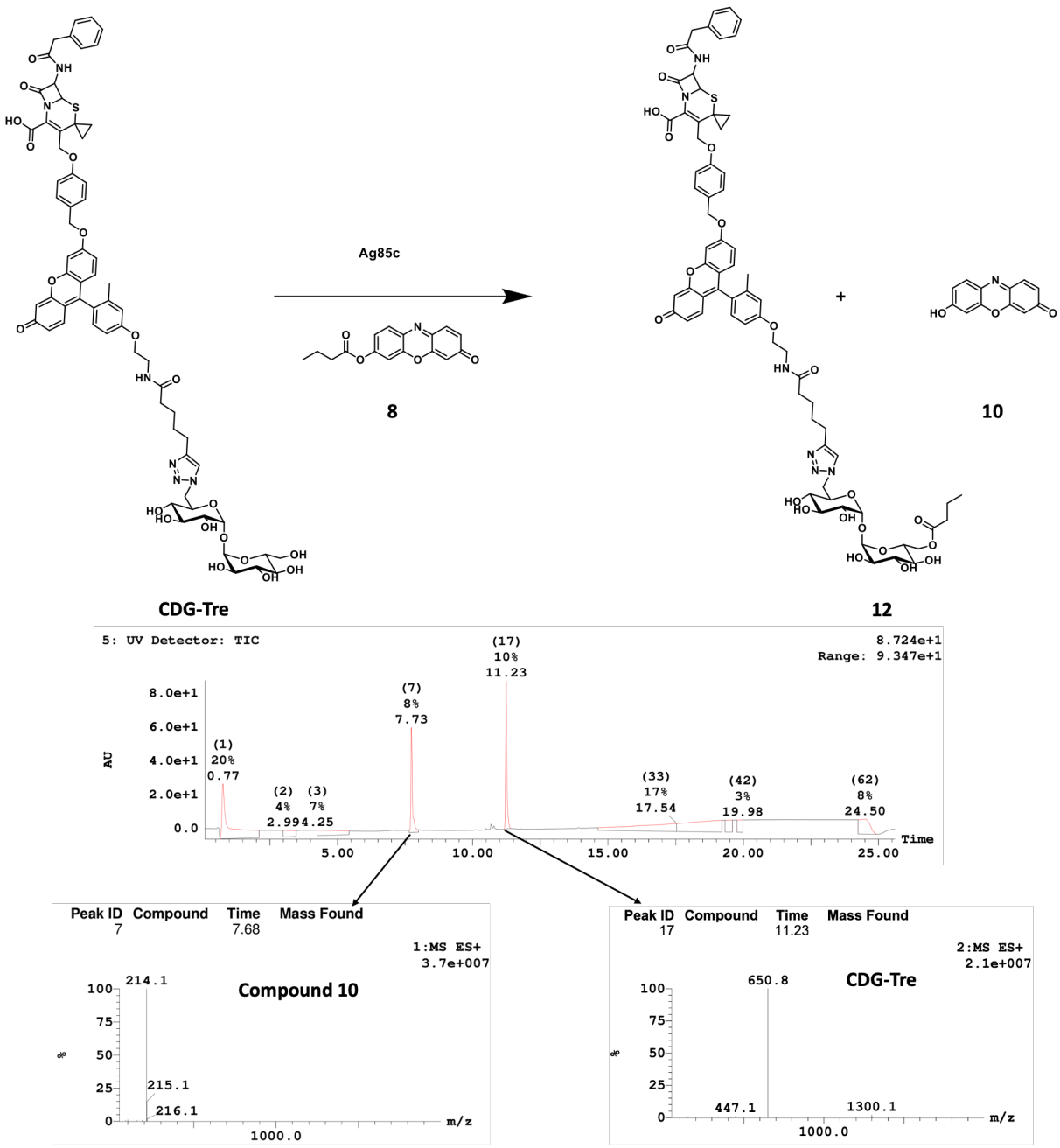


Fig. S8. BlaC catalyzes the conversion of CDG-Tre to 6-TG-Tre. a) CDG-Tre (200 nM) was incubated with 0.22 ng/μL BlaC at room temperature. Fluorescent intensity in arbitrary unit (a.u.) is monitored over time by a microplate reader (excitation: 490 nm). **b**) HPLC chromatograms before and after the BlaC treatment and the identity of the product peak (TG-Tre) was confirmed by LCMS.



	Expected Mass	Observed (M+1)
12	1369.46	N/A

Fig. S9. **LCMS analysis of Ag85c mediated transformation using CDG-Tre as an acyl group acceptor.** CDG-Tre (50 μM) and resorufin butyrate **8** (100 μM) were incubated with 500 nM of Ag85c for 6 h at 37°C and analyzed by LCMS. No butylrated product was observed. N/A: not detected.

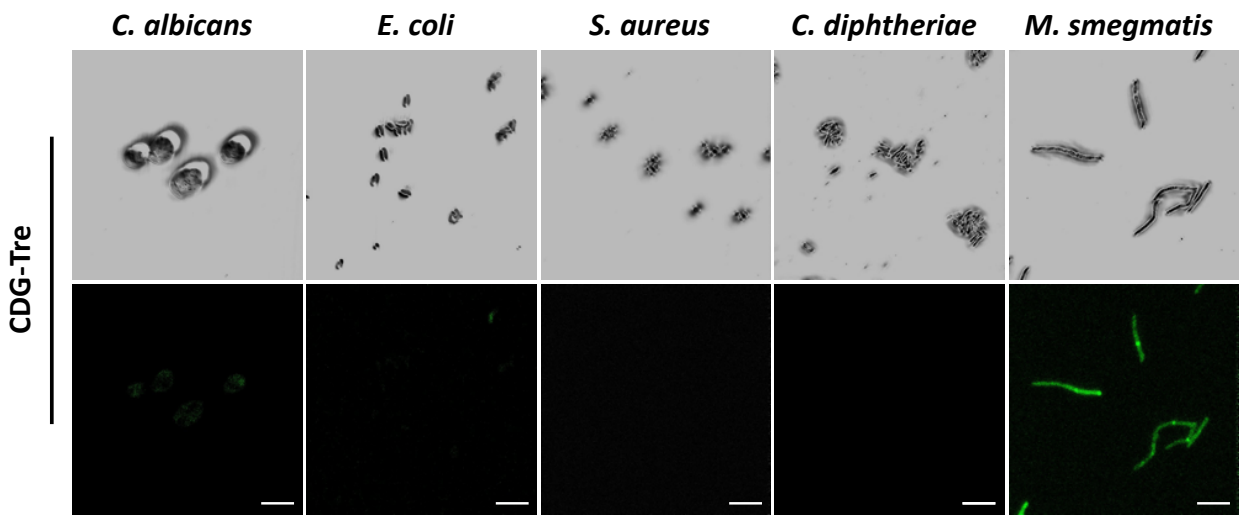


Fig. S10. Fluorescence analysis of different species of bacteria and fungi labeled with CDG-Tre. Confocal images (bright field and green fluorescence, 63X/oil, Ex-490 nm/Em-520 nm) of freshly cultured *C. albicans*, *E. coli* (TOP10), *S. aureus*, *C. diphtheriae* and *M. smegmatis* stained with 50 μM of CDG-Tre in PBS at 37°C for 2 h. Scale bars: 5 μm.

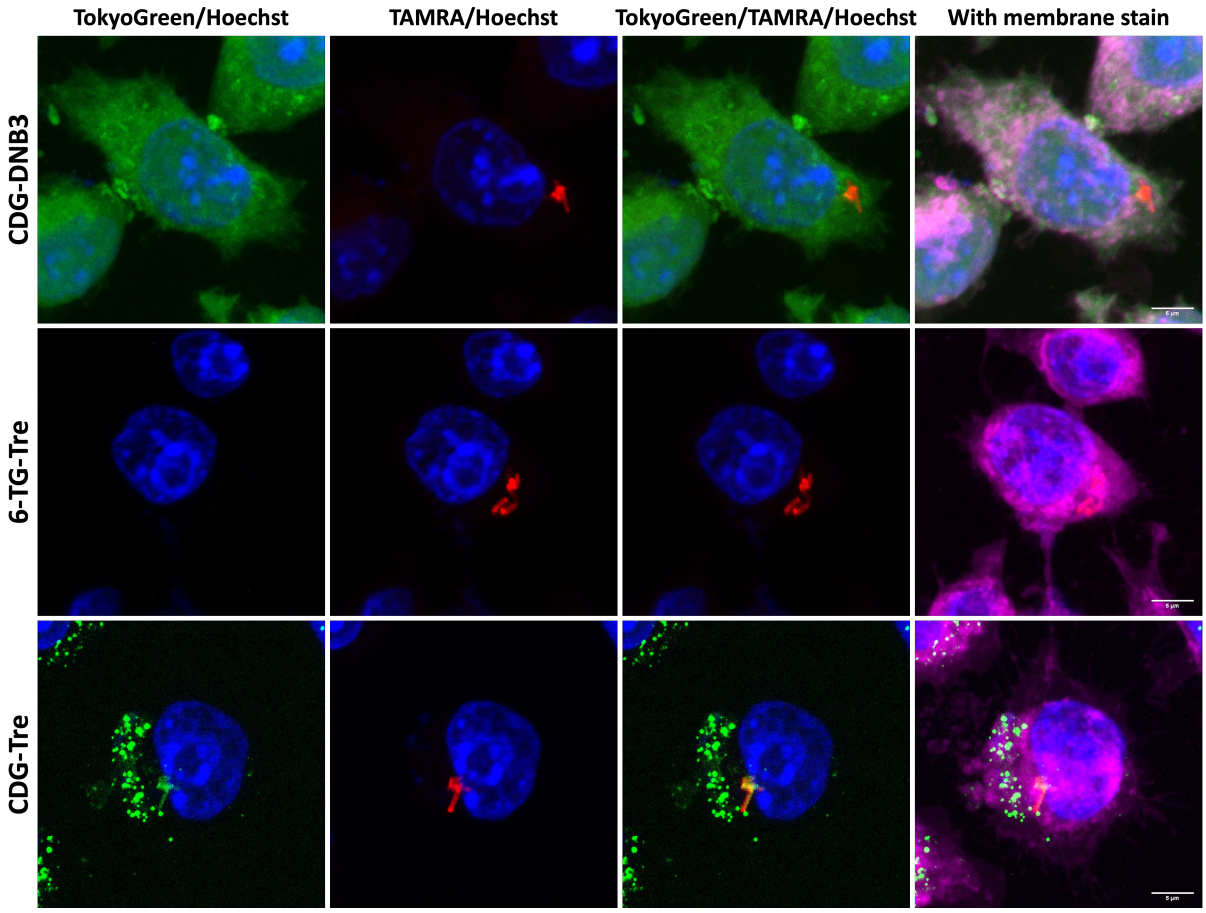


Fig. S11. Imaging of labeled BCG within macrophage phagosomes with CDG-DNB3, 6-TG-Tre and CDG-Tre. Freshly cultured BCG cells were pre-labeled with the red fluorescent TAMRA-Tre (50 μM in PBS) overnight then washed and incubated with mouse RAW264.7 macrophages at 10 to 1 ratio in complete medium for 6 h. Macrophages were washed with HBSS for three times, incubated with CDG-DNB3 (20 μM), 6-TG-Tre (20 μM) and CDG-Tre (20 μM) in serum free medium for 4 h. Cells were further washed with HBSS three times then stained with Hoechst, and Deep Red plasma membrane stain and fixed in 10% neutral buffered formalin solution for 30 min before confocal imaging (Zeiss LSM710 inverted confocal microscope, oil/60X, Emission/Excitation filters: Hoechst: Ex350/Em460, TokyoGreen: Ex490/Em520, TAMRA: Ex560/Em580, Deep Red: Ex650/Ex670). Scale bars indicate 5 μm.

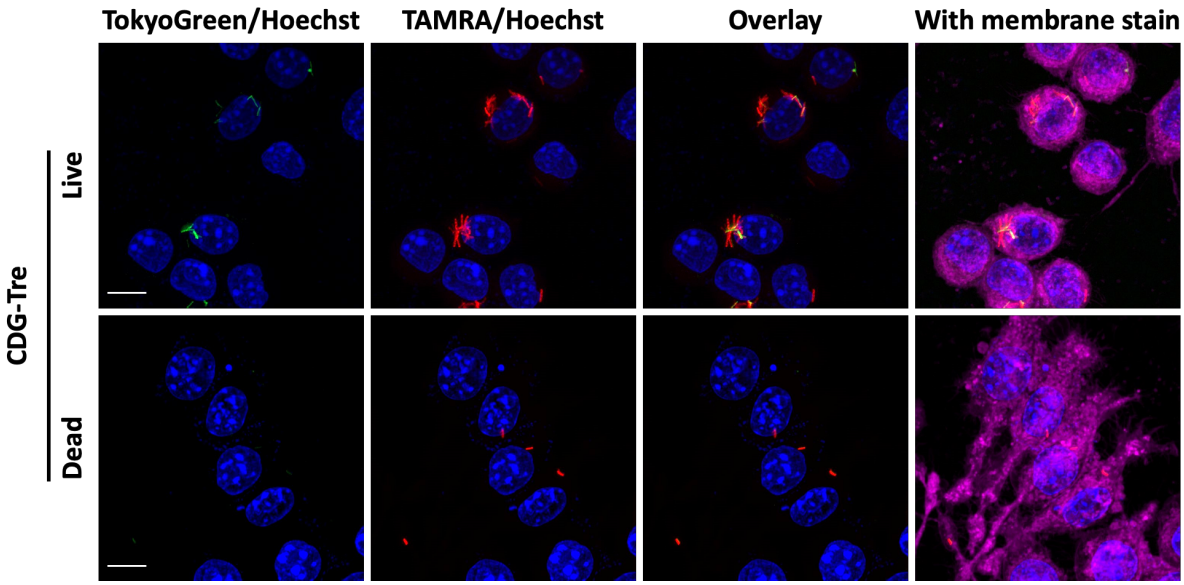


Fig. S12. Imaging of live or dead BCG cells within macrophage phagosomes labeled with by CDG-Tre. Freshly cultured BCG were pre-labeled with TAMRA-Tre (50 μM in PBS, red fluorescent) at 37 °C overnight. Dead (autoclaved: 120 °C for 20 min) BCG were pre-labeled non-specifically with the red fluorescent AlexaFluor594 conjugated to anti-CD3 antibody (2 μg/ml in PBS) overnight. Bacteria were washed and incubated with macrophage at 10 to 1 ratio in complete medium for 6 h. Macrophages were washed with HBSS for three times, incubated with CDG-Tre (2 μM) in serum free medium for 22 h then further incubated in complete medium overnight. Cells were stained with Hoechst and Deep Red Plasma membrane stain according to manufacturer's instructions, and fixed in 10% neutral buffered formalin solution for 30 min before confocal imaging (Zeiss LSM710 inverted confocal microscope, oil/40X, Hoechst: Ex350/Em460, TokyoGreen: Ex490/Em520, TAMRA: Ex560/Em580, Deep Red: Ex650/Ex670). Scale bars: 10 μm.

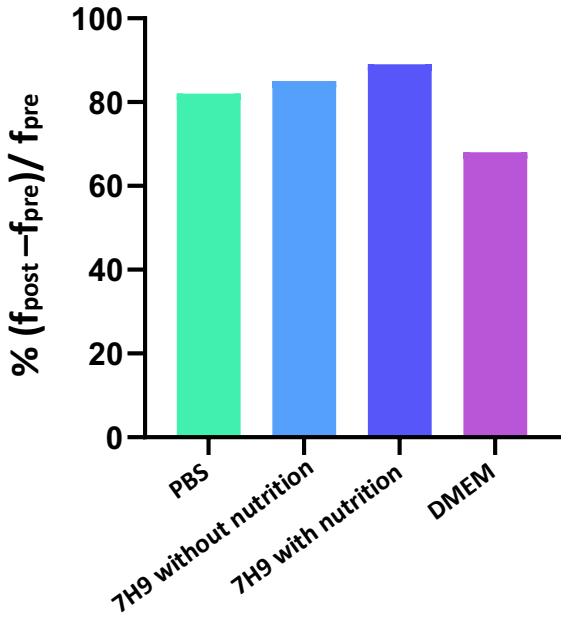


Fig. S13. Stability analysis of CDG-Tre. CDG-Tre (20 μM) was incubated in PBS, 7H9 with or without nutrition supplement (M0553 Middlebrook ADC Growth Supplement, sigma Aldrich), and DMEM for 22 h at 37°C followed by analysis via HPLC. The peak areas of CDG-Tre pre- and post- incubation in these solutions were compared.

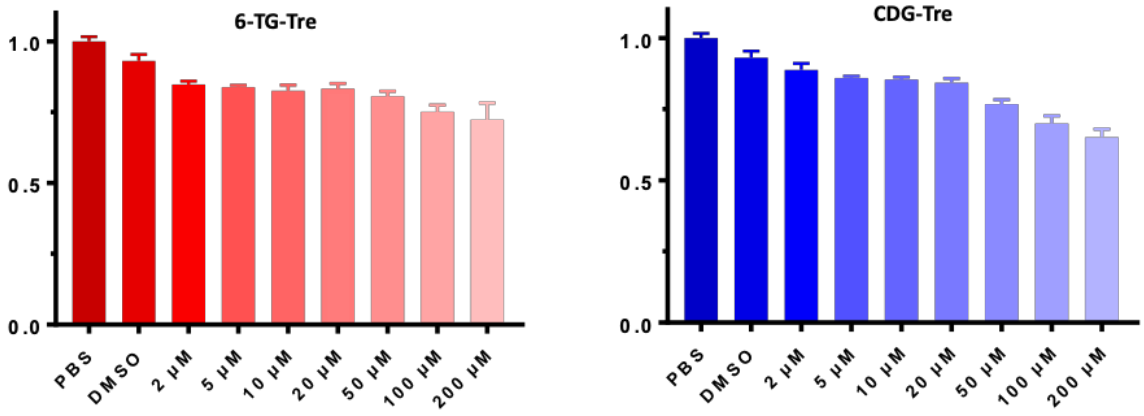
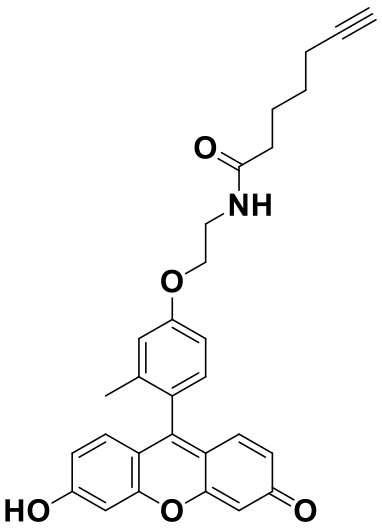
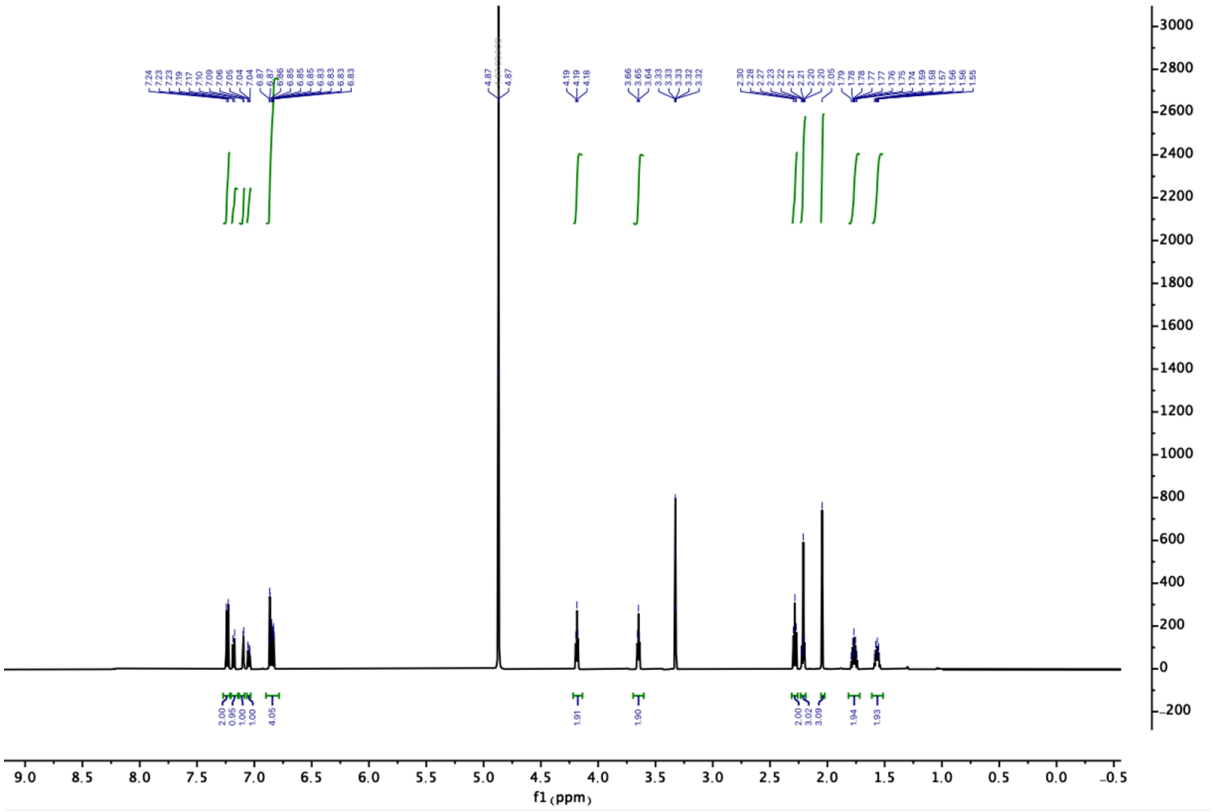


Fig. S14. Cell viability study of 6-TG-Tre and CDG-Tre. Viability assay (MTS) with 6-TG-Tre and CDG-Tre (dissolved in 0.5% DMSO) treated macrophages for 22 h. The average reading of PBS treated macrophages was arbitrarily set as 1 to normalize relative viability. Error bars represent standard deviation of three independent measurements.





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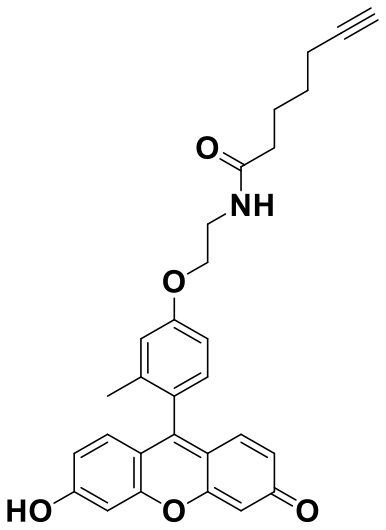

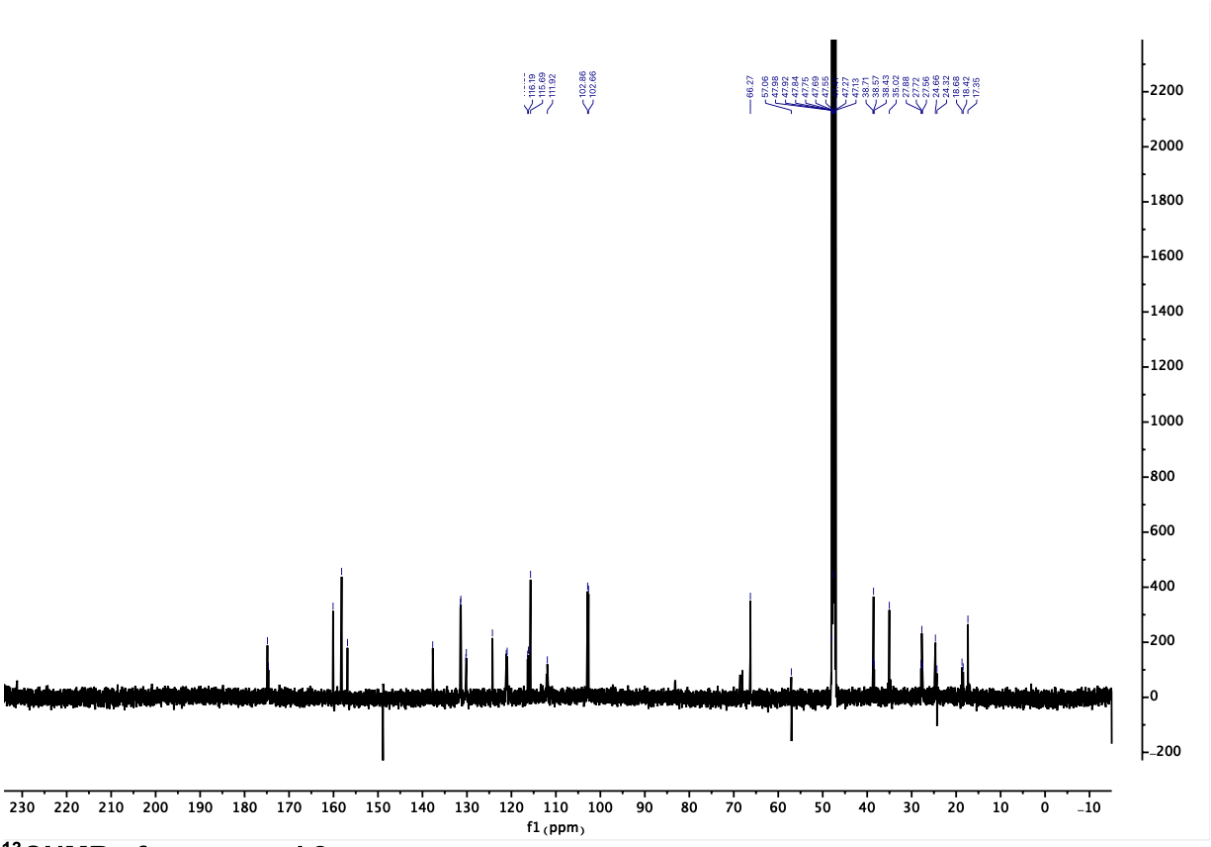

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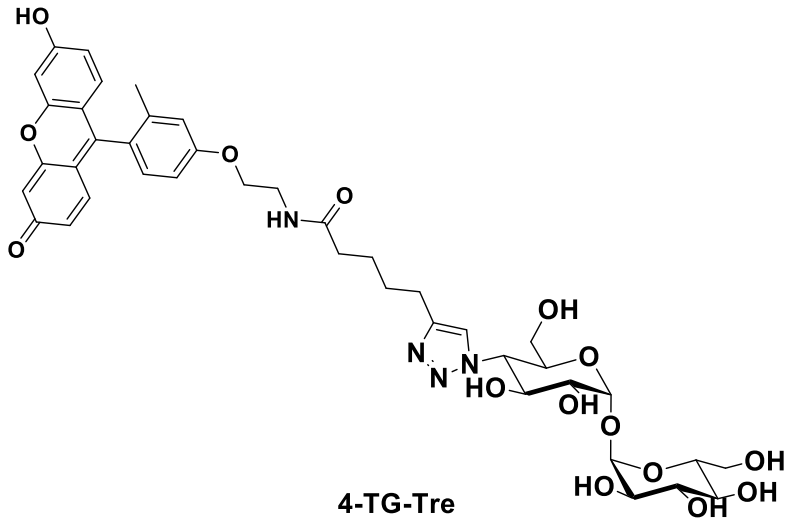
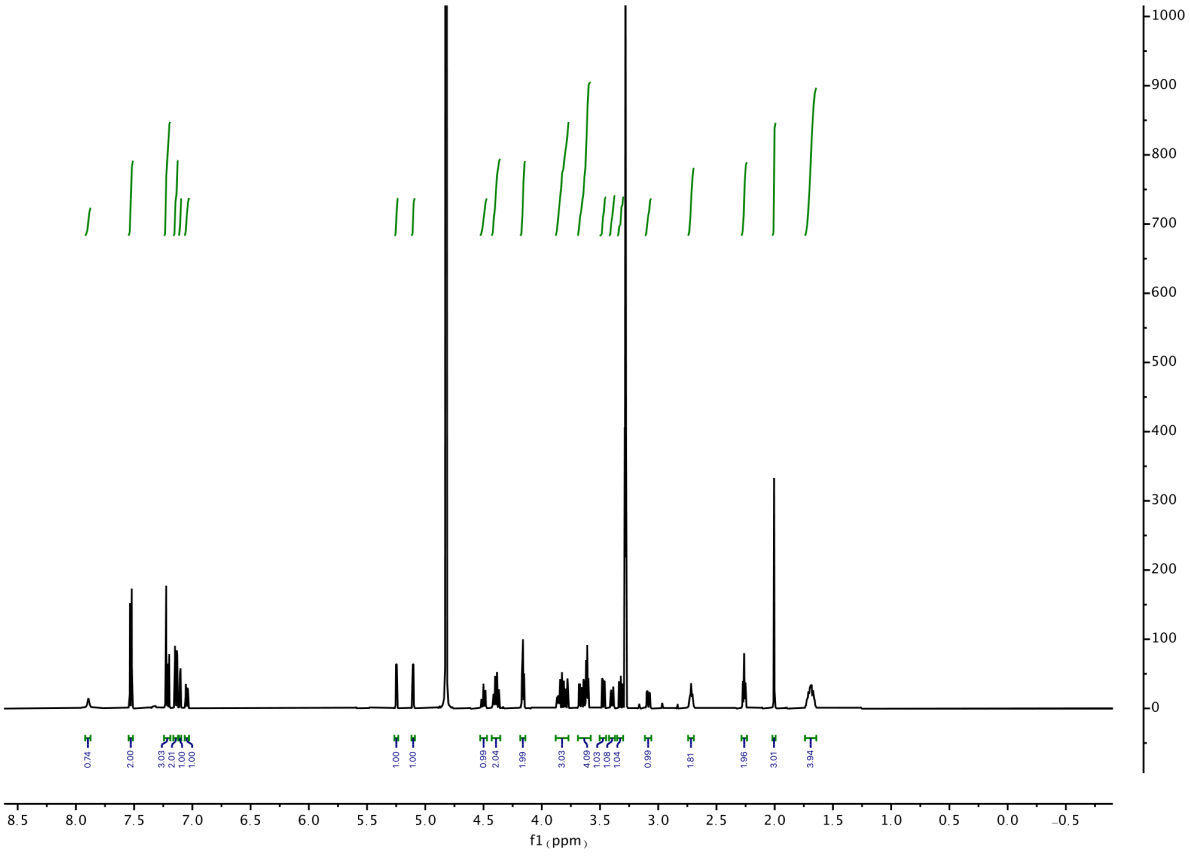


4-TG-Tre



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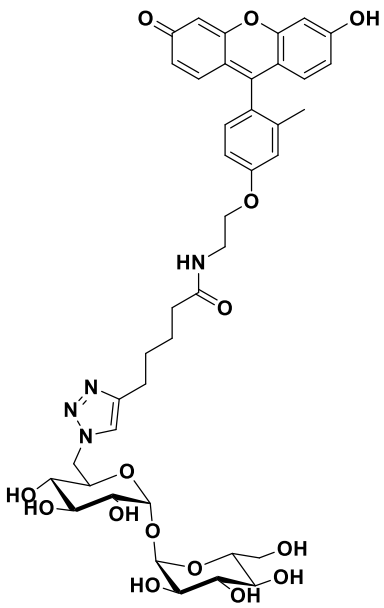
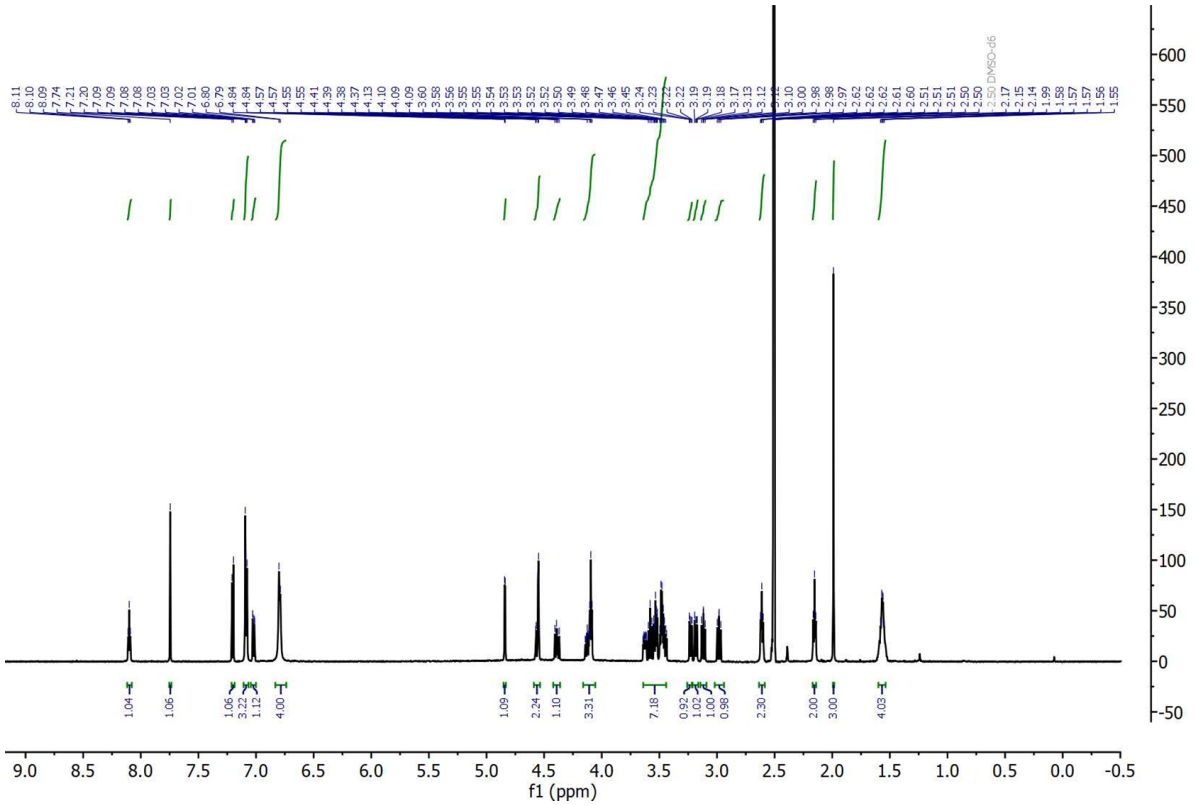


6-TG-Tre



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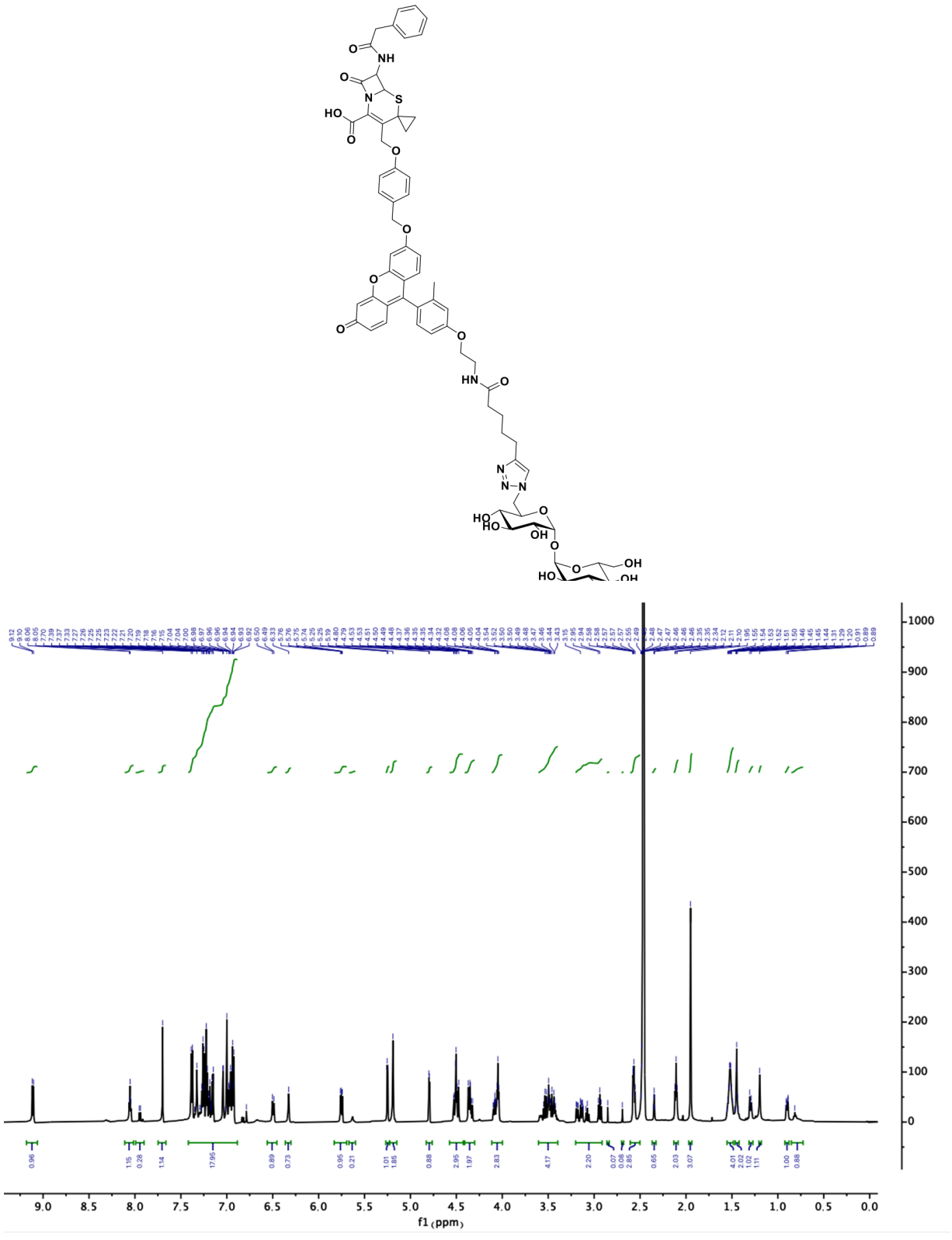

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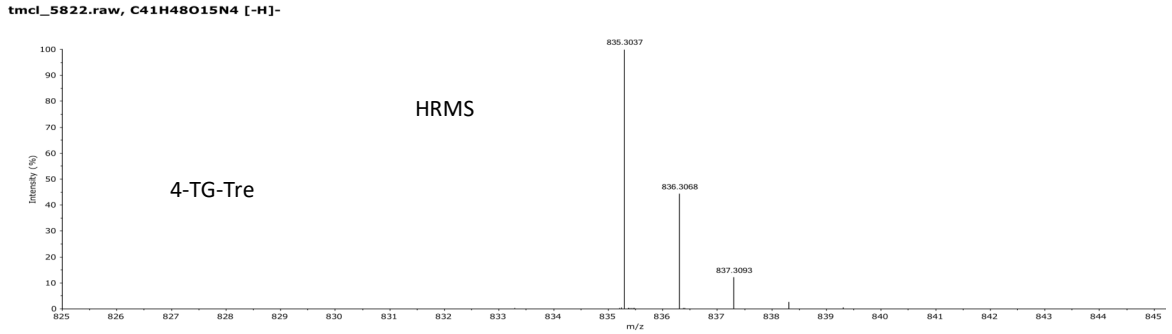

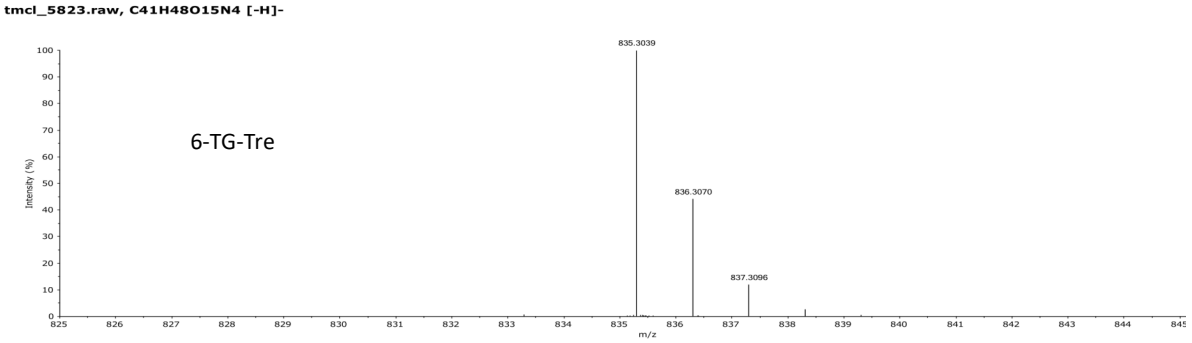

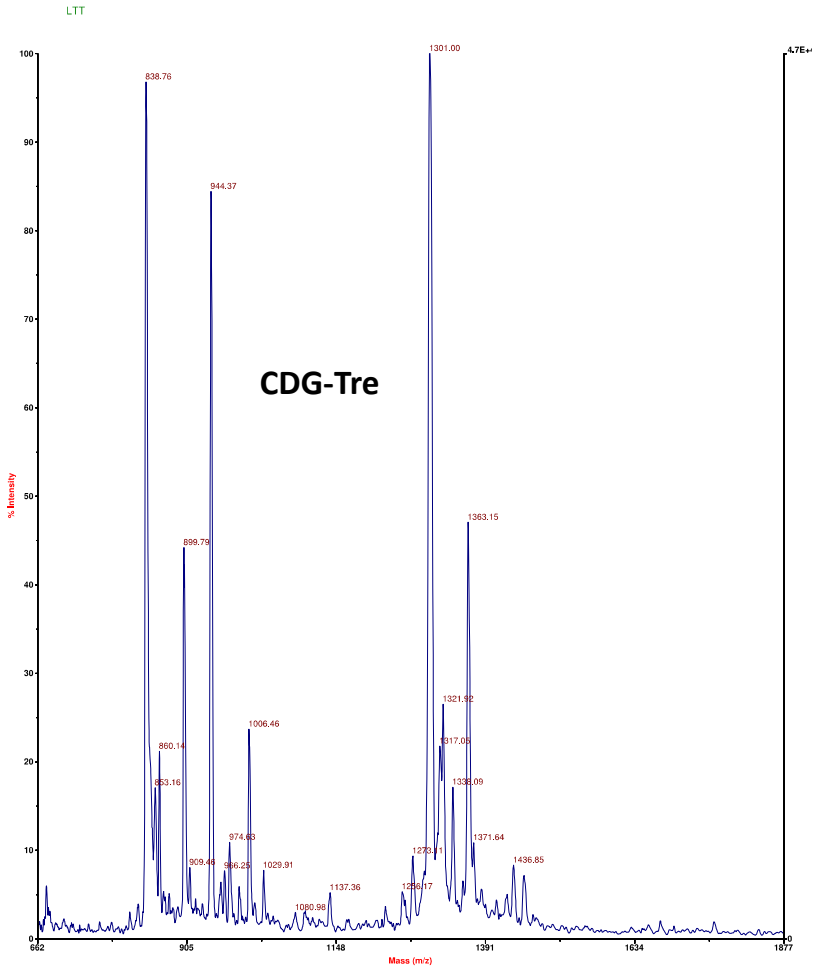

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HRMS of compounds TG-Tre and MALDI-TOF of CDG-Tre

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