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Chemical remodeling of the mycomembrane with chain-truncated lipids sensitizes mycobacteria to rifampicin

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Supplementary figures

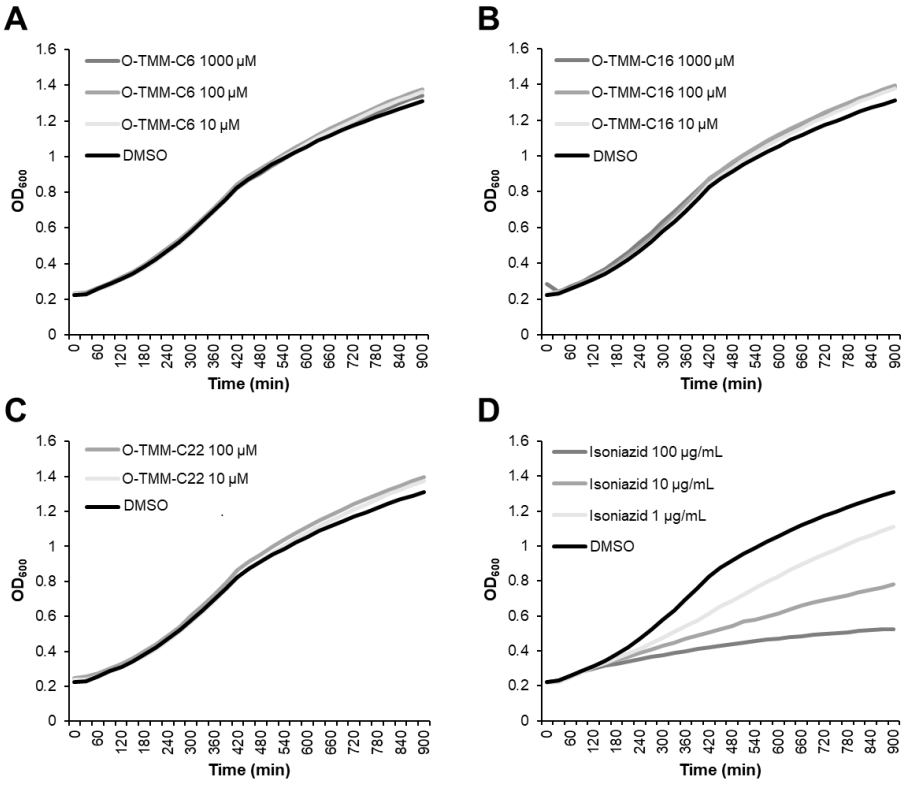

Figure S1. Growth inhibition evaluation of trehalose monomycolate (TMM) analogues. Growth curves for O-TMM-C6 (**A**), O-TMM-C16 (**B**), O-TMM-C22 (**C**), and positive control isoniazid (INH) (**D**). *M. smegmatis* was cultured in 7H9 liquid growth medium containing the indicated concentration of TMM analogue, isoniazid, or DMSO control. Cultures were incubated at 37 °C with shaking for 15 h and optical density at 600 nm (OD_{600}) was measured every 30 min to obtain the growth curves shown. Data shown are average values of three technical replicates and are representative of at least two independent experiments. O-TMM-C22 was insoluble at 1 mM in the assays.

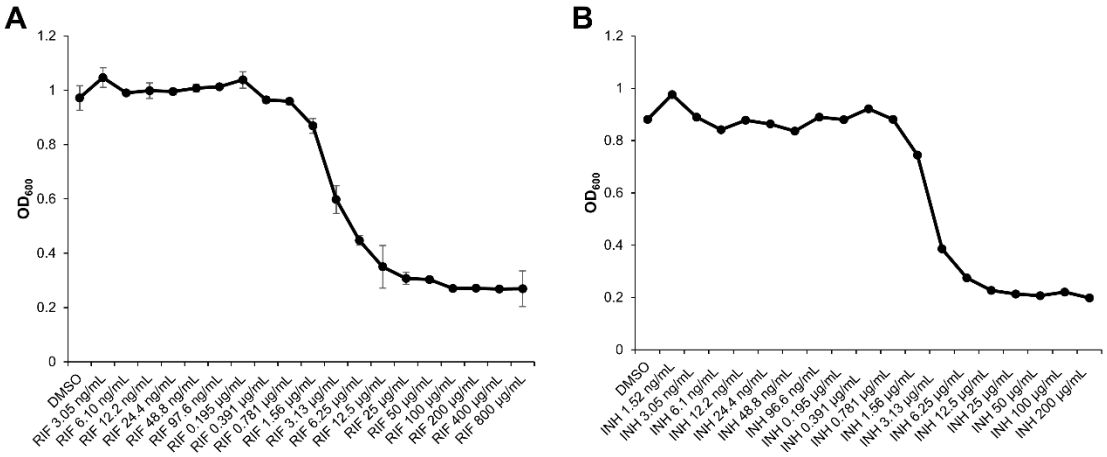

Figure S2. Minimum inhibitory concentration (MIC) determination of (A) rifampicin (RIF) and (B) isoniazid (INH). *M. smegmatis* was cultured in 7H9 liquid growth medium containing the indicated concentration of RIF, INH, or control. Cultures were incubated at 37 °C with shaking for 24 h and optical density at 600 nm (OD_{600}) was measured. Error bars represent the standard deviation of three technical replicates. Data shown are average values of three technical replicates and are representative of at least two independent experiments.

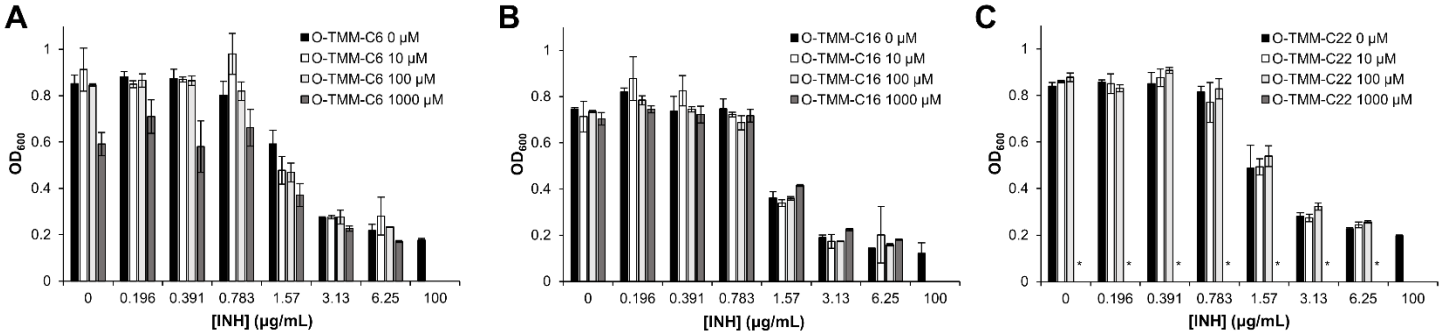

Figure S3. Co-treatment of *Msmeg* with TMM analogues and INH. *Msmeg* was cultured in 96-well microplates in 7H9 liquid growth medium containing (A) O-TMM-C6 (**1**), (B) O-TMM-C16 (**2**), or O-TMM-C22 (**3**) at the indicated concentrations along with INH at various sub-MIC-range concentrations or 0 µg/mL as the negative control and 100 µg/mL as the positive control. After 24 h, growth was assessed by measuring OD_{600} using a plate reader. Data shown are average values of three technical replicates and are representative of at least two independent experiments. Error bars represent the standard deviation of three replicates. *O-TMM-C22 was insoluble in the assay at 1 mM concentration.

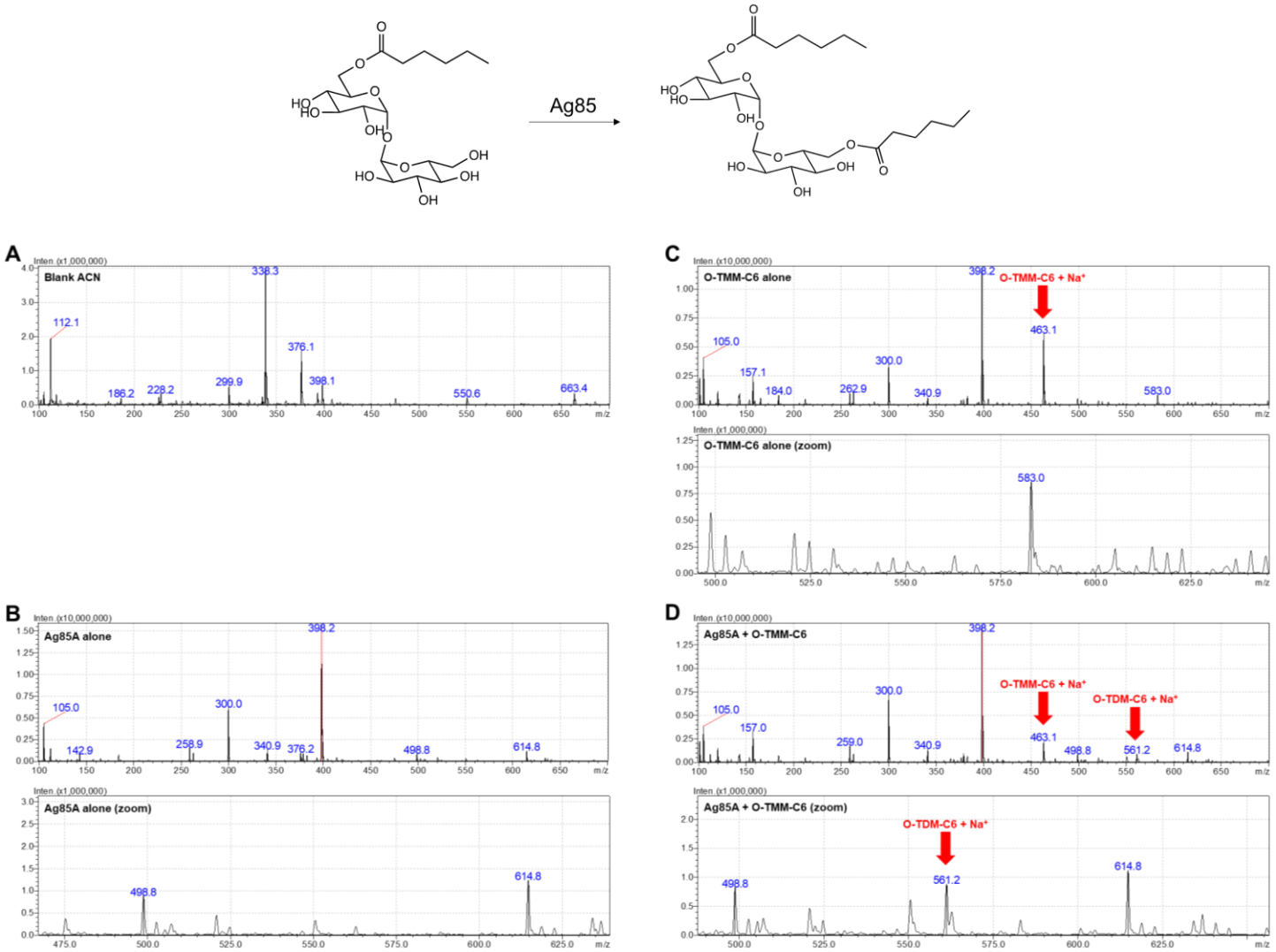

Figure S4. Ag85A catalyzes acyl transfer using O-TMM-C6 as a substrate to generate chain-truncated TDM analogue product. Recombinant Ag85A was incubated in the presence or absence of O-TMM-C6 at 37 °C for 15 min followed by 16 h at 4 °C, after which the reaction was quenched and analyzed by LC-MS. Top, reaction scheme. (A) Acetonitrile (ACN) blank. (B) Ag85A only negative control. (C) O-TMM-C6 only negative control (O-TMM-C6 ESI MS m/z: $[M+Na]^+$ calcd for $C_{18}H_{32}O_{12}Na$ 463.2; found 463.1). (D) Ag85A + O-TMM-C6 reaction (O-TDM-C6 ESI MS m/z: $[M+Na]^+$ calcd for $C_{24}H_{42}O_{13}Na$ 561.3; found 561.2).

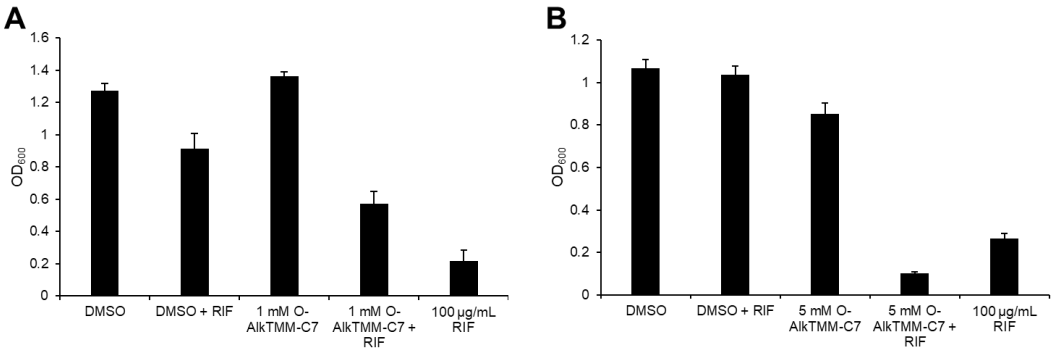

Figure S5. Pre-treatment of *Msmeg* with O-AlkTMM-C7 sensitizes bacteria to RIF. *Msmeg* was cultured in 96-well microplates in 7H9 medium containing (A) 1 mM or (B) 5 mM O-AlkTMM-C7 (**4**) or left untreated (DMSO control) for the indicated durations. Cells were then washed, incubated for 24 h in RIF (0.78 µg/mL) or left alone as control, and OD_{600} was measured. Data shown are average values of three technical replicates and are representative of two independent experiments. Error bars represent the standard deviation of three technical replicates.

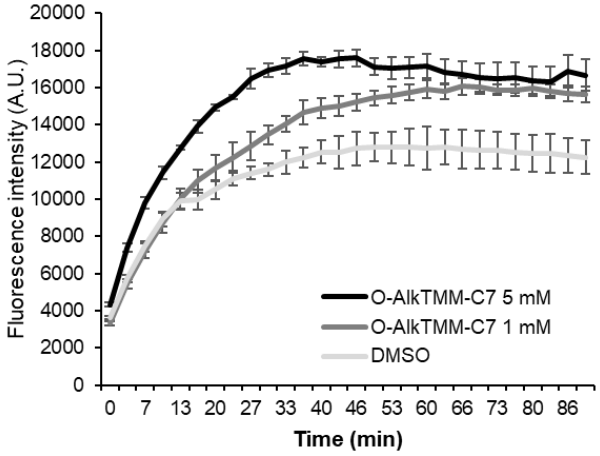

Figure S6. Pre-treatment of *Msmeg* with O-AlkTMM-C7 enhances cellular permeability. *Msmeg* was pre-treated with 1 mM or 5 mM O-AlkTMM-C7 (**4**) for 8 h, then treated with ethidium bromide and fluorescence (Ex 535 nm/Em 595 nm) was monitored. Data shown are average values of three technical replicates and are representative of two independent experiments. Error bars represent the standard deviation of three technical replicates.

Materials and methods

General methods for synthesis. Materials and reagents were obtained from commercial sources without further purification unless otherwise noted. Anhydrous solvents were obtained either commercially or from an alumina column solvent purification system. All reactions were carried out in oven-dried glassware under inert gas unless otherwise noted. Analytical thin-layer chromatography (TLC) was performed on glass-backed silica gel 60 Å plates (thickness 250 μm) and detected by charring with 5% H_2SO_4 in EtOH. Column chromatography was performed using flash-grade silica gel 32–63 μm (230–400 mesh). Nuclear magnetic resonance (NMR) spectra were obtained on a Varian Mercury 300 instrument.

General procedure for the synthesis of TMM analogues. This procedure generally followed the method of Sarpe *et al*.[1] An oven-dried round-bottom flask was charged with *N*,*N'*-dicyclohexylcarbodiimide (DCC) (2.0–4.0 equiv) and *N*,*N'*-dimethylaminopyridine (DMAP) (1.1 equiv). After drying the reagents under high vacuum and placing the flask under an argon atmosphere, anhydrous CH_2Cl_2 was added and the mixture was cooled to 0 °C. To the stirring solution was added the carboxylic acid (1.1–2.2 equiv) followed by slow, dropwise addition of a freshly prepared solution of 2,3,4,2',3',4'-hexakis-*O*-(trimethylsilyl)-α,α-trehalose[1] (1 equiv) in anhydrous CH_2Cl_2 (2 mL). The reaction mixture was stirred and gradually allowed to warm to room temperature. After TLC analysis showed generation of the monoester as a major product (approximately 4–6 h), the reaction was quenched by addition of excess CH_3OH and concentrated by rotary evaporation. After resuspension of the crude product in CH_2Cl_2, the insoluble urea byproduct was removed by filtration. The filtrate containing crude product was concentrated by rotary evaporation and purified by silica gel chromatography (hexanes/ethyl acetate containing 1% Et_3N) to give the monoester intermediate. The intermediate was dissolved in a mixture of CH_3OH/CH_2Cl_2 (2:1) and placed under an argon atmosphere. Dowex 50WX8-400 H^+ ion-exchange resin was added and the reaction was stirred for 2 h at room temperature, until TLC (CH_2Cl_2/CH_3OH 2:1) indicated that the reaction was complete. After the ion-exchange resin was filtered off, the filtrates were concentrated by rotary evaporation to give the desired TMM analogue as a white solid.

6-O-(hexanoyl)-α,α-D-trehalose (O-TMM-C6). O-TMM-C6 was synthesized according to the general procedure above using DCC (0.712 g, 3.46 mmol), DMAP (0.387 g, 1.73 mmol), hexanoic acid (0.31 g, 2.6 mmol), and 2,3,4,2',3',4'-hexakis-*O*-(trimethylsilyl)-α,α-trehalose (1.27 g, 1.64 mmol) to yield O-TMM-C6 (0.176

g, 23% over two steps). NMR data matched those reported in the literature by Paul *et al*.[2]

6-O-(palmitoyl)-α,α-D-trehalose (O-TMM-C16). O-TMM-C16 was synthesized according to the general procedure above as we previously reported.[3]

6-O-(behenoyl)-α,α-D-trehalose (O-TMM-C22). O-TMM-C22 was synthesized according to the general procedure above using DCC (0.71 g, 3.40 mmol), DMAP (0.11 g, 0.89 mmol), palmitic acid (0.60 g, 1.8 mmol), and 2,3,4,2',3',4'-hexakis-*O*-(trimethylsilyl)-α,α-trehalose (0.61 g, 0.79 mmol). Tetrahydrofuran was used as solvent instead of CH_2Cl_2. The reaction yielded O-TMM-C22 (0.127 g, 23% over two steps. NMR data matched those reported in the literature by Stocker *et al*.[4]

General procedures for bacterial culture experiments. *Mycobacterium smegmatis* mc^2155 (*Msmeg*) starter cultures were generated by inoculating a single isolated colony from a freshly streaked LB agar plate into 3 mL Middlebrook 7H9 liquid medium supplemented with ADC (albumin, dextrose, and catalase), 0.5% glycerol, and 0.05% Tween-80 in a culture tube. Starter cultures were incubated at 37 °C with shaking until reaching mid-logarithmic phase and then diluted with liquid medium to the desired density for initiating experiments. Stock solutions of TMM analogues were prepared in DMSO, RIF was prepared in 1:1 DMSO and 7H9 liquid medium, and INH was prepared in 7H9 liquid medium. All experiments were conducted in flat-bottomed 96-well microplates, to which appropriate volumes of bacterial cells, growth medium, and compound stocks were added to achieve a final volume 200 µL, the specified final concentrations of compounds, and a final DMSO concentration of 2% for all experimental and control conditions unless otherwise noted. Empty wells in the microplate were filled with 200 µL deionized water to minimize evaporation during incubation periods. Cultures in microplates were incubated at 37 °C with shaking for the durations specified. For wash steps, cells were transferred to a v-bottom 96-well microplate, centrifuged (4000 rpm, 5 min, room temperature), and washed with phosphate-buffered saline (PBS). OD_{600} and fluorescence readings were taken in a Tecan F200 Pro multimodal microplate reader. All experiments were conducted in technical triplicate and data shown are representative of three independent experiments.

Procedure for TMM analogue and antibiotic co-treatment assay. *Msmeg* starter culture was diluted with 7H9 liquid medium to an OD_{600} of 0.2. Appropriate volumes of (i) TMM analogue stock solution, (ii) antibiotic stock solution, and/or DMSO were added to the wells of a sterile flat-bottom polystyrene 96-well plate. Aliquots

of the *Msmeg* cell suspension were then added to the wells to obtain the desired final volume (200 µL), OD_{600} (~0.2), concentration of TMM analogue (0–1000 µM), and concentration of antibiotic (0–12.5 µg/mL). As a positive control, 100 µg/mL of the antibiotic (~10x MIC) was used. As negative controls, cells were treated with TMM analogue alone, antibiotic alone, or left untreated as DMSO controls. As a blank, 7H9 liquid medium was used. The contents of each well were mixed thoroughly by pipetting up and down, a lid was placed on the 96-well plate, and the plate was incubated at 37 °C with shaking in a humidity chamber for 24 h. The contents of each well were mixed by pipetting up and down, then end-point OD_{600} reading was taken.

Procedure for TMM analogue pre-treatment assay. *Msmeg* starter culture was diluted with 7H9 liquid medium to an OD_{600} of 0.05 and added to wells in a sterile polystyrene 96-well plate. A lid was placed on the plate and it was incubated at 37 °C with shaking in a humidity chamber for the 8 h pre-treatment period. During the pre-treatment incubation period, at varying timepoints (0–8 h), appropriate volumes of TMM analogue stock solution or DMSO were added to the wells in a sterile environment, then incubation was resumed. After a total incubation time of 8 h, cells were washed three times and resuspended in 7H9 liquid medium. A starting OD_{600} reading was obtained to ensure each well had comparable cell densities before the second incubation. Next, appropriate volumes of RIF stock solution or DMSO were added to wells achieve a final concentration of 0 or 0.78 µg/mL RIF. As a positive control, 100 µg/mL of RIF (~10x MIC) was used. As negative controls, cells were treated with DMSO in both steps (“DMSO” in Figure 3A), DMSO in pre-treatment followed by RIF in the second step (“DMSO + RIF” in Figure 3A), or TMM analogue in pre-treatment followed by DMSO in the second step (“8 h TMM Pre” in Figure 3A). As a blank, 7H9 liquid medium was used. The contents of each well were mixed thoroughly by pipetting up and down, a lid was placed on the 96-well plate, and the plate was incubated at 37 °C with shaking in a humidity chamber for 24 h. The contents of each well were mixed by pipetting up and down, then end-point OD_{600} reading was taken. In parallel, a comparison to the co-treatment experiment was performed, in which cells were treated with both TMM analogue and RIF for 24 h.

Procedure for ethidium bromide permeability assay. *Msmeg* starter culture was diluted with 7H9 liquid medium to an OD_{600} of 0.05. Appropriate volumes of TMM analogue stock solution or DMSO were added to the wells of a sterile flat-bottom polystyrene 96-well plate. Aliquots of the *Msmeg* cell suspension were then added to the wells to obtain the desired final volume (200 µL), OD_{600} (0.05), and concentration of TMM analogue

(0–5000 μM). The contents of each well were mixed thoroughly by pipetting up and down, a lid was placed on the 96-well plate, and the plate was incubated at 37 °C with shaking in a humidity chamber for various durations (0–8 h). When the incubation period was over, cells were washed one time, resuspended in 198 μL PBS, and transferred to a 96-well black opaque flat-bottom 96-well microplate wells containing 2 μL of 500 μM ethidium bromide stock solution in DMSO, giving a final ethidium bromide concentration of 5 μM and final DMSO concentration of 1%. The contents of each well were mixed thoroughly by pipetting up and down, then the plate was incubated at room temperature with shaking for 90 min, during which and fluorescence intensity readings (excitation 535 nm, emission 595 nm) were taken every 3 min.

Expression and purification of Ag85A. BL21 *Escherichia coli* cells (New England BioLabs, Ipswich, MA, USA) were transformed with a codon optimized plasmid (pDR32-Ag85A) encoding wild-type *M. tuberculosis* Ag85A protein tagged with six histidine (HIS) residues at the N-terminus. Cells were cultured in LB media at 37 °C to reach $A_{600\ nm}$ = 0.6, followed by incubation at 16 °C for induction of protein expression by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (GoldBio, St. Louis, MO, USA). Cells were harvested by centrifugation after 20-24 hours, resuspended in 20 mM Tris buffer (pH 8.0) (ThermoScientific, Ward Hill, MA, USA) and lysed via addition of 10 μM lysozyme (MP Biomedicals, Irvine, CA, USA) and 100 μM DNase I (GoldBio), followed by sonication (Misonix 3000) and centrifugation for 45 min (10,000 rpm, 4 °C). Clarified lysate was filtered with a 0.2 mm filter and applied to an immobilized Cobalt affinity column (5 mL, Cytiva, Marlborough, Massachusetts, USA) and washed with 10 column volumes of buffer A (20 mM Tris pH 8.0 and 5 mM imidazole (TCI, Portland, OR, USA)). Protein was eluted isocratically from the column with buffer B (20 mM Tris pH 8.0 and 150 mM imidazole). The resulting HIS-tagged Ag85A was treated with rhinovirus 3C protease, dialyzed overnight against a 20 mM Tris pH 8.0 buffer including 0.3 mM tris(2-carboxyethyl)phosphine (TCEP)-HCl (GoldBio), and reapplied to the immobilized Cobalt affinity column. Column flow-through containing 3C Protease-cleaved Ag85A lacking the HIS-tag was collected, concentrated by ultrafiltration, and applied to a Superdex 200 Increase 10/300 column (Cytiva) for further purification via size exclusion chromatography. Protein purity was confirmed via SDS-PAGE analysis and estimated to be greater than 95% pure. Protein concentration was determined by measuring absorbance at 280 and 260 nm.

Enzymatic reactions and sample preparation. Ag85A-mediated transfer of hexanoate from

donor/acceptor O-TMM-C6 to form C6-TMM-C6 was initiated by addition of Ag85A (1 μM) to a mixture containing O-TMM-C6 (1 mM) in a 50 mM sodium phosphate buffer (pH 7.5). The reactions were incubated at 37 °C for 15 min followed by 16 h incubation at 4 °C. Reactions were terminated by addition of 2x volume of ice-cold acetonitrile (ACN) and centrifuged for 20 min (15,000 rpm, 4 °C) to remove precipitated protein. The resulting supernatant was further diluted 20-fold with ACN before subjection to mass spectrometric analysis. Sample controls lacking enzyme or substrate were processed in parallel.

Liquid chromatography-mass spectrometry (LC-MS) analysis. LC-MS grade acetonitrile (ACN) and formic acid (FA) were purchased from Fisher Scientific (Fair Lawn NJ, USA). Purified water was obtained from Barnstead GenPure water purification system (ThermoScientific, Waltham, MA, USA). A Shimadzu Nexera UPLC (Ultra Performance Liquid Chromatography, Shimadzu Scientific Instruments, Columbia, MD, USA) system, consisting of two pumps (LC-30 AD) and autosampler (SIL-30AD) were utilized for chromatographic separation. The mobile phase consisted of 0.1% FA in water (mobile phase A) and 100% ACN (mobile phase B). The pumps were operated in isocratic mode (50% of mobile phase B) at a combined flow rate of 0.3 mL/min. A total injection volume of 2 µL was utilized for all samples. Detection was performed with a Shimadzu 8060 NX MS system (Shimadzu Scientific Instruments) equipped with a dual ion source and was operated in the ESI positive mode for the detection of all analytes. LabSolutions software (Version 5.8) was used to optimize temperature and gas flow rates. Final parameters were: interface temperature (350 °C), desolvation line (250 °C), heat block temperature (400 °C) nebulizing gas flow rate (2 L/min), heating gas (10 L/min), and drying gas flow rates (10 L/min). For detection of analytes, a Q3 scan was performed within a scan range of 100-700 m/z and the post-run module was used for the identification of the parent ions masses for each analyte.

References

1. Sarpe, V. A.; Kulkarni, S. S., Synthesis of Maradolipid. *J. Org. Chem.* **2011,** *76* (16), 6866-6870.
2. Paul, N. K.; Twibanire, J. D.; Grindley, T. B., Direct Synthesis of Maradolipids and Other Trehalose 6-Monoesters and 6,6'-Diesters. *J. Org. Chem.* **2012,** *78* (2), 363-369.
3. Kavunja, H. W.; Biegas, K. J.; Banahene, N.; Stewart, J. A.; Piligian, B. F.; Groenevelt, J. M.; Sein, C. E.; Morita, Y. S.; Niederweis, M.; Siegrist, M. S.; Swarts, B. M., Photoactivatable Glycolipid Probes for Identifying Mycolate–Protein Interactions in Live Mycobacteria. *J. Am. Chem. Soc.* **2020,** *142* (17), 7725-7731.
4. Stocker, B. L.; Khan, A. A.; Chee, S. H.; Kamena, F.; Timmer, M. S. M., On One Leg: Trehalose Monoesters Activate Macrophages in a Mincle-Dependant Manner. *ChemBioChem* **2014,** *15* (3), 382-388.