Supporting Information

for

A FRET-Based Fluorogenic Trehalose Dimycolate Analogue for Probing Mycomembrane-Remodeling Enzymes of Mycobacteria

Nathan J. Holmes,1,‡ Herbert W. Kavunja,1,‡ Yong Yang,2 B. Dillon Vannest,1 Claudia N. Ramsey,1 Dana M. Gepford,1 Nicholas Banahene,1 Anne W. Poston,1 Brent F. Piligian,1 Donald R. Ronning,3 Anil K. Ojha,2,4 and Benjamin M. Swarts1,*

1Department of Chemistry and Biochemistry, Central Michigan University, Mount Pleasant, Michigan, 48859, USA

2Division of Genetics, Wadsworth Center, New York State Department of Health, Albany, New York 12208

3Department of Chemistry and Biochemistry, University of Toledo, Toledo, Ohio 43606-3390, USA

4Department of Biomedical Sciences, University at Albany, New York, 12208

‡These authors contributed equally to this work

*Corresponding author: E-mail: ben.swarts@cmich.edu
Table of Contents

I. Supplementary Schemes, Figures, and Tables
    Scheme S1. Synthesis of FITC-TMM (fluorescent product of FRET-TDM cleavage)  S3
    Figure S1. Background fluorescence of FRET-TDM in various buffers and media  S3
    Figure S2. Optimization of FRET-TDM and Tdmh concentrations  S4
    Table S1. List of mycobacterial strains used in this study  S5
    Figure S3. FRET-TDM activation in starved *Msmeg*  S6
    Figure S4. FRET-TDM activation in starved *Mtb*  S7
    Figure S5. Time-dependent FRET-TDM activation by hydrolase panel  S8
    Figure S6. Time-dependent FRET-TDM activation by bacterial cell panel  S8
    Figures S7–S20. NMR Spectra  S9

II. References  S16
I. Supplementary Schemes and Figures

*Scheme S1*. Synthesis of FITC-TMM (6), the fluorescent product of FRET-TDM cleavage: (a) DCC, DMAP, 10-azidodecanoic acid, CH₂Cl₂; (b) Dowex 50WX8-400 H⁺ resin, CH₃OH, 44% over two steps; (c) Pd/C, H₂, CH₂Cl₂:CH₃OH (2:1), 99%; (d) FITC, Et₃N, DMF, CH₃OH, 85%.

*Figure S1*. Background fluorescence of FRET-TDM in various buffers and media. 1 µM FRET-TDM (or no probe) was incubated in the indicated buffers and media at 37 °C and fluorescence (Ex/Em 485/535) was continuously monitored over 60 min in a plate reader. Fluorescence values shown are the mean of three replicate experiments. A.u, arbitrary units.
**Figure S2.** Optimization of FRET-TDM and Tdmh concentrations for enzymatic assays. (A) Varied concentrations (0–100 µM) of FRET-TDM were incubated in the presence of Tdmh (10 µg/mL) in Tris-HCl buffer at 37 °C for 75 min, then fluorescence (Ex/Em 485/535) was analyzed in a plate reader. (B) Varied concentrations (0–50 µg/mL) of recombinant *Msmeg* Tdmh were incubated in the presence of FRET-TDM (10 µM) in Tris-HCl buffer at 37 °C for 75 min, then fluorescence (Ex/Em 485/535) was analyzed in a plate reader. Error bars represent the standard deviation of three replicate experiments.
### Table S1. List of mycobacterial strains used in this study.

| Name           | Remarks                                                                 | References                                      |
|----------------|-------------------------------------------------------------------------|-------------------------------------------------|
| mc²155         | High-Frequency Transformation strain of M. smegmatis                   | (Ojha et al., Cell 2005)¹                         |
| ΔMSMEG_1529    | deletion of MSMEG_1529 in mc²155, hyg',                                 | (Ojha et al., JBC 2010)²                         |
| ΔMSMEG_1529comp| ΔMsme_1529 with pMsme_1529, hyg', kan',                                 | (Ojha et al., JBC 2010)²                         |
| mc²7000        | M. tuberculosis H37Rv:ΔRD1:ΔpanCD                                        | (Ojha et al., Mol Microbiol 2008)³               |
| mc²7000: ΔtdmhMtb | Isogenic deletion of Mtb_Rv3451 in mc²7000, hyg',                        | (Yang et al., Cell Host and Microbe 2014)⁴       |
| mc²7000: ΔtdmhMtb: p5152 | ΔtdmhMtb with p5152(pMH94 + Rv3451-52 + 470bp ups @ SacI and XbaI), hyg', kan' | (Yang et al., Cell Host and Microbe 2014)⁴       |
| mc²7000: ΔtdmhMtb: pLAM12 | ΔtdmhMtb with pLAM12, hyg', kan'                                     | (Yang et al., Cell Host and Microbe 2014)⁴       |
| mc²7000: ΔtdmhMtb: pAO10 | ΔtdmhMtb with pAO10(pLAM12 + tdmhMtb @NdeI & EcoRI), hyg', kan'             | (Yang et al., Cell Host and Microbe 2014)⁴       |
Figure S3. FRET-TDM activation in starved Msmeg. (A) Evaluation of time-dependent FRET-TDM activation in Msmeg grown in nutrient-rich or starvation conditions with immediate probe addition. Msmeg was cultured in M63 medium to an OD\textsubscript{600} of 0.5–1.0, then cells were washed, normalized to OD\textsubscript{600} = 1.0, and incubated at 37 °C in either M63 medium or phosphate-buffered saline (PBS) in the presence of FRET-TDM (10 µM). Fluorescence (Ex/Em 485/535) was monitored continuously in a plate reader over 15 hrs. Solid lines, rich medium; dashed lines, starved medium. (B) Evaluation of time-dependent FRET-TDM activation in Msmeg grown in starvation medium with probe addition following 96 hrs of starvation. From an Msmeg culture growing in M63 medium at an OD\textsubscript{600} of 0.5–1.0, cells were washed and incubated at 37 °C in PBS for 96 hrs. Cells were washed in PBS, then FRET-TDM was added (10 µM) and the cells were incubated at 37 °C with continuous fluorescence monitoring (Ex/Em 485/535) over 15 hrs. For both (A) and (B), fluorescence values shown are the mean of three replicate experiments.
**Figure S4.** FRET-TDM activation in starved *Mtb.* (A–D) Evaluation of time-dependent FRET-TDM activation in *Mtb* mc²7000 strains grown in nutrient-rich (A) or starvation (C–D) conditions. For (A), *Mtb* strains were first cultured in 7H9 with OADC medium to an OD₆₀₀ of 0.5–1.0, then cells were washed and diluted 1:100 into Sauton’s medium and incubated at 37 °C in the presence of FRET-TDM (10 µM). Fluorescence (Ex/Em 485/535) was assessed in a plate reader at 0, 3, 6, 18, and 24 hr time points. For (C–D), exponential phase cultures of *Mtb* were harvested and washed once with PBS containing 0.05% Tween-80 (PBST), then starved in PBST at 37 °C for 4 h (B), 24 h (C), or 48 h (D) prior to washing the cells and initiating FRET-TDM assays as just described. (E) shows data from (A–D) as a plot of FRET-TDM fluorescence as a function of PBS starvation time. For all plots, fluorescence values shown are the mean of three replicate experiments and error bars (mostly obscured by the markers) represent the standard deviation. Controls are FRET-TDM only (10 µM) in the appropriate medium.
Figure S5. Plot of time-dependent fluorescence turn-on of FRET-TDM by the indicated mycobacterial hydrolase (Tdmh or Ag85C) or commercial hydrolases, as compared to the probe-only control. All experiments used 10 µg/mL enzyme in Tris-HCl buffer. Mean values from three replicate experiments are shown.

Figure S6. Plot of time-dependent fluorescence turn-on of FRET-TDM by whole cells of various bacterial species in PBS, as compared to the probe-only control. Mean values from three replicate experiments are shown.
Figure S7. Compound 2 $^1$H NMR (500 MHz).

Figure S8. Compound 2 $^{13}$C NMR (125 MHz).
Figure S9. Compound 3 $^1$H NMR (500 MHz).

Figure S10. Compound 3 $^{13}$C NMR (125 MHz).
Figure S11. Compound 4 $^1$H NMR (500 MHz).

Figure S12. Compound 4 $^{13}$H NMR (125 MHz).
**Figure S13.** Compound 1 (FRET-TDM) $^1$H NMR (500 MHz).

**Figure S14.** Compound 1 (FRET-TDM) $^{13}$C NMR (125 MHz).
Figure S15. 6-O-(10-azidodecanoyl)-α,α-D-trehalose $^1$H NMR (500 MHz).

Figure S16. 6-O-(10-azidodecanoyl)-α,α-D-trehalose $^{13}$C NMR (125 MHz).
Figure S17. Compound 5 $^1$H NMR (500 MHz).

Figure S18. Compound 5 $^{13}$C NMR (125 MHz).
Figure S19. FITC-TMM (6) $^1$H NMR (500 MHz).

Figure S20. FITC-TMM (6) $^{13}$C NMR (125 MHz).
II. References

(1) Ojha, A.; Anand, M.; Bhatt, A.; Kremer, L.; Jacobs Jr., W. R.; Hatfull, G. F. GroEL1: A Dedicated Chaperone Involved in Mycolic Acid Biosynthesis during Biofilm Formation in Mycobacteria. *Cell* 2005, *123*, 861–873.

(2) Ojha, A. K.; Trivelli, X.; Guerardel, Y.; Kremer, L.; Hatfull, G. F. Enzymatic Hydrolysis of Trehalose Dimycolate Releases Free Mycolic Acids during Mycobacterial Growth in Biofilms. *J. Biol. Chem.* 2010, 285, 17380–17389.

(3) Ojha, A. K.; Baughn, A. D.; Sambandan, D.; Hsu, T.; Trivelli, X.; Guerardel, Y.; Alahari, A.; Kremer, L.; Jacobs, W. R.; Hatfull, G. F. Growth of Mycobacterium Tuberculosis Biofilms Containing Free Mycolic Acids and Harbouring Drug-Tolerant Bacteria. *Mol. Microbiol.* 2008, 69, 164–174.

(4) Yang, Y.; Kulka, K.; Montelaro, R. C.; Reinhart, T. A.; Sissons, J.; Aderem, A.; Ojha, A. K. A Hydrolase of Trehalose Dimycolate Induces Nutrient Influx and Stress Sensitivity to Balance Intracellular Growth of Mycobacterium Tuberculosis. *Cell Host Microbe* 2014, *15*, 153–163.