Supplemental Information

Imaging Mycobacterial Growth and Division with a Fluorogenic Probe

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Supplemental materials and methods

General information for chemical synthesis

Solvents were purified according to published guidelines (1). All reactions were conducted under a nitrogen atmosphere in oven-dried glassware unless otherwise stated. Analytical thin layer chromatography (TLC) was carried out on E. Merck (Darmstadt) TLC plates pre-coated with silica gel 60 F254 (250 μm layer thickness). Analyte visualization was accomplished using a UV lamp or by charring with phosphomolybdic acid solution. Flash column chromatography was performed with Silicycle flash silica gel (40–63 μm, 60 Å pore size) using the quoted eluent.

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz spectrometer (acquired at 400 MHz for ^{1}H NMR and 101 MHz for ^{13}C NMR), or a 500 MHz spectrometer (acquired at 500 MHz for ^{1}H NMR and 126 MHz for ^{13}C NMR). Chemical shifts are reported relative to residual solvent peaks in parts per million ($CDCl_3$: ^{1}H, 7.27, ^{13}C, 77.23; CD_3OD: ^{1}H, 3.31, ^{13}C, 49.15). Peak multiplicity is reported as singlet (s), doublet (d), triplet (t), quartet (q), pentet (p), or some combination of these. Coupling constants (*J*) are reported in Hertz (Hz) and rounded to the nearest 0.1 Hz. High-resolution mass spectra (HRMS) were obtained on an electrospray ionization-time of flight (ESI-TOF) Micromass LCT mass spectrometer.

BODIPY-FL alkyne was purchased from Setareh Biotech (Eugene, OR) and DABCYL-OH from TCI (Japan) – all other chemical reagents were purchased from Sigma Aldrich (Milwaukee, WI) and used as received. 2,3,4,2',3',4'-Hexakis-*O*-(trimethylsilyl)-α,α-trehalose **2** and 15-azidopentadecanoic acid were synthesized according to published procedures (2, 3).

Detailed synthetic procedures

*Synthesis of 6-O-(15-[BODIPY-FL]-pentadeconyl)-6'-O-DABCYL-α,α-trehalose/QTF **1*** (See Figure S1 for overall route)

Dowex-50WX8-200 ion exchange resin (24 mg) was added to a solution of compound **5** (7.8 mg, 0.0048 mmol) in methanol (1.35 mL) and stirred at rt for 30 min. The resin was removed by filtration (washing with methanol) and the filtrate was concentrated under reduced pressure. Purification of the resulting residue by column chromatography (SiO_2, 10→15% $MeOH/CH_2Cl_2$) afforded the title compound **1** (4.3 mg, 75%) as an orange solid: R_f = 0.47 (15% $MeOH/CH_2Cl_2$); ^{1}H NMR (500 MHz, CD_3OD) δ 8.18 – 8.10 (m, 2H), 7.90 – 7.80 (m, 4H), 7.72 (s, 1H), 7.39 (s, 1H), 6.95 (d, *J* = 4.0 Hz, 1H), 6.86 – 6.79 (m, 2H), 6.27 (d, *J* = 4.0 Hz, 1H), 6.19 (s, 1H), 5.11 (t, *J* = 4.1 Hz, 2H), 4.60 (dd, *J* = 11.9, 2.1 Hz, 1H), 4.48 (dd, *J* = 11.8, 5.4 Hz, 1H), 4.42 (s, 2H), 4.37 (dd, *J* = 11.9, 2.2 Hz, 1H), 4.29 (t, *J* = 7.1 Hz, 2H), 4.24 – 4.16 (m, 2H), 4.03 (ddd, *J* = 10.2, 5.5, 2.2 Hz, 1H), 3.82 (ddd, *J* = 15.1, 9.7, 8.9 Hz, 2H), 3.56 – 3.46 (m, 3H), 3.34 (dd, *J* = 10.1, 8.9 Hz, 2H), 3.23 (t, *J* = 7.6 Hz, 2H), 3.10 (s, 6H), 2.64 (t, *J* = 7.6 Hz, 2H), 2.49 (s, 3H), 2.32 (t, *J* = 7.4 Hz, 2H), 2.26 (s, 3H), 1.80 (p, *J* = 7.1 Hz, 2H), 1.59 (p, *J* = 7.3 Hz, 2H), 1.30 – 1.18 (m, 20H) ppm; ^{13}C NMR (126 MHz, CD_3OD) δ 175.5, 174.6, 167.5, 161.4, 158.4, 157.5, 154.8, 146.3, 145.8, 144.9, 136.5, 134.8, 131.7, 131.4, 129.5, 126.6, 125.7, 124.1, 123.0, 121.4, 117.7, 112.6, 95.3, 95.2, 74.7, 74.6, 73.2, 73.2, 72.0, 72.0, 71.6, 71.5, 65.3, 64.4, 51.4, 40.4, 35.8, 35.7, 35.1, 31.3, 30.7, 30.7, 30.6, 30.6, 30.5, 30.5, 30.3, 30.1, 30.1, 27.5, 26.1, 25.6, 14.9, 11.2 ppm; HRMS (ESI-TOF$^+$) calcd for $C_{59}H_{80}{}^{10}BF_2N_9O_{14}$ ($M+H^+$) 1187.5995, found 1187.6007.

*Synthesis of 6'-O-DABCYL-2,3,4,2',3',4'-hexakis-O-(trimethylsilyl)-α,α-trehalose **3***
DABCYL-OH (83 mg, 0.31 mmol), EDC•HCl (109 mg, 0.57 mmol) and DMAP (16 mg, 0.13 mmol) were dissolved in dry CH_2Cl_2 (1 mL) and stirred at rt for 15 min, then cooled to 0 °C. A solution of compound **2** (200 mg, 0.26 mmol) in dry CH_2Cl_2 (1 mL) was added dropwise via cannula, and the reaction mixture stirred at rt for 20 h and then concentrated under reduced pressure. Purification of the resulting residue by column chromatography (SiO_2, 5→20% EtOAc/hexanes) afforded the title compound **3** (103 mg, 31%) as an orange solid: R_f = 0.38 (20% EtOAc/hexanes); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 – 8.12 (m, 2H), 7.94 – 7.85 (m, 4H), 6.83 – 6.70 (m, 2H), 4.96 (d, *J* = 3.1 Hz, 2H), 4.61 (dd, *J* = 12.0, 2.4 Hz, 1H), 4.31 (dd, *J* = 12.0, 3.7 Hz, 1H), 4.13 (dq, *J* = 8.4, 2.7 Hz, 1H), 3.97 (t, *J* = 8.9 Hz, 1H), 3.92 (t, *J* = 8.9 Hz, 1H), 3.85 (dt, *J* = 9.5, 3.4 Hz, 1H), 3.76 – 3.63 (m, 3H), 3.53 – 3.41 (m, 3H), 3.11 (s, 6H), 1.76 (dd, *J* = 7.4, 5.3 Hz, 1H), 0.18 (s, 9H), 0.17 (s, 9H), 0.16 (s, 9H), 0.15 (s, 9H), 0.14 (s, 9H), 0.14 (s, 9H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.3, 156.3, 153.1, 143.9, 130.8, 130.1, 125.7, 122.2, 111.6, 94.8, 94.6, 73.8, 73.5, 73.1, 73.0, 72.8, 72.1, 71.5, 71.1, 63.9, 61.8, 40.5, 1.3, 1.2, 1.1, 1.0, 0.4, 0.3 ppm; HRMS (ESI-TOF$^+$) calcd for $C_{45}H_{83}N_3O_{12}Si_6$ ($M+H^+$) 1026.4666, found 1026.4641.

*Synthesis of 6-O-(15-azidopentadeconyl)-6'-O-DABCYL-2,3,4,2',3',4'-hexakis-O-(trimethylsilyl)-α,α-trehalose **4***
15-Azidopentadecanoic acid (22 mg, 0.078 mmol), EDC•HCl (30 mg, 0.156 mmol) and DMAP (5.7 mg, 0.047 mmol) were dissolved in dry CH_2Cl_2 (0.5 mL) and stirred at rt for 15 min. A solution of compound **3** (40 mg, 0.039 mmol) in dry CH_2Cl_2 (1 mL) was added dropwise via cannula, and the reaction mixture stirred at rt for 16 h and then concentrated under reduced pressure. Purification of the resulting residue by column chromatography (SiO_2, 5% EtOAc/hexanes) afforded the title compound **4** (24 mg, 48%) as an orange solid: R_f = 0.33 (10% EtOAc/hexanes); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (m, 2H), 7.95 – 7.84 (m, 4H), 6.80 – 6.73 (m, 2H), 4.97 (t, *J* = 3.6 Hz, 2H), 4.60 (dd, *J* = 12.0, 2.4 Hz, 1H), 4.29 (ddd, *J* = 11.2, 7.9, 2.8 Hz, 2H), 4.16 – 3.90 (m, 5H), 3.67 (t, *J* = 9.0 Hz, 1H), 3.54 – 3.43 (m, 3H), 3.24 (t, *J* = 7.0 Hz, 2H), 3.12 (s, 6H), 2.34 (td, *J* = 7.5, 3.5 Hz, 2H), 1.66 – 1.52 (m, 4H), 1.39 – 1.22 (m, 20H), 0.18 (s, 9H), 0.16 (s, 9H), 0.16 (s, 9H), 0.14 (s, 9H), 0.14 (s, 9H), 0.14 (s, 9H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.9, 166.3, 156.3, 153.1, 143.9, 130.8, 130.1, 125.7, 122.1, 111.6, 94.7, 73.8, 73.6, 72.9, 72.1, 71.0, 70.9, 64.0, 63.5, 51.6, 40.5, 34.3, 29.8, 29.8, 29.8 29.7, 29.6, 29.6, 29.5, 29.3, 29.3, 29.0, 26.9, 24.9, 1.3, 1.2, 1.1, 1.1, 0.4, 0.4 ppm; HRMS (ESI-TOF$^+$) calcd for $C_{60}H_{110}N_6O_{13}Si_6$ ($M+H^+$) 1291.6820, found 1291.6833.

*Synthesis of 6-O-(15-[BODIPY-FL]-pentadeconyl)-6'-O-DABCYL-2,3,4,2',3',4'-hexakis-O-(trimethylsilyl)-α,α-trehalose **5***
Compound **4** (7.9 mg, 0.006 mmol) and BODIPY-FL alkyne (2 mg, 0.006 mmol) were dissolved in 5:5:1 *t*-BuOH:H_2O:CH_2Cl_2 (1.1 mL total). To this solution were added TBTA (645 μg in 64.5 μL CH_2Cl_2, 0.0012 mmol), sodium ascorbate (12 μL of a 0.1M aqueous solution, 0.0012 mmol), and $CuSO_4$•$5H_2O$ (6 μL of a 0.1M aqueous solution, 0.0006 mmol). The resulting mixture was stirred vigorously in the dark for 16 h, and then concentrated under reduced pressure. Purification of the resulting residue by column chromatography (SiO_2, 40→90% EtOAc/hexanes) afforded the title compound **5** (7.8 mg, 79%) as an orange solid: R_f = 0.17 (50% EtOAc/hexanes); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.18 – 8.13 (m, 2H), 7.93 – 7.85 (m, 4H), 7.45 (s, 1H), 7.06 (s, 1H), 6.84 (d, *J* = 4.0 Hz, 1H), 6.81 – 6.72 (m, 2H), 6.24 (m, 2H), 6.11 (s, 1H), 4.96 (dd, *J* = 5.2, 3.1 Hz, 2H), 4.60 (dd, *J* = 11.9, 2.4 Hz, 1H), 4.47 (d, *J* = 5.8 Hz, 2H), 4.33 – 4.24 (m, 4H), 4.13 (dt, *J* = 9.5, 3.0 Hz, 1H), 4.06 (dd, *J* = 11.8, 4.5 Hz, 1H), 4.02 (ddd, *J* = 9.6, 4.5, 2.3 Hz, 1H), 3.96 (t, *J* = 8.9 Hz, 1H), 3.93 (t, *J* = 8.9 Hz, 1H), 3.67 (t, *J* = 9.1

Hz, 1H), 3.54 – 3.43 (m, 3H), 3.27 (t, *J* = 7.5 Hz, 2H), 3.11 (s, 6H), 2.64 (t, *J* = 7.6 Hz, 2H), 2.55 (s, 3H), 2.39 – 2.28 (m, 2H), 2.24 (s, 3H), 1.86 (q, *J* = 7.3 Hz, 2H), 1.67 – 1.54 (m, 4H), 1.25 – 1.23 (m, 18H), 0.17 (s, 9H), 0.16 (s, 9H), 0.16 (s, 9H), 0.14 (s, 9H), 0.14 (s, 9H), 0.13 (s, 9H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.9, 171.9, 166.3, 160.6, 157.2, 156.3, 153.1, 144.9, 144.1, 143.9, 135.4, 133.5, 130.8, 130.1, 128.2, 125.7, 123.9, 122.2, 120.7, 117.4, 111.6, 94.7, 73.8, 73.6, 72.9, 72.8, 72.1, 72.1, 71.0, 70.9, 64.0, 63.5, 50.5, 40.4, 36.0, 35.2, 34.3, 32.1, 30.4, 29.9, 29.9, 29.8, 29.7, 29.6, 29.6, 29.5, 29.3, 29.2, 26.7, 24.9, 22.9, 15.1, 14.3, 11.5, 1.3, 1.2, 1.1, 1.1, 0.4, 0.4 ppm; HRMS (ESI-TOF$^+$) calcd for $C_{77}H_{128}{}^{11}BF_2N_9O_{14}Si_6$ (M+H$^+$) 1620.8281, found 1620.8303.

Synthesis of 6'-O-DABCYL-α,α-trehalose/Q-Tre ***6***

Dowex-50WX8-200 ion exchange resin (50 mg) was added to a solution of compound **3** (15 mg, 0.015 mmol) in methanol (3 mL) and stirred at rt for 15 min. The resin was removed by filtration (washing with 4:1 MeOH:1M HCl (aq.)) and the filtrate was concentrated under reduced pressure. Purification of the resulting residue by column chromatography (SiO_2, 10:89:1→15:84:1 MeOH:CH_2Cl_2:H_2O) afforded the title compound **6** (2.6 mg, 30%) as a red solid: R_f = 0.12 (15:84:1 MeOH:CH_2Cl_2:H_2O); ^{1}H NMR (500 MHz, CD_3OD) δ 8.20 – 8.12 (m, 2H), 7.93 – 7.80 (m, 4H), 6.89 – 6.82 (m, 2H), 5.14 (t, *J* = 3.9 Hz, 2H), 4.61 (dd, *J* = 11.8, 2.2 Hz, 1H), 4.50 (dd, *J* = 11.8, 5.5 Hz, 1H), 4.21 (ddd, *J* = 10.1, 5.4, 2.1 Hz, 1H), 3.87 – 3.77 (m, 4H), 3.67 (dd, *J* = 11.9, 5.4 Hz, 1H), 3.55 (dd, *J* = 9.7, 3.8 Hz, 1H), 3.52 – 3.45 (m, 2H), 3.33 (m, 1H), 3.12 (s, 6H) ppm; ^{13}C NMR (126 MHz, CD_3OD) δ 167.6, 157.6, 154.8, 144.9, 131.7, 131.4, 126.6, 122.9, 112.6, 95.3, 95.1, 74.7, 74.6, 73.9, 73.2, 72.1, 71.9, 71.6, 65.3, 62.6, 40.4 ppm; HRMS (ESI-TOF$^+$) calcd for $C_{27}H_{35}N_3O_{12}$ (M+H$^+$) 594.2294, found 594.2288.

Synthesis of 15-(BODIPY-FL)-pentadecanoic acid/Lipid-FL ***7***

15-Azidopentadecanoic acid (1.7 mg, 0.006 mmol) and BODIPY-FL alkyne (2 mg, 0.006 mmol) were dissolved in 5:5:1 *t*-BuOH:H_2O:CH_2Cl_2 (1.1 mL total). To this solution were added TBTA (645 μg in 64.5 μL CH_2Cl_2, 0.0012 mmol), sodium ascorbate (12 μL of a 0.1M aqueous solution, 0.0012 mmol), and $CuSO_4$•$5H_2O$ (6 μL of a 0.1M aqueous solution, 0.0006 mmol). The resulting mixture was stirred vigorously in the dark for 16 h, and then concentrated under reduced pressure. Purification of the resulting residue by column chromatography (SiO_2, 40→100% EtOAc/hexanes, then 0→10% MeOH/EtOAc) afforded the title compound **7** (3.5 mg, 94%) as an orange solid: R_f = 0.11 (50% EtOAc/hexanes); ^{1}H NMR (500 MHz, CD_3OD) δ 7.74 (s, 1H), 7.43 (s, 1H), 6.98 (d, *J* = 4.1 Hz, 1H), 6.29 (d, *J* = 4.0 Hz, 1H), 6.22 (s, 1H), 5.55 (s, 1H), 4.43 (s, 2H), 4.34 (t, *J* = 7.1 Hz, 2H), 3.72 (s, 1H), 3.24 (t, *J* = 7.6 Hz, 2H), 2.65 (t, *J* = 7.6 Hz, 2H), 2.51 (s, 3H), 2.31 – 2.25 (m, 5H), 1.85 (p, *J* = 7.1 Hz, 2H), 1.59 (h, *J* = 7.2 Hz, 2H), 1.28 (m, 20H) ppm; ^{13}C NMR (126 MHz, CD_3OD) δ 177.8, 174.6, 161.4, 158.4, 146.3, 130.0, 129.6, 129.1, 125.8, 125.5, 124.1, 121.4, 117.7, 55.0, 51.4, 35.8, 35.7, 35.0, 31.3, 30.7, 30.7, 30.6, 30.6, 30.6, 30.4, 30.3, 30.1, 27.5, 26.1, 25.6, 14.9, 11.2 ppm; HRMS (ESI-TOF$^+$) calcd for $C_{32}H_{47}{}^{10}BF_2N_6O_3$ (M+H$^+$) 612.3880, found 612.3880.

Synthesis of 15-(BODIPY-FL)- pentadecanoate/Lipid-FL-OMe ***8***

To a stirred solution of *15-(BODIPY-FL)-pentadecanoic acid* (1 mg, 0.00163 mmol) in 1:1 PhMe:MeOH (325 μL total) was added TMS-diazomethane (5 μL of a 2.0M solution in Hexanes). This solution was stirred for 1 h at rt and quenched with 10 μL of 10% AcOH/MeOH. The quenched solution was concentrated under reduced pressure. Purification of the resulting residue by column chromatography (SiO_2, 0→3% MeOH/CH_2Cl_2) afforded the title compound **8** (85 mg, 83%): R_f = 0.28 (5% MeOH/DCM). ^{1}H NMR (500 MHz, CD_3OD) δ 6.84 (d, *J* = 4.0 Hz, 1H), 6.23 (d, *J* = 4.0 Hz, 1H), 6.21 (t, *J* = 5.4 Hz, 1H), 6.12 (s, 1H), 4.48 (d, *J* = 5.8 Hz, 2H),

4.29 (t, *J* = 7.3 Hz, 2H), 3.66 (s, 3H), 3.27 (t, *J* = 7.6 Hz, 2H), 2.64 (t, *J* = 7.6 Hz, 2H), 2.55 (s, 3H), 2.30 (t, *J* = 7.6 Hz, 2H), 2.25 (s, 3H), 1.92 – 1.83 (m, 2H), 1.64 – 1.59 (m, 2H), 1.37 – 1.19 (m, 20H). ^{13}C NMR (126 MHz, CD_3OD) δ 174.37, 171.76, 158.89, 157.06, 144.77, 143.97, 139.76, 137.59, 133.32, 128.03, 123.77, 122.03, 120.53, 117.26, 51.45, 50.36, 35.84, 35.11, 34.13, 30.29, 29.71, 29.60, 29.58, 29.52, 29.44, 29.39, 29.26, 29.16, 29.01, 26.52, 24.97, 24.80, 14.97, 14.13, 11.33. HRMS (ESI-TOF$^+$) calcd for $C_{33}H_{49}{}^{10}BF_2N_6O_3$ ($M+H^+$) 627.4006, found 627.4012.

General information for biochemical assays

Stock solutions were prepared, sterile-filtered, for all chemical reagents used in biochemical assays: QTF **1** (5 mM in DMSO for kinetic assays, 500 μM in DMSO for all other assays), Q-Tre **6** (1 mM in DMSO), Lipid-FL **7** (1 mM in DMSO), trehalose (100 mM in H_2O), ebselen (1 mM in DMSO) and tetrahydrolipstatin (THL, 1mM in DMSO). DMSO stocks were stored at –20 °C and further diluted before use, to maintain a constant 2% DMSO concentration in all assays. Ebselen was purchased from VWR and supplied by TCI America. Tetrahydrolipstatin was purchased from Sigma Aldrich and supplied by Roche. HADA, hydroxycoumarin-carbonyl-amino-D-alanine was generated by the Weibel Group (11.8 mM in DMSO) using published procedures (4). *In vitro* assays with purified Ag85 proteins were performed in aqueous NaH_2PO_4/Na_2HPO_4 buffer (20 mM, pH = 7.2), whereas assays involving *M. smegmatis* culture filtrate proteins used aqueous NH_4HCO_3 buffer (10 mM, pH = 8.0).

For bacterial assays, the strains employed include *Mycobacterium smegmatis* mc^{2}155, *Corynebacterium glutamicum* ATCC13032, *Escherichia coli* K12 MG1655 and *Bacillus subtilis* 168. *M. smegmatis* was cultured in either LB liquid medium, M63 liquid medium containing 0.2% Glycerol, 1 mM $MgSO_4$ and 0.02% Tween-80, or Middlebrook 7H9 liquid medium (containing 10% ADC, 0.2% glycerol and 0.05% Tween-80). *C. glutamicum*, *E. coli* and *B. subtilis* were all cultured in LB liquid medium. Generally, starter cultures were prepared by inoculating the desired growth medium in a baffled shake flask with a single colony from a freshly-streaked agar plate. Starter cultures were incubated at 37 °C with shaking until saturation, then re-inoculated and grown to mid-logarithmic phase (determined by OD_{600} measurement on an Amersham Ultrospec 10 cell density meter), and finally diluted with additional liquid medium to the quoted optical density for each assay. When cells were induced with acetamide, 0.2%, they were grown in either M63 media or 7H9 without ADC.

Purification of Ag85 proteins (mycolyltransferases)

M. tuberculosis Ag85A, Ag85B, and Ag85C

The following reagents were obtained through BEI Resources, NIAID, NIH:
Ag85A (Gene Rv3804c), Purified Native Protein from *Mycobacterium tuberculosis*, Strain H37Rv, Catalog Number: NR-14856

Ag85B (Gene Rv1886c), Purified Native Protein from *Mycobacterium tuberculosis*, Strain H37Rv, Catalog Number: NR-14857

Ag85C (Gene Rv0129c), Purified Native Protein from *Mycobacterium tuberculosis*, Strain H37Rv, Catalog Number: NR-14858

Upon receipt, lyophilized native Ag85 proteins were thawed and suspended in aqueous NaH_2PO_4/Na_2HPO_4 buffer (20 mM, pH = 7.2) to a concentration of 10 μM for storage in aliquots at –80 °C. *M. tb*. Ag85A–C proteins were thawed as needed, and further diluted with NaH_2PO_4 / Na_2HPO_4 buffer (20 mM, pH = 7.2) immediately prior to use.

Cloning of M. smegmatis mycolyltransferases proteins
The following mycolyltransferase proteins were produced as C-terminal mCherry or hexahistidine tag fusions; Msmeg_3580, Msmeg_6398, Msmeg_6399, Msmeg_6583. Signal sequence truncations or signal sequence mutants were subcloned from the relevant C-terminal mCherry plasmids for Msmeg_3580, Msmeg_6398, Msmeg_6399.

Vectors encoding C-Terminal mCherry M. smegmatis mycolyltransferase fusion proteins
To generate C-terminal mCherry fusions to *M. smegmatis* Ag85 proteins, a linker-mCherry construct was codon optimized for *M. smegmatis* and ordered as a G-block from IDT with added overhangs for an isothermal reaction with a linearized Plam12 vector (Addgene, plasmid 26908) (5'-AGCTGCAGAATTCGAAG-3', 5'-TGGACTCCCTTTCTCTTATC-3') to generate a Plam12-mCherry plasmid. Each individual *M. smegmatis* Ag85 gene was amplified from isolated genomic *M. smegmatis* mc²155 DNA with the following primers 3580 (5'-GATAAGAGAAAGGGAGTCCATTGGTTGCCGTTATGAAGTTCATTCGAG-3', 5'-GGACCCGG ACGCACGAGCACCCGCTGGATGTC-3'), 6398 (5'-GATAAGAGAAAGGGAGTCCAATGAAGTTC GTTGGGAGAATGCGCGG-3', 5'-GGACCCGGACGCACCAGCACGCGCTGCAGGTC-3'), 6399 (5'-GATAAGAGAAAGGGAGTCCAATGGCACCCATGGTGAGC-3', 5'-GGACCCGGACG CACCAGCGAAGCGATCAGGTCAG-3'), 6583 (5'-GATAAGAGAAAGGGAGTCCAGTGAAGCCCT CGTTTTTCTC-3', 5'-GGACCCGGACGCACGAGGACCCGCAACAGATC-3'). These DNA fragments were joined with linearized a pLAM12-mCherry vector (5'-GGTGCGTCCGGGT CC-3', 5'-TGGACTCCCTTTCTCTTATCGGGTG-3') by isothermal annealing with a Gibson Assembly master mix (NEB). Plasmids were transformed into DH5α *E. coli.* Vectors encoding C-terminal mCherry fusions to *M. smegmatis* Ag85 proteins were confirmed by DNA sequence analysis at the UW-Madison Biotechnology Center. Plasmids were then transformed into *M. smegmatis* mc²155 for homologous expression.

Signal sequence mutants of C-terminal mCherry Fusion *M. smegmatis* Ag85 proteins:
Each C-terminal mCherry fusion vector encoding *M. smegmatis* mycolyltransferases (Msmeg-_3580, Msmeg_6398, Msmeg_6399) was modified by inverse PCR such that the construct replaces twin-arginine motifs (RR) with lysine. The following primers were used: Msmeg_358 0MC KK (5'-/5phos/AAGAAATTTGTGATCGGTGCCCT-3', 5'-/5phos/CAGGGCTTTCCATGCT G-3'), Msmeg_6398 MC KK (5'-/5phos/AAGAAACTGACGGTTGCGGTC -3', 5'-/5phos/ CGACAGACCTGCCGC-3'), Msmeg_6399 MC KK (5'-/5phos/-3', 5'-/5phos/CAGACCGGATAC GCGCA-3') Msmeg_3580MC Δss (5'-/5phos/TTTTCCCGTCCTGGTCTG-3', 5'-/5phos/CAATG GACTCCCTTTCTCTTATCGG-3'), Msmeg_6398 MC Δss (5'-/5phos/TGGTCTCGACCCGGC-3', 5'/5phos/CATTGGACTCCCTTTCTCTTATCGG-3'), Msmeg_6399 MC Δss (5'-/5phos/TTTTCG CGTGAGGGCC-3', 5'-/5phos/CATTGGACTCCCTTTCTCTTATCGG-3'. Plasmids were transformed into DH5α *E. coli.* Vectors encoding signal sequence variants of C-terminal mCherry fusions to *M. smegmatis* Ag85 proteins were confirmed by DNA sequence analysis at the UW-Madison Biotechnology Center. Plasmids encoding the mycolyltransferase variants were then transformed into *M. smegmatis* mc²155 for mycolyltransferase production.

C-Terminal hexahistidine-tagged M. smegmatis mycolyltransferases
The genes encoding Msmeg_3580, Msmeg_6398, Msmeg_6399, Msmeg_6583 were amplified by inverse PCR from previously generated constructs encoding *M. smegmatis* mycolyltransferase C-terminal mCherry fusions with the following phosphorylated primers, 5'-/5phos/TAATAAAGCTGCAGAATTCGAAGCTTAT-3' and 5'-/5phos/ATGGTGGTGATGATGGT GCGACGCCCCGGC-3'. Template plasmid was digested with DPNI (NEB), and amplified products were self-ligated with T4 DNA ligase (Fermentas) and transformed into DH5α E. *coli.*

Vectors encoding C-terminal hexahistidine tag *M. smegmatis* mycolyltransferase proteins were confirmed by DNA sequence analysis at the UW-Madison Biotechnology Center. Plasmids were then transformed into *M. smegmatis* mc^2155 for homologous expression.

Production of M. smegmatis mycolyltransferases
M. smegmatis strains harboring vectors encoding C-terminal hexahistidine tag *M. smegmatis* mycolyltransferases were grown to saturation overnight in M63 liquid medium containing 0.2% glycerol, 1 mM $MgSO_4$ and 0.02% Tween-80. Cultures were diluted 1:200 in M63 liquid medium containing 0.2% glycerol, 1mM $MgSO_4$ and 0.02% Tween-80 and induced at OD_{600} 0.2 with 0.2% acetamide for 6-10 hours at 30 °C. Cells were pelleted, and supernatants were collected and filtered through a 0.2-micron filter. Collected culture filtrates containing secreted C-terminal hexahistidine tagged mycolyltransferase proteins were concentrated with 10 kDa molecular weight filter devices at 4 °C. Concentrated protein filtrates were incubated with Prometheus Magnetic agarose derivatized with nitrilotriacetic acid (Genesee Scientific) according to manufacturer's instructions. Proteins were eluted with 500 mM imidazole and eluted proteins were subsequently dialyzed against NaH_2PO_4 / Na_2HPO_4 buffer (20 mM, pH = 7.2) in a 10 kDa dialysis cassette (Pierce). Protein concentration was determined by NanoOrange® Protein Quantitation. The resulting protein solutions were then diluted with NaH_2PO_4/Na_2HPO_4 buffer (20 mM, pH = 7.2) to a final concentration of 20 nM, and temporarily stored at 4 °C until use (within 48 h). Purity was assessed by polyacrylamide gel electrophoresis and staining by Sypro Ruby (see Figure S9).

For immunoblotting, SDS/PAGE sample buffer was added to *M. smegmatis* cells lysed by bead beating or to concentrated media filtrate (3k MWCO) and then samples were loaded onto a 4-15% Tris-HCl gel. Samples were then transferred onto 0.45 μm PVDF membrane (Millipore) and blocked for 2 h at rt with a solution of Tris-buffered saline and 0.1% Tween 20 (TBS-T) containing 5% nonfat milk. The membrane was exposed to at 1:10000 dilution of rabbit polyclonal anti-mCherry (ab167453, Abcam) for 1hr at room temperature in TBS-T solution with 3% milk. After rinsing three times in TBS-T for 5 min, the blot was treated at rt with a 1:10000 dilution of HRP-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) in TBS-T solution containing 3% milk for 1 h at rt. The membrane was washed with TBS-T and visualized with chemiluminescent substrate (ECL prime, GE) on an ImageQuant LAS 4000 (GE Healthcare).

Fluorescence assays
Fluorescence measurements were acquired on a Tecan Infinite M1000 Pro microplate reader. Assays requiring only fluorescence reads were performed in Corning black 96-well half-area microplates. Assays that involve tandem fluorescence and absorbance reads were performed in Corning black 96-well clear-bottom plates. To monitor BODIPY-FL fluorescence, samples were excited at 503 nm, and detected (a minimum of 4 reads per well) at a single emission wavelength of 515 nm in QTF cleavage assays or between 510 – 600 nm when acquiring full emission spectra. Fluorimeter gain and Z-position were optimized to wells containing the lipid-FL standard. Settings were kept constant across replicates and in comparable experiments. Fluorescence emission intensities are reported in relative fluorescence units (RFU).

Emission spectra and quenching efficiency
A black 96-well half-area microplate was used to measure the fluorescence emission of a QTF concentration series. Measurements were carried out analogously with a concentration series consisting of equimolar mixtures of cleavage standards Q-Tre and Lipid-FL. Stock solutions of

QTF, Q-Tre and Lipid-FL in DMSO were prepared, and transferred into wells containing aqueous NaH_2PO_4/Na_2HPO_4 buffer (20 mM, pH = 7.2), to give final concentrations of 100 nM, 250 nM, 500 nM, 1 μM, 2.5 μM, 5 μM and 10 μM for all analytes at a final well volume of 100 μL and DMSO concentration of 2%. Fluorescence emission at 515 nm following excitation at 503 nm was recorded at 37 °C for each series and plotted versus analyte concentration to determine quenching efficiency (see Figure S5). Fluorescence emission scans (510 – 600 nm) were also acquired for the 1 μM samples (Figure S3).

End-point assays with Ag85 proteins

A black 96-well half-area microplate was used to fluorescence resulting from QTF exposure to purified *M. tuberculosis* or *M. smegmatis* mycolyltransferases proteins. Additionally, the effect of added trehalose, THL or ebselen on this fluorescence was assessed. The following stock solutions were prepared: QTF (100 μM in DMSO), ebselen (1 mM in DMSO) and THL (1 mM in DMSO), and trehalose (100 mM in H_2O). Solutions of *M. tuberculosis* Ag85A–C (400 nM) and *M. smegmatis* proteins (20 nM) in aqueous NaH_2PO_4/Na_2HPO_4 buffer (20 mM, pH = 7.2) were also prepared. Wells (n = 3) were filled with enzyme solution, phosphate buffer, and trehalose solution where appropriate (Ag85A–C wells only), followed by either DMSO (0.5 μL), THL (0.5 μL) or ebselen (0.5 μL). The total volume of all wells was 49.5 μL. Sample plates were subjected to shaking for 2 h at 37 °C before the addition of 0.5 μL of QTF stock solution. All wells were at a final volume of 50 μL (phosphate buffer with 2% DMSO) with Ag85 and QTF concentrations of 20 nM and 1 μM, respectively. Each Ag85 protein was assayed with and without trehalose (5 mM), THL (10 μM), or ebselen (10 μM). Fluorescence emission at 515 nm was recorded immediately to acquire a zero-time point level and after 20 h with shaking at 37 °C.

Kinetic assays with M. tuberculosis Ag85 A–C

A black 96-well half-area microplate was used to measure initial reaction velocities for varying concentrations of QTF with a fixed concentration (1 μM) of each Ag85 isoform. Stock solutions of QTF were prepared (0.05, 0.125, 0.25, 0.375, 0.5, 1.25, 2.5 and 5 μM in DMSO), along with a solution of Ag85A, Ag85B or Ag85C (1 μM) in aqueous NaH_2PO_4/Na_2HPO_4 buffer (20 mM, pH = 7.2). Wells (n = 3) were filled with phosphate buffer (93 μL) and Ag85 solution (5 μL), and the plate incubated at 37 °C for 15 min. Reactions were initiated by the addition of 2 μL QTF solution, to give final concentrations of 50 nM Ag85, and 1, 2.5, 5, 7.5, 10, 25, 50 and 100 μM QTF. Wells were mixed thoroughly, and fluorescence emission monitored continuously for 20 min.

Plots of fluorescence (RFU) versus time (s) were generated using Microsoft Excel, and the slope of the data was used to calculate initial reaction velocities (in nM s–1), after conversion from RFUs to concentration using a BODIPY-FL standard curve (10 – 500 nM). At QTF concentrations exceeding 10 μM, the measured reaction velocities were distorted by an "inner filter effect" (IFE), in which the fluorescence of the generated Lipid-FL product undergoes intramolecular quenching in the presence of high concentrations of substrate. We therefore applied Kohlbrenner's method to correct the initial velocity data (5). After applying this correction, the data were fit to the Michaelis-Menten equation using GraphPad Prism 6, to determine K_m (app) and k_{cat} (app) values.

In-culture bacterial growth assay

Cultures of *M. smegmatis* or *C. glutamicum* were grown in LB media to mid-logarithmic phase and diluted to an OD_{600} of 0.05. A black 96-well clear-bottom plate was set up to monitor fluorescence in the presence of QTF during bacterial growth. Wells (n = 3) were filled with diluted bacteria (98 μL), and 2 μL of a 125 μM QTF DMSO stock solution (or 2 μL DMSO for negative control wells) was added to give a final QTF concentration of 2.5 μM, a final well volume of 100 μL, and DMSO concentration of 2%. The plate was incubated with shaking at 37 °C for reported time. Fluorescence emission at 515 nm and absorbance at 600 nm was recorded at specified intervals.

Flow cytometry and fluorescence microscopy

E. coli, *B. subtilis* and *C. glutamicum* were cultured in LB + 0.05% Tween-80. All cultures were grown at 30 °C in a shaking incubator overnight to saturation. A portion of the culture (3 mL) was then diluted to OD_{600} = 0.02 in 10 mL baffled flasks (3 independent samples) and grown to an OD_{600} of 0.2, whereupon 1 μM QTF was added to initiate labeling. Aliquots (500 μL) were removed for analysis at OD_{600} = 0.8 (2 doubling times). The *E. coli*, *B. subtilis* and *C. glutamicum* samples were pelleted and resuspended in 500 μL of 1% paraformaldehyde in PBS. Samples were fixed at rt for 30-60 min, quenched with 500 μL of 1 M lysine solution, and pelleted and resuspended in 1X PBS for analysis by flow cytometry (Figure S8, Left column). We also performed analysis of two control samples to establish background fluorescence values of cells alone (auto-fluorescence) or cells that were fixed and then incubated with probe (background). For the auto-fluorescence control, each strain at OD_{600} = 0.2 was fixed in the absence of probe (- QTF) (Figure S8, Center column). For background control experiments, each strain at OD_{600} = 0.2 was fixed (which should disrupt mycolyltransferase activity) and then exposed to QTF (1 μM for 30 m at 30 °C). These control samples were then spun to pellet the cells and resuspended once (+ QTF) (Figure S8, Right column).

M. smegmatis was cultured in 7H9 with ADC component and 0.05% Tween-80 and grown at 30 °C in a shaking incubator overnight to saturation. A portion of the culture (3 mL) was diluted to OD_{600} = 0.02 in 10 mL baffled flasks (3 independent samples) and grown to an OD_{600} of 0.2, whereupon 1 μM QTF was added to initiate labeling. 500 μL aliquots were removed for analysis at OD_{600} =1.2 (3 doubling times). We found that *M. smegmatis* cells under these conditions did remain intact after fixation. Therefore, flow cytometry of *M. smegmatis* was performed on live cells in 7H9 + ADC/Tween-80. Aliquots of *M. smegmatis* at OD_{600} = 1.2 were analyzed immediately by flow cytometry (Figure S8, Left column). We also performed analysis two negative control samples to establish background fluorescence values of cells alone (auto-fluorescence) or cells that were incubated with probe and washed at incubation times insufficient for labeling (background). For auto-fluorescence controls, *M. smegmatis* at OD_{600} = 0.2 was analyzed by flow cytometry in the absence of probe (- QTF) (Figure S8, Center column). For background control, *M. smegmatis* at OD_{600} = 0.2 were washed to remove secreted mycolyltransferases, incubated with 1 μM probe for 15 m at 30 °C then spun and resuspended once and analyzed by flow cytometry (+ QTF) (Figure S8, Right column).

For flow cytometry, cells were diluted as necessary. Flow cytometry was performed on a BD Accuri C6 for 100,000 events with 7,000 threshold (*E. coli*, *B. subtilis* and *C. glutamicum*) or 15,000 (*M. smegmatis*). Flow cytometry analysis was performed in triplicate on three independent cultures for each strain, representative histograms and scatter plots are shown. All flow cytometry data was processed in Flowjo (FlowJo LLC).

For analysis by microscopy, cell aliquots were taken directly from samples of *E. coli*, *B. subtilis*, *C. glutamicum (*OD_{600}=0.8) or *M. smegmatis* (OD_{600} = 1.2) analyzed by flow cytometry. Each sample was spotted onto a glass-bottomed microwell dish (MatTek corporation) and covered with a 1% (w/v) agarose pad. Images were collected at rt with a Nikon A1 laser scanning confocal microscope (Nikon Instruments). Images were acquired with a Nikon plan apo 100/1.4 oil objective with a 1.2-AU pinhole diameter and NIS-elements C software (Nikon Instruments). Identical acquisition settings were used for samples and controls. Brightness and contrast were identically adjusted with the open-source Fiji distribution of ImageJ. Images were then converted to an RGB format to preserve normalization and then assembled into panels. Images are representative of greater than five fields of view.

Mixed culture assay

A vector encoding pEKEX2-mCherry was prepared using primers described in Table S4 by isothermal annealing with a Gibson Assembly master mix (NEB) and the resulting construct was transformed into DH5α. Vectors correctly encoding pEKEX2-mCherry were confirmed by DNA sequence analysis at the UW-Madison Biotechnology Center. Plasmids were then passaged through dam-/dcm- competent *E. coli* (NEB). dam-/dcm- DNA was methylated in-vitro by GpC Methylase (M.CviPI) (Zymo) and transformed into *C. glutamicum* for expression.

E. coli, B. subtilis and *C. glutamicum/*pEKEX2-mCherry were grown individually in LB to late logarithmic phase, OD_{600} = 0.5-1.0. Each culture was diluted and grown to OD_{600} = 0.1. Strains were mixed 1:1:2 *E. coli*: *B. subtilis*: *C. glutamicum* (the increased ratio of *C. glutamicum* employed accounts for the slower growth rate) or maintained individually. To each sample, 1 μM QTF was added, and cells were grown for ~2 h at 37 °C. Cells were spotted without washing onto a glass-bottomed microwell dish (MatTek corporation) and covered with a 1% (w/v) agarose pad. Images were collected at room temperature with a Nikon A1 laser scanning confocal microscope (Nikon Instruments). Images were acquired with a Nikon plan apo 100/1.4 oil objective with a 1.2-AU pinhole diameter and NIS-elements C software (Nikon Instruments) with appropriate filter sets. Images were processed in the open-source Fiji distribution of ImageJ.

Isolation of *M. smegmatis* culture filtrate proteins (CFPs)

CFPs were isolated from cultures by growing *M. smegmatis* mc^2155 in 7H9 with 0.2% glycerol. Cells were pelleted at 3,000 x g and supernatants were collected and filtered through a 0.2-micron filter. CFPs were then concentrated using a membrane with a molecular weight cutoff of 10 kDa and dialyzed into NH_4HCO_3 buffer (10 mM, pH = 7.4). Protein in the isolated culture filtrate batches was quantified by BCA, aliquoted, snap frozen in liquid nitrogen, and then stored at -80 °C. Aliquots of CFPs were thawed as needed and used immediately.

End point fluorescence assay with *M. smegmatis* CFP

A black 96-well half-area microplate was used to measure fluorescence derived from QTF in the presence of *M. smegmatis* CFP with and without ebselen. Stock solutions of CFP (200 μg/mL in 10 mM aqueous NH_4HCO_3 buffer), QTF (100 μM in DMSO) and ebselen (1 mM in DMSO) were prepared. Three sets of wells (n = 3) were filled with 97 μL aqueous NH_4HCO_3 buffer (10 mM, pH = 8.0). To the first set was added 1 μL of *M. smegmatis* culture filtrate protein (200 ng) and 1 μL of DMSO. To a second set, 1 μL of *M. smegmatis* CFP solution (200 ng), 1 μL of ebselen stock solution (10 μM final) was added. Finally, the third set (negative control wells) was treated with 1 μL of buffer and 1 μL of DMSO. The plate was incubated with shaking for 2 h at 37 °C, then 1 μL of QTF stock solution (1 μM final) was added to all wells,

bringing the final volume to 100 μL. Fluorescence emission at 515 nm was recorded immediately to acquire a zero-time point, and again after 12 h incubation at 37 °C with shaking.

Native-PAGE in-gel activity assay

In-gel activity assays were performed by Blue Native Page Polyacrylamide Gel Electrophoresis (Invitrogen, NativePAGE™ Gel system). Briefly, CFP samples (3-9 μg) were mixed with 4x NativePage sample buffer and loaded onto a 4-16% BIS-TRIS gel. Gels were run on ice for 60 minutes at 150V and 90 minutes at 250V using 1x NativePAGE Light Blue Cathode Buffer to reduce background. Gels were rinsed with 1X PBS (pH 7.4) for 5 min to reduce background. Gels were placed on the fluorescence glass imaging surface of GE Typhoon FLA 9000 Gel Imaging Scanner and immersed in 2.5 μM QTF in 1X PBS (pH 7.4) to identify those proteins that can process QTF. Fluorescence was revealed upon scanning, and the resulting images were processed with the open-source Fiji distribution of ImageJ.

Identification of proteins by mass spectrometry

Enzymatic "in gel" digestion

"In gel" digestion and mass spectrometric analysis were carried out at the Mass Spectrometry Facility [Biotechnology Center, UW-Madison]. The digestion was performed as outlined on the website: http://www.biotech.wisc.edu/ServicesResearch/MassSpec/ingel.htm. In short, Coomassie Blue R-250-stained gel bands were de-stained twice for 5 min in methanol/water/NH_4HCO_3 [50%:50%:100 mM], dehydrated for 5 min in acetonitrile/water /NH_4HCO_3 [50%:50%:25 mM], final dehydration was repeated once more for 1 min in 100% acetonitrile, dried in a Speed-Vac for 2 min, and treated to 25 mM dithiothreitol in 25 mM NH_4HCO_3 for 30 min at 56 °C. The samples were then subjected to the alkylating agent iodoacetamide (55 mM in 25 mM aqueous NH_4HCO_3) in darkness at rt for 30 min. After exposure, samples were washed in water (2 x 30 sec, equilibrated in 25 mM aqueous NH_4HCO_3 for 1 min, dehydrated for 5 min in acetonitrile/water/NH_4HCO_3 (50%:50%:25 mM) exposed for 30 sec to 100% acetonitrile, dried again and treated to trypsin solution (20 μL of a solution containing 10 ng/μl trypsin Gold (Promega) in 25 mM NH_4HCO_3/0.01% ProteaseMAX w/v (Promega)). An additional 30 μL of digestion solution (25 mM NH_4HCO_3/0.01% ProteaseMAX w/v) was added to facilitate complete rehydration for peptide extraction. The digestion was conducted for 3 h at 42 °C. Peptides generated from digestion were transferred to a new tube and acidified with 2.5% TFA [trifluoroacetic acid] to 0.3% final. Degraded ProteaseMAX was removed via centrifugation [max speed, 10 min] and the peptides solid phase extracted (*ZipTip® C18* pipette tips Millipore, Billerica, MA).

NanoLC-MS/MS

Peptides were analyzed by nanoLC-MS/MS using the Agilent 1100 nanoflow system (Agilent) connected to a new generation hybrid linear ion trap-orbitrap mass spectrometer (LTQ-Orbitrap Elite™, Thermo Fisher Scientific) equipped with an EASY-Spray™ electrospray source. Peptides were separated prior to mass spectral analysis by chromatography using capillary emitter column (PepMap® C18, 3 μM, 100Å, 150x0.075 mm, Thermo Fisher Scientific) onto which 2 μL of extracted peptides was automatically loaded. The NanoHPLC system delivered solvents A: 0.1% (v/v) formic acid , and B: 99.9% (v/v) acetonitrile, 0.1% (v/v) formic acid at 0.50 μL/min to load the peptides (over a 30 min period) and 0.3 μL/min to elute peptides directly into the nano-electrospray with a gradual gradient from 3% (v/v) B to 30% (v/v) B over 77 min concluding with a 5 min fast gradient from 30% (v/v) B to 50% (v/v) B at which time a 5 min flush using 50-95% (v/v) B took place. As peptides eluted from the HPLC-column/electrospray source, survey MS scans were acquired in the Orbitrap with a resolution

of 120,000 followed by MS2 fragmentation of 20 most intense peptides, by ion count, detected in the MS1 scan from 300 to 2000 m/z; redundancy was limited by dynamic exclusion.

Data analysis
Raw MS/MS data were converted to mgf file format using MSConvert (ProteoWizard: Open Source Software for Rapid Proteomics Tools Development). The resulting mgf files were used to search against the *M. smegmatis* acid sequence database containing a list of common contaminants (6,756 total entries) using in-house *Mascot* search engine 2.2.07 [Matrix Science] with variable methionine oxidation with asparagine and glutamine deamidation plus fixed cysteine carbamidomethylation. Peptide mass tolerance was set at 15 ppm and fragment mass at 0.6 Da. Protein annotations, significance of identification, and spectral based quantification was done with the help of scaffold software (version 4.3.2, Proteome Software Inc., Portland, OR). Protein identifications were accepted if they could be established at greater than 80% probability within 1% false discovery rate and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (6). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

HRMS analysis of QTF cleavage by *M. smegmatis* CFP
To a 500 μL Eppendorf tube was added 94 μL of aqueous NH_4HCO_3 buffer (10 mM, pH = 8.0), 5 μL of *M. smegmatis* CFP solution (200 μg/mL in 10 mM aqueous NH_4HCO_3 buffer), and 1 μL of 5 mM QTF DMSO stock solution, giving final concentrations of 10 μg/mL *M. smegmatis* CFP and 50 μM QTF. To serve as a negative control, 99 μL of buffer and 1 μL of QTF solution were added to a second tube. Both samples were incubated at 37 °C with shaking for five days, then analyzed without further manipulation on an electrospray ionization-time of flight (ESI-TOF) Micromass LCT mass spectrometer in positive ion mode.

TLC analysis of lipid extracts from QTF-treated bacteria
Cultures (5 mL) of *M. smegmatis* in Middlebrook 7H9 liquid medium (containing 10% ADC, 0.2% glycerol and 0.05% Tween-80) and *C. glutamicum* in LB liquid medium, both at an OD_{600} of 0.2 in 12 mL aerated culture tubes, were treated with either 50 μL DMSO (for negative control cultures) or 50 μL of 5 mM QTF stock, giving final concentrations of 5 μM QTF and 1% DMSO. Bacteria were incubated at 37 °C until the culture reached an OD_{600} of 0.9 (~7 h for *C. glutamicum*, ~19 h for *M. smegmatis*). Bacteria were then pelleted by centrifugation (3000 x g, 5 min, rt), and washed three times with PBS containing 0.05% BSA. Each cell pellet was suspended in 2:1 chloroform:methanol (3 mL), transferred to a 10 mL glass culture tube equipped with a stir bar, and stirred vigorously for 16 h to extract soluble cell wall lipids. After extraction, each sample was pelleted (3000 x g, 5 min, rt) and washed twice with 2:1 chloroform:methanol (2 mL).

The organic extracts were combined and concentrated to dryness under reduced pressure, and the residue was resuspended in THF (200 μL). A portion of each sample (15 μL) was spotted on silica gel thin layer chromatography plates. Samples were then eluted using chloroform/methanol/acetone (90:15:10) and imaged on a Typhoon FLA 9000 fluorescence scanner.

To release covalently attached cell wall mycolates from AG, isolated cell wall pellets were resuspended in 1 mL 5% aq. TBAH solution, mixtures were sealed and stirred overnight with heating to 100 °C in an oil bath. After stirring samples were cooled to rt. 500 uL water, 500 uL

DCM and 50 uL methyl iodide (10:10:1) were added and mixture was stirred vigorously at room temperature for 90 min. This protocol was also used to assess BODIPY stability under saponification conditions. Samples were allowed to separate and bottom layer of DCM was removed, concentrated and resuspended in 50 uL of chloroform. A portion of each sample was spotted on silica gel thin layer chromatography plates. Samples were then eluted using 10% methanol in chloroform and imaged on a Typhoon FLA 9000 fluorescence scanner.

Live-cell imaging

Microfluidic device fabrication and operation

All devices were fabricated using standard soft lithography (7). Briefly, the master was patterned on a silicon wafer as three layers of SU-8 photoresist (Microchem, Newton, MA, USA) using a laser pattern generator (Heidelberg Instruments, Heidelberg, Germany). The height of each layer of photoresist was calibrated using a stylus profilometer (Tencor, Milpitas, CA, USA). The first layer was spun to a height of approximately 600 nm using SU-8 2000.5. This layer was used to pattern the bypass channels that surrounded each observation area and enabled fresh media to continuously flow across the cells during our measurements. A second layer of SU-8 2000.5 was deposited on top of the first to create a combined thickness of 1.2 μm. This layer was used to create the observation areas where cells were trapped during our measurements. Finally, a third layer of SU-8 3025 (approximately 25 μm tall) was used to create the main channel network that integrated all of the observation areas. The compound 1,1,2,2-tetradecafluorotrichlorosilane (Gelest Inc., Morristown, NJ, USA) was deposited on the master under vacuum for 4 h prior to embossing. Poly(dimethylsiloxane) (PDMS) was cast at a 10:1 ratio (base to crosslinking agent; Sylgard 184, Dow Corning, Midland, MI, USA) on the master to a depth of ~4 mm; the polymer was then cured at 100°C for 4 h. The cured PDMS replica was carefully removed from the master and trimmed to a suitable footprint. A 1 mm diameter bore was used to punch each inlet. For final device assembly, the PDMS replica was irreversibly plasma bonded (Harrick Plasma, Ithaca, NY, USA) to a clean glass coverslip.

Prior to operating the microfluidic device, a fresh culture of *M. smegmatis* was grown from a single colony at 37 °C in 7H9 with ADC component to an absorbance at 600 nm of roughly 1 (Abs_{600} ~ 1). A portion of the culture was collected using a benchtop centrifuge (6000 RPM for 10 min) and was re-suspended in fresh media to Abs_{600} = 1 to wash the cells and standardize the cell concentration. This cell suspension was diluted 1:100 before it was flowed through the microfluidic device. Just prior to loading the cell suspension on-chip, plain fresh media was pumped through the device using a syringe pump (Harvard Apparatus, Holliston, MA, USA) until all of the channels were completely filled with liquid. Once filled, the 1:100 cell suspension was pumped through the device (at a flow rate of 50 μL/h) until the majority of the observation areas contained a small number of cells. After seeding the device with cells, fresh growth media was pumped continuously through the device at 20 μL/h and the cells were cultured on-chip for 12 h at room temperature before they were exposed to QTF. After this culturing period, QTF was added to the media at a concentration of 250nM and was continuously supplied to the cells (at 20 μL/h) for the remainder of the experiment.

To visualize QTF dynamics, the whole microfluidic device was mounted on an inverted microscope (Nikon Ti Eclipse; Nikon Instruments Inc, Tokyo, Japan) equipped with a CoolSNAP HQ² camera (Photometrics, Munich, Germany). Time-lapse images were taken immediately after the addition of the probe, and images were acquired every 10 min for up to 24 h. All images were acquired through a Nikon Plan APO 100x oil immersion objective. Phase contrast and epifluorescence images were taken at every time point. All epifluorescence images were exposed for 2 sec with an appropriate filter set. To mitigate photobleaching, the fluorescence light source was attenuated to 50% of its maximum intensity. Fresh media and

probe was continuously supplied to the cells throughout the time-lapse at a flow rate of 20 μL/h.

All time-lapse images were processed using a custom R script and the Imager package (8). Briefly, to both flatten and reduce background signal, we defined the background using a low-pass filter: every image was transformed with an isotropic Gaussian blur with a large kernel size (sigma = 30 px). These background images were then subtracted from each raw image. To improve contrast, pixel intensity cutoffs were defined manually, and the distribution (histogram) of pixel intensities was stretched identically for each image. Finally, the pixels were binned (2 x 2) to reduce the overall file size.

M. smegmatis HADA and QTF microscopy and colocalization

M. smegmatis from a single colony was grown in 7H9 with ADC component aerobically overnight at 37 °C to saturation. Cells were diluted into fresh media, and QTF was added at 1 μM final concentration when cells were at an absorbance of 0.2 at 600 nm. Cells were exposed to QTF for at least two doubling times in the presence of QTF. HADA was added at a concentration of 500 μM for 30 m (10% of the doubling time). Cells were then washed twice in 7H9 with ADC component, spotted onto a glass-bottomed microwell dish (MatTek corporation), and covered with a 1% (w/v) agarose pad. Images were collected at room temperature with a Nikon A1 laser scanning confocal microscope (Nikon Instruments). Images were acquired with a Nikon plan apo 100/1.4 oil objective with a 1.2-AU pinhole diameter and NIS-elements C software (Nikon Instruments) with appropriate filter sets. Brightness and contrast were identically adjusted within each channel with the open-source Fiji distribution of ImageJ. To perform colocalization analysis, individual bacteria displaying QTF and/or HADA fluorescence were analyzed in the open-source Fiji distribution of ImageJ using the Plot Profile function. For each bacterium, the maximum fluorescence of each plot was collected. The maximum fluorescence levels were normalized to the highest fluorescence value and plotted against its position along the long axis of the bacterium, which was normalized to 1. The analysis was performed on the listed number of individual bacteria and expressed as mean ±SD (Standard Deviation). Plots were generated using R/ggplot2 (9).

M. smegmatis mCherry fusion protein microscopy

M. smegmatis harboring Ag85-mCherry fusion plasmids was cultured in M63 + 0.02% Tween-80 and induced in early logarithmic phase, OD_{600} = 0.3 with 0.2% acetamide for 6 hours 37°C. For analysis by microscopy, cell aliquots were spotted onto a glass-bottomed microwell dish (MatTek corporation) and covered with a 1% (w/v) agarose pad. Images were collected at room temperature with a Nikon A1 laser scanning confocal microscope (Nikon Instruments). Images were acquired with a Nikon plan apo 100/1.4 oil objective with a 1.2-AU pinhole diameter and NIS-elements C software (Nikon Instruments) with appropriate filter sets. Identical acquisition settings were used for all samples. Brightness and contrast were identically adjusted with the open-source Fiji distribution of ImageJ. Protein distribution was analyzed using the Plot Profile function in the open-source Fiji distribution of ImageJ. For each bacterium, the maximum fluorescence of each plot was collected. The maximum fluorescence levels were normalized to the highest fluorescence value and plotted against its position along the long axis of the bacterium. Heat maps were generated in Microsoft Excel for the listed number of cells analyzed (30-50).

M. smegmatis Msmeg_6398-mCherry and QTF analyzed by microscopy

To image QTF colocalization with *M. smegmatis* expressing Msmeg_6398-mCherry, we used a modified agar pad method (10). Briefly, *M. smegmatis* harboring a plasmid encoding an

mCherry fusion to Msmeg_6398 was grown in 7H9 without ADC and supplemented with kanamycin at 25 μg/mL aerobically overnight at 37°C. Cells were diluted into fresh 7H9 without ADC with kanamycin at 25 μg/mL and sub-cultured to mid-logarithmic density as determined by absorbance at 600 nm (0.4-0.6). A portion of the culture (200uL) was spotted onto a glass-bottomed microwell dish (MatTek corporation). The solution was allowed to settle for 10 min and then aspirated to leave a thin layer on cells. Molten 0.6% top agar (2 mL) in 7H9 without ADC component and supplemented with kanamycin at 25 μg/mL and 1 μM QTF was poured over MatTek dish containing cells after cooling to 37°C. Agar was allowed to solidify, and cells were incubated at room temperature for 12-15 hours prior to imaging.

MatTek dishes containing cells were imaged with a Nikon A1 laser scanning confocal microscope (Nikon Instruments). Images were acquired with a Nikon plan apo 100/1.4 oil objective with a 1.2-AU pinhole diameter and NIS-elements C software (Nikon Instruments) using appropriate filter sets and High Sensitivity GaAsP detectors. Brightness and contrast were identically adjusted all images with the open-source Fiji distribution of ImageJ.

To perform colocalization analysis, images of individual bacteria labeled with QTF and expressing Msmeg_6398-mCherry fusion fluorescence were analyzed in the open-source Fiji distribution of ImageJ using the Plot Profile function. For each bacterium, the maximum fluorescence of each plot was collected. The maximum fluorescence levels were normalized to the highest fluorescence value and plotted against its position along the long axis of the bacterium, which was normalized to 1. The analysis was performed on the listed number of individual bacteria and expressed as mean ± SD (Standard Deviation). Plots were generated using R/ggplot2 (9).

Supplemental Figures

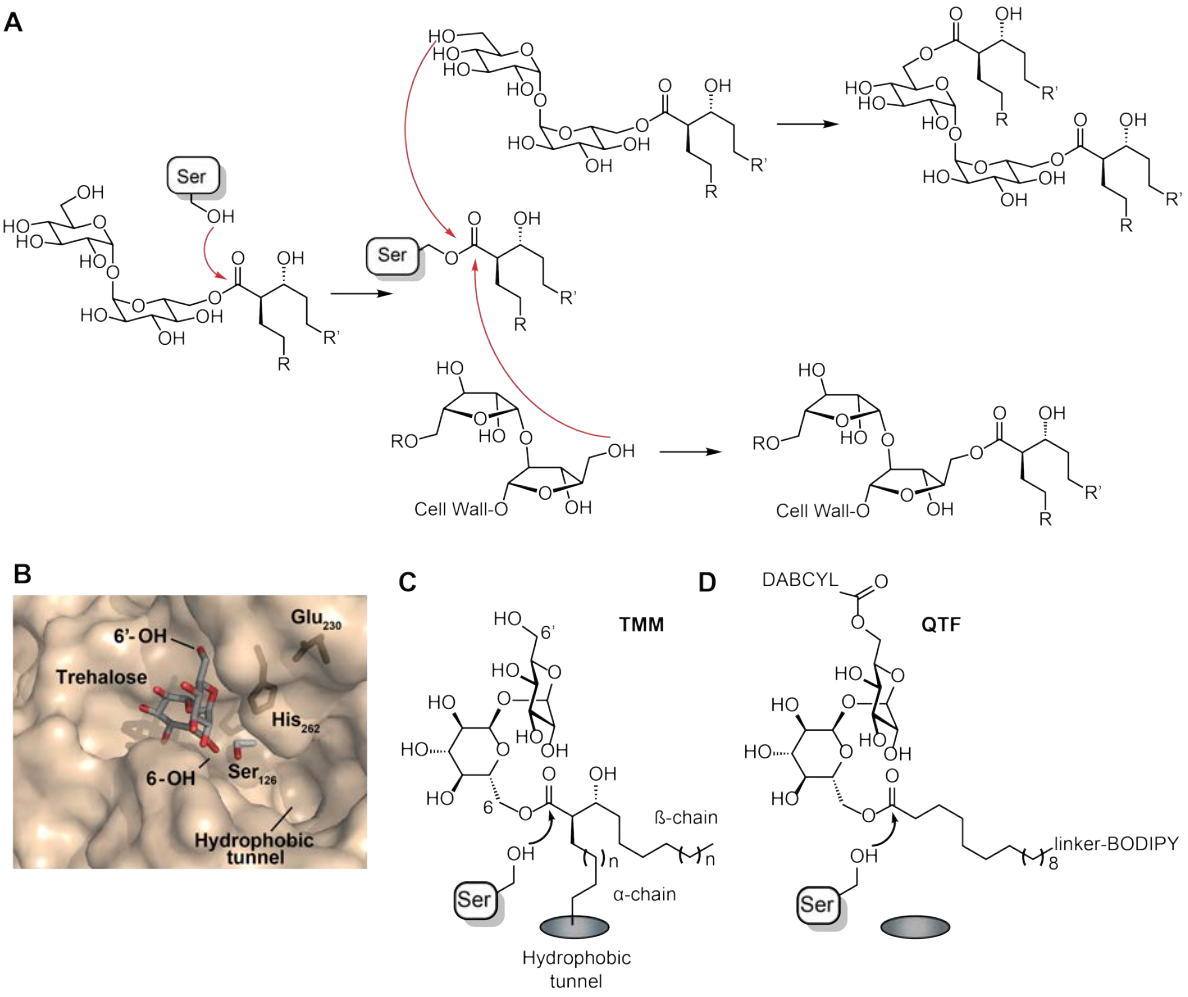

Figure S1. Design of fluorescence turn-on probe QTF. A) Ag85 bound acyl enzyme intermediate can form TDM and mAG. B) Structure of the mycolyltransferase Ag85B active site showing the binding orientation of trehalose (PDB ID: 1FOP) (31). The trehalose 6-OH is proximal to the catalytic serine residue and postulated mycolate binding site, while the 6'-OH points toward the donor saccharide pocket. C) Inferred binding orientation of mycolyl donor trehalose monomycolate (TMM) prior to enzymatic cleavage by Ag85. D) QTF is designed to mimic TMM binding with the DABCYL and BODIPY-FL chromophores at the 6'-OH position and lipid terminus, respectively.

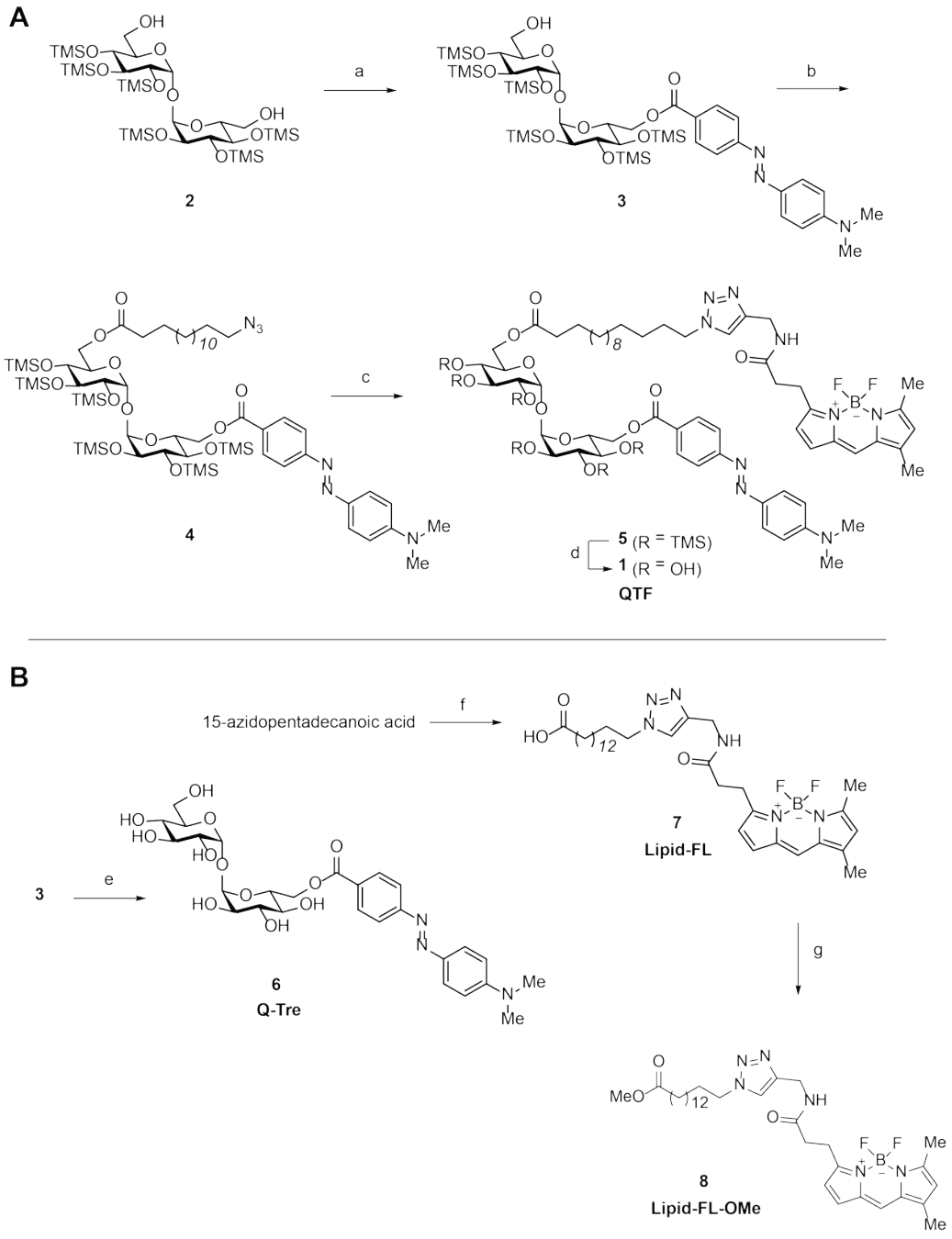

Figure S2. Synthesis of QTF and standards: Synthetic route for the preparation of f A) QTF **1** and B) cleavage standards Q-Tre **6** & Lipid-FL **7**. Reagents and conditions: a) DABCYL-OH, EDC•HCl, DMAP, CH_2Cl_2, rt, 16 h, 31%; b) 15-Azidopentadecanoic acid, EDC•HCl, DMAP, CH_2Cl_2, rt, 16 h, 48%; c) BODIPY-FL–alkyne, $CuSO_4$•5H2O, sodium ascorbate, TBTA, t-BuOH:H2O:CH2Cl2 (5:5:1), rt, 24 h, 79%; d) Dowex-H+, MeOH, rt, 30 min, 75%; e) Dowex-H^+, MeOH, rt, 15 min, 30%; f) BODIPY-FL–alkyne, $CuSO_4$•5H2O, sodium ascorbate, TBTA, t-BuOH:H2O:CH_2Cl_2 (5:5:1), rt, 16 h, 94%; g) TMS-diazomethane, 1:1 PhMe:MeOH, rt, 1 h, 83%.

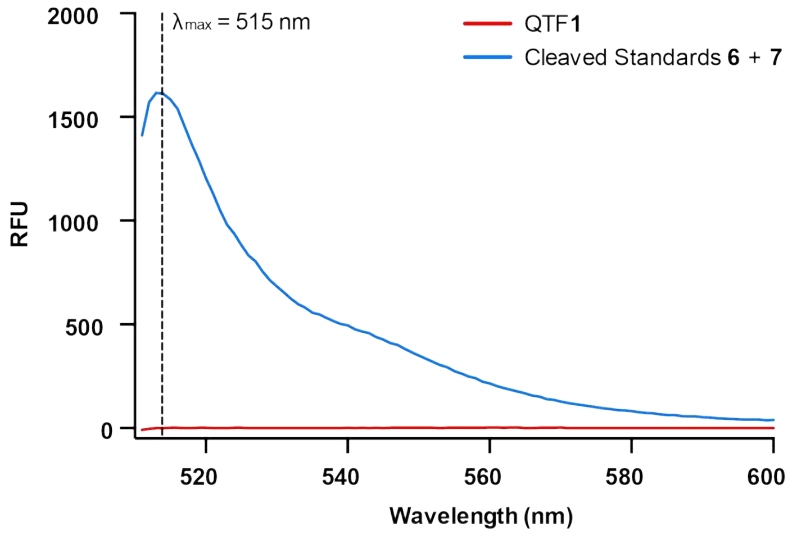

Figure S3. Fluorescence emission spectra of intact and cleaved QTF. Plot of fluorescence emission intensity versus wavelength for QTF (1 μM, red line) and an equimolar mixture of cleavage standards Q-Tre (1 μM) and Lipid-FL (1 μM) (blue line), in aqueous NaH_2PO_4/Na_2HPO_4 buffer (20 mM, pH = 7.2) containing 2% DMSO at 37 °C. QTF fluorescence intensity was normalized to 1 RFU at 515 nm. The cleaved standard mixture is >1500-fold more fluorescent than the intact probe. λ_{max} (ex) = 503 nm, λ_{max} (em) = 515 nm.

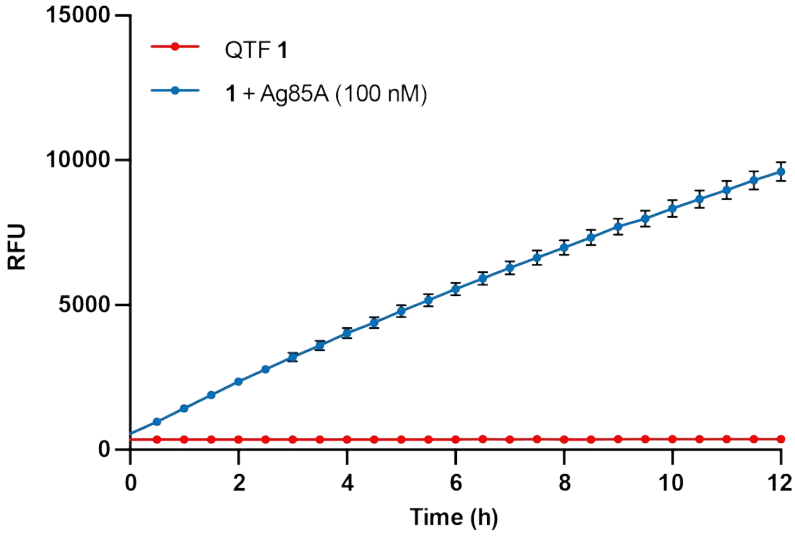

Figure S4. Aqueous stability of QTF. Plot of fluorescence emission intensity versus time incubation of QTF (1 μM, red line) for 12 h in aqueous NaH_2PO_4/Na_2HPO_4 buffer (20 mM, pH = 7.2) containing 2% DMSO at 37 °C. No increase in fluorescence emission is observed, indicating spontaneous hydrolysis does not occur. For comparison, the blue line reflects the

fluorescence emission generated during cleavage of QTF by Ag85A (100 nM) under otherwise identical conditions. λ_{max} (ex) = 503 nm, λ_{max} (em) = 515 nm.

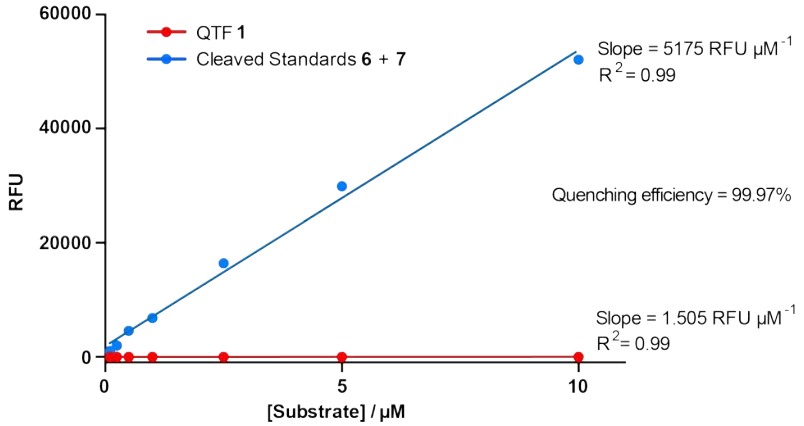

Figure S5. Determination of quenching efficiency in QTF. Plot of fluorescence emission intensity versus concentration for QTF (red line) and an equimolar mixture of the cleavage standards Q-Tre and Lipid-FL (blue line), in aqueous NaH_2PO_4/Na_2HPO_4 buffer (20 mM, pH = 7.2) containing 2% DMSO at 37 °C. Fluorescence increases linearly with concentration up to 10 µM in each case, and the ratio of slopes reveals that quenching is 99.97% efficient in intact QTF **1**. λ_{max} (ex) = 503 nm, λ_{max} (em) = 515 nm.

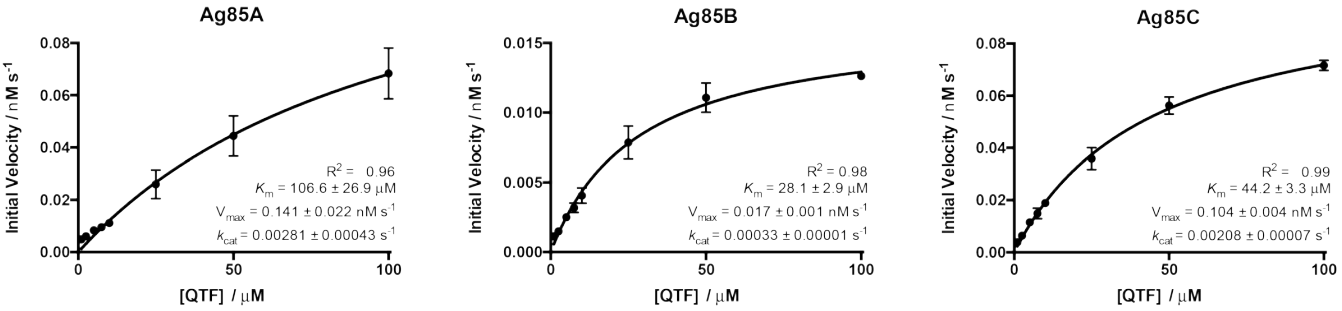

Figure S6. Kinetic analysis of QTF cleavage by *M. tb*. Ag85A–C. Plots of initial reaction velocities against substrate concentration for cleavage of QTF by purified native Ag85A, Ag85B, and Ag85C from *M. tuberculosis*. Fitting of the data to the Michaelis-Menten equation allowed K_m and k_{cat} to be determined (see Table S1 and Supporting Information page 9-10). Error bars represent the standard error from three replicate experiments. Assay conditions: [Ag85] = 50 nM, [QTF **1**] = 1–100 µM, in aqueous NaH_2PO_4/Na_2HPO_4 buffer (20 mM, pH = 7.2) containing 2% DMSO at 37 °C.

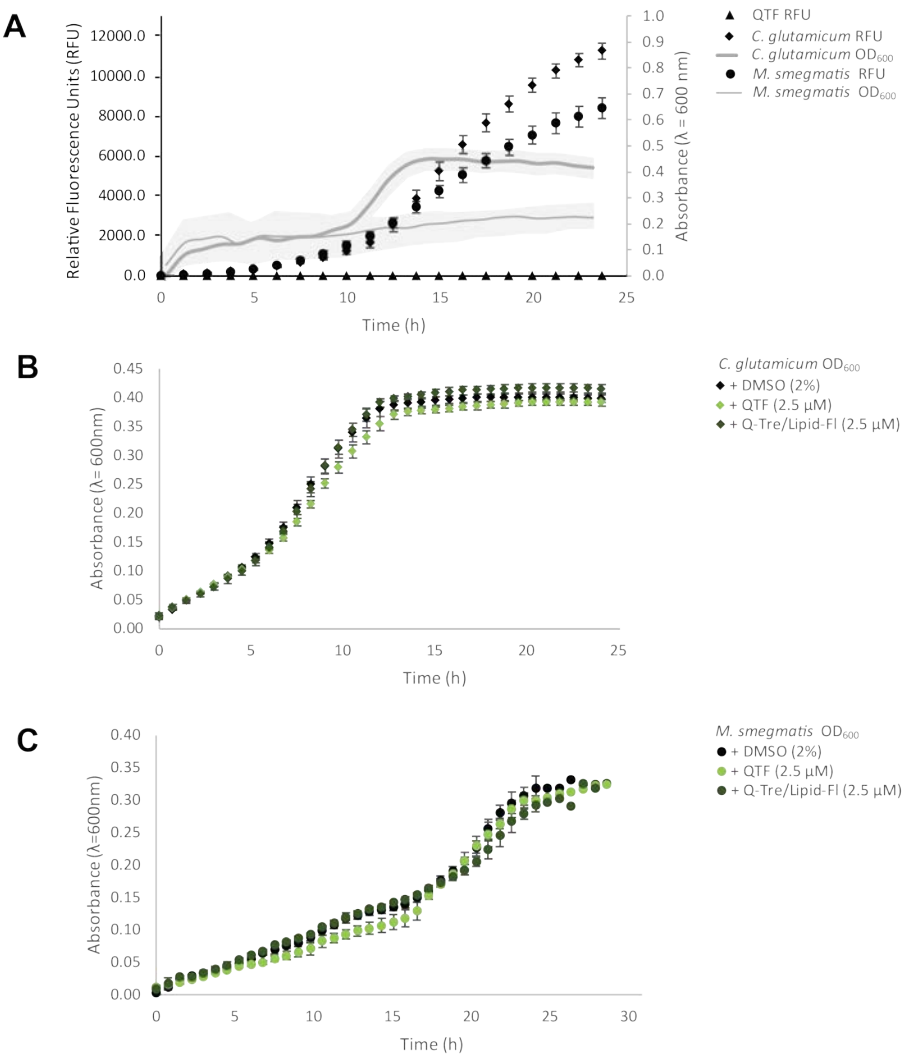

Figure S7. In culture growth of *M. smegmatis* and *C. glutamicum* with QTF. A) Plots of fluorescence emission intensity versus time for QTF (2.5 µM) incubated alone (triangles) or in the presence of *M. smegmatis* (circles) or *C. glutamicum* (diamonds) bacteria, in a 96-well plate in LB media at 37 °C for 24 h. Growth of either bacteria triggers an increase in fluorescence. Plots of optical density versus time for the growth of *M. smegmatis* (thick grey line) or *C. glutamicum* (thin grey line) in the presence of QTF (2.5 µM), acquired through tandem OD_{600} absorbance measurement during the same experiment. B) OD_{600} absorbance measurement of *C. glutamicum* with 2.5 µM QTF (dark green diamonds) or with 2% DMSO (black diamonds) or a 2.5 µM mixture of QTF cleavage standards Q-Tre and Lipid-Fl (light green diamonds), under same conditions as panel A, indicating QTF or its cleavage standards (reaction products) does not affect the growth of *C. glutamicum*. C) OD_{600} absorbance measurement of *M. smegmatis* with 2.5 µM QTF (dark green circles) or with 2% DMSO (black circles) or a 2.5 µM mixture of QTF cleavage standards Q-Tre and Lipid-Fl (light green circles), under same conditions as panel A, indicating QTF or its cleavage standards (reaction products) does not affect the growth of *M. smegmatis*. Error bars depict the standard deviation from three replicate experiments. RFU = relative fluorescence units.

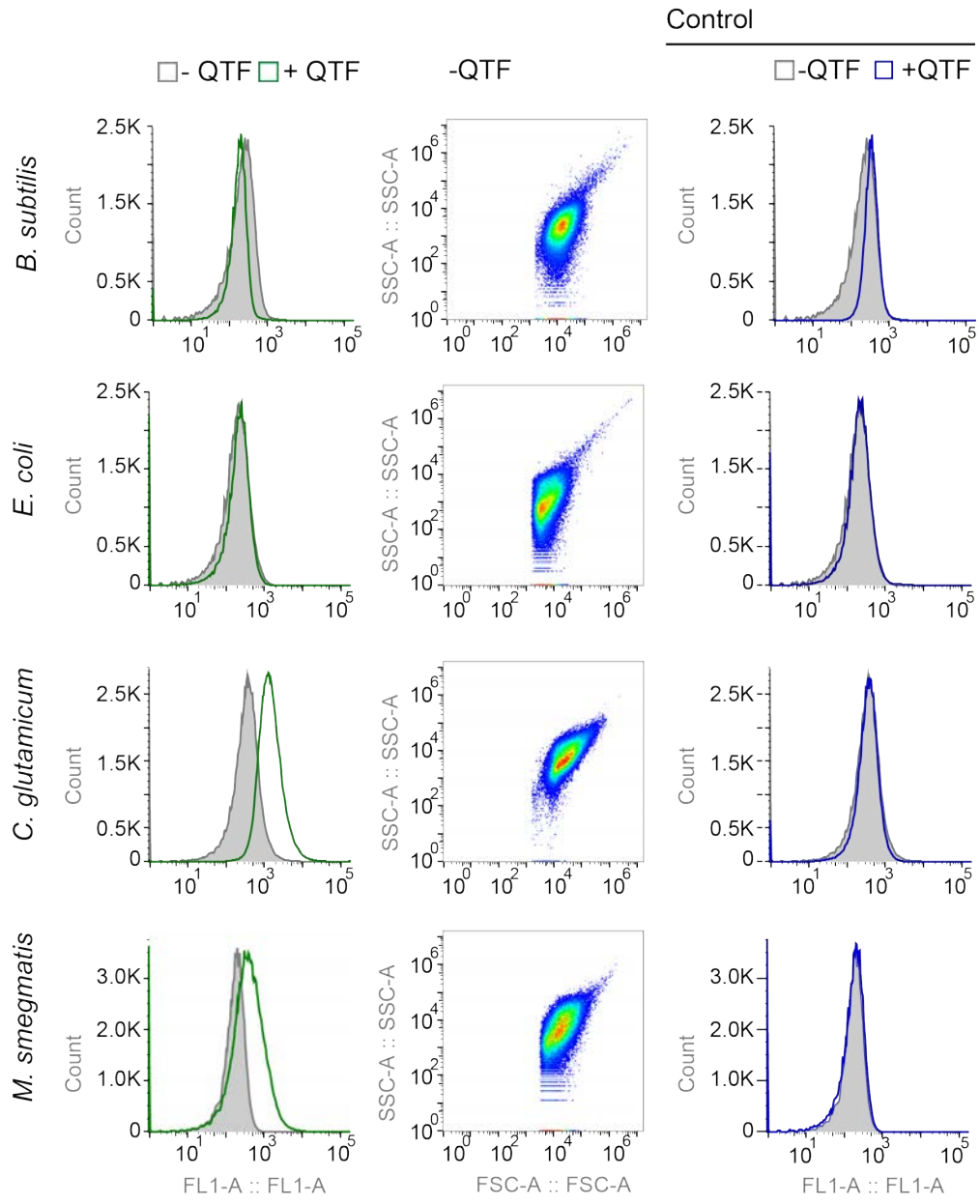

Figure S8. Flow cytometry histograms/scatter plots. Left panel histogram depicts data from bacteria incubated with QTF for 2-3 doubling times (+ QTF) relative to that from untreated bacteria (- QTF). Representative scatter plots are shown for bacteria alone (Center). Right panel histogram depicts controls for QTF labeling; including, bacteria alone (- QTF) for auto fluorescence and bacteria that were briefly exposed to QTF for 15-30 minutes at 30 °C either fixed or live (*M. smegmatis*) (+ QTF) as a background control. Plots are representative of 2-3 independent experiments for each strain and condition.

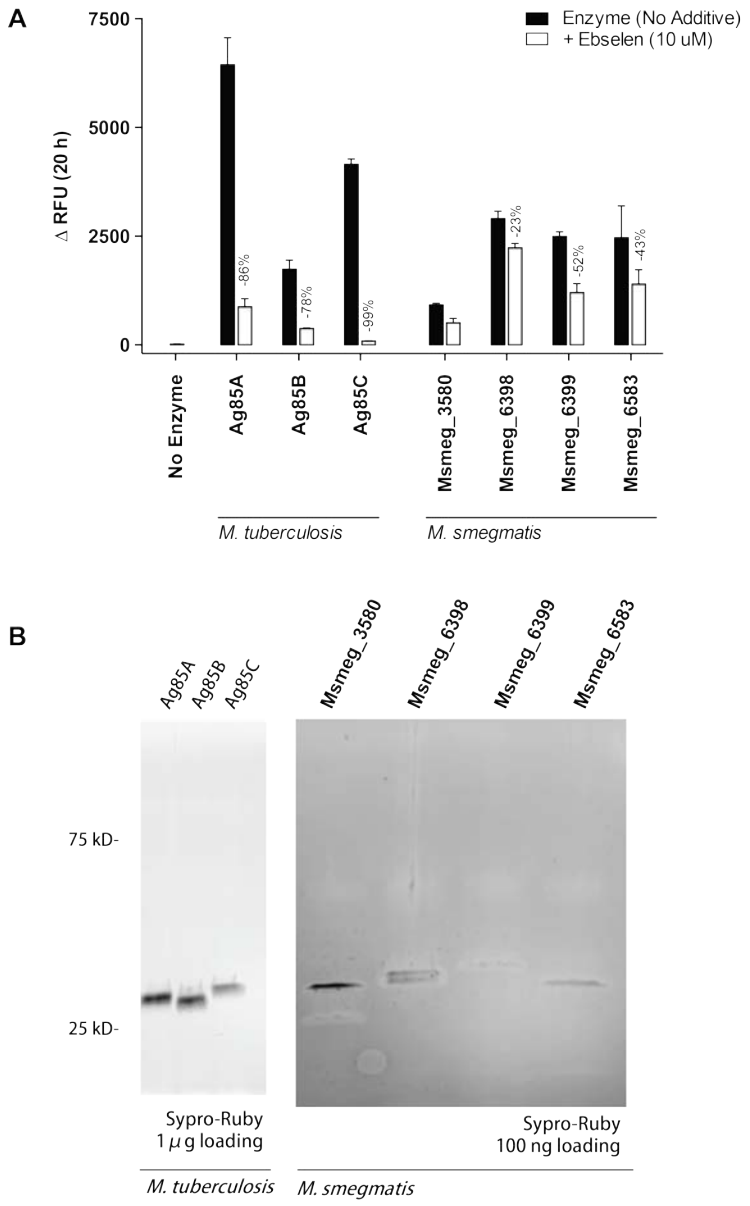

Figure S9. Inhibition of *M. tb.* and *M. smegmatis* Ag85 proteins by ebselen. A) Relative increases in fluorescence emission following incubation of QTF (1 μM) with purified *M. tuberculosis* and *M. smegmatis* Ag85 proteins (20 nM) for 20 h in aqueous NaH_2PO_4/Na_2HPO_4 buffer (20 mM, pH = 7.2) containing 2% DMSO at 37 °C. QTF is turned over by tested proteins (black bars) and inhibited following a 2 h pretreatment of each enzyme with 10 μM ebselen (white bars). Inhibition by ebselen was less effective for *M. smegmatis* proteins compared to *M. tb*. Ag85A–C. Percent reduction in QTF turnover is shown for each protein. RFU = relative fluorescence units. B) SDS-PAGE gel demonstrating the purity mycolyltransferases, stained with Sypro Ruby.

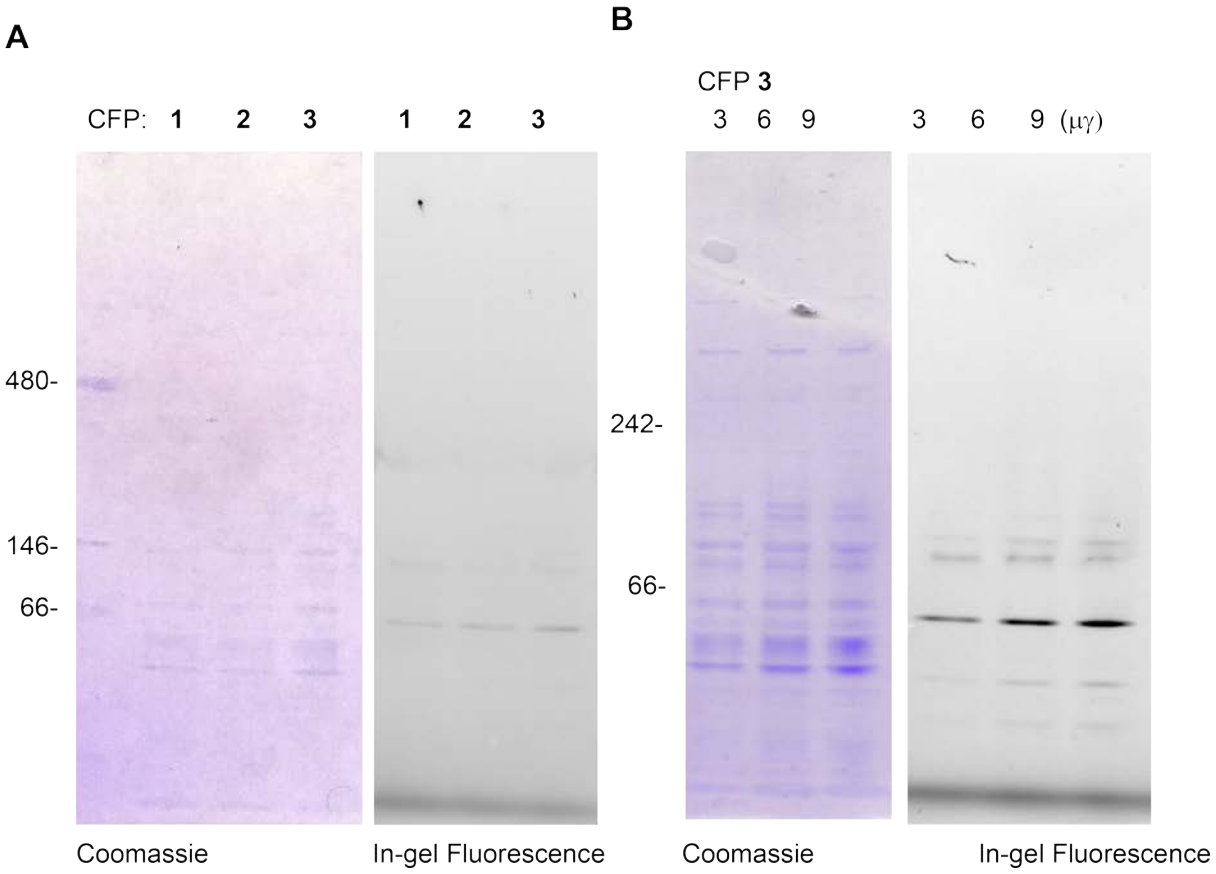

Figure S10. Isolated culture filtrate proteins are robust batch to batch. A) In-gel fluorescence and Coomassie-stained native PAGE gel derived from analysis of 3 independently isolated *M. smegmatis* culture filtrate protein batches (CFP 1, CFP 2, CFP3). Each lane contains 3.5 μg of total protein. Samples were incubated with QTF (1 μM). B) Expanded gel from Figure 3D: CFP 3 was loaded at 3, 6 and 9 μg. The gel was incubated with QTF (1 μM). The in-gel digests were taken from the lane loaded with 9 μg CFP as indicated in Figure 3D.

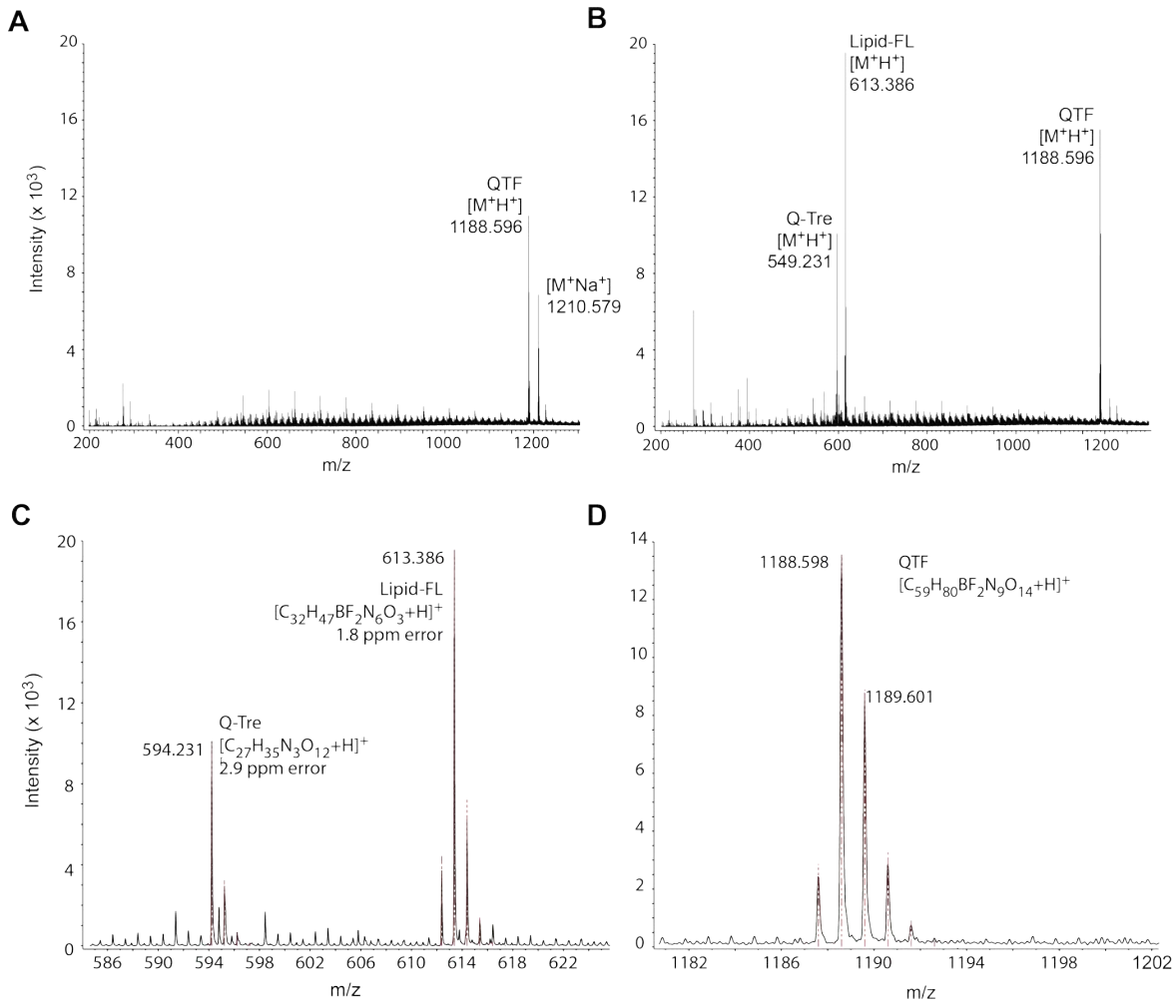

Figure S11. HRMS analysis of QTF products from *M. smegmatis* CFP exposure. A) High-resolution mass spectrum of QTF (100 μM) after incubation in aqueous NH_4HCO_3 buffer (10 mM, pH = 8.0) at 37 °C for 5 days. B) HRMS analysis following exposure of QTF (50 μM) to *M. smegmatis* CFP (1 μg). The masses of the expected cleavage standards Q-Tre, Lipid-FL , and intact QTF are depicted. C–D) Expansions of relevant regions that show the isotope patterns for [M+H⁺] ions of QTF, Q-Tre and Lipid-FL match the predicted distributions (red).

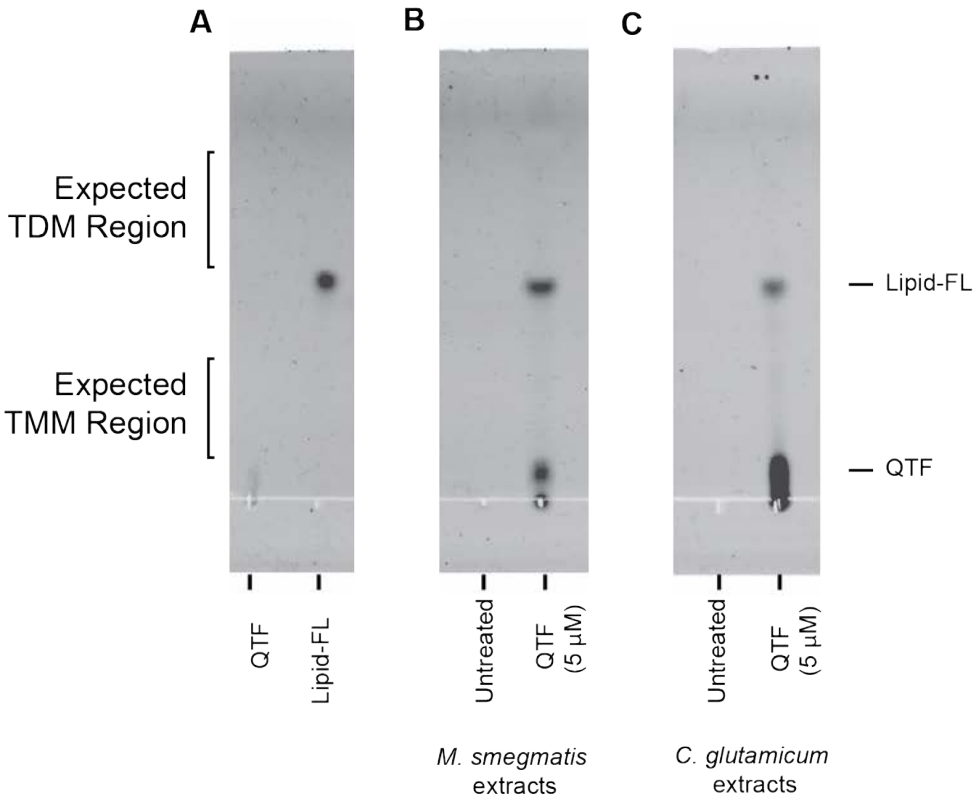

Figure S12. TLC analysis of membrane extracts from cells treated with QTF. Fluorescence images taken following thin-layer chromatographic (TLC) resolution of A) QTF and Lipid-FL standards alongside membrane extracts from B) *M. smegmatis* and C) *C. glutamicum* cells cultured with QTF (5 μM) until mid-logarithmic phase. Intact QTF and the fluorescent cleavage product Lipid-FL were recovered both species. No other fluorescent compounds or products were detected. Membrane extracts were obtained using the method on page S12-13 TLC plates were eluted in $CHCl_3$/MeOH/acetone (90:15:10).

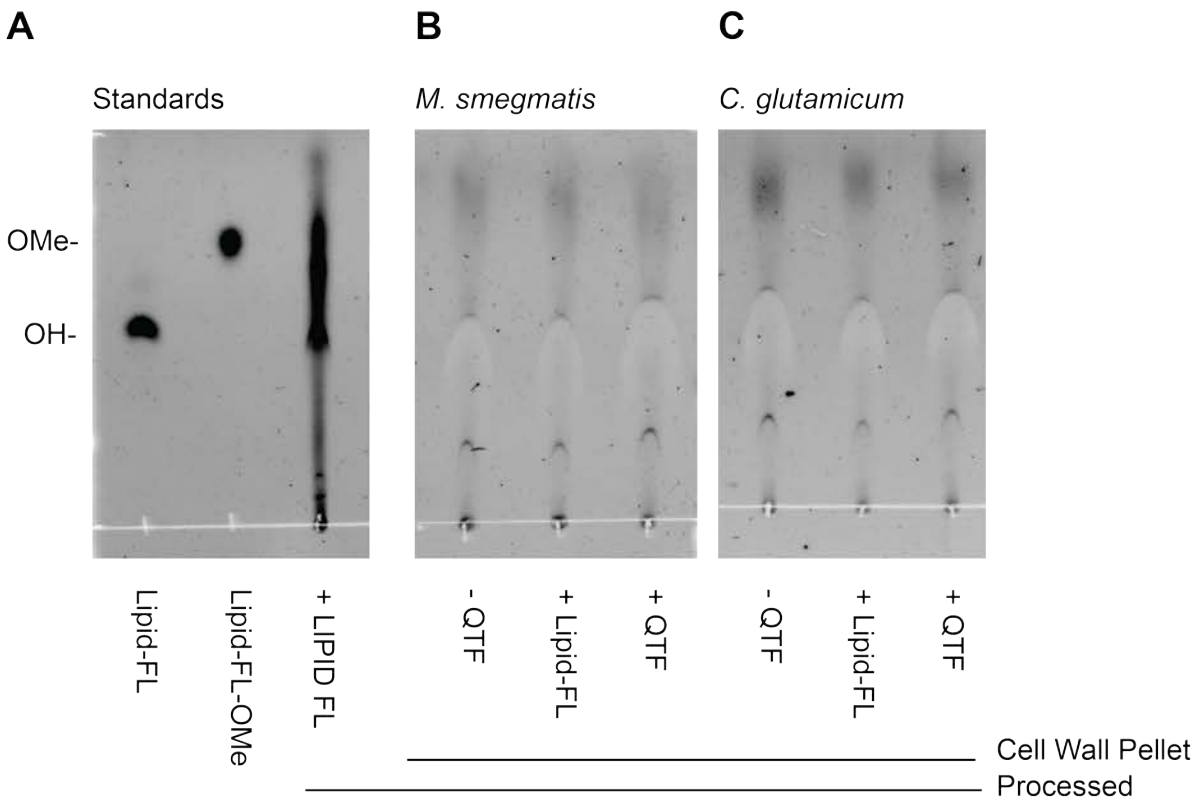

Figure S13. TLC analysis of isolated cell walls from cells treated with QTF. Fluorescence images taken following thin-layer chromatographic (TLC) resolution of A) Standards of Lipid-Fl, Lipid-FL-OMe and Lipid-FL (after saponification and methylation) alongside released cell wall mycolates from B) *M. smegmatis* and C) *C. glutamicum* cells cultured without QTF, with Lipid-FL (5 μM) or QTF (5 μM) until mid-logarithmic phase. No other fluorescent compounds or products were detected. Membrane extracts were obtained using the method on page S12-13 TLC plates were eluted in 10% MeOH in $CHCl_3$.

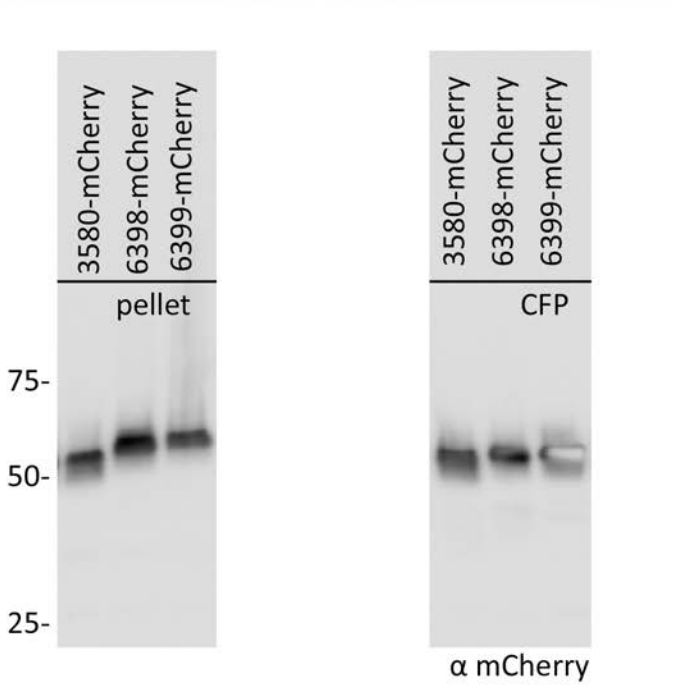

Figure S14. Production of *M. smegmatis* Ag85 C-terminal mCherry fusion proteins. Western blot of recovered pellets and concentrated secreted protein fractions. *M. smegmatis* cells were grown in M63 to mid-logarithmic growth phase (OD_{600} = 0.4-0.6) and induced with 0.2% acetamide for 6 hours at 30 °C. Western blot was probed with anti-mCherry antibody (1:10000, Abcam ab167453). mCherry-mycolyltransferase fusion proteins are secreted and intact.

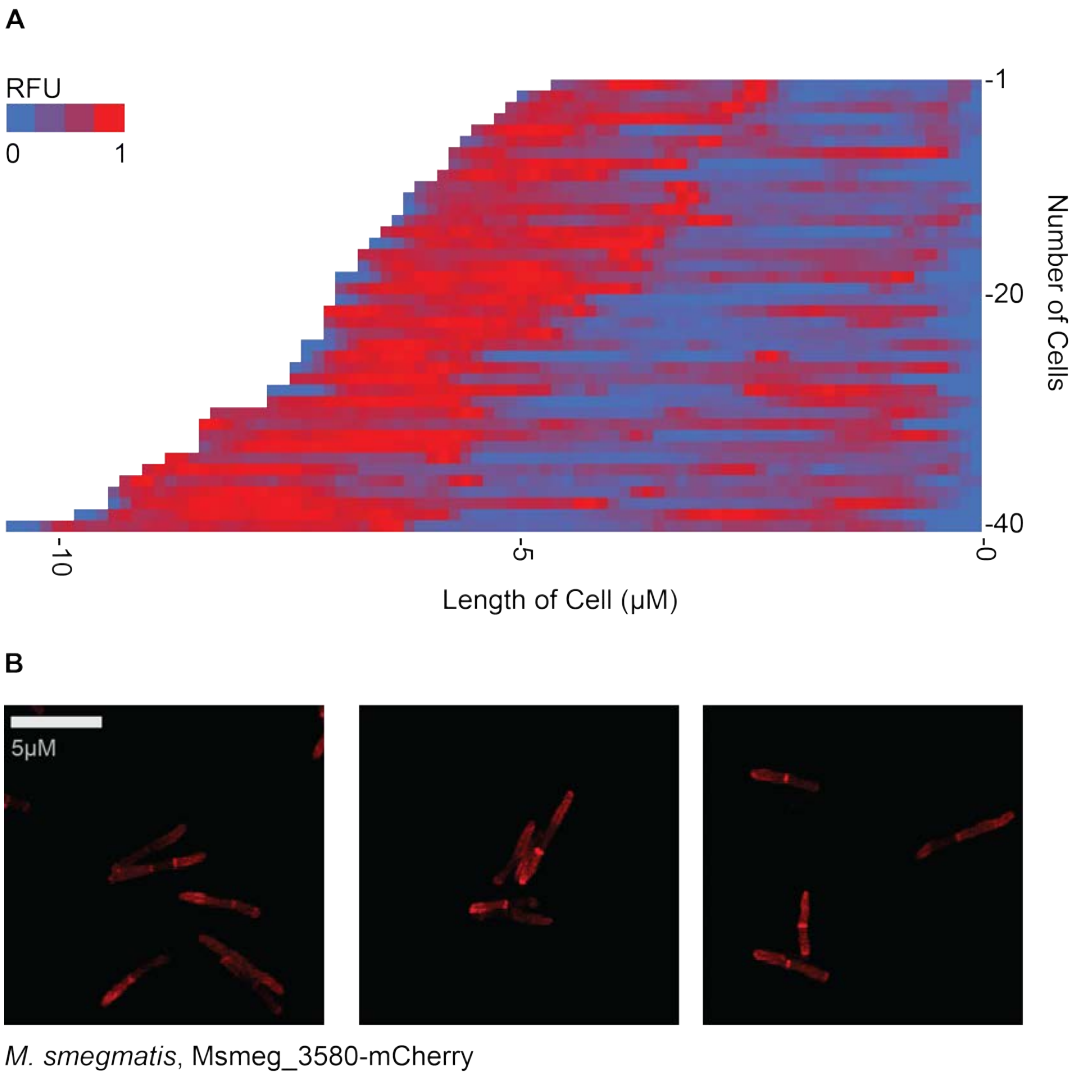

Figure S15. Msmeg-3580-mCherry localizes asymmetrically at poles. Msmeg_3580-mCherry expression in *M. smegmatis*. *M. smegmatis* cells were grown in M63 to mid-logarithmic growth phase (OD_{600} = 0.4-0.6) and induced with 0.2% acetamide for 6 h at 30 °C then imaged by confocal analysis. A) Image analysis of cell length and fluorescence distribution reveals spatial localization of Msmeg_3580-mCherry. Demograph of mCherry fluorescence (N = 40) represent cells sorted by length vertically ascending, shortest (top) to longest (bottom). Fluorescence intensities are presented as heatmaps (blue to red). Note asymmetric fluorescence of staining of Msmeg_3580 (mCherry) at cell poles. B) Representative microscopy images of Msmeg_3580-mCherry expressed in *M. smegmatis*

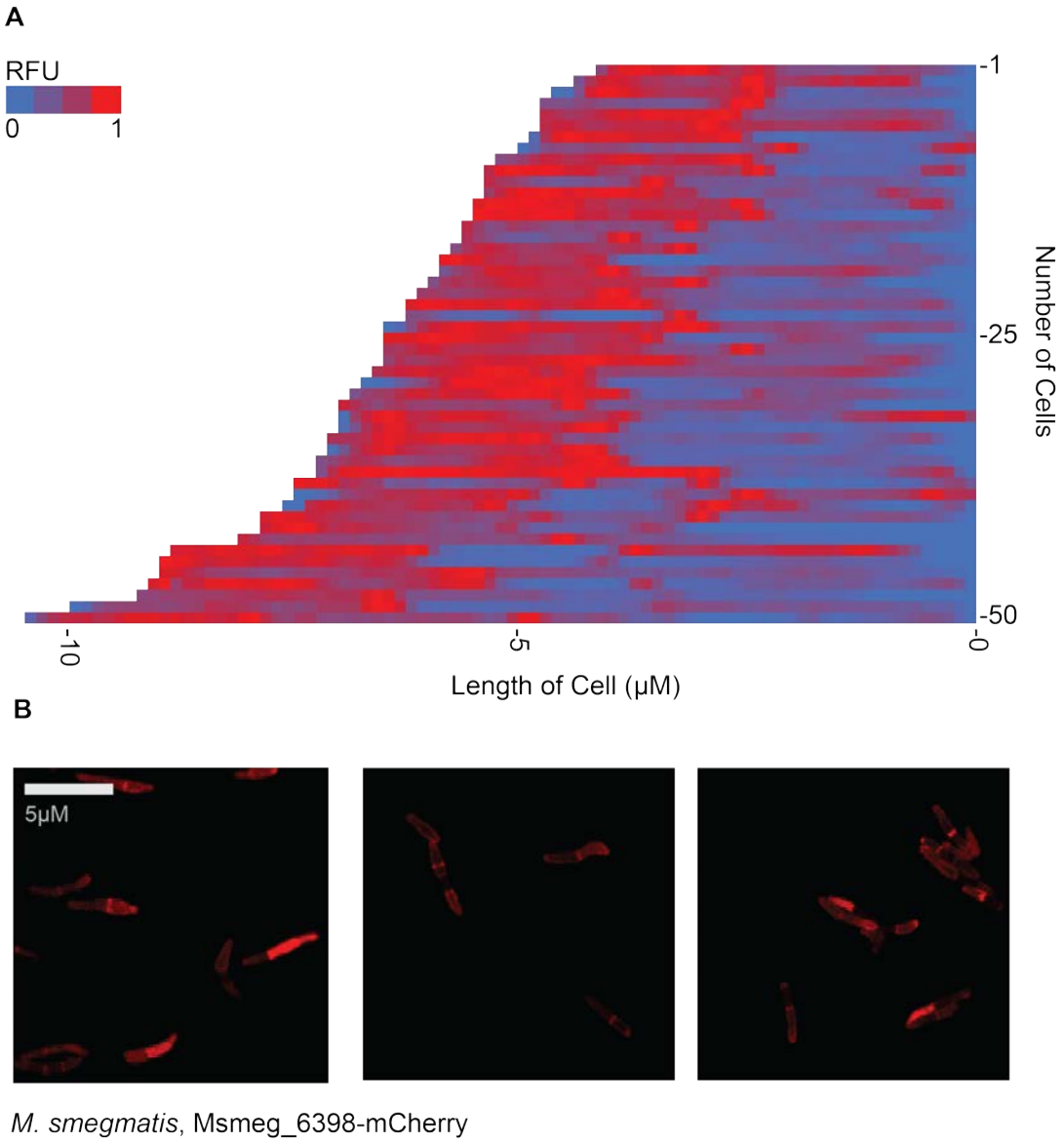

Figure S16. Msmeg-6398-mCherry localizes asymmetrically at poles. Msmeg_6398-mCherry expression in *M. smegmatis*. *M. smegmatis* cells were grown in M63 to mid-logarithmic growth phase (OD_{600} = 0.4-0.6) and induced with 0.2% acetamide for 6 hours at 30°C then imaged by confocal analysis. A) Image analysis of cell length and fluorescence distribution reveals spatial localization of Msmeg_6398-mCherry. Demograph of mCherry fluorescence (N =50) represent cells sorted by length vertically ascending, shortest (top) to longest (bottom). Fluorescence intensities are presented as heatmaps (blue to red). Note asymmetric fluorescence of staining of Msmeg_6398 (mCherry) at cell poles. B) Representative microscopy images of Msmeg_6398-mCherry expressed in *M. smegmatis*

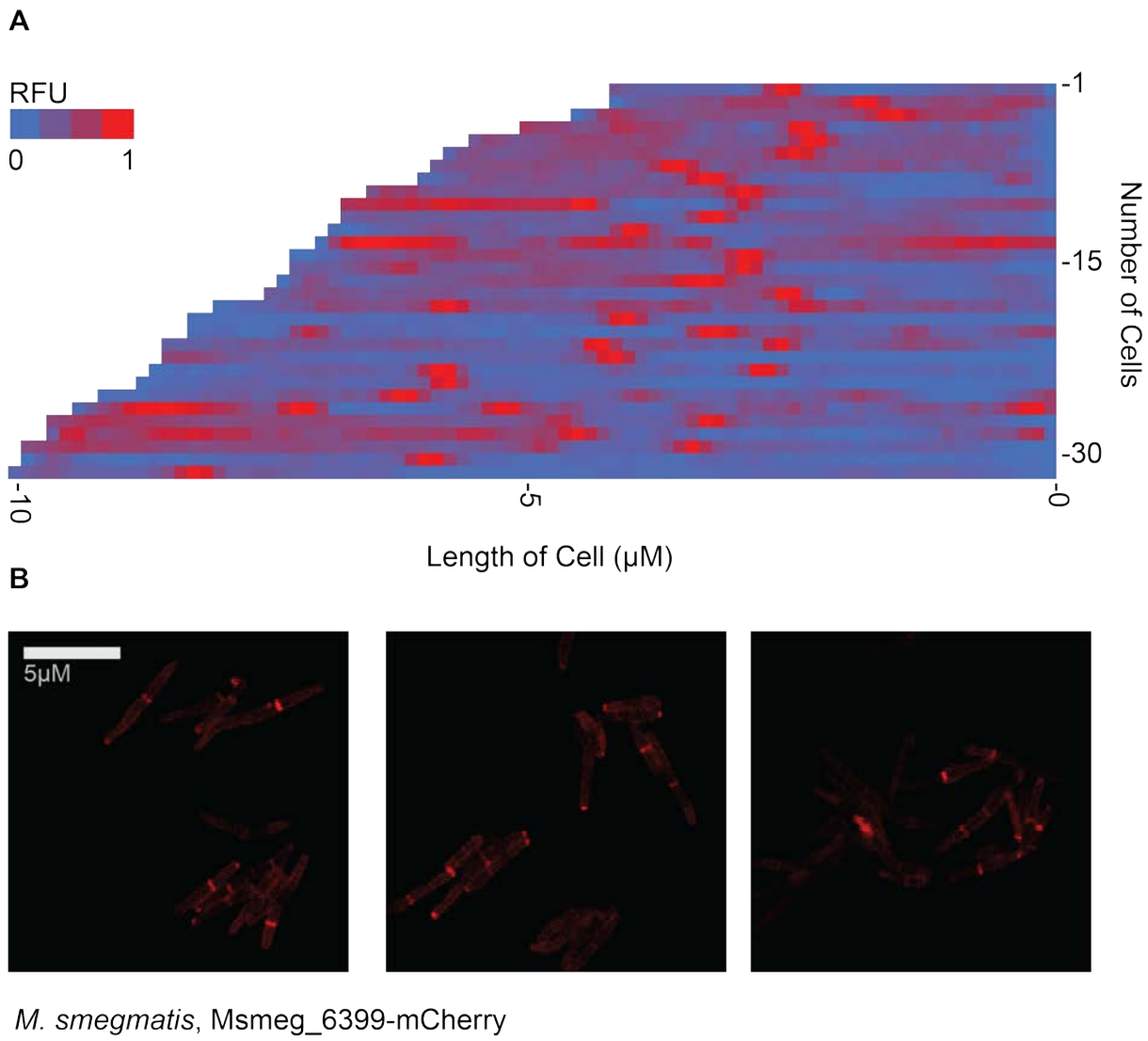

Figure S17. Msmeg-6399-mCherry localizes to cell septum. Msmeg_6399-mCherry expression in *M. smegmatis*. *M. smegmatis* cells were grown in M63 to mid-logarithmic growth phase (OD_{600} = 0.4-0.6) and induced with 0.2% acetamide for 6 hours at 30°C then imaged by confocal analysis. A) Image analysis of cell length and fluorescence distribution reveals spatial localization of Msmeg_6399-mCherry. Demograph of mCherry fluorescence (N = 30) represent cells sorted by length vertically ascending, shortest (top) to longest (bottom). Fluorescence intensities are presented as heatmaps (blue to red). Note fluorescence of staining of Msmeg_6399 (mCherry) at cell septum. B) Representative microscopy images of Msmeg_6399-mCherry expressed in *M. smegmatis*

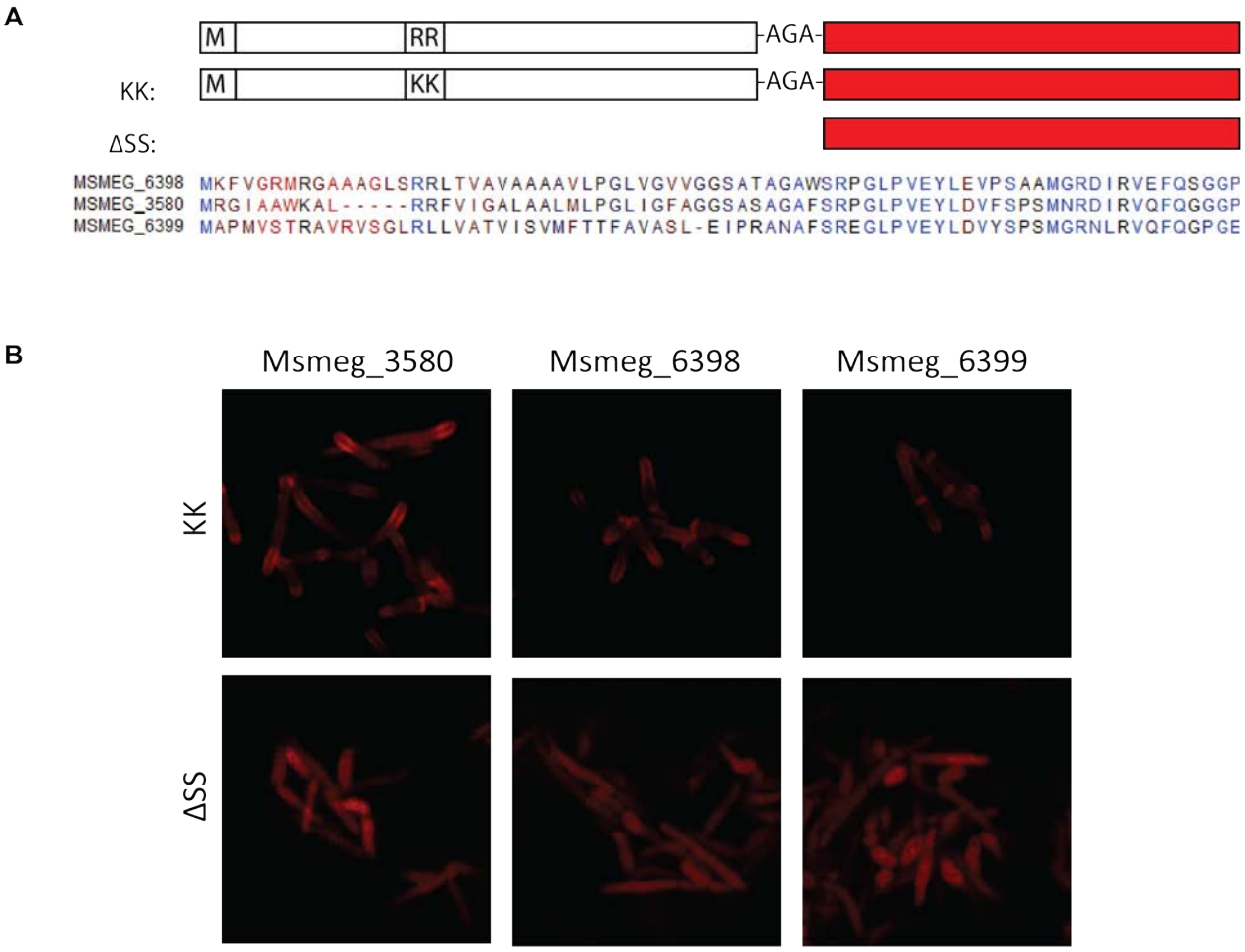

Figure S18. Microscopic images depicting the localization of *M. smegmatis* C-terminal mCherry mycolyltransferase signal sequence variants. A) Schematic depicting the signal sequence variants generated for *M. smegmatis* mycolyltransferases. B) Confocal microscopy images of *M. smegmatis* cells harboring indicated plasmids. Cells were grown in M63 to mid-logarithmic growth phase (OD_{600} = 0.4-0.6) and induced with 0.2% acetamide for 6 hours at 30 °C then imaged by confocal analysis. Note polar localization of Msmeg_3580 and Msmeg_6398 mcherry fluorescence that the cell poles and Msmeg_6399 mCherry fluorescence at the cell septum in twin-lysine signal sequence variants (TAT disruption), as was observed for WT signal sequences (Figure S15-17). Deletion of signal sequences resulted in fluorescent cytoplasm and no observable localization.

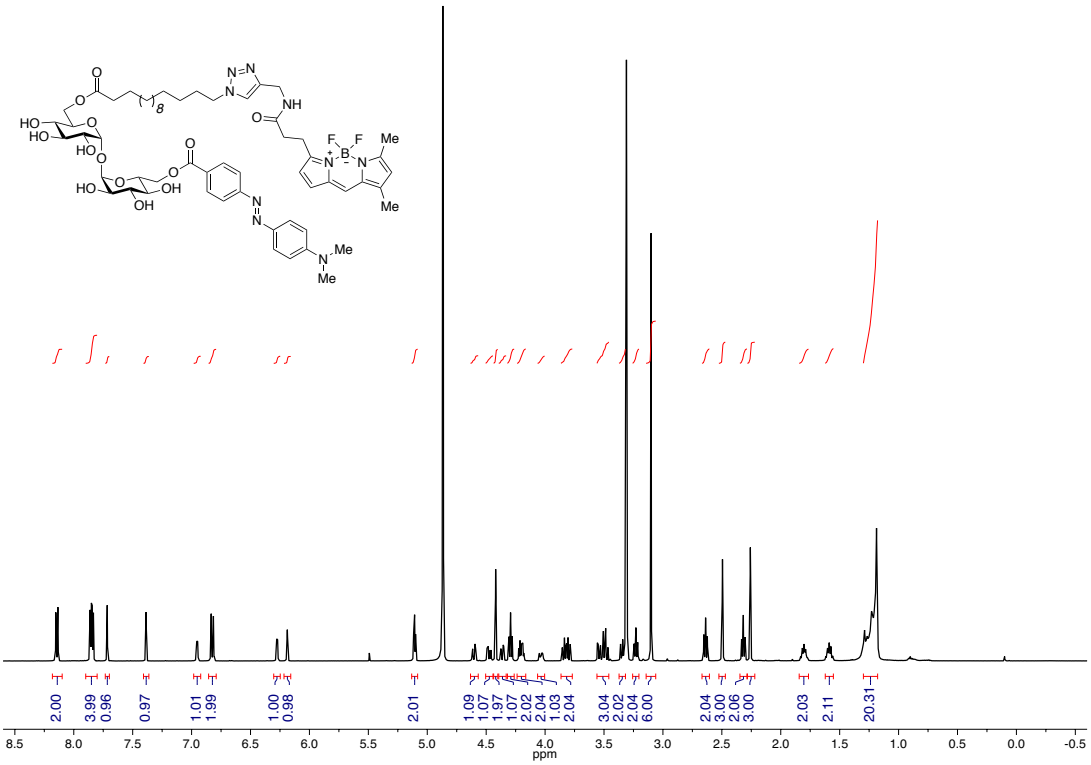

Figure S19. ^{1}H NMR Spectrum of QTF **1** (500 MHz, CD_3OD)

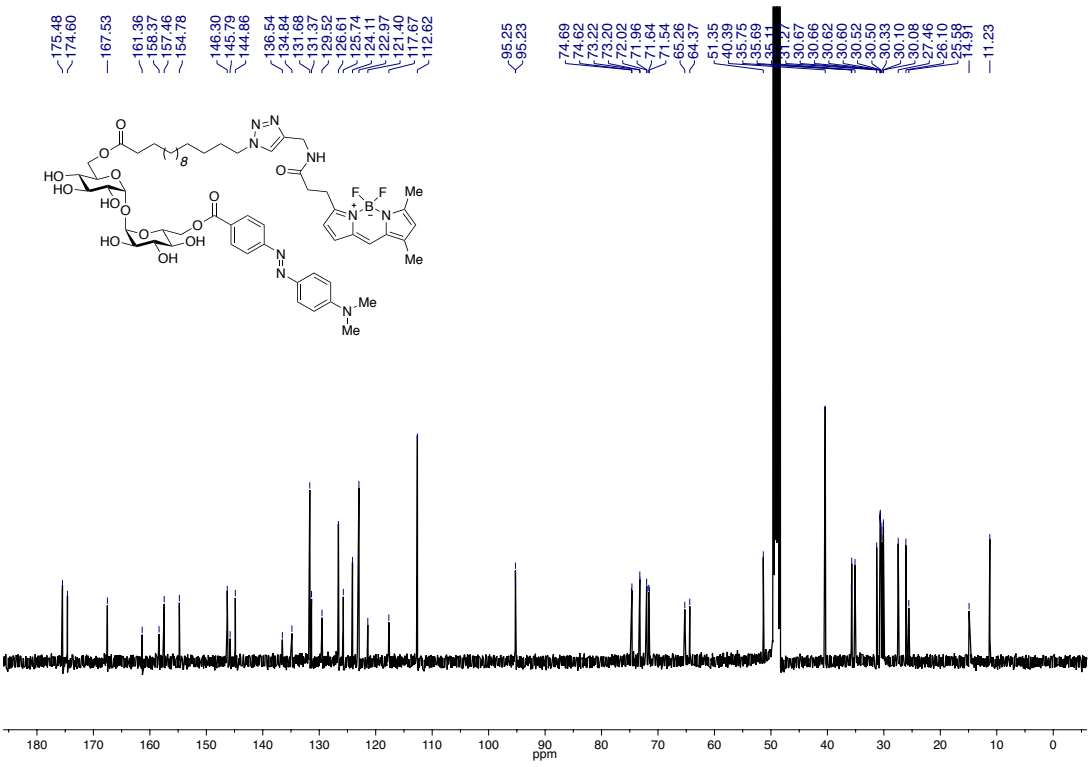

Figure S20. ^{13}C NMR of QTF **1** (126 MHz, CD_3OD)

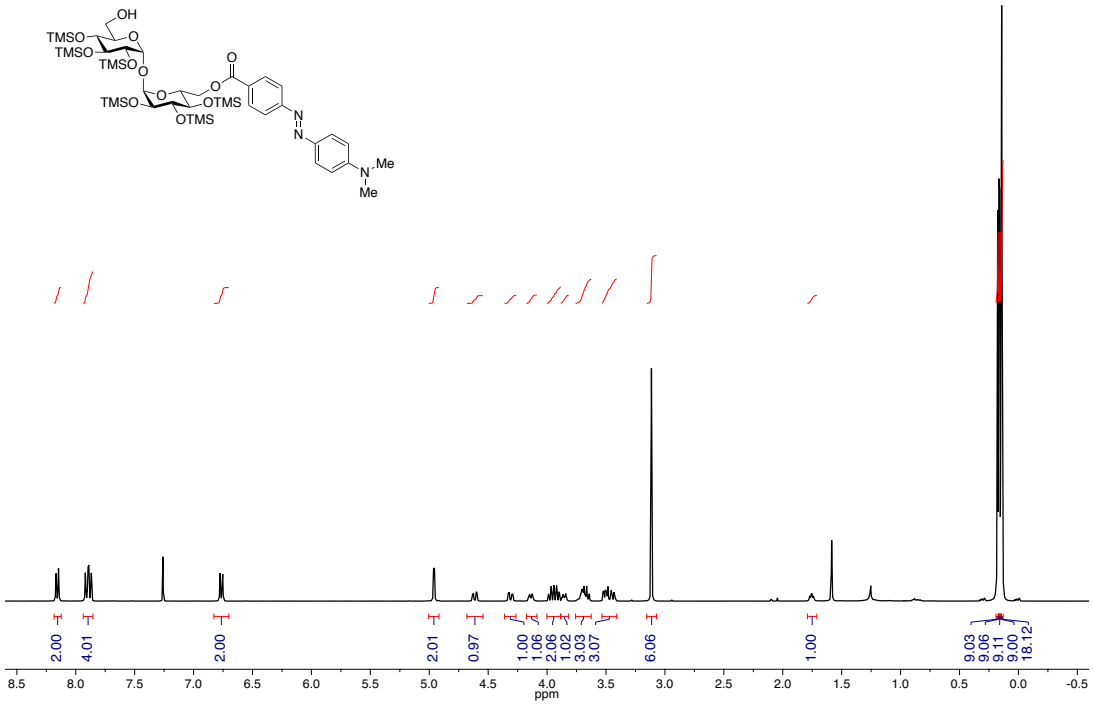

Figure S21. ^{1}H NMR spectrum of compound **3** (400 MHz, $CDCl_3$)

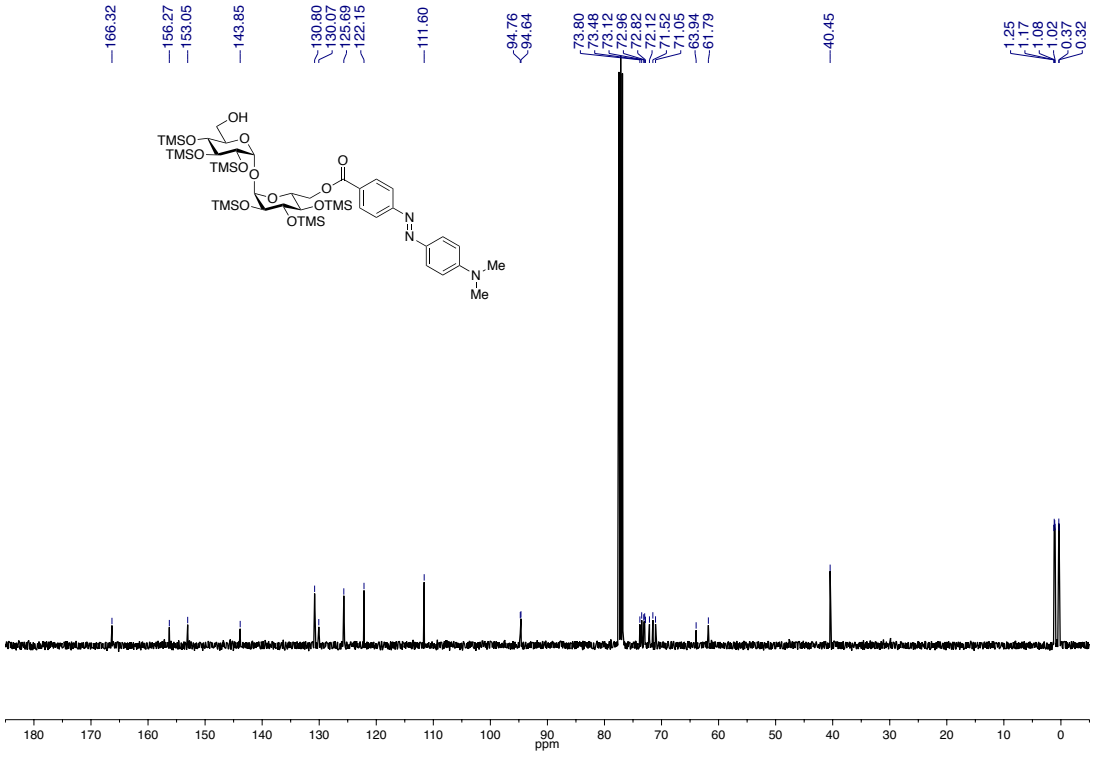

Figure S22. ^{13}C NMR spectrum of compound **3** (101 MHz, $CDCl_3$)

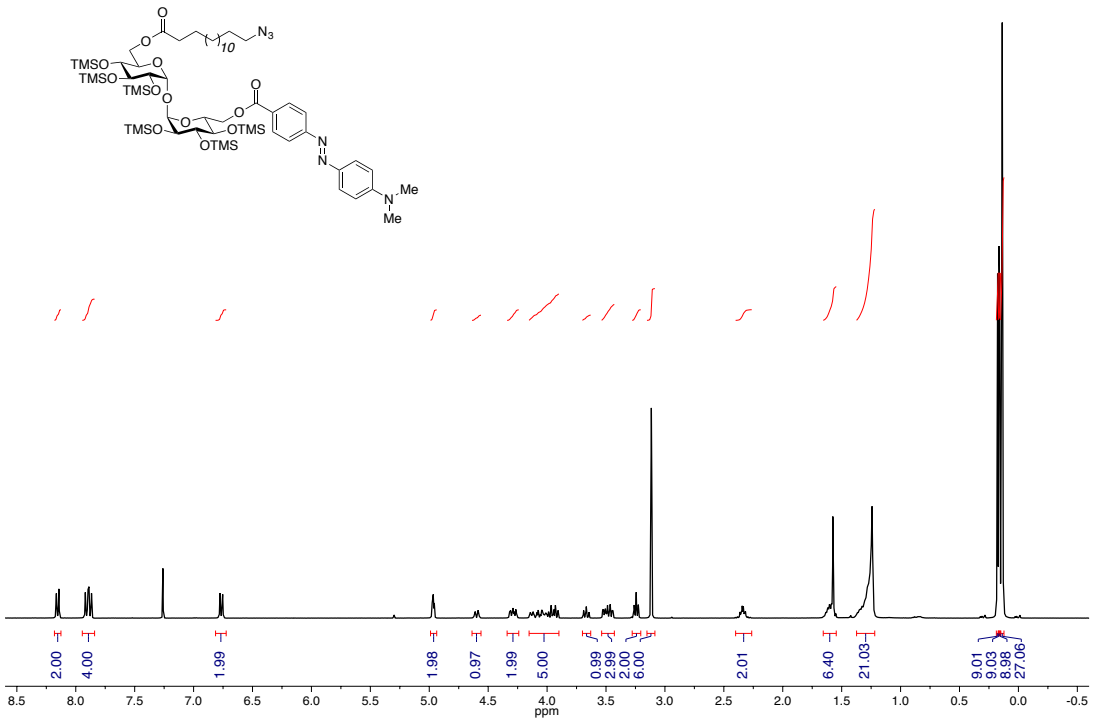

Figure S23. ^{1}H NMR spectrum of compound **4** (400 MHz, $CDCl_3$)

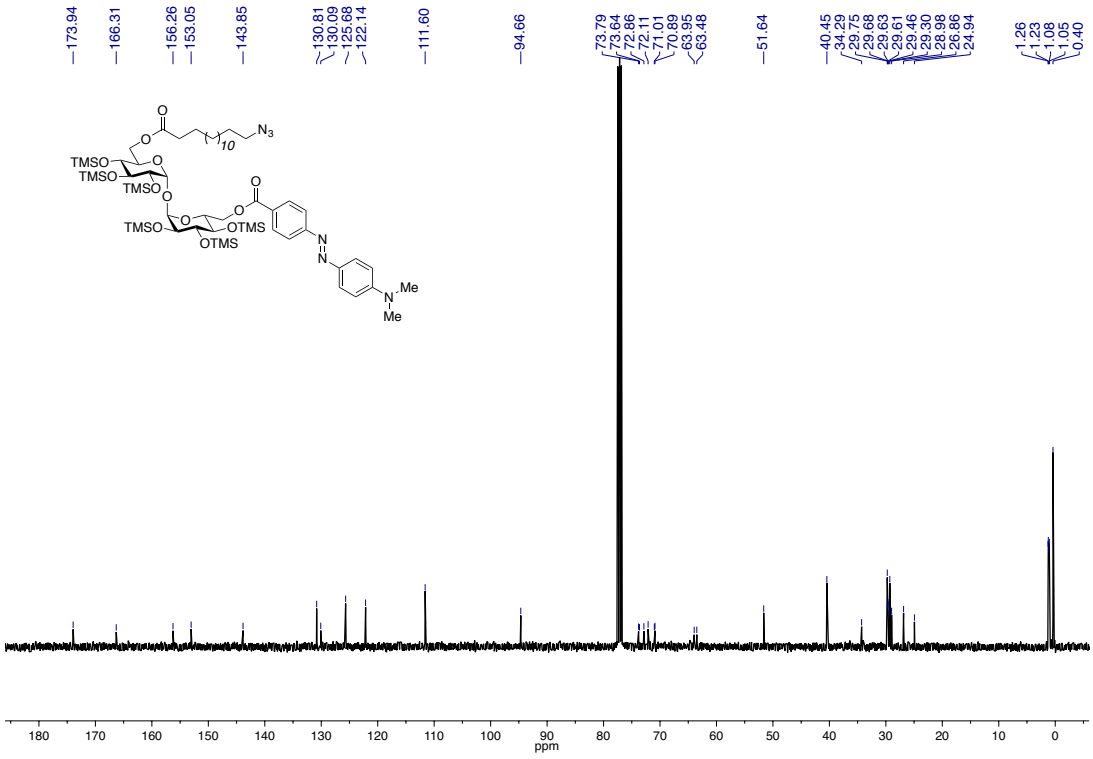

Figure S24. ^{13}C NMR spectrum of compound **4** (101 MHz, $CDCl_3$)

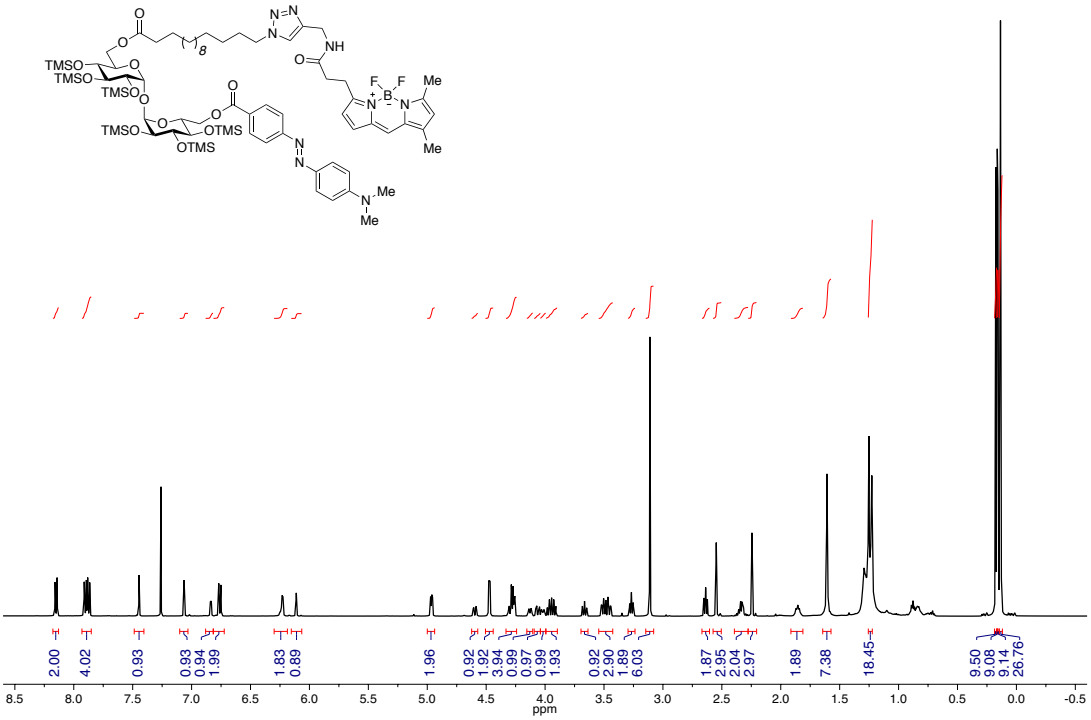

Figure S25. ^{1}H NMR spectrum of compound **5** (500 MHz, $CDCl_3$)

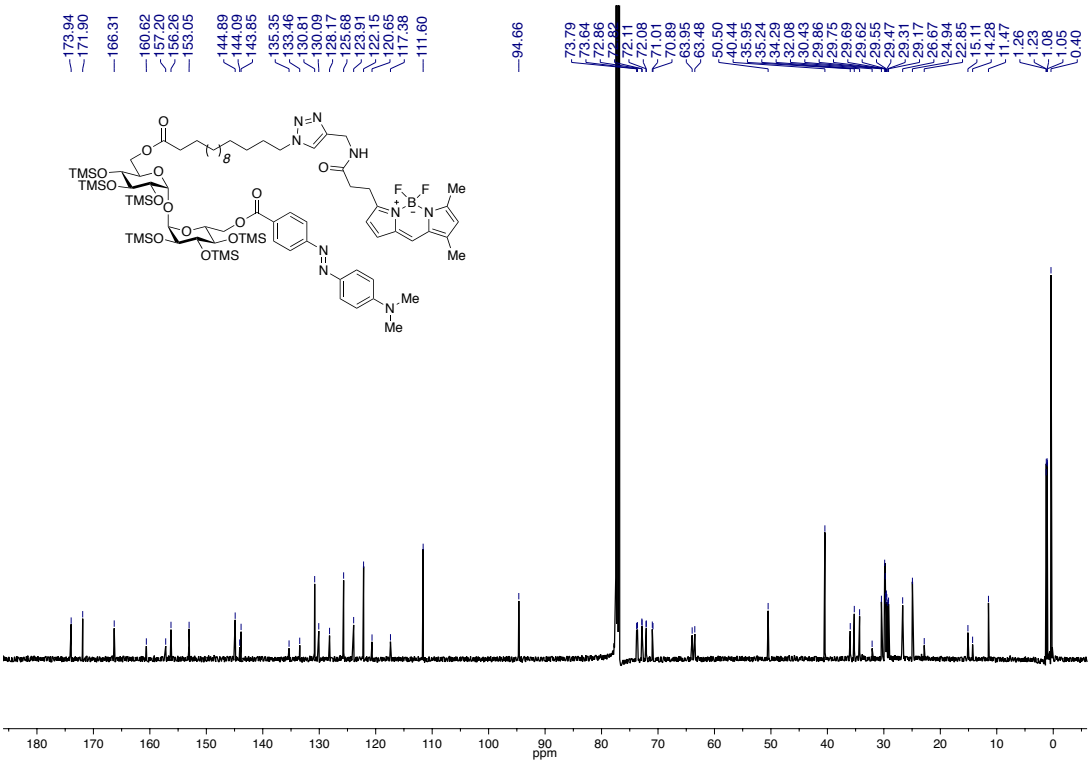

Figure S26. ^{13}C NMR spectrum of compound **5** (126 MHz, $CDCl_3$)

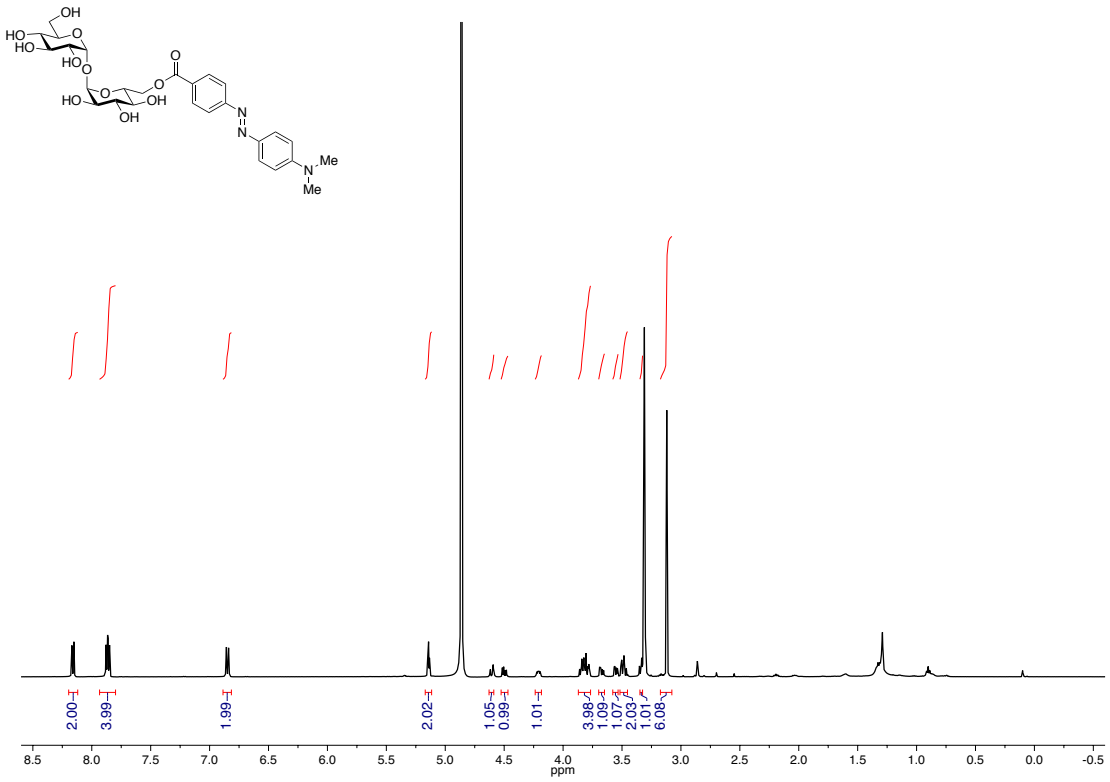

Figure S27. ^{1}H NMR spectrum of Q-Tre **6** (500 MHz, CD_3OD)

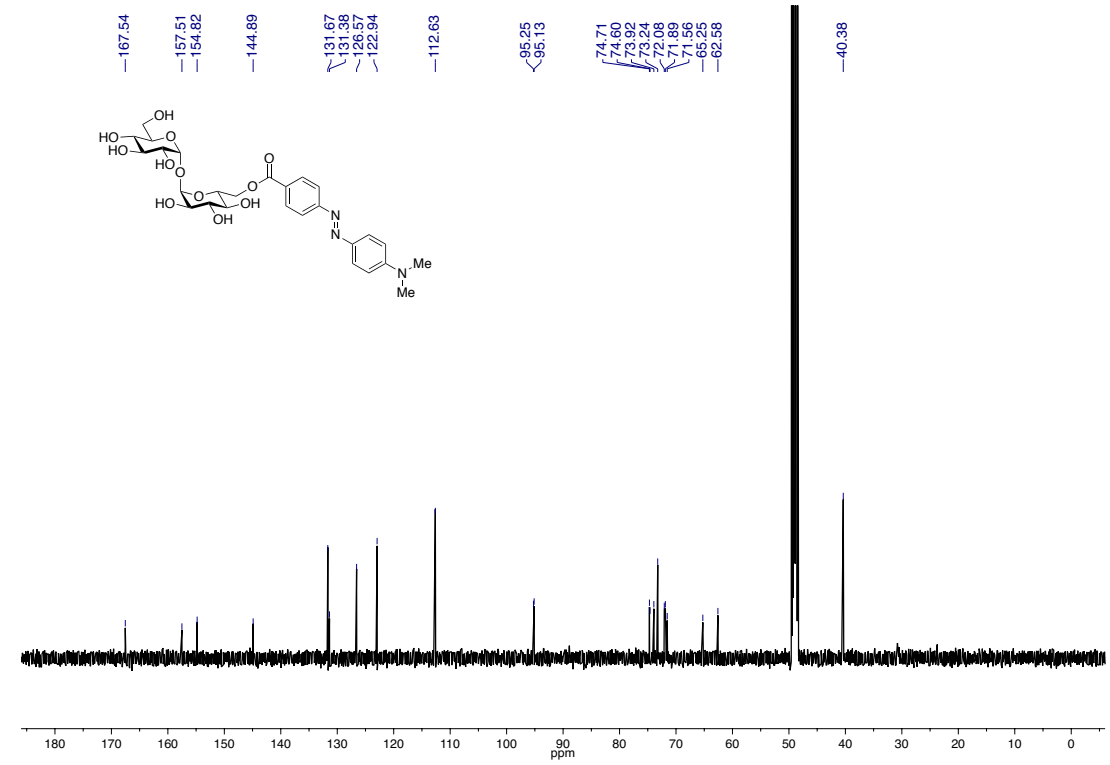

Figure S28. ^{13}C NMR spectrum of Q-Tre **6** (126 MHz, CD_3OD)

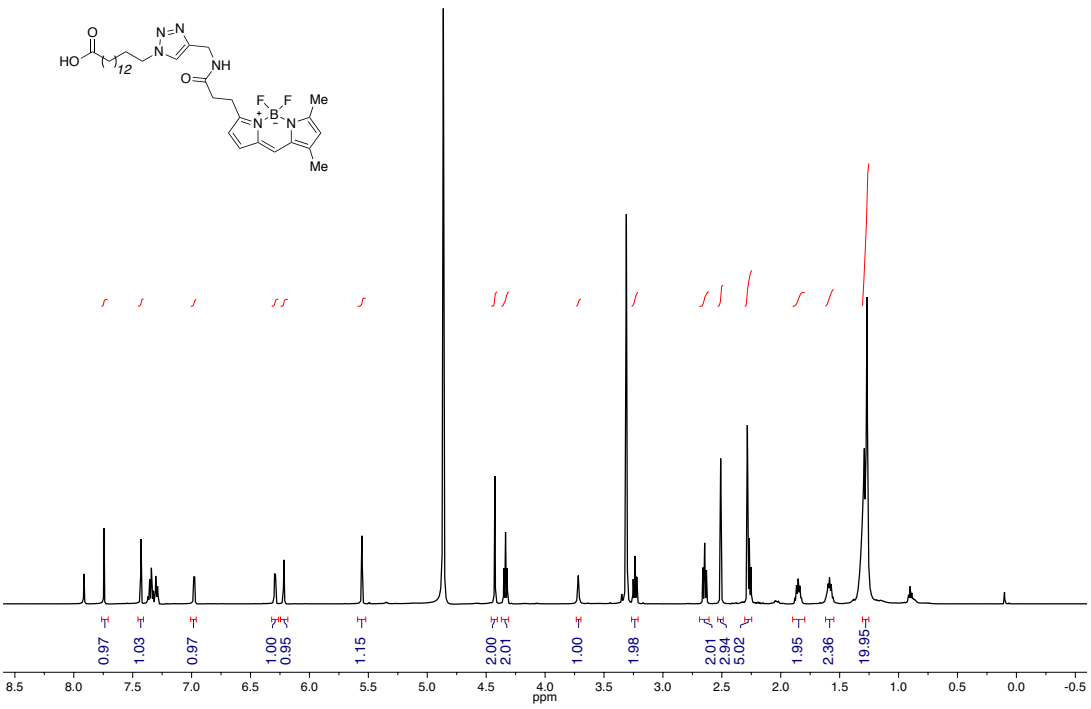

Figure S29. ^{1}H NMR spectrum of Lipid-FL **7** (500 MHz, CD_3OD)

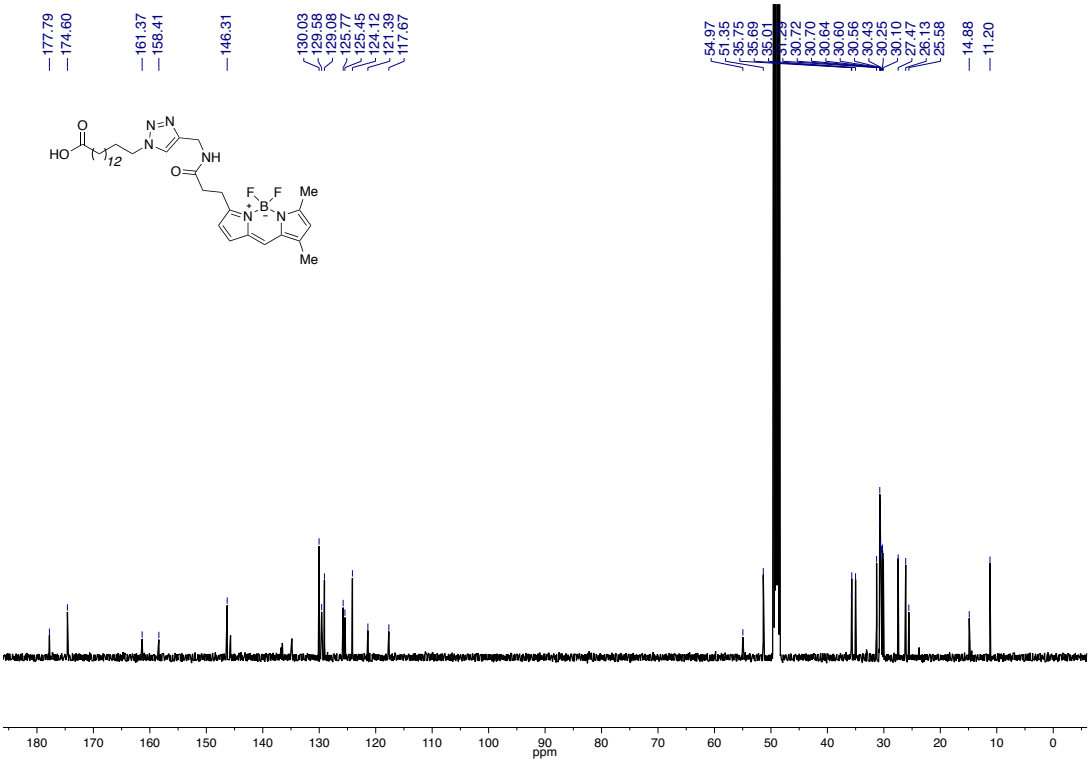

Figure S30:·^{13}C NMR spectrum of Lipid-FL **7** (126 MHz, CD_3OD)

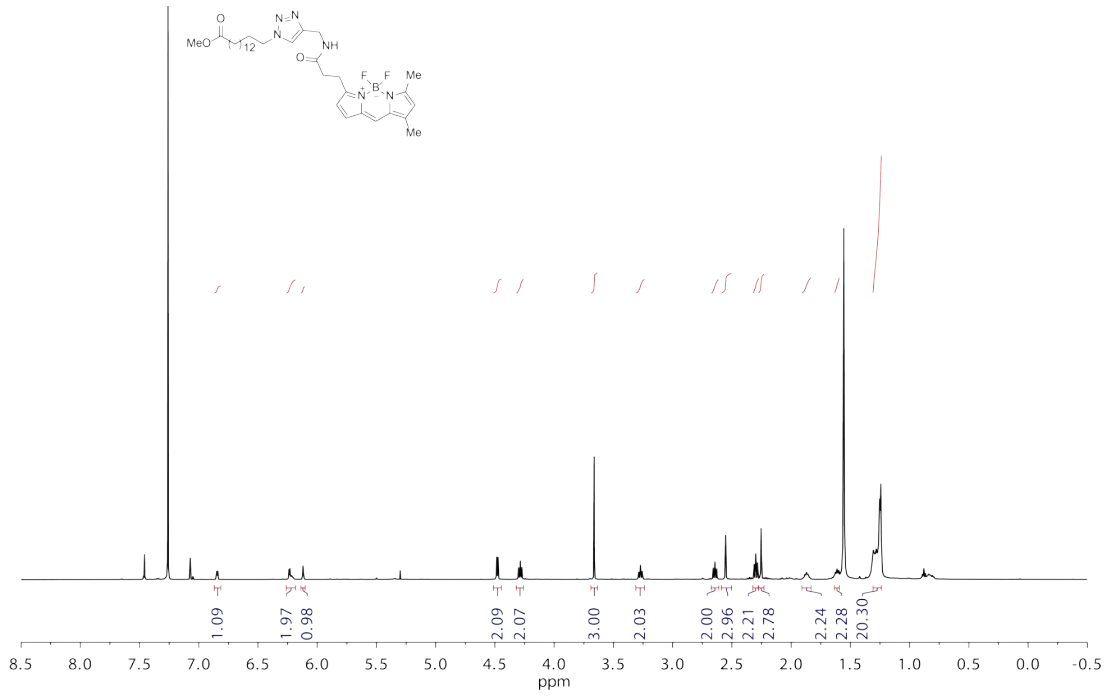

Figure S31: ^{1}H NMR spectrum of Lipid-FL-OMe **8** (126 MHz, CD_3OD)

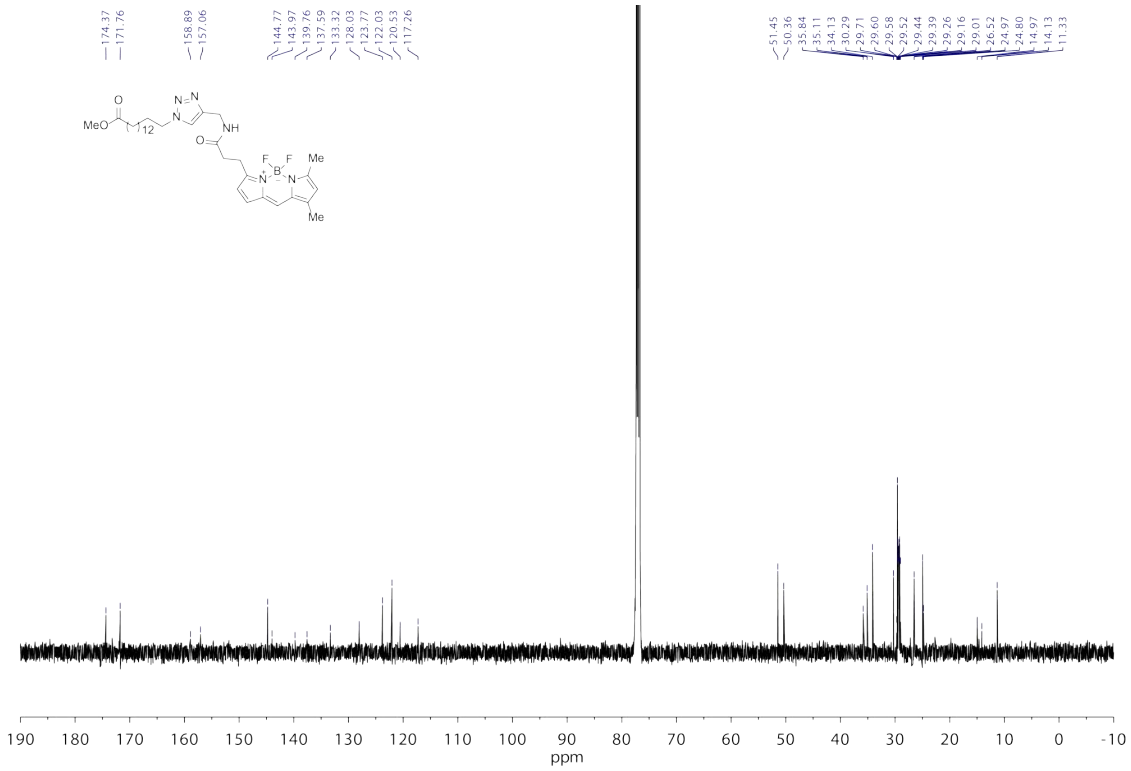

Figure S32: ^{13}C NMR spectrum of Lipid-FL-OMe **8** (126 MHz, CD_3OD)

Supplemental Tables

Table S1. Kinetic parameters for hydrolytic cleavage of QTF by purified native Ag85A, Ag85B and Ag85C from *M.tb*. K_m and k_{cat} were determined through Michaelis-Menten analysis of initial reaction velocities. Assay conditions: [Ag85] = 50 nM, [QTF] = 1–100 μM, 37 °C in 20 mM phosphate buffer (pH = 7.2).

M.tb Ag85 protein	K_m (app) (μM)	k_{cat} (app) (s^{-1})	k_{cat} (app) / K_m(app) (M^{-1} s^{-1})
A	107 ± 27	2.81×10^{-3}	26
B	28 ± 3	0.33×10^{-3}	12
C	44 ± 3	2.08×10^{-3}	48

Table S2. *M. smegmatis* Ag85 proteins identified in extracted fluorescent bands

band no.	Protein name	accession number	% seq. coverage	Peptide sequence	Total spectrum count	Peptide id . probability	Best Mascot Ion score
1	antigen 85 (Msmeg_2078)	118473709	8.0%	ANDMWGPTEDPNSAWK	3	97.1%	35.4
			8.0%	ANDMWGPTEDPNSAWKR	3	99.6%	23.4
			8.0%	NDPMVQIPR	3	99.7%	32.3
1	antigen 85 (Msmeg_3580)	118467737	10.3%	NDPMVNINQLVANNTR	2	98.9%	30.2
			10.3%	VQFQGGGPHAVYLLDGLR	2	99.7%	38.7
1	antigen 85 (Msmeg_6398)	118468744	24.5%	ADDMWGSTNDPNNAWK	5	99.1%	27.5
			24.5%	ANDPTENVATIANNGTR	5	99.7%	60.6
			24.5%	FLEGFVCR	5	99.0%	28
			24.5%	IWVYCGNGKPGELGGTDLPAK	5	99.7%	37.6
			24.5%	VEFQSGGPGAPALYLLDGMR	5	88.9%	20.9
2	antigen 85 (Msmeg_6398)	118468744	13.4%	ANDPTENVATIANNGTR	3	89.7%	26.4
			13.4%	FLEGFVCR	3	99.0%	31.1
			13.4%	VEFQSGGPGAPALYLLDGMR	3	84.5%	18.2
3	antigen 85 (Msmeg_2078)	118473709	28.6%	ANDMWGPTEDPNSAWK	16	99.7%	31.3
			28.6%	ANDMWGPTEDPNSAWKR	16	99.7%	37.8
			28.6%	DVAATGNAAIGLSMAGSAALILAAYHPDR	16	98.6%	28.2
			28.6%	ELQAMVPDLQR	16	99.7%	51.3
			28.6%	LVANNTR	16	90.0%	27.8
			28.6%	NDPMVQIPR	16	99.7%	34.5
			28.6%	VQFQSGGPGSHAVYLLDGLR	16	99.5%	20.6
3	antigen 85 (Msmeg_3580)	118467737	13.4%	GNGQDYTYK	8	99.7%	52.7
			13.4%	NDPMVNINQLVANNTR	8	99.7%	87.7
			13.4%	RNDPMVNINQLVANNTR	8	84.6%	15.7
			13.4%	VQFQGGGPHAVYLLDGLR	8	84.7%	14.9

Table S3. other proteins identified in extracted fluorescent bands

Protein name	Msmeg_	accession number	Exclusive unique peptide count	band	secreted?
glycoside hydrolase; trehalase	Msmeg_4535	118470232	31	1	y
lipolytic protein G-D-S-L; arylesterase	Msmeg_6317	118468600	5	1	y
antigen 85	Msmeg_6398	118468744	5	1	y
hypothetical protein; hydrolase	Msmeg_2074	118470246	3	1	y
antigen 85	Msmeg_2078	118473709	3	1	y
antigen 85	Msmeg_3580	118467737	2	1	y
hypothetical protein; hydrolase	Msmeg_2074	118470246	12	2	y
antigen 85	Msmeg_6398	118468744	3	2	y
serine esterase, cutinase (cut1)	Msmeg_0194	118470941	3	2	y
antigen 85	Msmeg_2078	118473709	7	3	y
antigen 85	Msmeg_3580	118467737	4	3	y
lipolytic protein G-D-S-L; arylesterase	Msmeg_6317	118468600	4	3	y
(treZ) trehalohydrolase	Msmeg_3184	118469689	4	3	y

Table S4. Primers and plasmids used in this study

Primers and Gblocks	
linker-mCherry (Msm)	5'-GATAAGAGAAAGGGAGTCCAGGTGCGTCCGGGTCCGCCGGGGCGT CGGTCTCGAAGGGCGAGGAGGACAATATGGCGATTATCAAGGAATTC ATGCGGTTCAAGGTCCACATGGAAGGTTCCGTCAACGGTCATGAGTT TGAAATTGAAGGGGAGGGGGAAGGGCGGCCCTACGAGGGCACCCAG ACGGCGAAACTGAAAGTGACCAAGGGGGGCCCCTTGCCGTTCGCCTG GGATATTTTGTCGCCCCAATTTATGTATGGCTCGAAAGCGTATGTGAA GCACCCAGCGGATATTCCAGATTACTTGAAGCTCAGCTTTCCCGAGG GTTTCAAATGGGAACGTGTGATGAACTTTGAAGACGGGGGGGTGGT GACCGTGACCCAGGACAGCAGCCTGCAAGACGGGGAGTTCATTTACA AAGTCAAACTCCGGGGCACGAATTTTCCGAGCGATGGGCCGGTCATG CAAAAGAAGACGATGGGTTGGGAAGCCAGCTCCGAACGCATGTATCC GGAGGATGGGGCGCTCAAGGGCGAGATCAAGCAACGTCTGAAACTCA AGGACGGTGGCCACTACGACGCGGAGGTGAAAACGACGTACAAAGC GAAAAAGCCGGTCCAGCTGCCGGGGGCCTACAATGTGAATATTAAGC TCGATATTACGTCCCACAATGAGGATTATACCATTGTGGAACAATACG AACGCGCGGAAGGTCGTCATTCCACGGGGGGGATGGACGAGCTCTAT AAGTAATAAAGCTGCAGAATTCGAAGCTT-3'
pLAM12 inv F	5'-AGCTGCAGAATTCGAAG-3'
pLAM12 inv R	5'-TGGACTCCCTTTCTCTTATC-3'
pLAM12 MC inv F	5'-GGTGCGTCCGGGTCC-3'
pLAM12 MC inv R	5'-TGGACTCCCTTTCTCTTATCGGGTG-3'
Msmeg_3580 F	5'-GATAAGAGAAAGGGAGTCCATTGGTTGCCGTTATGAAGTTCATTCGAG-3'
Msmeg_3580 R	5'-GGACCCGGACGCACGAGCACCCGCTGGATGTC-3'
Msmeg_6398 F	5'-GATAAGAGAAAGGGAGTCCAATGAAGTTCGTTGGGAGAATGCGCGG-3'
Msmeg_6398 R	5'-GGACCCGGACGCACCAGCACGCGCTGCAGGTC-3'
Msmeg_6399 F	5'-GATAAGAGAAAGGGAGTCCAATGGCACCCATGGTGAGC-3'
Msmeg_6399 R	5'-GGACCCGGACGCACCAGCGAAGCGATCAGGTCAG-3'
Msmeg_6583 F	5'-GATAAGAGAAAGGGAGTCCAGTGAAGCCCTCGTTTTTCTC-3'
Msmeg_6583 R	5'-GGACCCGGACGCACGAGGACCCGCAACAGATC-3'
H6 F	5'-/5phos/TAATAAAGCTGCAGAATTCGAAGCTTAT-3'
H6 R	5'-/5phos/ATGGTGGTGATGATGGTG CGACGCCCCGGC-3'
pLAM12 ATG-MC inv F	5'-/5phos/ATGGGTGCGTCCGGGTC-3'
pLAM12 ATG-MC inv R	5'-/5phos/TGGACTCCCTTTCTCTTATCGGG-3'
pLAM12_3580MC KK F	5'-/5phos/AAGAAATTTGTGATCGGTGCCCT-3'
pLAM12_3580 MC KK R	5'-/5phos/CAGGGCTTTCCATGCTG-3'
pLAM12_6398 MC KK F	5'-/5phos/AAGAAACTGACGGTTGCGGTC -3'
pLAM12_6398 MC KK R	5'-/5phos/ CGACAGACCTGCCGC-3'
pLAM12_6399 MC KK F	5'-/5phos/AAACTGCTGGTGGCGACAGT-3'
pLAM12_6399 MC KK R	5'-/5phos/CAGACCGGATACGCGCA-3'
pLAM12_3580MC Δss F	5'-/5phos/TTTTCCCGTCCTGGTCTG-3'
pLAM12_3580 MC Δss R	5'-/5phos/CAATGGACTCCCTTTCTCTTATCGG-3'
pLAM12_6398 MC Δss F	5'-/5phos/TGGTCTCGACCCGGC-3'

pLAM12_6398 MC Δss R	5’-/5phos/CATTGGACTCCCTTTCTCTTATCGG-3’
pLAM12_6399 MC Δss F	5’-/5phos/TTTTCGCGTGAGGGCC-3’
pLAM12_6399 MC Δss R	5’-/5phos/CATTGGACTCCCTTTCTCTTATCGG-3’
linker-mCherry (Cgl)	5’- GGAGCTTCTGGTTCTGCTGGAGCGTCAGTCTCGAAGGGCGAGGAGGA CAATATGGCGATTATCAAGGAATTCATGCGGTTCAAGGTCCACATGG AAGGTTCCGTCAACGGTCATGAGTTTGAAATTGAAGGGGAGGGGGAA GGGCGGCCCTACGAGGGCACCCAGACGGCGAAACTGAAAGTGACCAA GGGGGGCCCCTTGCCGTTCGCCTGGGATATTTTGTCGCCCCAATTTAT GTATGGCTCGAAAGCGTATGTGAAGCACCCAGCGGATATTCCAGATT ACTTGAAGCTCAGCTTTCCCGAGGGTTTCAAATGGGAACGTGTGATG AACTTTGAAGACGGGGGGGTGGTGACCGTGACCCAGGACAGCAGCCT GCAAGACGGGGAGTTCATTTACAAAGTCAAACTCCGGGGCACGAATT TTCCGAGCGATGGGCCGGTCATGCAAAAGAAGACGATGGGTTGGGA AGCCAGCTCCGAACGCATGTATCCGGAGGATGGGGCGCTCAAGGGCG AGATCAAGCAACGTCTGAAACTCAAGGACGGTGGCCACTACGACGCG GAGGTGAAAACGACGTACAAAGCGAAAAAGCCGGTCCAGCTGCCGG GGGCCTACAATGTGAATATTAAGCTCGATATTACGTCCCACAATGAG GATTATACCATTGTGGAACAATACGAACGCGCGGAAGGTCGTCATTC CACGGGGGGGATGGACGAGCTCTATAAGTAAAAGATATGACCATG-3’
pEKEx2 F	5’-ATTACGCCAAGCTTGCATGC-3’
pEKEx2 R	5’-CCACACATTATACGAGCCGAT-3’
Cgl-linker-mCherry F	5’- ATCGGCTCGTATAATGTGTGGGGAGCTTCTGGTTCTGC-3’
Cgl-linker-mCherry R	5’- GCATGCAAGCTTGGCGTAATCATGGTCATATCTTTTACTTATAGA-3’

plasmids	properties	source
pLAM12	Kan^R; acetamidase promoter, mycobacterial origin of replication	(11)
pLAM12-mCherry	pLAM 12 with C terminus mCherry (for fusion)	this work
pLAM12-ATG-mCherry	pLAM12 with C terminus mCherry (for expression)	this work
pLAM12-3580-mCherry	pLAM 12 with C terminus mCherry and Msmeg_3580	this work
pLAM12-6398-mCherry	pLAM 12 with C terminus mCherry and Msmeg_6398	this work
pLAM12-6399-mCherry	pLAM 12 with C terminus mCherry and Msmeg_6399	this work
pLAM12-ss(kk)-3580-mCherry	pLAM12 with C terminus mCherry and Msmeg_3580 and signal sequence R to K substitution	this work
pLAM12-ss(kk)-6398-mCherry	pLAM 12 with C terminus mCherry and Msmeg_6398 and signal sequence R to K substitution	this work
pLAM12-ss(kk)-6399-mCherry	pLAM 12 with C terminus mCherry and Msmeg_6399 and signal sequence R to K substitution	this work
pLAM12-Δss-3580-mCherry	pLAM 12 with C terminus mCherry and Msmeg_3580 and no signal sequence	this work

pLAM12-Δss-6398-mCherry	pLAM 12 with C terminus mCherry and Msmeg_6398 and no signal sequence	this work
pLAM12-Δss-6399-mCherry	pLAM 12 with C terminus mCherry and Msmeg_6399 and no signal sequence	this work
pLAM12-3580-H6	pLAM 12 with C terminus 6xHis tag and Msmeg_3580	this work
pLAM12-6398-H6	pLAM 12 with C terminus 6xHis tag and Msmeg_6398	this work
pLAM12-6399-H6	pLAM 12 with C terminus 6xHis tag and Msmeg_6399	this work
pLAM12-6583-H6	pLAM12 with C terminus 6xHis tag and Msmeg_6583	this work
pEKEx2	Kan^R; pTac promoter, *E. coli* origin of replication, *C. glutamicum* origin of replication	(12)
pEKEx2-mCherry	pEKEx2 expressing mCherry	this work

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